CHARACTERIZATION OF A PUTATIVE QUATERNARY NEUTRALIZING EPITOPE
ON THE LASA VIRUS GLYCOPROTEIN

AN ABSTRACT

SUBMITTED ON THE TWENTY-SECOND DAY OF APRIL 2015
TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE SCHOOL OF MEDICINE
OF TULANE UNIVERSITY
FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY

BY

Benjamin T. Bradley

APPROVED:

Director, James E. Robinson, M.D.

Bruce A. Bunnell, Ph.D.

Robert F. Garry, Ph.D.

Cindy A. Morris, Ph.D.

John S. Schneefelin, M.D.
Abstract

Lassa virus (LASV) is the causative agent of Lassa Fever (LF), an acute and occasionally fatal disease with hemorrhagic features. Current treatments for LF are limited to ribavirin and supportive care. The objective of this dissertation project was to characterize a human monoclonal antibody (mAb) with the potential to treat LASV infections. This antibody, mAb 8.9F, was isolated from the serum of a convalescent patient and has been shown to protect guinea pigs from lethal LASV challenge. Our approach focused on confirming that mAb 8.9F bound a quaternary neutralizing epitope (QNE), determining which residues comprised the epitope, and examining how mutations to critical residues would impact viral fitness. Our results showed that mAb 8.9F shared a number of features similar to previously characterized QNEs including broad neutralization of viral subtypes and high sensitivity to epitope disruption by detergents. We found that mAb 8.9F recognizes sites on both the GP1 and GP2 subunits of the LASV glycoprotein. Both subunits dissociate from the antibody at similar rates under chaotropic conditions, suggesting that these subunits have equal binding affinity with mAb 8.9F. To identify amino acids important to the epitope, we used a method of knockout site-directed mutagenesis that replaced sequences of LASV glycoprotein with homologous sequences from lymphocytic choriomeningitis virus. We created single mutations for those regions unreactive with mAb 8.9F and identified H124F, P145R, and F147N as mutations capable of abrogating mAb 8.9F reactivity. Viral fitness of these mutants was evaluated by measuring glycoprotein processing and viral infectivity. Our results showed that mutations abrogating mAb 8.9F binding did not
significantly inhibit glycoprotein production, processing, or surface transport; neither was pseudovirus formation affected. However, these mutations did significantly reduce pseudovirus infectivity suggesting that mutants escaping mAb 8.9F neutralization may be less fit. The role of N-linked glycosylation in mAb 8.9F recognition was also examined but results proved inconclusive. In summary, this work provides a detailed analysis of the first documented QNE found on the Lassa virus glycoprotein. This work will help direct rational vaccine design and post-exposure antibody therapy.
Acknowledgements

The research contained within this dissertation would not have been possible without the generosity and faith displayed by Dr. James Robinson. Upon the departure of my previous advisor, I was left with two years of unpublished research and no one within the Immunology and Microbiology Department willing to support me. To say I was devastated would be an understatement. As early as high school I remember being drawn to infectious disease, and viruses in particular. I was amazed at the hidden intricacy of these non-living pathogens. Also during this time, I became interested in medicine and the treatment of disease. While I had no illusions about the difficulty of curing viral diseases, the Sisyphean task was entrancing nonetheless. When I no longer had the faculty support to become a virologist, I was forced to idly watch as my dream was torn from me. Fortunately, Dr. James Robinson, my advisor and director of the Physician Scientist Program, had begun a collaboration with Dr. Robert Garry examining the antibody response of humans following Lassa virus infection. While I offered to work on this project without receiving any stipend, Dr. Robinson and Dr. Garry were able to find the funds necessary to support me. For this, I am indebted to both of these men in ways that are impossible to repay. I can only hope that my accomplishments as physician and mentor will serve as a testament to their generosity.

I also want to thank the other members of my dissertation committee. In addition to Dr. Robinson and Dr. Garry, Drs. Bruce Bunnell, Cindy Morris, and John Schieffelin provided guidance and support throughout my Ph.D. years. In particular I
am grateful to Dr. Bunnell whose lab I worked in as an undergraduate at Tulane. He provided me with encouragement and exposure necessary to quickly develop my technical laboratory skills. It is very unlikely I would have been accepted to any M.D./Ph.D. programs without the experiences I gained from him and his excellent lab members. Also, I am grateful to Dr. Jeffery Gimble, M.D./Ph.D. who provided me with my very first research experience as a senior graduating from high school. His mentorship was instrumental in teaching me the basics of good laboratory practices as well as the importance of well-designed studies. As a result of working with him, I began to consider pursuing a career as a physician scientist.

Additional thanks go to all the members of the Robinson Lab both past and present. Debra Elliott, Julie Rouelle, Ashely Smira, Landon vom Steeg, and Matt Gleaton. Entering a new lab is always a rough transition, but they made it as painless as possible by welcoming me with open arms. In particular, I am thankful for the advice and troubleshooting Debra provided me. Even when she was extremely busy, she was willing to take the time to help me logically think through whatever difficulty I was having with an experiment. Her technical and organizational abilities are invaluable assets within the Robinson Lab.

Always encouraging and supporting me have been a group of friends unlike any other. Eric Couper, Steven Dailey, Sean Gahagan, Kevin Hanegan, and Dustin Kingsmill have helped shape me since I first met some of them over a decade ago. The quality of their character is unlike many others. They consistently motivate me to push myself further while reminding me to maintain a sense of compassion to the world around me. It is with absolute sincerity that I say how excited I am to see the
amazing things they will accomplish. They are all unique, passionate, and caring. You would almost expect that one would become an MD/PhD if surrounded by such fertile minds. It seems glib to say that chance brought these people into my life. Perhaps there's a natural resonance that people of conviction have. If so, I'm thankful that they noticed mine.

Finally, I offer my deepest and most heartfelt thanks to my parents, Tom and Germaine Bradley. As a researcher, we always seek to understand the mechanism behind a process. It is never sufficient to associate a cause and effect, the pathway must also be elucidated. However, in regards to my parents, there exists no test, no device that could identify the limitless source from which their love arises. The process of life seems inextricably tied to love. It is a force capable of overcoming all boundaries of the body and mind. It propels us to understand our existence outside the context of a single entity doomed to struggle. In love, we are intertwined into others and transcend the concerns, anxieties, and selfishness of our singular being. We then understand that our work and our care ripple through time. Kindness, like a gene, can be passed on and built upon. It can shape an environment and protect us from harm. Of the good that I’ve done and goals that I’ve accomplished, they are my parents’ love translated into action.
“When they love you, and they will/
Tell ’em all they’ll love in my shadow/
And if they try to slow you down/
Tell ’em all to go to hell”

Japandroids “The House That Heaven Built”
TABLE OF CONTENTS

Acknowledgements ii

List of Tables viii

List of Figures ix

Introduction 1
- Lassa Virus: Basic Virology and Replication Cycle 1
- Glycoprotein Structure and Viral Entry 3
- Epidemiology 6
- Clinical Features 8
- Pathology and Immune Response 10
- Treatment and Prevention 14
- Significance 18
- Hypothesis 18
- Specific Aims 19
- Figures 21

General Materials and Methods 28

Chapter 1: Characterizing Features of mAb 8.9F Consistent with Recognition of a Quaternary Neutralizing Epitope 38
- Introduction 39
- Materials and Methods 43
- Results 46
- Discussion 54
- Figures 60

Chapter 2: Identification of Amino Acids and N-glycans Mediating mAb 8.9F Epitope Recognition 71
- Introduction 72
- Materials and Methods 77
- Results 79
- Discussion 84
- Figures 92

Chapter 3: Effect of mAb 8.9F-abrogating Mutations on Glycoprotein Processing and Pseudovirus Function 112
- Introduction 113
- Materials and Methods 119
- Results 121
- Discussion 124
- Figures 128
Chapter 4: Role of N-linked Glycosylation in Lassa
Virus Glycoprotein Processing and Function 136

Introduction 137
Materials and Methods 144
Results 146
Discussion 154
Figures 165

Summary 180

List of References 190
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Members of the <em>Arenaviridae</em> family</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>LCMV-LASV short segment mutants primers</td>
<td>93-98</td>
</tr>
<tr>
<td>3</td>
<td>Primers used for single amino acid substitutions</td>
<td>103-104</td>
</tr>
<tr>
<td>4</td>
<td>Primer sequences for serine/threonine to alanine mutations</td>
<td>109</td>
</tr>
<tr>
<td>5</td>
<td>List of primers for asparagine to glutamine substitutions</td>
<td>165</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Electron micrograph of LCMV</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>Lassa virus structure and genome</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>Replication and glycoprotein synthesis of arenaviruses</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>Organization of Lassa virus glycoprotein precursor</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>mAb 8.9F neutralizes Josiah pseudoviruses at a concentration similar to other highly neutralizing mAbs</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>mAb 8.9F neutralizes representative strains from each of the four lineages of Lassa virus</td>
<td>61</td>
</tr>
<tr>
<td>7</td>
<td>mAb 8.9F is unable to react with solubilized protein on ELISA</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>mAb 8.9F binding is detectable following incubation with unfixed cells</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>RIPA treatment of GPC-transfected cells or pseudovirus eliminates mAb 8.9F binding</td>
<td>64</td>
</tr>
<tr>
<td>10</td>
<td>mAb 8.9F precipitates the GP1 and GP2 subunits</td>
<td>65</td>
</tr>
<tr>
<td>11</td>
<td>Sodium thiocyanate treatment of antibody-antigen complexes</td>
<td>66</td>
</tr>
<tr>
<td>12</td>
<td>Densitometric analysis of NaSCN blots</td>
<td>67</td>
</tr>
<tr>
<td>13</td>
<td>Treatment of cells with PF-429242 reduces GPC cleavage</td>
<td>68</td>
</tr>
<tr>
<td>14</td>
<td>Surface binding of monoclonal antibodies to PF-429242 treated cells</td>
<td>69</td>
</tr>
<tr>
<td>15</td>
<td>Immunofluorescent images of PF-429242 treated cells</td>
<td>70</td>
</tr>
<tr>
<td>16</td>
<td>Sequence alignment of Lassa virus glycoprotein with LCMV glycoprotein</td>
<td>92</td>
</tr>
<tr>
<td>17</td>
<td>LCMV pseudoviruses are not neutralized by mAb 8.9F but are neutralized by 12.1F</td>
<td>99</td>
</tr>
<tr>
<td>Page</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Flow cytometry data for short sequence mutants</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>mAb 8.9F unreactive short sequence mutants produce cleaved glycoprotein</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Immunofluorescent images of mAb 8.9F-negative short segment mutants</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>H124F, P145R, and F147N mutations prevent mAb 8.9F surface recognition on transfected cells</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Immunofluorescent images of mAb 8.9F-unreactive mutants</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Slot blot of mAb 8.9F-unreactive pseudoviruses</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Neutralization assay of H124, P145R, and F147N pseudoviruses with mAb 8.9F and 12.1F</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>T81A and S169A eliminate mAb 8.9F surface binding</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Slot blot of S/T -&gt; A mutations probed with mAb 8.9F and 12.1F</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Mechanisms of viral antigenic escape</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Mutations abrogating mAb 8.9F binding do not significantly affect GPC cleavage</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Surface transport of GP1 for mAb 8.9F-unreactive mutants is comparable to wild type</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>The S255T mutation produces no detectable GPC or GP2 signal</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Immunofluorescent images of 250 RDIYIS single mutations</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Mutations eliminating mAb 8.9F binding do not prevent glycoprotein incorporation into pseudoviruses</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Pseudoviruses with mAb 8.9F-abrogating mutations are able to bind Vero E6 cells</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Mutations affecting mAb 8.9F binding reduce pseudovirus infectivity</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>Glycoprotein cleavage of N-glycosylation mutants</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Measurement of GPC cleavage by N-glycosylation mutants</td>
<td>167</td>
</tr>
<tr>
<td>37</td>
<td>Measurement of total glycoprotein production by N-glycosylation mutants</td>
<td>168</td>
</tr>
<tr>
<td>38</td>
<td>Glycoprotein surface expression of N-glycosylation mutants</td>
<td>169</td>
</tr>
<tr>
<td>39</td>
<td>Surface immunofluorescent images of N-glycan mutants staining with mAb 8.9F</td>
<td>170</td>
</tr>
<tr>
<td>40</td>
<td>Incorporation of GP2 in mutant pseudoviruses</td>
<td>171</td>
</tr>
<tr>
<td>41</td>
<td>Densitometric analysis of Figure 40</td>
<td>172</td>
</tr>
<tr>
<td>42</td>
<td>Correlation of glycoprotein cleavage to GP2 incorporation in pseudoviruses</td>
<td>173</td>
</tr>
<tr>
<td>43</td>
<td>Titration of pseudoviruses with N-glycan mutations</td>
<td>174</td>
</tr>
<tr>
<td>44</td>
<td>Correlation plots of pseudovirus infectivity versus GP2 incorporation and glycoprotein cleavage</td>
<td>175</td>
</tr>
<tr>
<td>45</td>
<td>mAb 8.9F and 12.1F surface staining of N-glycan mutants</td>
<td>176</td>
</tr>
<tr>
<td>46</td>
<td>Slot blot of pseudoviruses with N-glycan deletions</td>
<td>177</td>
</tr>
<tr>
<td>47</td>
<td>Paired data for GPC cleavage of mutations at individual N-glycosylation sites</td>
<td>178</td>
</tr>
<tr>
<td>48</td>
<td>Paired infectivity data of mutations at individual N-glycosylation sites</td>
<td>179</td>
</tr>
</tbody>
</table>
INTRODUCTION

Lassa virus: Virology and Replication Cycle

Lassa virus is a member of the family Arenaviridae which includes Lymphocytic choriomeningitis virus (LCMV) and Tacaribe virus (Table 1). Arenaviruses are named after their sandy appearance when viewed by electron microscopy caused by the presence of large numbers of ribosomes (Figure 1). Members of the Arenaviridae family are broadly divided into two groups based on their serology: New World (or Tacaribe serocomplex) and Old World (or Lassa-Lymphocytic choriomeningitis serocomplex). Structurally, these viruses are pleiomorphic and range in size from 40nm to 300nm. The outside of the virus is composed of a host-derived lipid envelope that is punctuated with viral glycoprotein 1 (GP1) and glycoprotein 2 (GP2) trimers at regular intervals (Figure 2A). These GP complexes are associated with the Z protein located under the envelope. The Z protein has been found to play a major role in ribonucleoprotein incorporation into virions and membrane budding.

The Lassa virus genome consists of two ambisense, single-stranded RNA segments: L and S (Figure 2B). The L segment is approximately 7.3kb in length and codes for the RNA-dependent RNA polymerase (RdRP or L polymerase) and the Z protein. The S segment is 3.4kb in length and encodes the nucleoprotein (NP) and the glycoprotein precursor (GPC). The L and NP viral mRNA is directly transcribed from the Lassa virus genome. Following RdRP protein synthesis, the viral antigenome is produced and the viral mRNA for GPC and Z proteins are transcribed.
Dividing the coding regions within each RNA segment is a stem-loop that functions in stalling the L protein 10.

The genetic information of arenaviruses is stored within the virus as a complex of RNA, NP, and L, known as the ribonucleoprotein (RNP). In order for efficient virus production and infectivity, specific interactions between the RNP subunits are required. The RNA genomic segments have complimentary 3’ and 5’ regions which lead to the formation of a panhandle structure 11. This structure is hypothesized to facilitate encapsidation and formation of the circular nucleocapsid that has been observed on electron microscopy 12,13. Based on crystallographic data, a basic pocket within the 5’ region of NP catalyzes RNA binding 14. NP-RNA binding elicits structural changes within the protein that promotes the formation of a polymer of NP protecting the length of the RNA 15. The L protein, which interacts with the both NP and viral RNA, has also been identified as a crucial part of the RNP 16. Upon entry into the cytoplasm, the L protein functions in both replication and transcription 17.

In order to translate viral mRNA, arenaviruses utilize a cap snatching mechanism. Cap snatching refers to the mechanism by which a 5’ cap is harvested from cellular mRNA and attached to viral mRNA to allow for protein translation. Studies have shown that the N-terminal region of the L protein contains an endonuclease structurally similar to those previously identified in other viral species 18. During the cap-snatching process, N-terminal region of the L protein binds to cellular RNA transcripts and hydrolyzes the first ten to twenty amino acids including the m(7)Gp capping nucleotide 19. In addition to the L protein, NP has also
been shown to have cap-binding properties despite being dissimilar to other known cap-binding proteins \(^{20}\). NP appears to help stabilize the cleaved nucleotides and catalyze attachment to viral mRNA. Following capping, host ribosomes translate the viral mRNA thus enabling the production of viral proteins.

**Glycoprotein Structure and Viral Entry**

The glycoprotein precursor (GPC) of Lassa virus is contained within the S gene segment and is translated as a single polypeptide. The GPC contains three functional subunits: the stable signal peptide (SSP), glycoprotein 1 (GP1), and glycoprotein 2 (GP2). Maturation of the polypeptide is initiated by host signal proteases that cleave the SSP subunit. Following transport to the ER, GP1-GP2 cleavage is accomplished by the subtilisin-kexin isoenzyme 1/site 1 protease (SKI1/S1P) which normally functions in cellular lipid metabolism \(^{21}\). Arenavirus GP1-GP2 cleavage is necessary in order for the formation of infectious particles and viral replication \(^{21,22}\). It is interesting to note that when cleavage is inhibited, unprocessed GPC is transported to the surface but is not incorporated into budding virions \(^{21}\). The GP1-GP2 cleavage motif is highly conserved between arenavirus, suggesting that SKI1/SP1 is active in the processing most arenavirus glycoproteins \(^{23}\). Based on these observations, researchers hypothesize that selective inhibition of SKI1/S1P could be a target for therapeutic intervention during arenavirus infections. \(^{24,25}\) A schematic of the GPC polypeptide subunits, along with cleavage and glycosylation sites are provided by Figure 4.

In comparison to other signal peptides, arenavirus SSPs are unusually long and stable \(^{26,27}\). Most signal peptides are degraded shortly after protein processing,
the arenavirus SSP, however, is transported to the surface of infected cells where it maintains stable interactions with the cytoplasmic region of GP2 \(^{28}\). The SSP has been shown to have a role in directing ER transport of the GPC and in regulating cleavage of the GP1 and GP2 subunits \(^{29,30}\). The SSP may also play a role in the formation of stable trimers on the virion surface \(^{31}\).

Lassa virus GP1 and GP2 are the main viral proteins involved in cell binding and entry \(^{32}\). The glycoprotein complex is arranged as trimeric spikes on the surface of virions. The extracellular GP1 subunit is anchored to the virion through non-covalent interactions with the transmembrane protein GP2. GP1 functions as the receptor binding subunit of arenaviruses \(^{33}\). All arenaviruses are capable of binding the alpha-dystroglycan receptor and some New World viruses can also use transferritin-1 \(^{34}\). While alpha-dystroglycan may serve as the cellular receptor, other proteins have been found that aid in infectivity by increasing cell-virus interactions. For example, interactions between GP1 and dendritic cell-expressed DC-SIGN have been implicated in viral aggregation on the cell surface \(^{35}\).

Following alpha-dystroglycan receptor binding, virions are endocytosed via an internalization pathway that is independent of caveolin, clathrin, dynamin, and actin. However, components of the endosomal sorting complex required for transport (ESCRT) pathway have been demonstrated as necessary for productive Lassa virus infection \(^{36}\). After endosomal acidification begins, a pH-dependent, trigger-induced switch of GP1 to the internal cellular receptor LAMP-1 occurs \(^{37}\). As LAMP-1 is predominantly expressed in lysosomes, this receptor switch is believed to be the final step necessary concentration viruses within lysosomal compartments
Acidification also plays an important role in activating GP2, which serves as a class I fusion protein. GP2 is believed to function in a manner similar to that of other class I fusion proteins. When exposed to acidic conditions, a trigger-induced conformational change occurs in the protein leading to the formation a coiled coil structure. The ends of the coil extend cause membrane fusion by bringing the virus and lysosomal surfaces in close enough contact to trigger spontaneous fusion. At this point in the infection process, the viral RNA, L protein and NP are released into the cytoplasm initiating viral replication (Figure 3).

Despite only recently solving the crystal structure of LCMV GP2, arenavirus fusion proteins have long been researched. Early studies found that treating LCMV virions with acidic buffers inactivated the virus and exposed sequestered GP2 epitopes. Later structural comparisons between arenaviruses and filoviruses demonstrated significant similarities in glycoprotein arrangement and sequence similarity. As the glycoprotein structure for several filoviruses had been solved, this study gave additional support to the theory of the arenavirus GP2 subunit as a class I fusion protein. Later, structural analysis of recombinantly expressed GP2 ectodomain showed the formation of trimeric all-alpha complexes which was highly suggestive of a class I fusion protein. Upon crystallographic analysis of post-fusion GP2, a classic coiled coil structure was exhibited along with stabilizing intrachain salt bridges. Based on these observations, arenavirus glycoproteins have been classified as type I fusion proteins similar to those found in HIV and influenza.

**Epidemiology**
Lassa virus is named after the village in Nigeria where the first case, a 69-year old nurse infected in January of 1969, was documented. The patient initially presented with fever and sore throat. As her condition deteriorated, she was transported to a larger hospital in Jos, Nigeria where two additional nurses were infected. Since this first documented case, Lassa virus infections have been identified throughout West Africa including regions of Sierra Leone, Guinea, Liberia, and Cote d'Ivoire.

Arenaviruses are maintained and spread through rodent reservoirs. The rodent genus *Mastomys*, also known as the multimammate rat, has been identified as the reservoir for Lassa virus. The virus was originally believed to be harbored within the *natalensis* species, but the complicated taxonomy of the *Mastomys* genus hindered efforts to resolve this issue. Currently there are eight recognized *Mastomys* species of which several inhabit Lassa virus-endemic regions. Early studies using hemoglobin electrophoresis to type species found *M. erythroleucus* and *M. huberti* as possible hosts. Further complicating matters, the term *Mastomys natalensis* has been used to refer to multiple species in southern Africa that exist outside of Lassa virus endemic regions. In spite of these difficulties, recent advances in genetic characterization have helped confirm *M. natalensis* as the most probable vector.

Current research suggests Lassa virus is maintained within the rodent population though vertical and horizontal transmission. Experimental infection of neonatal *Mastomys* establishes a persistent infection and infected animals were found to shed live virus. In conjunction with this finding, naïve adults infected
with Lassa virus developed a disseminated infection lasting up to 30 days while virus persisted in some organs up to 103 days post-infection \(^{53}\). In addition to efficiently shedding virus, \textit{M. natalensis} exhibits relatively little histopathological change follow infection. While one study found smaller, more frequent inflammatory lesions in the tissue of infected animals, other studies have demonstrated persistent infection to have no influence on the fitness of the animal \(^{50,54}\). These findings suggest \textit{M. natalensis} is an ideal host reservoir for Lassa virus.

Studies have shown a close association between \textit{Mastomys natalensis} infestation and Lassa virus infection \(^{49}\). In areas where Lassa fever is endemic, \textit{M. natalensis} is the primary rodent found in homes and infected animals tend to cluster within a small number of homes per village \(^{50}\). Several methods of virus transmission to humans have been identified. Because infected mice efficiently shed virus in their urine and feces, the primary mechanism of infection is believed to result from the ingestion of contaminated food \(^{49}\). The feasibility of Lassa virus infection via food sources is further reinforced by the observation that monkeys and mice can be infected intragastrically by LCMV, a related arenavirus \(^{55,56}\). Lassa virus is stable when aerosolized, and animal transmission models have shown Lassa virus to spread effectively via the airborne route \(^{57,58}\). Because \textit{Mastomys natalensis} is consumed for food in regions of West Africa, humans may also become infected through contact with infected animal body fluids or consumption of meat \(^{59}\).

**Clinical Features**

Lassa virus is predicted to infect 100,000 to 300,000 people annually with 5,000 deaths \(^{60}\). Estimates of the seroprevalence of Lassa virus antibodies vary
considerably and this is most likely due to geographic influences on *M. natalensis* prevalence. The high seroprevalence of Lassa virus antibodies in endemic areas suggests that most infections are subclinical. Furthermore, studies analyzing the seroconversion rate of people in endemic areas show a rate between 5% and 20% with a smaller portion of those individuals self-reporting fever or other symptoms associated with Lassa virus infection. Again, this suggests the majority of Lassa virus infections are asymptomatic to mild. While the overall case fatality rate of Lassa virus infections are less than 5%, transmission and mortality rates have been shown to increase during nosocomial outbreaks with up to 65% of cases being fatal. The elevated mortality in these outbreaks may have been due to immunologically compromised patients becoming infected as well as poor hygienic practices among hospital staff. Studies have shown this increased risk to be eliminated when healthcare providers used good hygienic practices for infection control.

While early detection of Lassa virus infection has been shown as critical for reducing fatal cases, early clinical features are indistinguishable from many other acute viral infections. Presentation of symptoms begins seven to 18 days post-infection and includes fever, malaise, and weakness. Additional symptoms that may develop during illness include vomiting, diarrhea, and tinnitus. However, these symptoms are also frequently present in other non-Lassa fever viral infections and make diagnosing Lassa fever based on clinical presentation alone difficult. While gross hemorrhaging is infrequent, subtle signs of vascular dysregulation such as edema, conjunctival injection, and pleural effusions are present in severe cases. In
patients that survive infection, resolution of symptoms normally begins after eight to ten days. Patients with severe or fatal cases of Lassa fever see a worsening of symptoms between days six and ten. The illness may progress to include pulmonary edema, encephalopathy, and shock. Resolution of symptoms is achieved by fourteen days post infection; however, sensoneural deafness may be a sequela of Lassa fever in up to 30% of cases. Following clearance from the blood, the virus may continue to be shed in the urine and detectable in the cerebrospinal fluid.

In assessing disease severity and outcome, levels of virus in the blood have been the most predictive. Patients whose tissue culture infectious dose (TCID$_{50}$)/ml was above $10^3$ at any point during their illness had a 3.7 times greater mortality rate relative to admitted patients whose viremia remained below that threshold. Fatal cases were also more often associated with high levels of liver enzymes in the blood. In combination with a TCID$_{50}$/ml greater than $10^3$, serum levels of aspartate aminotransferase (AST) over 150 IU/L upon initial hospital admission were associated with 78% mortality. Death occurs after a mean duration of twelve days and levels of viremia were found to range from $10^3$- $10^8$ TCID$_{50}$/ml. Viremia tends to increase before plateauing late in the course of fatal disease. Fatal cases also showed disseminated spread with virus detected in the liver, spleen, lymph nodes, and brain. In patients that survived, viremia plateaus around day six and reaches undetectable levels in blood three weeks after symptoms begin.

**Pathology and Immune Response**

Determining the exact pathological mechanisms involved in fatal cases of Lassa fever has proven elusive. Based on the lack of gross pathological findings in
addition to the high levels of viremia found in fatal cases, dysregulation of cytokine expression has been proposed as a likely mechanism for disease in Lassa fever though it has not been conclusively proven. During a study of two imported Lassa fever cases, levels of TNF-a and IFN-g were measured over the course of the illness. In the first patient, cytokine levels were found to peak shortly before fatal hemorrhagic shock. However, the second fatal case monitored in the study did not have elevated levels of proinflammatory cytokines. Another study found no statistically significant difference in the levels of proinflammatory cytokines between non-fatal, fatal, or non-Lassa fever infections. It is unclear how important these two cytokines are in Lassa virus pathogenesis. Perhaps TNF-a and IFN-g play a role in some fatal infections displaying hemorrhagic symptoms, but not others. Alternatively, these cytokines may be elevated in all cases but the increase may only occur during a brief window preceding death. More conclusive findings have come from examining IL-8 and IL-10. These two cytokines are suppressors of the cellular immune response. Studies have shown that low levels of IL-8 and IL-10 are associated with fatal outcomes in Lassa fever. Further confirming a link between IL-8 and Lassa virus pathogenesis is data showing in vitro infected macrophages downregulate IL-8 expression.

In addition to cytokine dysregulation, Lassa virus pathology may also result from impairment of cell-mediated adaptive immunity. Dendritic cells are one of the key antigen presenting cells that play a role in early detection of Lassa virus infection. When dendritic cells are infected in vitro, Lassa virus fails to upregulate the expression of two co-stimulatory molecules for naïve T-cell activation, CD86 and
Furthermore, Lassa virus-infected dendritic cells induced proliferation of T-cells at a level lower than uninfected controls. The importance of cell-mediated immunity in clearing infection was also supported by studies investigating Mopeia virus clearance. Mopeia shares 75% genetic similarity to Lassa virus but does not cause clinical disease in humans. Mopeia virus was found to induce higher levels of TNF-a in monocytes and induce a stronger T-cell response in infected dendritic cells. While activation of T-cell responses are important for virus clearance, a dual role of T-cells as inhibitors and enhancers of pathogenesis has been demonstrated in mice. Experimentally infected wild-type mice are able to clear Lassa virus within several days; however, transgenic mice expressing human MHC-I molecules experienced high levels of viremia suggesting MHC-I stimulation of T-cells plays a role in virus clearance. Additionally, when the transgenic mice were depleted of T-cells, levels of serum AST remained within the normal range and animals did not show overt signs of disease. These observations were made in spite of the fact that these mice showed high levels of viremia. Thus, T-cells seem to play a role both in the pathogenesis of Lassa fever and in controlling virus spread. Additional research evaluating the specific role of T-cell subsets and co-stimulatory molecules will provide a more complete picture of the immunological subtleties governing Lassa virus infection and disease.

In contrast to T-cells, humoral-mediated immunity does not appear to be a major factor in survival following primary Lassa virus infection. Previous studies have found infection to elicit production of IgM and IgG antibodies early in infection with nearly 90% of infected patients producing both antibody classes two weeks
after symptom onset. It is unlikely that the antibodies produced during this period are neutralizing as they are in circulation at the same time as the virus. Similar data showing poor antibody neutralization has been demonstrated in experimentally infected monkeys and guinea pigs. In the experiments done by Baize, et. al., IgM and IgG levels were found to peak by day 16 in both fatal and non-fatal cases with those animals that survived having slightly earlier and higher antibody peaks. No neutralizing antibodies were detected early in infection. The results of these studies have led to the conclusion that antibodies play a minor role during primary infection. However, this should not discount the use of high-affinity antibodies in preventing infection if administered prophylactically or elicited by a recombinant vaccine. Antibodies are generated against N, Z, and both GP subunits and have been shown to protect animals in passive transfer experiments. Interestingly, neutralizing antibodies following Lassa virus infection arise weeks to months after convalescence. The slow increase in neutralizing antibody activity suggests that low-level viral persistence may continue stimulating the immune system following the resolution of symptoms. To that end, Lassa virus has been isolated in the urine and cerebrospinal fluid of infected humans after viral clearance from the blood and viral RNA has been isolated from animal tissue up to 112 days post-inoculation. Additionally, the immunological memory following Lassa virus infection is believed to be a long-lived response. Serum samples from patients infected over 40 years ago with no subsequent exposure have been shown to be reactive on antigen ELISA.

**Treatment and Prevention**
While several classes of small-molecule inhibitors are under investigation as therapeutic treatments for arenavirus infections, ribavirin is currently the most widely tested and studied \(^{87-89}\). Ribavirin (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was originally developed as a synthetic nucleoside against DNA viruses \(^{90}\). This treatment has shown protective effects against Lassa virus infection \textit{in vitro}, in animal studies, and clinical trials. In cell-culture assays, ribavirin inhibited Lassa virus replication at concentrations between 10\(\mu\)g/ml and 50\(\mu\)g/ml \(^{91}\). For animal studies, rhesus macaques were infected with 1.2\(\times\)10\(^6\) PFU and experimental groups were placed on ribavirin therapy immediately following inoculation or 5 days post-infection. Treatment groups were administered a bolus of ribavirin followed by maintenance doses every 8 hours. In the group treated at day 0, all monkeys survived with mild symptoms and significantly lower viremia compared to controls. The group treated 5 days post-infection had more severe symptoms and viremia levels were similar to untreated controls until day 10 post-infection \(^{92}\).

The importance of initiating ribavirin treatment early in disease was further confirmed by data from human infections. When ribavirin therapy was started within six days of the onset of symptoms, the risk of mortality within two high risk groups, those with AST > 150U/l or those with TCID\(_{50}\)/ml >10\(^{3.6}\), was reduced from 55% to 5% and 76% to 9% respectively. Additionally, even if therapy was started 7 days after symptom onset the risk was still reduced to 26% and 47% \(^{67}\). In a study of two imported cases of Lassa fever where ribavirin therapy was started 11 days post-symptom onset, one case showed a slight decrease in viremia but both cases
proved fatal \textsuperscript{73}. Based on these observations, the early identification and rapid treatment with ribavirin appear to be necessary for its effectiveness. Studies measuring the utility of ribavirin for Lassa virus post-exposure prophylaxis have been inconclusive. This is primarily due to the remote disease distribution and infrequent number of high-risk exposures \textsuperscript{93}. Side effects of ribavirin treatment are relatively mild and non-specific, however an episode of rigor late in treatment has been documented \textsuperscript{93,94}.

The exact mechanism of action by which ribavirin is able to inhibit arenavirus spread is still under investigation. Several mechanisms have been proposed based on \textit{in vitro} data. Complicating matters, studies have found different viruses are inhibited by different mechanisms \textsuperscript{95}. The majority of viruses are inhibited by one of three mechanisms: depletion of free GTP, inhibition of RNA-dependent RNA polymerase (RdRp), or lethal mutagenesis via ribavirin incorporation \textsuperscript{96-98}. Studies examining the effect of ribavirin on LCMV replication found high mutagenic activity that was unrelated to the decrease in cellular GTP. These results suggest that ribavirin's mechanism of action in arenavirus infections is due in part to induction of RdRp-mediated replication errors \textsuperscript{99}. These errors result in lethal mutagenesis or error catastrophe, a condition in which the number of genomic errors prevents stable replication and the population becomes extinct.

Neutralizing antibodies, either in purified form or as convalescent serum, have also been investigated as a treatment option for Lassa fever. Experimental studies have shown passive immunization to protect macaques and guinea pigs
from lethal virus challenge. These studies found several important factors implicated in effective antibody therapy. One was the \( \log_{10} \) plaque-forming units neutralizing index (LNI) that measures serum neutralization. LNI was more predictive for determining effective treatment than the indirect fluorescent antibody (IFA) titer that measures total antibody binding irrespective of neutralization potential. When guinea pigs were experimentally infected, the group receiving serum with LNI=4.8 and IFA=320 survived whereas fatal infection occurred in the group treated with LNI=0.6 and IFA=640 plasma. Similarly when cynomolgus macaques were lethally challenged, the groups receiving LNI=4.1 at 1ml/kg and LNI=2.6 at 3ml/kg were protected. Matching the viral strain of the convalescent serum to the infecting strain of virus is also important in determining the effectiveness of passive antibody therapy. Despite the wealth of data on convalescent serum in animal studies, data on the treatment of human infections is relatively poor. Two case studies have suggested a role for convalescent serum in reducing the severity of Lassa fever. However, the studies either involved a small number of patients or no control group. In addition, no clinical measurements were given in regards to virus titer or antibody levels of the treated patients. In a later case-control study enrolling a large number of patients, convalescent serum was found to have no significant effect in patients with indicators of severe disease (AST>150 IU or TCID\(_{50}/\)ml> \( 10^{3.6} \)). One caveat to this study was that convalescent plasma was only measured for IFA and not LNI. This may have led to the use of poorly neutralizing samples.
Significance

Lassa fever is a serious public health concern in West Africa. Current treatments may provide some relief from disease burden, but they are costly and require treatment immediately after infection. Vaccination and treatment with neutralizing antibodies provide additional methods to lower disease severity. Crucial to the development of effective vaccine treatments is the identification and classification of highly neutralizing antibodies. However, relatively little work has been done to characterize the antibody response generated following Lassa virus infection as the disease primarily affects developing countries within a small region. Our lab has extensive experience in isolating monoclonal antibodies and mapping epitopes of human immunodeficiency virus. By using these previously refined techniques to analyze the antibody response of convalescent patients, we identified several neutralizing antibodies with therapeutic potential. This work aims to characterize one of the antibodies we isolated, named mAb 8.9F, as recognizing a quaternary neutralizing epitope.

Hypothesis

We hypothesize that monoclonal antibody 8.9F recognizes a quaternary neutralizing epitope present on the functional glycoprotein subunit of Lassa virus. Mutations that abrogate antibody binding will also have an impact on glycoprotein function.
Specific Aims

The overall goal of this dissertation work was to characterize a potential therapeutic antibody to Lassa virus and to examine its unique epitope structure. We proposed that this unique antibody recognized a quaternary neutralizing epitope and that amino acid mutations abrogating binding would also significantly impact glycoprotein function. The overall objective of this dissertation project was to classify the structure of the first documented quaternary neutralizing epitope to Lassa virus. We accomplished our objective by using human-derived monoclonal antibodies to Lassa virus in conjunction with recombinant glycoprotein expression to elucidate mAb 8.9F epitope by completing the following specific aims:

Specific Aim 1. Analyze the functional characteristics of mAb 8.9F

1.1 Measure 8.9F reactivity and neutralization against LASV

1.2 Determine the glycoprotein subunit recognized by mAb 8.9F and its relative affinity

1.3 Determine importance of glycoprotein processing in mAb 8.9F recognition

Specific Aim 2. Identify key residues mediating mAb 8.9F recognition

2.1 Identify single bases mediating mAb 8.9F recognition

2.2 Determine if N-linked glycans affect mAb 8.9F reactivity
Specific Aim 3. Examine the impact of mAb 8.9F-abrogating mutations on viral fitness

3.1 Measure changes in glycoprotein processing and pseudovirus function of mAb 8.9F-unreactive mutants

3.2 Examine changes in glycoprotein processing and infectivity caused by deletion of N-glycosylation sites
<table>
<thead>
<tr>
<th>Virus</th>
<th>Distribution</th>
<th>Principal vertebrate host</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old world species:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCM</td>
<td>Worldwide</td>
<td><em>Mus musculus</em></td>
<td>ARMSTRONG and LILLIE (1934)</td>
</tr>
<tr>
<td>Lassa</td>
<td>West Africa</td>
<td><em>Mastomys natalensis</em></td>
<td>BUCKLEY and CASALS (1970)</td>
</tr>
<tr>
<td>Mobala</td>
<td>Central Africa Republic</td>
<td><em>Praomys jacksonii</em></td>
<td>GONZALEZ et al. (1983)</td>
</tr>
<tr>
<td>Mopeia</td>
<td>Mozambique</td>
<td><em>Mastomys natalensis</em></td>
<td>WULFF et al. (1977)</td>
</tr>
<tr>
<td>New world species: Tacaribe complex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amapari</td>
<td>Brazil</td>
<td><em>Oryzomys goeldi</em></td>
<td>PINHEIRO et al. (1966)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Neacomys guianae</em></td>
<td></td>
</tr>
<tr>
<td>Flexal</td>
<td>Brazil</td>
<td><em>Oryzomys species</em></td>
<td>PINHEIRO et al. (1977)</td>
</tr>
<tr>
<td>Junin</td>
<td>Argentina</td>
<td><em>Calomys laucha</em></td>
<td>PARODI et al. (1958)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Calomys musculinus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Akodon azarae</em></td>
<td></td>
</tr>
<tr>
<td>Latino</td>
<td>Bolivia</td>
<td><em>Calomys callosus</em></td>
<td>JOHNSON et al. (1973)</td>
</tr>
<tr>
<td>Machupo</td>
<td>Bolivia</td>
<td><em>Calomys callosus</em></td>
<td>JOHNSON et al. (1966)</td>
</tr>
<tr>
<td>Parana</td>
<td>Paraguay</td>
<td><em>Oryzomys buccinatus</em></td>
<td>WEBB et al. (1970)</td>
</tr>
<tr>
<td>Pichinde</td>
<td>Colombia</td>
<td><em>Oryzomys albigularis</em></td>
<td>TRAPIDO and SANMARTIN (1971)</td>
</tr>
<tr>
<td>Tacaribe</td>
<td>Trinidad</td>
<td><em>Antibesius literates</em></td>
<td>DOWNS et al. (1963)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Antibesius jamaicensis</em></td>
<td></td>
</tr>
<tr>
<td>Tamiami</td>
<td>Florida</td>
<td><em>Sigmoidon hispideis</em></td>
<td>CALISHER et al. (1970)</td>
</tr>
</tbody>
</table>

Table 1. Members of the *Arenaviridae* family.
Adapted from ²
Figure 1. Electron micrograph of LCMV.
Adapted from 1. (A) Shows spherical virions budding from the cell surface. (B) Shows sandy interior of virions caused by the incorporation of host ribosomes.
Figure 2. Lassa virus structure and genome. Figures adapted from 103,104.
Figure 3. Replication and glycoprotein synthesis of arenaviruses.
Adapted from [32].
Figure 4. Organization of Lassa virus glycoprotein precursor. Adapted from 26.
General Materials and Methods

Biological safety

For all experiments described below, strict adherence to the regulations described by Tulane’s Institution Review Board for Biosafety and the United States Centers for Disease Control was maintained. Personal protective equipment consisting of a lab coat and eye protection was used during BSL-2 level work. Liquids from pseudovirus experiments were inactivated in Vespene and solid waste was autoclaved before disposal in biohazardous waste containers.

Cell culture

Human epithelial kidney 293T cells were purchased from the American Type Culture Collection (ATCC)\textsuperscript{105-107}. Cells were maintained in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and Anti-Anti (100x) (Gibco). African green monkey kidney cells (Vero) were purchased from ATCC\textsuperscript{108}. Cells were maintained in DMEM with 10%FBS and Anti-Anti. TZM-bl cells were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH and courtesy of Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc\textsuperscript{109-112}. Cells were maintained in RPMI 1640 media supplemented with 10% FBS and Anti-Anti. MS40L cells used for B cell feeder cultures (kindly provided by Xin Luo, Virginia Polytechnic Institute, Blacksburg, VA), were maintained in Iscove’s Modified Dulbecco’s medium plus 10% FCS and antibiotics\textsuperscript{113}.

Cells were subcultured when approaching 90% confluency. Existing media was first removed and properly discarded. For a 75 cm\textsuperscript{2} flask, 10 ml of pre-warmed PBS was added to the flask and rotated to remove any dead or floating cells. Cells
were incubated in 3 mL of 0.25% trypsin-EDTA for five minutes at 37 degrees Celsius (C) after removing the PBS. 6 mL of FBS was added to further detach the cells following trypsin incubation and the entire volume was transferred to a 15mL conical tube. Cells were spun down at 300g for 7 minutes before being resuspended in complete growth media. Cells were sub-cultured at a ratio between 1:3 and 1:6 depending on future experiments.

**Viral protein expression plasmids**

The full-length expression plasmids of the Lassa virus glycoproteins used in these experiments were generated from the following strains: Josiah (lineage IV GenBank: AY628203.1), Nigeria A19 (lineage III GenBank: GU481072.1), Nigeria 237 (lineage II GenBank: KM822029.1), and Pinneo (lineage I GenBank: AY628207.1). These sequences were cloned in to either the pCAGGS vector for ELISA study or pLM2, a PBR322 based vector that we also use for MAb heavy and light chain expression. The SG3Δenv plasmid was obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH and courtesy of Drs. John C. Kappes and Xiaoyun Wu.112,114

Stocks of plasmid DNA was generated by transfecting 30uL of JM109 cells with 200ng of DNA following cloning of Lassa virus GPC genes into the appropriate expression vector. The mixture was incubated for 20 minutes on ice before being transferred to a 42C heat block for 30 seconds. Cells were rested on ice for 2 minutes then 500uL S.O.C. Medium (Invitrogen) was added. Cells were placed in a 37C rotating shaker for 1 hour. Following incubation, 50uL of cells were streaked on an LB plate containing ampicillin. Plates were incubated overnight at 37C. Individual
colonies were then selected and placed into 5mL Polystyrene round-bottom tubes (BD Falcon) containing 2ml of LB broth plus 1ug/ml of Ampicillin. Tubes were incubated in a 37C rotating shaker overnight. Plasmid DNA was isolated using the Zippy Plasmid Miniprep Kit (Zymo Research, Irvine, CA). Plasmid DNA was stored at -20C.

**Transfection**

HEK293T cells were grown to 75% confluency before transfection for expression of recombinant Lassa virus glycoprotein. 400ul of DMEM was added to sterile 1mL titer tubes (Bio-Rad) and 3ug of plasmid DNA was added when transfecting an individual well of a 6-well plate. 10uL of polyethylenimine (PEI), prepared as a stock solution of 1ug/ml of PEI Max (Polysciences, Inc.) in sterile water, was added to the plasmid DNA and incubated for 15 minutes at room temperature. Media was removed from the well and cells were washed with PBS before addition of the transfection solution. The solution was placed drop-wise over the entire surface of the cells and the plate was gently rocked before being placed in a 37C incubator for 4 hours. Following the incubation, 2mL of DMEM + 5% FBS was added to the cells. 48 hours post-transfection, cells were used for experimentation.

A transfection method similar to the one listed above was used to generate pseudoviruses. The major difference for pseudovirus generation was the use of 1.5ug GPC in addition to 1.5ug of pSG3 DNA when preparing the transfection reagent. All of the subsequent steps were the same as the glycoprotein-only transfections.

**Pseudovirus harvest and purification**
Pseudoviruses were harvested twice from transfected HEK293T cells. The first harvest was 24 hours post-transfection. Fresh complete media was added to the cells and they were incubated for an additional 48 hours before the second harvest. Pseudovirus-containing supernatant was temporarily stored at 4C until titration for each of the time points was completed. If the supernatants from the different time points showed similar infectivity, they were pooled together. Pseudoviruses were first cleared of cellular debris by filtration through a 0.45um membrane. Next, the pseudovirus was pelleted using PEG-it precipitation. The 5x PEG-it reagent was added and diluted to 1x in the pseudovirus supernatant and incubated overnight at 4C. The following day the solution was spun at 1500g for 30 minutes. The supernatant was poured into a new tube and spun for an additional 5 minutes at 1500g. The viral pellets were resuspended in PBS overnight.

Additional purification through a sucrose gradient was performed for pseudoviruses used in the slot blot assay in order to remove soluble GP1 that is shed by cells transfected with full-length glycoprotein. A solution of 30% sucrose in sterile water was prepared. 1.25ml of sucrose was added to a 5mL ultracentrifuge tube (Beckman Coulter) and 3.75mL of the viral supernatant was carefully layered on top. The solution was spun in a Beckman Coulter SW60Ti rotor at 27,000g for 2 hours at 4C. Following centrifugation, the supernatant was carefully removed via aspiration as to not disturb the viral pellet. This step was repeated until 12mL of viral supernatant had been centrifuged. The viral pellet was resuspended in 1mL of PBS overnight at 4C. Concentrated pseudovirus was then titrated and then stored at -80C in 200uL aliquots.
**Pseudovirus titration**

A TZM-bl based assay was used in order to assess pseudovirus infectivity. To prepared samples, 50uL of DMEM were added to each well of a 96-well black plate (Nunc). 100uL of supernatant were added to every third column of the plate and mixed well by pipetting. Using an 8-channel multichannel pipette, 50uL of virus from the first well was transferred to the next well and mixed. This step was repeated once more in order to create a dilution series of pseudovirus ranging from 1:2 to 1:8. The final 50ul was discarded from the last well. Then TZM-bl cells were seeded in the plates at a concentration of 5x10^5 cells/well in 100uL DMEM+10% FBS. Cells were incubated for 72 hours at 37C+5% CO2. Luciferase activity was measured by removing 150ul of media and replacing it with 100uL of Brite-glo working reagent (Promega). Cells were incubated in the lysis buffer for 2 minutes before being measured with a Wallac 1420 Multilabel Counter (PerkinElmer: Waltham, MA).

For the neutralization assay, monoclonal antibodies were serial diluted in 96-well plates containing 50ul DMEM. 50ul of pseudovirus, generally diluted to 50,000 RLU, was mixed with the antibodies and allowed to react for 1h at 37C. Following antibody binding, 100ul of the mixture was added to 100ul of TZM-bl cells as described above.

**Site-directed mutagenesis**

Changes to the Lassa virus glycoprotein were introduced via a site-directed mutagenesis protocol similar to that described by Liu, et. al. 117. This protocol adapts the Stratagene QuikChange Site-Directed Mutagenesis (La Jolla, CA) system to allow
for the insertion, deletion, or substitution of up to 25 residues. Primers are designed as described by Naismith, et. al.\textsuperscript{117}. For single substitutions, the region of insertion was first identified. When designing the forward primer, we first identified a region of approximately 15 bases with a melting temperature (Tm) of 40C, and the bases containing the desired mutations were substituted into the original sequence. Next, a region of approximately 25 bases with a melting temperature of 60C was added to the 3’ end. A complementary method was used in designing the reverse primer. A 25 base pair segment with Tm of 60C was used for the 5’ end followed by the desired mutations. The 3’ end of the reverse primer was made of a 15bp section with a Tm of 40C. An online tool OligoCalc (Northwestern University) was used to create the reverse primer sequence used for mutagenesis. Primers were synthesized via IDT Technologies and diluted to 100uM in ultra-pure water for storage at -20C.

The cloning reaction was prepared as follows and is based on the protocol described in the Invitrogen AccuPrime \textit{Pfx} DNA Polymerase manual. All steps were performed on ice. 2.5ul of 10x Accuprime \textit{Pfx} Reaction Mix was added to a sterile PCR tube followed by 1ul each of forward and reverse primers at 25uM stock solution. Next, 100ng of template DNA and 0.5ul of \textit{Pfx} DNA Polymerase were added and the solution was mixed via pipetting. Finally, the reaction volume was brought to 25ul using sterile, distilled water. Tubes were briefly vortexed to collect the sample at the bottom of the tubes. Reaction mixes were run in an Invitrogen thermocycler. The template strands were denatured at 95C for 2 minutes followed by a 20-cycle amplification procedure (15s at 95C, 62C for 30s, 68C for 6 minutes). Reaction mixture was kept at 4C until ready for further purification.
**PCR reaction purification**

The Macherey-Nagel NucleoSpin Gel and PCR Clean-up Kit (Germany) was used to purify PCR reactions and remove enzymes and free nucleotides. 40ul of NTI reagent was added to the PCR mix following completion of the PCR reaction. Samples were loaded on NucleoSpin columns and centrifuged for 30s. The silica membrane was washed twice with 350ul of NT3 reagent by spinning at 11,000g for 30s on a benchtop centrifuge. Filtrate was discarded and the column was spun once more for 1 minute at 11,000g to dry the membrane. DNA was eluted by addition of 20ul sterile, distilled water. The water was allowed to incubate on the membrane for 2 minutes at room temperature before being eluted into a 1.5mL tube. Eluted DNA was stored at -20C.

For gel purification, DNA was extracted by identifying the band of interested using UV light. The gel section was identified, excised with a clean blade, and added to a 1.5mL tube. A volume of NTI equal to two times the weight of the gel segment was added to the tube. The gel was solubilized by incubation at 50C in a heat block for 10 minutes. Following solubilization, the process for DNA extraction as described above was followed.

**Western blot**

Cellular protein was isolated from cells and analyzed by western blotting. Cell monolayers were washed twice with PBS to remove media and cellular debris. Then RIPA buffer or 1% TritonX was added to the monolayer and allowed to incubate for 10 minutes on ice. Cells were removed from the plate by scraping or pipetting and added to 1.5mL tubes. Tubes were spun down at 10,000g on a
benchtop centrifuge to remove cellular debris. Then, 4x sample buffer was added to clarified protein lysate and mixed well. The sample was heated for 10 minutes at 80°C and allowed to cool before loading into the gel. The wells were washed with loading buffer before adding 20μl of protein sample per well when using 10-well gels. 2μL of LiCor protein standard also loaded in the gel and used as a reference for samples. Gels were normally run at a constant 80V for 20 minutes before increasing the voltage to 120V until the loading buffer approached the end of the gel.

Gels were transferred to nitrocellulose using the BD iBlot system. The membrane was removed from the system, trimmed, and blocked in PBS + 0.5% Tween 20 + 5% milk for one hour at room temperature. Primary antibody diluted in PBS + 0.5% Tween 20 + 5% milk and added to the blot and placed on a rocker for 1 hour at room temperature or overnight at 4°C. The blot was washed three times with PBS + 0.5% Tween 20 for 5 minutes at room temperature. Secondary antibody was diluted in PBS + 0.5% Tween 20 + 5% milk, added to the blot, and rocked at room temperature for one hour while covered. The blot was washed three times with PBS + 0.5% Tween 20 for 5 minutes at room temperature then kept in PBS until it was time to develop the blot. The blot was developed using the LiCor Odyssey scanner. For reading 700nm antibodies, the gain was set to 4.0 and for reading 800nm antibodies the gain was set at 5.0. Scanning was done at medium resolution with a 0um offset. Following image acquisition, blots were analyzed using the ImageStudio 4.0 (LI-COR Inc., Lincoln, NE).

**Immunofluorescent microscopy**
The following protocol was used to generate indirect immunofluorescent images of transfected cells. 48-hours post transfection, cells were detached via incubation with trypsin. Trypsin was inactivated using FBS and the cells were spun down at 1,000 g for 5 minutes in a 15mL conical tube. The cell pellet was then washed twice with PBS and spun down as previously described. Most of the PBS was removed from the cell pellet before resuspending the cells by flicking the tube. Following resuspension, a 20μL pipette tip was used to smear the cells on an 8-well glass slide. The cells were then allowed to dry on the glass for 10 minutes or until any visible liquid had evaporated. For the remainder of the staining processed, slides were kept in a wet chamber to prevent desiccation. The cells were blocked using DMEM+10% FBS for 30 minutes at room temperature. PBS was used to wash the cells three times before proceeding. Primary antibody was prepared at 10μg/ml in DMEM+10% FBS and added to individual wells on the slide. Primary incubation was done at room temperature for one hour. Cells were washed three times in PBS. FITC-conjugated anti-Human IgG Fc antibodies were used for secondary staining. The secondary antibody was prepared at a 1:1,000 dilution in DMEM+10% FBS and added to slides for 30 minutes at room temperature while protected from light. Slides were again washed in PBS. DAPI stain was prepared by diluting the stock solution in water and filtering through a .22μm SteriFlip tube. DAPI stain was added to the cells for 5 minutes before being washed in PBS. Finally mounting media was added to the wells and a coverslip placed on top.
Images were obtained on a Nikon inverted microscope using brightfield, FITC, and DAPI channels. Image files were saved as TIFF files and processed using the ImageJ software.

Flow Cytometry

Flow cytometry was used to measure surface-expressed protein as well as total protein in permeabilized cells. 48 hours post-transfection, 293T cells were detached with trypsin and inactivated with FBS. Cells were placed in 15mL conical tubes and spun down at 300g for 5 minutes. The pellet was resuspended in MACS buffer and washed twice. If the cells were being measured for total protein expression, then they were permeabilized with 0.1% Triton X diluted in PBS at this time. The primary antibody was diluted to the appropriate concentration in MACS buffer. Cells were resuspended in the primary antibody and gently rocked for 30 minutes on ice. The cells were again washed thoroughly in MACS buffer before addition of the secondary antibody. Secondary antibody incubation was performed on ice for 30 minutes with rocking. After the washing steps, the cells were fixed using a 4% paraformaldehyde solution for 10 minutes at room temperature. Cells were stored in the dark at 4C until it was time to analyze the samples. Data acquisition from flow cytometry experiments was completed on the BD LSRFortessa (San Jose, CA) cell analyzer. The parameters acquired included forward scatter, side scatter, and FITC signal strength. 20,000 events were measured for each sample. The data was then analyzed using FlowJo 6 (Ashland, OR) software.

Immunoprecipitation
Immunoprecipitation was used to measure antibody binding of surface-expressed glycoprotein. 48 hours post-transfection, 293T cells were detached with trypsin and inactivated with FBS. Cells were placed in 15mL conical tubes and spun down at 300g for 5 minutes. The pellet was resuspended in PBS and washed twice. Samples were aliquoted to 1.5mL tubes and spun down to remove the PBS. The primary antibodies being tested were diluted to 5ug/ml in PBS and used to resuspend the cell pellet. The antibody-cell mix was placed on a shaker for 45 minutes at room temperature before being washed with PBS. Subsequently, the cell-antibody complexes were lysed by incubation in 200uL RIPA buffer for 10 minutes at room temperature. During the primary antibody incubation, 20uL of magnetic Protein A beads (company) were aliquoted in a 1.5mL tube and washed with PBS + 0.02% Tween20. The washing solution was removed by placing the tube on a magnetic holder. The clarified cell lysate was mixed with the Protein A beads and incubated on a rocker for 30 minutes at room temperature. Unbound protein was removed by washing the beads twice in PBS + 0.02% Tween20. 100ul of 4x sample buffer diluted in ultrapure water was added to each of the samples and then heated at 80C for 10 minutes in order to dissociate the protein from the beads. The sample buffer was then analyzed by western blotting as described above.

**Slot Blot**

The Bio-Rad Bio-Bot Microfiltration Apparatus (Hercules, CA) was assembled according to the manufacture's instructions before proceeding with the experiment. 200ul of pseudovirus-containing inoculum was added to each of the wells before applying a gently vacuum to clear the wells. This process was repeated to load a
total of 600ul of pseudovirus supernatant and ensure strong signal strength. The wells were washed twice with 200ul of PBS to flush all antigen through the membrane. The apparatus was then disassembled and the nitrocellulose membrane was blocked in PBS + 0.5% Tween 20 + 5% milk. The remaining steps for antibody incubation and visualization of the blot follow those described in the western blot section.

**Statistical Analysis**

Data analysis was performed using the Prism 6 software (GraphPad Software Inc., La Jolla, CA). The threshold for significance was set at 95% (p < 0.05) for all experiments. Comparisons between different glycoprotein mutants relative to the wild type were performed using a ordinary one-way ANOVA. Virus titration and antibody neutralization experiments were done in triplicate and repeated at least twice.
CHAPTER 1

Characterizing Features of mAb 8.9F Consistent with Recognition of a Quaternary Neutralizing Epitope

Specific Aim 1.1 and 1.2
Introduction

Lassa virus (LV) infects over 300,000 people a year and is the cause of approximately 5,000 deaths \(^6^\). Current treatment methods are underdeveloped. While ribavirin has shown an effect on improving survival of Lassa fever (LF) patients, there are several shortcomings in regard to its utility. In particular, ribavirin must be administered early in infection \(^6^\). This may not be possible in LV-endemic areas, as diagnostic tools and treatment centers are sparsely distributed. Convalescent serum from LV-infected monkeys showed a protective effect but only when therapy was initiated up to five days post-infection \(^8^\). However, convalescent serum requires temperature-controlled storage and a continual supply of highly neutralizing serum. Due to these limitations of current treatments, there is a profound need for an effective and easily administrable therapy. The research presented in this chapter seeks to characterize a monoclonal antibody directed against Lassa virus that may provide not only an effective post-exposure prophylactic treatment, but also inform future vaccine design.

In this chapter we seek to characterize mAb 8.9F a neutralizing monoclonal antibody that recognizes a unique epitope structure known as a quaternary neutralizing epitope (QNE). While research on Lassa virus antibodies is sparse and no quaternary neutralizing antibodies against the virus have been described, the past ten years of research into the QNEs of other viruses provides a framework for developing experiments to elucidate the epitope structure of mAb 8.9F. Current work in the field of QNE-recognizing antibodies will first be reviewed.
The most well characterized quaternary neutralizing antibodies have been identified in response to human immunodeficiency virus (HIV) infection. In 2005, Gorny, et. al. published a paper where they characterized mAb 2909. This antibody was isolated from peripheral blood mononuclear cells (PBMCs) and transformed into a heteromyleoma cell line. The PBMCs originated from a patient with a 15-year HIV infection and five years of detectable viral load. The antibody was titrated against the SF162 strain in two different neutralization assays and showed high potency in both. Interestingly, when mAb 2909 was tested against solubilized antigen on ELISA, there was no detectable signal. These same observations held true when reactivity of mAb 2909 was tested against pseudovirions expressing glycoprotein monomers. Thus, it was concluded that the epitope recognized by mAb 2909 was only present on the intact virus structure. Further support to the complex nature of the antibody epitope was provided by competition experiments demonstrating regions of the CD4bd, V2, and V3 of gp120 that mediated antibody binding. Earlier studies on HIV antibodies generated in chimpanzees had suggested that highly neutralizing antibodies may depend on complex epitopes.

Following the initial identification of an HIV quaternary neutralizing epitope, this class of antibodies has rapidly expanded. Antibodies recognizing a QNE similar to 2909 in SHIV-infected macaques have been characterized. Additionally, a large family of antibodies similar to 2909 has been isolated from 'elite neutralizers', that is patients whose antibody responses put them in the top 1% of HIV-1 neutralizers. Antibody isolation from these patients have yielded two antibodies, PG9 and PG16,
that are capable of neutralizing up to 80% of circulating primary HIV-1 isolates. This wide reactivity is due in part to their ability to recognize a conserved glycosylated residue at position 160 that mAb 2909 does not bind. Despite differences in neutralization, mAb 2909 and PG16 are closely related in their recognition sites as confirmed by the crystal structures of the antibodies in complex with HIV glycoprotein. Both mAb 2909 and PG16 recognize a similar epitope found on V2-V3 with the major difference being the requirement for an asparagine at position 160 for PG16. Furthermore, both antibodies are structurally similar in terms of their binding pocket and relative charge. A negatively charged residue at position 100 is important for each antibody to bind.

Quaternary neutralizing epitopes are not only determined by the amino acid sequence, but also cleavage, glycosylation, formation of multimers have been found to play a role in antibody recognition. When Walker, et. al. first identified PG9 and PG16 they sought to determine if the epitope consisted of a single monomer in its membrane-stable conformation or if the epitope was composed of two adjacent subunits. Based on the results from a chimeric transfection of cells with two different types of gp120, one that bound PG9/16 and one that did not, it was determined the antibodies recognized single subunits in the context of the native virion. Attempts to develop a vaccine based on soluble gp120 aided in the understanding of the relationship between antigen stability and neutralizing antibody responses. Early HIV trimer-based vaccines showed poor efficacy due to their unstable nature. Dissociation would generate an overwhelming number of monomers that would redirect the antibody response to produce predominantly
non-neutralizing ones. Also, non-neutralizing HIV-1 antibodies preferentially bound uncleaved gp140 over the cleaved version. This is in contrast to the neutralizing antibodies that were found to bind cleaved and uncleaved gp140 with similar affinity. This suggests a cleaved trimer is the ideal immunogen for eliciting neutralizing antibodies against HIV-1. The role of glycoprotein cleavage in QNE antibody recognition was further clarified by the generation of a stable, cleaved gp120 trimer. This recombinant protein strongly bound all broadly neutralizing antibodies while recognizing very few non-neutralizing antibodies. The relationship between glycoprotein cleavage, multimer formation, and broadly neutralizing antibody recognition was used to inform the design of our experiments testing Lassa virus mAb 8.9F.

Based on the significant therapeutic opportunities provided by broadly neutralizing HIV mAbs, it is unsurprising to learn that researchers have begun to investigate putative quaternary neutralizing epitopes present on other viral antigens. Currently, the most well characterized non-HIV-1 QNE antibody is mAb 1F4 found against Dengue virus. mAb 1F4 was isolated from the serum of a DENV-1 infected patient. In contrast to the HIV QNE antibodies that recognize multiple strains, mAb 1F4 only reacted with the DENV-1 serotype. It is unclear whether this is a broad trend of Dengue antibodies or if additional high-throughput studies are needed to identify pan-neutralizing antibodies. In regards to epitope recognition, mAb 1F4 is similar to its HIV counterparts in that it binds a single monomer of the E protein dimer. Quaternary neutralizing epitopes have also
been documented in West Nile virus, Yellow Fever virus, and respiratory syncitial virus (RSV) \(^{133-135}\).

The utility of antibodies that react with QNEs is a burgeoning area of antiviral research and characterizing these epitopes will help inform vaccine design. This chapter seeks to characterize mAb 8.9F as an antibody that recognizes a QNE present on the Lassa virus glycoprotein. We will examine which glycoprotein subunits are included in the epitope and the role glycoprotein processing plays in antibody recognition.

**Methods**

**Cell Culture**

See General Materials and Methods

**Pseudovirus Neutralization Assay**

We used the standard protocol described in the General Materials and Methods section for testing the neutralization of mAb 8.9F versus other neutralizing antibodies. The antibody was diluted in half-log intervals from 0.02 to 20ug/ml. Neutralization experiments were repeated in triplicate.

**Immunoprecipitation**

The protocol used for immunoprecipitation of glycoprotein subunits is described in the General Materials and Methods section. Western blots were probed for GP1 with a mix of 2.4F, 3.3B, and 19.5A at 2ug/ml while blots probed for GP2 used a mix of 4.1F, 13.4E, and 24.6C at 2ug/ml. LiCor Goat anti-Human IgG Fc 680RD antibody diluted 1:10,000 was used for secondary staining and visualization.
**Immunofluorescence microscopy**

See General Materials and Methods. For the experiments in this chapter, a basic indirect fluorescent method was used. The primary antibody incubated with the sample is specified in the figure legend while the secondary antibody used was a Goat anti-Human IgG Fc antibody conjugated to FITC.

**ELISA**

Collaborators at Scripps Research Institute generated recombinant protein. Recombinant GP1, GP2, and GP1-GP2 lacking the transmembrane domain were produced in insect and mammalian cells. The protein was diluted to 5ug/ml in 0.1M sodium carbonate (pH 9.6) buffer. 50ul of sample were added to each well of a 96-well ELISA plate. The plates were incubated overnight in 4C to allow for antigen binding. The following day, plates were thoroughly washed with TBST. Plates were then blocked for 1 hour at 37C with 250ul of PBST+5% milk. Plates were thoroughly washed. 100ul of antibody at 5ug/ml was added to each well and incubated with the antigen for 1 hour at 37C. Detection was performed with 100 μL/well of HRP-conjugated goat α-human IgG antibody reagent (KPL)) diluted to 1:2500 in blocking buffer. After 1 h incubation, 100 μL/well of TMB-H2O2 substrate (Sigma) was added, and the plates were incubated for 5 min. The reaction was stopped by adding 100 μL/well of TMB stop solution (KPL) and read at 450 nm.

**Sodium Thiocyanate Incubation**

Incubation of immune complexes with sodium thiocyanate was used to evaluate the strength of antibody-antigen interactions. The protocol used is similar to the one described for standard immunoprecipitation. Transfected cells were
incubated with monoclonal antibodies diluted to 10ug/ml in MACS Buffer (PBS +1% BSA +Sodium Azide) for 30 minutes on ice. Following incubation, cells were spun down and resuspended in 100ul of RIPA buffer. Cells were lysed for 10 minutes at room temperature. Cellular debris was spun down at 13,000g for 10 minutes. Clarified lysate was added to Protein A magnetic beads and incubated at room temperature with rocking for 45 minutes. Supernatant was removed using a magnet, and different concentrations of NaSCN diluted in PBS were added to the beads. Following one hour of NaSCN incubation, beads were thoroughly washed before eluting bound protein in sample buffer. The standard western blot protocol was followed after protein elution.

**PF-429242 Treatment of Cells**

PF-429242 was obtained from Sigma-Aldrich and reconstituted in sterile water to make a 100mM stock solution. The stock solution was diluted to a working solution of 1mM when preparing samples. Transfection media was removed two hours after addition to the cells and replaced with DMEM+10% FBS containing the inhibitor. Half-log dilutions of 30, 10, 3, 1, and 0.3uM were used for these experiments. Cells were allowed to grow in PF-429242 treated media for 48 hours before beginning experiments.

**Results**

*mAb 8.9F neutralizes Lassa pseudoviruses at levels similar to other mAbs and is reactive against representative samples of each Lassa virus strain*
In order to characterize the functional characteristics of mAb 8.9F, we tested mAb 8.9F neutralization of Lassa virus glycoprotein pseudotyped particles in comparison to a panel of other neutralizing monoclonal antibodies isolated by our lab. The antibodies were tested against the Lassa virus Josiah strain. The 50% inhibitory concentration (IC50) of mAb 8.9F against Lassa pseudovirus was calculated to be 0.247 ug/ml (Figure 5A). This value lies within the same range as other isolated monoclonal antibodies. mAb 19.7E, 12.1F, 37.2D, 25.10C, and 37.2G were tested for neutralization of pseudovirus Josiah and found to have IC50s of 5.908, 0.142, 0.559, 0.080, and 5.599 respectively (Figure 5B). Based on these results mAb 8.9F seems to neutralize pseudoviruses bearing the Josiah glycoprotein at levels similar to other highly-neutralizing antibodies including mAbs 12.1F, 37.2D, and 25.10C.

A characteristic of previously identified antibodies against QNEs is that they have the capacity to be broadly neutralizing. While the existing database of Lassa virus sequences are small compared to HIV, we wanted to test if mAb 8.9F would be able to neutralize pseudoparticles bearing glycoproteins from each lineage. We generated viruses pseudotyped with the Josiah, Nigeria A19, Nigeria 237, and Pinneo glycoprotein strains. Neutralization titers showed mAb 8.9F to have an IC50 of 0.247, 0.125, 0.182, and 0.403 ug/ml against Josiah, A19, 237, and Pinneo respectively (Figure 6). These results suggest that mAb 8.9F shares the same pan-neutralizing characteristics seen in other broadly neutralizing QNE antibodies.

**mAb 8.9F reactivity requires the glycoprotein multimer**
In order to characterize mAb 8.9F as recognizing a QNE, one of the most important factors is to demonstrate that the epitope is not present on single subunits expressed outside the context of the membrane-bound trimer. The inability of the QNE to be recognized on solubilized monomers has been demonstrated for HIV antibodies and Dengue virus antibodies\textsuperscript{121,132}. To demonstrate that mAb 8.9F recognizes an epitope consistent with quaternary structures, solubilized antigen was generated and analyzed on ELISA using a panel of monoclonal antibodies. Testing of a recombinant GP1-GP2 protein produced in insect cells showed high binding with other monoclonal antibodies regardless of neutralization capacity. However, mAb 8.9F and A32, an HIV mAb used as a negative control, were unable to generate a binding signal (Figure 7). To eliminate the possibility of a protein processing or folding error, ELISA studies were also completed using individual glycoprotein subunits and protein generated in mammalian cells (data not shown). Based on these results, the epitope recognized by mAb 8.9F is not present on recombinant monomers.

As we hypothesized that the epitope reacting with mAb 8.9F would be present on the quaternary structure of the glycoprotein, we examined whether mAb 8.9F reactivity with surface-expressed glycoprotein could be detected through immunofluorescence. 48hrs post transfection, unfixed cells were allowed to air dry on glass slides. The signal for mAb 8.9F was detected by incubation with a goat anti-human IgG antibody conjugated with FITC. Samples were observed at 10x and 40x for image generation. Upon microscopic examination of transfected cells, those stained with mAb 8.9F displayed a strongly positive signal. Based on the number of
positive cells at 10x magnification, transfection efficiency was approximately 40%. At the 40x resolution, staining had a pattern consistent with membrane expression (Figure 8). While the relatively low number of mAb 8.9F-positive cells suggests transfection efficiency or cellular processing of the glycoprotein may be inefficient, the staining pattern observed strongly suggests that the mAb 8.9F epitope is present on the surface of glycoprotein-expressing cells.

**Disruption of glycoprotein complexes in the membrane abrogates mAb 8.9F binding**

We performed a series of experiments testing the effect of RIPA buffer treatment on mAb 8.9F epitope recognition. These experiments were performed in order to demonstrate that disruption of glycoprotein complexes abrogated mAb 8.9F binding. Radioimmunoprecipitation assay buffer or RIPA is a detergent capable of solubilizing cell proteins including those located in the cell and nuclear membranes. The buffer contains both non-ionic and ionic detergents that make it stronger than other widely used lysis buffers such as NP-40 or Triton X-100. We selected this lysis buffer as it was strong enough to ensure that the glycoprotein found in the cell membrane would be adequately solubilized.

We first tested the effects of RIPA treatment on the binding of mAb 8.9F to membrane-expressed glycoprotein. In this experiment, mAb 8.9F was incubated with GPC-transfected cells before or after the cells were lysed in RIPA buffer. The lysates generated were incubated on magnetic Protein A beads to extract any protein bound to mAb 8.9F. The bound protein was then analyzed via western
blotting. The results of this experiment show that RIPA treatment before antibody-antigen incubation prevents mAb 8.9F immunoprecipitation. Conversely, mAb 12.1F was able to bind the glycoprotein regardless of RIPA treatment (Figure 9A). These results suggest that once the native glycoprotein conformation in the cell membrane is disrupted, the QNE is lost.

This data was further supported by an immunoprecipitation experiment examining mAb 8.9F binding of pseudoviruses. Using a vacuum manifold, pseudoviruses were treated with PBS or RIPA then affixed to nitrocellulose. Following blocking, strips of nitrocellulose were cut and incubated with different mAbs then developed. These results showed that RIPA treatment eliminated mAb 8.9F binding, but maintained reactivity with mAb 12.1F (GP1 specific) and 13.4E (GP2 specific) (Figure 9B). Based on the ELISA data and RIPA disruption experiments, mAb 8.9F exhibits features consistent with previously identified QNE reactive antibodies. Monomers of glycoprotein, whether generated by cellular lysis or bound to ELISA plates, do not react with mAb 8.9F. However, when the protein is expressed in the context of a plasma membrane, mAb 8.9F is capable of binding the glycoprotein.

*The QNE recognized by mAb 8.9F contains regions of the GP1 and GP2 subunits*

Based on currently published data, the epitopes recognized by QNE mAbs include a range of protein domains and spatial orientations. For the highly-neutralizing HIV mAb PG9, studies have shown that it recognizes an asymmetric epitope composed of two of the three gp120 monomers on the tip of the
glycoprotein spike. However, when the crystal structure was solved for the dengue mAb 1F4, it was determined that the QNE it recognized was found on the hinge region of a single E protein monomer. Since we were unable to investigate the stoichiometry of antibody binding to protomers, we decided to investigate which subunit of the Lassa virus glycoprotein was recognized by mAb 8.9F.

mAb 8.9F was used to immunoprecipitate Lassa virus glycoprotein from transfected cells. Blots were probed with GP1 and GP2 specific antibodies in order to determine the glycoprotein subunits present in the mAb 8.9F epitope. Based on these results, 8.9F appears to precipitate the GP1 and GP2 subunits of Lassa virus (Figure 10). As would be expected based on ELISA data, mAb 12.1F predominantly pulled down the GP1 subunit. However mAb 12.1F also precipitated a GP2 band. This may be due to either weak interactions between the GP1-GP2 subunits or the mAb 12.1F epitope may contain a small region of GP2. 13.4E precipitated GP2 only and with relatively low strength relative to 8.9F. Most likely, this is due to the fact that mAb 13.4E recognizes a linear epitope of the glycoprotein.

After determining mAb 8.9F could precipitate both glycoprotein subunits, we wanted to explore the relative strength with which the antibody bound each subunit. In doing so, this would eliminate the confounding factor of GP bands appearing on western blot due to GP-GP interactions as opposed to mAb-GP interactions. Increasing concentrations of sodium thiocyanate (NaSCN), a chaotropic agent, were used to disrupt any weak, non-covalent bonds between residual glycoprotein subunits. Disruption of mAb 8.9F complexes with NaSCN began at 1.5M NaSCN, and by 2.0M NaSCN less than 5% of the original signal was detectable on
western blot (Figure 11). Densitometric analysis of the blot showed that GP1 and GP2 dissociates from mAb 8.9F with similar kinetics. The concentrations at which mAb 8.9F was calculated to dissociate from 50% of the GP1 and GP2 subunits were calculated to be 1.8M and 1.7M, respectively. Also as expected, the GP2 band immunoprecipitated by mAb 12.1F was quickly eliminated on NaSCN incubation (Figure 12).

To understand how mAb 8.9F-glycoprotein dissociation compares to other neutralizing monoclonal antibodies isolated by our lab, we repeated the experiment described above using additional neutralizing antibodies. These results show that mAb 12.1F behaves as would be expected of a GP1-specific antibody. It quickly dissociated from GP2 at low NaSCN concentrations while the GP1 subunit was detected up to 4.0M. In contrast, the dissociation curve of mAb 37.2D appeared very similar to mAb 8.9F albeit dissociation occurred at lower NaSCN concentrations. Thus it is highly likely that the epitope recognized by mAb 37.2D includes regions of GP1 and GP2 (Figure 12).

Based on these results, the epitope of mAb 8.9F appears to include regions of the GP1 and GP2 subunits of Lassa virus. Additionally, the kinetics of mAb-antigen disruption by NaSCN further suggests the epitope of mAb 8.9F contains equal contributions from GP1 and GP2. NaSCN data also supports the hypothesis that mAb 12.1F recognizes GP1 and mAb 37.2D recognizes a non-quaternary epitope of GP1 and GP2.

*Cleavage of the glycoprotein is necessary for mAb 8.9F recognition*
While HIV gp140 cleavage was found to be unnecessary for PG9 and PG16 antibody recognition, we wanted to investigate what effects inhibition of GP1-GP2 cleavage would have on the reactivity of mAb 8.9F. In order to test this idea, we used a small molecule inhibitor PF-429294 that specifically blocks the site-1 protease responsible for glycoprotein processing in arenaviruses. PF-429242 is under investigation as an antiviral treatment as inhibition of glycoprotein cleavage has been associated with the inability to produce infectious viruses.

Initially, a western blot was run to assess the effect of PF-429242 treatment on production of Lassa virus glycoprotein in transfected cells. Following transfection, cells were maintained in media containing 0.3uM to 30uM of the inhibitor in half-log doses. PF-429242 began inhibiting cleavage at the lowest dose tested (0.3uM) and the GP2 band was no longer detectable after 1.0M (Figure 13). It is interesting to note that while PF-429242 has not been shown to have any cytotoxicity or impact on GPC production, treatment with the inhibitor did seem to reduce the strength of the GPC signal at 3, 10, and 30um. This may be the result of gel loading error or perhaps a build-up of uncleaved GPC in the cell resulted in proteolysis.

After clearly demonstrating PF-429242 inhibited glycoprotein cleavage, we next investigated how this treatment would affect the binding of mAb 8.9F and 12.1F to the surface of transfected cells. When analyzed by flow cytometry, mAb 8.9F showed decreased binding in response to increased levels of the inhibitor. The mAb 8.9F signal decreased to 55% at 0.3um and 30% at 1um. By 3uM PF-429242, the 8.9F signal was undetectable. This decrease in mAb 8.9F binding closely mirrors
the reduction in cleaved GP2 as seen on western blot. In contrast, mAb 12.1F surface binding did show a slight decrease in surface reactivity, but it never dropped below 50% (Figure 14). The decrease may have been due to the reduced amount of glycoprotein produced in cells treated with a high concentration of inhibitor. The results of these experiments were further confirmed by direct observation via immunofluorescence of PF-429242 treated cells that were stained with mAb 8.9F and 12.1F (Figure 15). Cells stained with mAb 8.9F lost positive signal after 1uM treatment whereas positive 12.1F stained cells could be detected up to 30um.

In conclusion, PF-429242 appears to be a potent inhibitor of glycoprotein cleavage that may also have some effect on increasing GPC degradation. The surface reactivity of mAb 8.9F appears to be directly related to production of cleaved glycoprotein. At levels of PF-429242 that completely inhibit GP2 detection, mAb 8.9F reactivity is also abolished. These results suggest that cleavage of the glycoprotein is necessary for exposing the mAb 8.9F epitope.

Discussion

Quaternary neutralizing antibodies are a recently discovered class of antibodies that preferentially recognize a complex epitope found on the surface of fully-processed protein multimers. While the neutralization breadth of these antibodies may vary, several subsets have been identified from the B cells of elite neutralizers of HIV that are capable of neutralizing up to 80% of tested strains at sub-micromolar levels. With these characteristics in mind, we sought to characterize mAb 8.9F, a Lassa virus neutralizing monoclonal antibody isolated
from the serum of a convalescent patient. Based on our preliminary characterization experiments, we discovered that the epitope recognized by mAb 8.9F has many characteristics similar to the quaternary neutralizing epitopes described on other viruses $^{133,135,139}$.

The first characteristic of mAb 8.9F tested was its potency and neutralization breadth. A basic pseudovirus neutralization assay was employed to measure antibody reactivity. When tested against the Josiah strain, mAb 8.9F had an IC50 of 0.2466 ug/ml. This value placed it among the most potent neutralizers (Figure 5). mAb 8.9F was also tested against a representative sample from each of the four identified Lassa virus lineages. In these tests, mAb 8.9F neutralized each of these samples at levels below 1ug/ml. While more strains of the virus will need to be tested in order to generate a full picture of mAb 8.9F neutralization ability, it is apparent that mAb 8.9F possesses the basic characteristics of a potent and broadly neutralizing antibody. Furthermore, it may be possible to identify a family of antibodies similar to mAb 8.9F via high-throughput screening techniques. This approach has been successfully used for HIV antibodies $^{124}$.

Perhaps the most characteristic feature of quaternary neutralizing epitopes are their stringent and labile requirements for recognition. In general, QNE antibodies poorly recognize soluble monomers in comparison to their activity against protomers within the native protein $^{123,132}$. This preference for native antigen was confirmed in our evaluation of mAb 8.9F. When we tested mAb 8.9F against solubilized antigen in ELISA, we did not detect any binding activity. This is in contrast to the other neutralizing mAbs tested (Figure 7). The GP2 sequence used
for generating recombinant GP2 and GP1-GP2 constructs lacked the transmembrane and intracellular domains. While it is not possible to exclude these regions as antigenic sites without testing the complete protein, it is unlikely that these regions would contain the mAb 8.9F epitope as they are not exposed on the cell surface. However, it has been discovered that individual subunits of the glycoprotein may direct proper folding of one another. In this case, the deleted regions of GP2 could influence mAb 8.9F epitope exposure.

Next, we wanted to demonstrate that mAb 8.9F binding could be visualized and that detergents easily disrupted the epitope. Cells transfected with full-length GPC showed strong membrane reactivity with mAb 8.9F when the cells were left untreated and allowed to air dry on slides (Figure 7). No fluorescent signal was observed if cells were fixed in 4% paraformaldehyde beforehand (data not shown). Next we tested whether mAb 8.9F could immunoprecipitate antigen when reacted with transfected cells or pseudoviruses. In both situations, mAb 8.9F was able to precipitate glycoprotein as determined on western blot. However, RIPA buffer treatment of cells prior to antibody incubation abolished mAb 8.9F antigen precipitation while other neutralizing antibodies were unaffected by RIPA treatment (Figure 8). In the pseudovirus western blot, the low levels of mAb 13.4E reactivity are likely due to poor epitope recognition. This is because the mAb 13.4E binds a linear epitope. Immunofluorescent reactivity and RIPA-mediated mAb 8.9F binding further demonstrate that the epitope recognized by mAb 8.9F is present only on the membrane surface as a complex.
Unfortunately, no crystal structure of mAb 8.9F bound to its epitope has been generated. For this reason, we investigated which of the glycoprotein subunits were immunoprecipitated following incubation with mAb 8.9F. Based on the crystal structure of QNEs from HIV, the trimer apex of gp120 seems to be the predominant target for antibodies. As Lassa virus glycoprotein creates a similar trimer to HIV with GP1 acting analogously to gp120 and GP2 to gp41, we hypothesized that mAb 8.9F would be directed towards GP1. However, when we tested cells with mAb 8.9F, 12.1F, and 13.4E, mAb 8.9F showed a strong signal for both GP1 and GP2 (Figure 10). Also, it was interesting to see a faint GP2 band immunoprecipitated by mAb 12.1F. GP2 may have been precipitated due to weak interactions with mAb 12.1F, or more likely, this band was due to non-covalent interactions with the precipitated GP1.

In order to further refine the immunoprecipitation data, we examined the impact of a chaotropic agent on antibody-antigen complex stability. Chaotropic agents such as sodium thiocyanate (NaSCN) reduce the non-covalent forces that stabilize protein structures. Thus, NaSCN could be used in order to disrupt residual GP1-GP2 interactions following IP and investigate the relative affinity with which different antibodies were able to bind the glycoprotein. Through this approach we found NaSCN disrupted mAb 8.9F binding of GP1 and GP2 at similar concentrations (Figure 11). This would suggest that the mAb 8.9F epitope has a relatively equal distribution between GP1 and GP2. These results are in contrast to the data from NaSCN treatment of mAb 12.1F complexes. When glycoprotein bound to mAb 12.1F was treated with NaSCN, the GP2 subunit hypothesized to be interacting only
though GP1 was quickly eliminated, whereas the GP1 subunit which mAb 12.1F strongly bound on ELISA was maintained up to 4.0M NaSCN. Additionally, mAb 37.2D, a neutralizing antibody that recognizes GP1 and GP2 on ELISA, was tested to compare its dissociation rates to mAb 8.9F. While the slope of the GP1 and GP2 dissociation curves were similar to mAb 8.9F, an overall lower concentration of NaSCN was required for mAb 37.2D-GP dissociation (Figure 12). This suggests that while mAb 37.2D likely binds both glycoprotein subunits, the antigen-antibody affinity is lower than that of mAb 8.9F. While more antibodies should be tested, we hypothesize that if a GP2 ELISA-reactive antibody were tested in this manner, the GP1 subunit would dissociate quickly while the GP2 subunit would remain bound past 3.0M NaSCN.

Finally, we wanted to test if glycoprotein cleavage was necessary for mAb 8.9F recognition. As the glycoprotein precursor is translated as a polypeptide that can be transported to the cell surface, it is conceivable that these precursor molecules could be integrated into the formation of glycoprotein trimers that are mAb 8.9F reactive. In the case of PG9 reactivity with HIV gp120, it was also found that PG9 could bind cleavage-defective gp160 molecules. While we could have tested this hypothesis through the generation of a cleavage deficient mutant, we were concerned that any base substitution within the GP1-GP2 cleavage site might have an unintended impact on processing or result in low levels of cleaved protein. For this reason, we used a small chemical inhibitor of the site-1-protease called PF-429242. Upon examination of PF-429242 treated cells by western blotting, we found a dose-dependent reduction in mAb 8.9F surface binding (Figure 13).
When mAb 12.1F surface binding was evaluated, there was a slight decrease in reactivity at high inhibitor concentrations despite a detectable signal remaining. These results suggest that mAb 8.9F requires cleaved glycoprotein trimers in order to recognize its epitope. The reduction of mAb 12.1F binding is less likely due to poor recognition of uncleaved GPC and is more likely due to lower GPC production or surface transport. This assumption is supported by a western blot of PF-429242 treated cells showing a lower GPC signal at high inhibitor concentrations. It is unclear if this decrease also affects the amount of glycoprotein transported to the surface. In order to address this issue a western blot of surface-biotinylated proteins following PF-429242 treatment could be performed.

Quaternary neutralizing epitopes are a relative new discovery with profound implications in the development of vaccination strategies. Using our lab's experience in the isolation of monoclonal antibodies, we found a likely candidate representing the first identified QNE on the Lassa virus glycoprotein. This epitope was recognized by an antibody, mAb 8.9F, that demonstrated broad and potent neutralizing effects. The recognized epitope is exposed on the surface of cells in the context of a fully processed glycoprotein trimer. The epitope recognized by mAb 8.9F contains regions of the GP1 and GP2 subunits, a finding that is in contrast to previously characterized QNEs.
Figures

A

B

<table>
<thead>
<tr>
<th></th>
<th>19.7E</th>
<th>12.1F</th>
<th>37.2D</th>
<th>25.10C</th>
<th>37.2G</th>
<th>8.9F</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (ug/ml)</td>
<td>12.68</td>
<td>0.1409</td>
<td>0.546</td>
<td>0.03288</td>
<td>12.26</td>
<td>0.2466</td>
</tr>
</tbody>
</table>

Figure 5. mAb 8.9F neutralizes Josiah pseudoviruses at a concentration similar to other highly neutralizing mAbs.
Pseudoviruses were generated using the Lassa virus Josiah strain glycoprotein and titrated in TZM-bl cells. Prior to infection, pseudoviruses were incubated with half-log dilutions of isolated monoclonal antibodies against Lassa virus. (A) Data shows results from triplicate experiments. (B) IC50 values were calculated for each mAb using Prism6.
Figure 6. mAb 8.9F neutralizes representative strains from each of the four lineages of Lassa virus.

Pseudoviruses representing each of the four identified Lassa virus lineages were titrated in TZM-bl cells. Prior to infection, pseudoviruses were incubated with half-log dilutions of mAb 8.9F. (A) Plot shows results from triplicate experiments. (B) IC50 values were calculated for each mAb using Prism6.
Figure 7. mAb 8.9F is unable to react with solubilized protein on ELISA. Recombinant full-length glycoprotein was generated in insect cells and immobilized on ELISA plates. Immobilized antigen was tested for reactivity against a panel of isolated monoclonal antibodies. Binding was quantified by measuring absorbance at 630nm.
Figure 8. mAb 8.9F binding is detectable following incubation with unfixed cells.
293T cells were transfected with full-length Lassa virus glycoprotein then allowed to air-dry on glass slides. Cells were incubated with mAb 8.9F and a FITC-conjugated secondary antibody. Images were generated on a Nikon microscope with NIS-Elements software. Images were taken with a 10x and 40x objective.
Figure 9. RIPA treatment of GPC-transfected cells or pseudovirus eliminates mAb 8.9F binding.

(A) Cells were transfected with full-length glycoprotein then incubated with mAb 8.9F before or after treatment with RIPA buffer. Following treatment, antigen bound to mAb 8.9F was measured using protein A beads and western blotting. (B) Pseudoviruses generated using the Josiah glycoprotein were affixed to nitrocellulose following treatment with RIPA or PBS. Reactivity of monoclonal antibodies against disrupted or intact virions was assessed via western blotting of membranes. Images for both blots were generated using the LiCor imaging system and ImageStudio software.
Figure 10. mAb 8.9F precipitates the GP1 and GP2 subunits.
Cells transfected with full-length glycoprotein were incubated with mAb 8.9F, 12.1f, or 13.4E. Antigen bound to each antibody was examined via immunoprecipitation. Bound antigen and lysate from non-precipitated, transfected cells were measured on western blot. A mix of mAbs 3.3B, 4.1F, 19.5A were used to measure GP1 levels while a mix of mAbs 2.4F and 24.6C measured GP2 levels.
Figure 11. Sodium thiocyanate treatment of antibody-antigen complexes.
GPC-transfected cells were incubated with (A) mAb 8.9F (B) 12.1F or (C) 37.2D followed by lysis and incubation on protein A beads. Following, protein A binding the beads were incubated in increasing concentrations of sodium thiocyanate for 1 hour. Beads were washed and eluted proteins were run on western blot. Blots were probed for the subunit indicated above.
Figure 12. Densitometric analysis of NaSCN blots.
Band intensities of western blots in Figure x were quantitated using ImageStudio Lite. Regions of interest were selected and local background was subtracted. Data was plotted using Prism 6 software.
Figure 13. Treatment of cells with PF-429242 reduces GPC cleavage. 293T cells were transfected with full-length glycoprotein. Media was replaced with fresh growth media containing the concentration of PF-429242 specified above. 48 hours post-transfection cells were lysed and run on western blot to assay for GP2 levels.
Figure 14. Surface binding of monoclonal antibodies to PF-429242 treated cells.
293T cells transfected with Lassa virus GPC were grown for 48 hours in media containing the PF-429242 inhibitor. Live cells were then stained with mAb 8.9F or 12.1F followed by fluorescent secondary antibody. Signal was measured using BD Fortessa flow cytometer and quantified using FlowJo software.
Figure 15. Immunofluorescent images of PF-429242 treated cells.
293T cells transfected with Lassa virus GPC were grown for 48 hours in media containing the PF-429242 inhibitor. Cells were then detached and allowed to air dry on glass slides. Cells were stained with mAb 8.9F or 12.1F followed by a FITC conjugated secondary antibody. Nuclei were visualized with DAPI. Images were takes on a Nikon microscope using a 20x objective. Images were processed using ImageJ software.
Chapter 2

Identification of Amino Acids and N-glycans Mediating mAb 8.9F Epitope Recognition

Specific Aims 2.1 and 2.2
**Introduction**

Epitope mapping is an important tool in the elucidation of B-cell epitopes. The results of mapping experiments have many downstream applications in regards to evaluating antibody responses following vaccination, developing molecular inhibitors, and understanding the pathogenic function of targeted epitopes. When reviewing epitope studies, it is important to remember that the term epitope may be used in reference to multiple different characteristics of the protein. It may refer to the surface protein, a domain of that protein, or the individual amino acids or atoms. In most B-cell epitope literature, the terms structural and functional epitopes are used to discriminate between those residues that are directly interacting and those which help stabilize the epitope structure but may not interact with the antibody. For purposes of this chapter, the word 'epitope' will primarily refer to the amino acids present in the glycoprotein of Lassa virus that are identified to have a structural or functional role in mAb 8.9F recognition.

Several different methods of epitope mapping exist, each with their own advantages and disadvantages. X-ray crystallography is perhaps the most precise method for determining both the epitope and interacting antibody residues. This method can be used to map antibody binding of peptides and glycans. The primary disadvantages in this approach are the time-consuming process of optimizing antigen crystallization as well as the need to produce large amounts of antigen. Deuterium exchange – mass spectrometry is a less exact, but more flexible platform for mapping epitopes when compared to x-ray crystallography. In principle, antibody-antigen binding prevents the exchange of hydrogen atoms in the amide
groups of the epitope when in a solution of deuterated water. The hydrogen-deuterium exchange rate of each amino acid residue can then be quantified via mass spectrometry. The most recently developed technique for epitope mapping involves measuring the antibody-induced alterations of an antigen’s nuclear magnetic resonance (NMR) spectrum. In order for this assay to work, a recombinant antigen is synthesized using isotopes of H, N, and C followed by measurement of its NMR profile. Following antibody binding, the NMR profile is again measured and the resulting changes in an individual atom’s resonance structure are used to determine interacting proteins or carbohydrates. The high level of technicality and the requirement to produce recombinant protein are the major roadblocks for NMR mapping.

While the previously mentioned techniques require highly specific and expensive equipment, epitope mapping via site-directed mutagenesis provides a low-cost and accurate approach. This method is able to identify amino acids and sites of glycan attachment that are involved in epitope recognition. The major shortcoming of mutagenesis is that it is time intensive; however, high-throughput methods are currently being developed. In our mapping experiments of the mAb 8.9F epitope, we used a mutagenesis-based approach. There are two main approaches in mutagenesis-based mapping. The first approach is known as alanine scanning mutagenesis. In this approach, a library of mutants containing single alanine substitutions is generated and scanned for reactivity against the antibodies of interest. The assumption is that if an antibody does not bind a mutant, then that position plays a role in stabilizing the epitope or directly interacting with the
antibody. The major shortcoming of this approach is that a quality control step ensuring the protein is properly folded is required. For antigens with a broad antibody library, this is less of a limiting factor. This method has been successful in the mapping of Dengue virus antibodies\(^\text{147}\).

The second method of B-cell epitope mapping via site-directed mutagenesis is to design antigenic mutations based on an orthologous or homologous structure. Peng, et. al. have demonstrated this approach in characterizing EphA2 recognition by mAb 1C1\(^\text{148}\). mAb 1C1 is a previously characterized antibody against the erythropoietin-producing hepatoma receptor A2, a molecule shown to be upregulated in cancer cells\(^\text{149,150}\). Because mAb 1C1 did not react with the related EphA4 receptor, the researches created a series of ‘knock-out’ and ‘knock-in’ mutants whereby short segments of amino acids were swapped between the two protein sequences. The rationale behind the experiment was that when the critical residues in EphA2 were knocked-out by EphA4 mutations, 1C1 binding would be abrogated and when those residues critical in EphA2 were knocked-in to EphA4 the binding of 1C1 to the receptor would be restored. The results of these mutagenesis experiments closely reflected the findings in x-ray crystallography.

Unfortunately the crystallographic structure of mAb 8.9F and the Lassa virus glycoprotein have proven difficult to ascertain. For this reason, our approach in mapping critical peptide residues in the epitope relied on ‘knock-out’ mutagenesis of the Lassa virus glycoprotein using the related arenavirus lymphocytic choriomeningitis virus (LCMV). Initially, short sequences containing LCMV bases were inserted in the LASV plasmid via site-directed mutagenesis. The mutants were then
scanned for mAb 8.9F reactivity while using mAb 12.1F as a positive control to confirm the protein was properly folded. Once segment mutants of interest were identified, single amino acid substitutions were introduced to find the specific bases responsible for mAb 8.9F epitope recognition.

Amino acids are not the only components recognized by antibodies. Glycans that are post-translationally attached to proteins may also constitute a partial (glycopeptidic) or complete B-cell epitope. The best-characterized glycan-dependent epitopes have been described in human immunodeficiency virus (HIV) infections. The major surface protein of HIV virions, gp120, has been found to be a heavily glycosylated molecule with glycans accounting for approximately half of its mass. The majority of the glycans attached to gp120 appear to be oligomannose chains. While many HIV neutralizing antibodies have been identified, the relatively low number of glycan-reactive antibodies may be related to the fact that glycosylated host proteins could cross react with antibodies generated against the pathogen. Of the glycan-reactive antibodies, several have been found with broadly neutralizing characteristics. The first glycan-dependent HIV epitope was described for mAb 2G12. Structural analysis found that the antibody bound two mannose residues in the D1 arm of the polysaccharide. Characterization of another HIV mAb, PG16, found the glycosylation site at position 160 was critical in antibody recognition. When this residue was substituted into a PG16-unreactive strain, pseudoviruses were effectively neutralized.

Glycan array probing and oligosaccharide competition for antibody binding are the primary methods used to determine the specific glycan composing the
epitope. In glycan array probing, synthesized or isolated glycans are attached to
glass slides in microspots. These spots are then reacted with fluorescently labeled
antibodies and analyzed via fluorescent detector. In oligosaccharide competition,
increasing concentrations of mutant glycans are analyzed for their ability to disrupt
antibody-antigen recognition. The glycan epitope is determined by those mutants
that most potently inhibit antigen binding. However, these assays are limited by
the difficulty in synthesizing the glycan of interest or in isolating it from the microbe
of interest.

As we do not have the tools to evaluate the contributions of oligosaccharides
to the mAb 8.9F epitope by the procedures described above, we instead used a site-
directed mutagenesis approach. Mutations were made to the N-X-S/T motif that
signals for glycan attachment in order to eliminate N-linked glycan attachment. The
primers were designed to swap the serine/threonine residues of each site for
alanine. Alanine was chosen as the replacement amino acid in order to preserve the
biochemical similarities of that position. Based on the success of site-directed
mutagenesis in mapping neutralizing antibody epitopes, we believe this approach
will provide a reliable way to identify residues that affect mAb 8.9F binding.

Methods

Cell culture

See General Materials and Methods

Alignment of LCMV-LASV sequences and primer design
In order to design the short segment mutant primers, the sequence of LCMV glycoprotein, Armstrong strain (accession number: P09991) was aligned to the sequence of LASV glycoprotein, Josiah strain (accession number: AAT49004). Alignment was accomplished by use of the Basic Local Alignment Search Tool (BLAST) available on the NCBI website (Figure 16)\(^{160}\). Following alignment, regions of up to six amino acids were identified that contained differing bases. Site-directed mutagenesis primers were then designed based on these regions (TABLE OF PRIMERS). A total of 61 primer sets were designed and used to mutate the full-length LASV glycoprotein. Of these reactions, 50 primer sets were able to introduce the correct mutations as determined by sequencing performed by Beckman Coulter Genomics (Danvers, MS). We designed primer F865 (5’- TAG AAT AAC ATC CAC TTT GCC TTT C -3’) to be used for sequencing reactions.

**Design of N-glycosylation mutants**

We used the NetNGlyc prediction software as well as previous research identifying glycosylation sites in the Josiah glycoprotein to locate residues suitable for site-directed mutagenesis\(^{161,162}\). When designing individual mutant primers, we replaced the serine or threonine of the Asn-X-Ser/Thr motif with an alanine residue. We included at least two base changes in the codon used to replace the existing sequence as a way to safeguard against spontaneous mutations reverting the glycoprotein to its wild type amino acid sequence.

**Flow Cytometry**

The basic protocol we used for analyzing cells via flow cytometry is described in the General Materials and Methods section. In this chapter we
measured cell-surface expressed glycoprotein and total cell glycoprotein. For the latter experiment, cells were permeabilized by incubation in 100µL of 0.1% Triton X before addition of the primary antibody. The primary antibodies used for these experiments were mAb 8.9F, 12.1F, or a mix of GP2 antibodies composed of mAb 4.1F, 13.4E, and 24.6C. The secondary antibody used for these experiments was a FITC-conjugated Goat anti-Human IgG Fc antibody diluted 1:500.

**Western blotting**

Protein lysate from cells and pseudoviruses were measured by western blot as described in the General Materials and Methods section. A mix of mAb 4.1F, 13.4E, and 24.6C antibodies at 2µg/ml was used to probe for GPC/GP2 cleavage. This was followed by incubation with IRDye 680RD Goat anti-Human IgG secondary diluted 1:10,000. mAb 183, a murine antibody against p24 was used as a loading control with pseudovirus samples. IRDye 800CW Goat anti-Mouse IgG diluted 1:10,000 was used for secondary staining. Ribosomal S3 protein was used as the loading control in experiments with cell extract. A rabbit anti-human S3 antibody (Pierce, Rockford, IL) diluted 1:1,000 followed by IRDye 800CW Goat anti-human antibody diluted 1:10,000 were used for detection.

**Indirect Immunofluorescence**

See General Materials and Methods and Chapter 1 for protocol. Antibodies used for staining are listed with the figure legend.

**Statistical Analysis**

Data analysis was performed using the Prism 6 software (GraphPad Software Inc., La Jolla, CA). The threshold for significance was set at 95% (p < 0.05) for all
experiments. Comparisons between different glycoprotein mutants relative to the wild type were performed using an ordinary one-way ANOVA. Virus titration and antibody neutralization experiments were done in triplicate and repeated at least twice.

**Results**

**LCMV pseudoviruses and surface-expressed glycoprotein do not react with mAb 8.9F**

In order to use the glycoprotein sequence of LCMV as our template for creating substitutions in the LASV glycoprotein, we first confirmed that pseudoviruses and surface-expressed glycoprotein had no reactivity with mAb 8.9F. When tested via pseudovirus neutralization assay, mAb 8.9F had no discernable effect on the infectivity of LCMV pseudoviruses (Figure 17). While it is possible that mAb 8.9F could bind LCMV pseudoviruses as a non-neutralizing antibody, we took an additional step to confirm that there was no reactivity by testing GPC-transfected cells for mAb 8.9F reactivity on immunofluorescence. Cells were fixed to glass slides via air-drying and stained with mAb 8.9F and 12.1F followed by a FITC-conjugated secondary. While reactivity with 12.1F was preserved, mAb 8.9F staining did not display a detectable signal.

**Short segment mutants 121 SDAHK, 145 PNFNQ, and 250 RDIYIS eliminate mAb 8.9F reactivity**
In order to elucidate which residues in LASV may be contributing to mAb 8.9F reactivity, we introduced short segments of the LCMV genome into the LASV plasmid to generate a library of experimental mutants. A total of 61 primer sets were designed to create unique mutants. Of these, we were able to generate 50 positive mutants when analyzed by genetic sequencing. 293T cells were transfected with mutant plasmids and antibody staining was measured via flow cytometry in order to assess which mutants may harbor regions of the mAb 8.9F epitope. To help sort mutants that may have displayed misfolded protein from those that were truly mAb 8.9F unreactive, we used mAb 12.1F as a marker for correct surface expression. Based on these results, 15 mutants were identified to be mAb 8.9F and 12.1F unreactive while three of the mutants were only unreactive with mAb 8.9F (Figure 18). Interestingly, a majority of the mutants that were unreactive with both antibodies were found in the GP2 region. The three mAb 8.9F unreactive mutants, 121 SDAHK, 145 PNFNQ, and 250 RDIYIS were all located in the GP1 region. As mAb 12.1F is primarily reactive with GP1, we also wanted to confirm that the GP2 region of the three mAb 8.9F negative mutants were being correctly produced and that the protein was not truncated, thus leading to ineffective mAb 8.9F binding. The protein from the unreactive short segment mutants was run on a western blot and probed with antibodies against linear GP2. These results showed that the GP2 in the mutant proteins was the same molecular weight as the protein produced in the wild type. This result suggested that GP1 mutations did not affect GP2 translation. For mutants 121 SDAHK and 145 PNFNQ the GPC signal was noticeably higher than the wild-type signal. A possible explanation for this observation is that the inserted mutations
affected the cleavage of full-length glycoprotein by host proteases. As a result of less efficient cleavage, a build up of the precursor protein was detected. To further confirm the results generated by flow cytometry, we transfected cells with the short segment mAb 8.9F unreactive mutants and observed them via immunofluorescent microscopy. For all three mutants, mAb 12.1F was detectable on the surface of cells while the mAb 8.9F signal was undetectable. Based on the results of these experiments, we conclude that the segments of 121 SDAHK, 145 PNFNQ, and 250 RDIYIS contain possible sites of mAb 8.9F recognition.

**H124F, P145R, and F147N mutations affect mAb 8.9F reactivity**

Following the identification of three regions within the glycoprotein that mediate mAb 8.9F binding, we created a series of mutants with single substitutions from the regions of interest (Table 3). These mutations were tested for mAb 8.9F reactivity in a similar manner as described for the short segment mutants. We found that mutants H124F, P145R, and F147N did not react with mAb 8.9F but did produce a detectable signal against mAb 12.1F when surface binding was measured with flow cytometry. One mutant, S225T, was unreactive with both mAb 8.9F and 12.1F (Figure 18). In addition to reactivity with cell-expressed glycoprotein, we examined the ability of antibodies to bind mutant pseudoviruses. Pseudoviruses were affixed to nitrocellulose via Bio-Rad slot blot and antibody reactivity was tested via western blot. Again, the results showed no reactivity with mAb 8.9F for the three point mutations. However, all three mutants and wild-type pseudovirus were detectible with mAb 12.1F and 13.4E. A32, an HIV antibody, was used as a
negative control (Figure 23). The mAb 12.1F and 13.4E signals for H124F and F147N pseudoviruses were comparable to that of the wild type while the signals for P145R pseudoviruses were visibly weaker. The ability of mAb 8.9F and 12.1F to neutralize pseudoviruses was tested to help eliminate concerns that the mutations introduced into the glycoprotein could disrupt the ability to form functional trimers. By clarifying whether mAb 8.9F was unreactive due to glycoprotein multimer destabilization or disruption of the epitope-antibody interaction, we could better understanding the importance of these mutations. When mAb 8.9F and 12.1F titration curves were generated against each of the pseudoviruses, we found mAb 12.1F showed a clear dose-dependent effect on all mutants and wild-type pseudovirus. In contrast, mAb 8.9F was able to effectively neutralize wild-type LASV pseudovirus but the results with pseudoviruses containing point mutations indicated mAb 8.9F was an ineffective neutralizer (Figure 24). Based on these experiments, we conclude residues H124, P145, and F147 are critical for the recognition of the mAb 8.9F epitope on the surface of transfected cells and pseudovirus particles. These mutations do not have a significant effect on detection by other antibodies and do not compromise the ability of the glycoprotein to form fusogenic trimers.

**Mutation of N-glycan attachment sites 79 and 167 affect mAb 8.9F reactivity**

As previously identified neutralizing antibodies against other viral pathogens have demonstrated epitopes comprised partly or entirely of glycans, we sought to investigate the role N-linked glycans have in mAb 8.9F epitope recognition. Previous studies have shown that LSAV GP1 is glycosylated at all possible N-linked sites.
As a result, a series of primers were designed to substitute alanine residues with the serine or threonine residue of the Asn-X-Ser/Thr glycosylation motif (Table 4). Site-directed mutagenesis and sequencing validated that all mutagenesis reactions were successful. mAb 8.9F reactivity to N-linked glycosylation mutations was first assessed by reactivity against cell-surface expressed glycoprotein via flow cytometry. T81A and S169A mutants demonstrated no reactivity against mAb 8.9F, S91A and T226A mutants exhibited low mAb 8.9F reactivity (below 40% as compared to wild type), and mutations T101A, S11A, and S121A did not appear to have a major effect on mAb 8.9F binding (Figure 25). While mutations T81A and S169A may play a role in the epitope recognized by mAb 8.9F, these mutations could have effects on other factors involved in protein expression and function. Future experiments will be needed to measure changes in glycoprotein cleavage and surface transport. If T81A and S169A mutations affect production of cleaved trimers or surface expression, then it would be reasonable to expect mAb 8.9F to be unreactive based on our previous findings.

In addition to mAb 8.9F reactivity with cell-surface expressed N-linked glycosylation mutations, we also determined antibody binding against mutant pseudoviruses. Generated pseudovirions were affixed to nitrocellulose via slot blot and stained with mAb 8.9F and 12.1F. For the S/T to A substitutions, T81A, S91A, S121A, and S169A showed no mAb 8.9F band. Mutants T226A had a weakly positive signal relative to the wild-type control. T101A and S111A had signals similar to the wild type. LCMV pseudovirus was used as a negative control for mAb 8.9F. The GP1 mAb 12.1F was used to help confirm that glycoprotein structure was maintained
and that pseudovirus was produced. T81A, S91A, and S169A displayed no visible band and T226A had a relatively weak signal. All other mutant pseudoviruses had a mAb 12.1F signal similar to the control (Figure 26). Additional experiments will be required to confirm that pseudoviruses with no signal were able to incorporate glycoprotein into their membrane. We conclude that T81A and S169A are capable of abrogating mAb 8.9F binding to cell surface and pseudoviruses but the mechanisms remain unclear. It may be the case that mAb 8.9F binding was inhibited due to changes in glycoprotein processing or multimer formation.

**Discussion**

Monoclonal antibodies play a central role in the development of antiviral therapies. In addition to their use in post-exposure prophylactic treatments and passive vaccination, identifying antibodies with broad reactivity and potent neutralization helps to inform effective vaccine design. As infection with naturally occurring viruses tends to induce a host of non-neutralizing and poorly neutralizing antibodies, an understanding of the critical residues displayed by pathogenic antigens that elicit neutralizing antibodies is essential for the field of vaccine design. Lassa virus, the causative agent of Lassa fever and approximately 5,000 deaths annually, has no clinically approved antibody therapy. Our previous aim identified the antibody mAb 8.9F as a broad and potent neutralizer with reactivity against a putative quaternary epitope. To help develop downstream applications, understanding key interacting regions on the viral antigen with mAb 8.9F is vital.
This chapter has attempted to identify residues in the glycoprotein of Lassa virus that mediate mAb 8.9F reactivity and possibly comprise the epitope.

Initial identification of bases in the glycoprotein that may affect mAb 8.9F binding was achieved through the use of a site-directed mutagenesis ‘knock-out’ approach. In this method, we wanted to identify a protein closely related to LASV GPC that was mAb 8.9F unreactive. Primers could then be designed via sequence alignment that would substitute amino acids from the unreactive protein into the LASV GPC. A likely candidate to design the knockout mutants around was the glycoprotein of lymphocytic choriomeningitis virus. Based on immunofluorescence and pseudovirus neutralization assays, we determined that the LCMV glycoprotein did not react with mAb 8.9F. However, mAb 12.1F was able to detect cell-surface expressed glycoprotein and neutralize LCMV pseudoviruses. Thus, LCMV was an appropriate template to use in mutant design as it allowed for elimination of the mAb 8.9F epitope but could be detected by another of our monoclonal antibodies to ensure correct expression and processing.

To design the necessary mutants used in the short-segment scanning experiments, we aligned the LCMV and LASV sequences using BLAST. Because of the large number of differences in bases between the two glycoproteins, we designed mutant primers consisting of one to seven sequence changes. This was done in order to reduce the total number of mutants generated and help improve efficiency in scanning. A total of 61 mutants were designed, however, only 50 mutants returned a positive result following sequencing. Of these negative mutations, two of the primer sets encoded insertions of six amino acids while the other four primer
sets encoded substitutions of two or three amino acids. When sequenced, the insertion mutants did not show the presence of any of the mutated residues, a likely reason for the negative results is due to the length of the primers. This issue could be resolved by designing two primer sets with three base substitutions. The other four mutants that were unsuccessfully cloned may be due to issues in the primer design. If the non-overlapping regions of the primer set contained a high degree of complementarity, then a primer dimer may form that would reduce the efficiency of the cloning reaction. Another complicating factor may have been the design of the sequencing primer. Some mutations at the terminal end of the protein were unable to be sequenced, as our primer's efficiency did not always permit full sequencing. This difficulty could easily be overcome with the design of an additional sequencing primer.

Scanning of the short segment mutants for mAb 8.9F reactivity was accomplished via flow cytometry. While analyzing these mutants we also included 12.1F as a control to check for correct protein folding. This experiment was able to identify three regions, 121 SDAHK, 145 PNFNQ, and 250 RDIYIS that were mAb 8.9F unreactive but still bound to mAb 12.1F. There were also several mutations that eliminated mAb 8.9F and 12.1F binding. We chose not to investigate these mutants further due to the possibility that they produced misfolded protein. However, these regions should not be completely ignored. Ideally, these mutants should be tested with a wider range of antibodies as it is possible that some mutants disrupted the mAb 12.1F epitope. Even though LCMV glycoprotein was found to bind mAb 12.1F, short segment LCMV-LASV mutants may require additional compensatory
mutations to restore antibody binding. Another likely explanation for the loss of mAb 8.9F and 12.1F reactivity in short segment mutants is that the substituted mutations affected the protein transport and processing pathways. If these mutations limited GPC transport to the cell membrane or increased proteasomal degradation, antibody detection levels would be low. This confounding factor could be addressed by biotinylation of surface proteins followed by immunoprecipitation in order to assess the surface expression of antibody-unreactive mutant glycoproteins.

Once we had identified three regions of the glycoprotein likely to play a role in the mAb 8.9F epitope we further refined our search by creating individual amino acid substitutions within each region. Again, we used an approach based on flow cytometry and immunofluorescence to identify the mutations of interest. Our search found that H124F, P145R, and F147N all had an effect on eliminating mAb 8.9F binding while allowing mAb 12.1F recognition. The most surprising results came from the individual mutations done in the 250 RDIYIS region. While this region had initially been identified as one that held a mAb 8.9F unreactive region but maintained its overall structure, the individual S255T mutation eliminated both mAb 8.9F and 12.1F binding. One possible explanation for this results is that when expanding the plasmid in bacterial cells a point mutation was introduced that terminated protein translation. If S255T is in fact unreactive to both of the antibodies tested, then perhaps the 250 RDIYIS region contains an additional base pair substitution that restores mAb 12.1F reactivity. Another possible explanation to this observation is that residues 251 and 252 may require deletion as the segment
mutant of 250 RDIYS substituted residues 250, 253-255 and deleted residues 251 and 252 while the single mutants did not delete these two residues.

When examining the effect H124F, P145R, and F147N mutations had on mAb 8.9F reactivity, we also wanted to understand how these mutations might affect antibody interaction with pseudovirus-associated glycoprotein. When filtered pseudovirus supernatant was bound to nitrocellulose, each of the mutants was unreactive with mAb 8.9F while displaying signal for mAb 12.1F and 13.4E. Of the mutants, H124F and F147N both appeared to have mAb 12.1F and 13.4E signals approximate to that of the wild type whereas P145R had a slightly weaker signal on western blot. P145R may have a weaker signal on western blot due to less efficient pseudovirus budding or lower cell surface transport of the glycoprotein. Poor glycoprotein transport in P145R seems to be supported by flow cytometry data that shows the surface signal of P145R is lower relative to H124F and F147N. It is also interesting to note that despite lower surface glycoprotein signals, H124F and F147N had slot blot signals similar to the wild type. This could be due to saturation of the nitrocellulose with viral antigen. Pseudovirus neutralization assay was used as an additional test to see how single base substitutions would affect mAb 8.9F and 12.1 neutralization curves. As would be expected, mAb 12.1F retained its ability to neutralize mutant pseudoviruses. The neutralization curve of H124F closely mirrored that of the wild-type pseudovirus. While P145R and F147N pseudoviruses showed a dose-dependent response to mAb 12.1F, only 80% and 70% of maximum neutralization was achieved at 10ug/ml. It is likely that if higher concentrations of the antibody were used, complete neutralization could be achieved. Alternatively,
incomplete neutralization could be a byproduct of the relative low pseudovirus titer affecting our calculations. The results of this experiment suggest that P145R and F147N may still bind mAb 12.1F but do so which a lower affinity and results in an increase in the IC50. Incubation with mAb 8.9F showed no discernable effect on the infectivity of mutant pseudovirus. Based on these results, we conclude that H124F, P145R, and F147N mutations are capable of disrupting the mAb 8.9F epitope.

Because glycans have also been demonstrated to have a role in epitope recognition by highly neutralizing antibodies, we examined the effect of mutating N-linked glycosylation sites by S/T to A mutations in the GP1 subunit. As was done with the single base substitutions, we used flow cytometry and slot blot analysis to determine if any glycans could completely abrogate mAb 8.9F reactivity. We found two mutations T81A and S169A that abrogated mAb 8.9F binding in both situations. However, pseudoviruses containing the mutations were unreactive with mAb 12.1F. These mutations may play a role in the epitope, but it is also likely that introduction of these mutations inhibited pseudovirus formation. Because S91A produced a signal when measured for mAb 8.9F binding by flow cytometry but produced no mAb 8.9F or 12.1F slot blot signal, we hypothesize that this N-linked glycan may also play a role in pseudovirus formation. The case for S121A is less clear. In this situation, mAb 8.9F reacted with surface protein but not the pseudovirus. However, mAb 12.1F still reacted with S121A pseudovirus suggesting that S121A is capable of forming pseudoviruses. In this situation, we hypothesize that either the glycoprotein undergoes some change during pseudovirus formation that eliminates the epitope or that the binding affinity is reduced to levels that are undetectable by slot blot. We
suspect the latter case is more likely. The T226A mutation exhibited a strong binding signal when measured for surface reactivity, but the mAb 8.9F band on slot blot was extremely faint. As the 12.1F band was also relatively weak compared to the control, we suspect T226A and reduced pseudovirus formation. Many of these results require further investigation as to how these mutations affect glycoprotein processing and function. In Chapter 4 we take a more detailed approach in exploring the impact of N-glycans deletions on Lassa virus glycoprotein.

mAb 8.9F is a promising antibody candidate for the treatment of Lassa virus infections. While post-exposure prophylactic treatment may be useful in situations of lab exposure, designing a vaccine capable of eliciting immunity in at-risk populations is critical for reducing disease burden. In this regard, finding the epitope that recognizes mAb 8.9F is an important step to help guide future vaccine development. In order to identify amino acid residues and glycans that may compose the epitope, we used a site-directed mutagenesis approach to scan the entire glycoprotein. In doing so we identified three amino acid mutations, H124F, P145R, F147N, that were capable of eliminating mAb 8.9F binding without destroying the overall glycoprotein conformation. Our results examining the effect of removing N-linked glycosylation sites is less clear. While we have identified two possible sites, more studies will be required before the effect of this mutation is clearly understood. While a mutagenesis-based approach to mapping does provide some insight into the epitope structure, these results will be most informative when used in conjunction with the crystal structure of Lassa virus glycoprotein. In this way a 3D structure can be created and the physical location of critical residues
identified in mutagenesis can be mapped. Further study of the mAb 8.9F epitope will be critical in the understanding of its therapeutic potential.
Figure 16. Sequence alignment of Lassa virus glycoprotein with LCMV glycoprotein.

The sequence of Lassa virus glycoprotein (accession number: AAT49004) was aligned with the sequence of the lymphocytic choriomeningitis virus (accession number: P09991) using the NCBI BLAST tool. Those regions highlighted in green were used to design the site-directed mutagenesis primers.
<table>
<thead>
<tr>
<th>Insertion</th>
<th>58 TTSL → GPDI</th>
<th>67 ELQTL → QFKSV</th>
<th>73 LNMET→FDMSH</th>
<th>80 M → L</th>
<th>84 LSCTK → NACSAN</th>
<th>96 MVG → SMG</th>
<th>99 NET → T_S</th>
<th>107 LTNT → FTND</th>
<th>114 NHK → SHN</th>
<th>121 SDAHK → TSAFN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GMYGLK</strong> insertion</td>
<td><strong>FP</strong></td>
<td>GTG GTA GGT CTT GCG GCA UGT ATG GTC TAA AAA CAA CCA GTC TTT ATA AAG</td>
<td><strong>RP</strong></td>
<td>CCG CAA GAC CTA CCA CAC AAC AGG AGG AAA GTG ACC AAA CCA ACA ACG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>TGG TAG GTC TGT G CTG GGT CCT GAT ATT TAT AAA GGG GTT TAT GAG CTT CAG ACT CTG G</td>
<td><strong>RP</strong></td>
<td>CAT AAA CCC CTT TAT AAA TAT CAG GAC CGC AAG ACC TAC CAC ACA ACA GGA G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>GGG GTT TAT CAA TTC AAG TCT GTT GAA CTA AAC ATG GAG ACA CTC AAT ATG ACC</td>
<td><strong>RP</strong></td>
<td>CTC CAT GTT TAG TTC AAC AGA CTT GAA TTG ATA AAC CCC TTT ATA AAG ACT GTG TGT GC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>CAG ACT CTG GAA TTC GAC atg AGT CAT CTC AAT ATG ACC ATG CCT CTG TCC TGC</td>
<td><strong>RP</strong></td>
<td>CAT GGT CAT ATT GAG ATG ACT CAT GTC GAA TTC CAG AGT CTG AAG CTC ATA AAG TTT ATA AAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>ATG GAG ACA CTC AAT TTA ACC ATG CCT CTG TCC TGC ACA AAC ACT</td>
<td><strong>RP</strong></td>
<td>GAG AGA GCC ATG GTT AAA TTG AGT GTC TCC ATG TTT AGT TCC AGA GTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>ATG ACC ATG CCT AAT GCT TGC TCT GCT CAT AAT AAC AAG ACT CAT TAT ATA ATG ACC ATG</td>
<td><strong>RP</strong></td>
<td>GCA AGC ATT AGG CAT GGT CAT AGG CAT GGT CAT ATT GAG TGT CTC CAT G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>CAT CAT TAT ATA TCT ATG GGC AAT GAG ACA GGA CTA GAA CTG AC</td>
<td><strong>RP</strong></td>
<td>CTC TCT CAT TGC CCA TAG ATA TAT AAT GAT GAC TGT TGT TCT TGG TGC AGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>ATA ATG GTG GGC ACT TCT GGA CTA GAA CTG ACC TTG ACC AAC AC</td>
<td><strong>RP</strong></td>
<td>CAG TCT TAG TCC AGA AGT GCC CAC CAT TAT ATA ATG ATG ACT GTT GTT C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>CTA GAA CTG ACC TTC ACC AAC GAT AGC ATT ATT AAT AAT CAC AAA TTT TGC AAT CTG</td>
<td><strong>RP</strong></td>
<td>GAT TAA TAA TGC TAT CGT TGG TGA AGG TCA GTT CTA GTC CTG TCT CAT TG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>CAC GAG CAT TAT TAG TCA CAA TTT TTG CAA TCT GTC TGA TGC CCA CAA AAA GAA C</td>
<td><strong>RP</strong></td>
<td>GAT TGC AAA AAT TGT GAC TAA TAA TGC TCG TGT TGG TCA AGG TCA GTC C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FP</strong></td>
<td>CAA ATT TTG CAA TCT G ACT AGT GCC TTT AAC AAG AAC CTC TAT GAC CAC GCT CTT ATG AG</td>
<td><strong>RP</strong></td>
<td>GAG GGT CTT GTT AAA GGC ACT AGT CAG ATT GCA AAA TTT GTG ATT AAT AAT GCT CGT G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124 HK -&gt; FN</td>
<td>FP</td>
<td>CTG TCT GAT GCC TTT AAC AAG AAC CTC TAT GAC CAC GCT CTT ATG AGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>127 NLY -&gt; KTF</td>
<td>FP</td>
<td>GAT GCC CAC AAA AAG AAA ACC TTT GAC CAC GCT CTT ATG AGC ATA ATC TCA AC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>132 A -&gt; T</td>
<td>FP</td>
<td>CTC TAT GAC CAC ACT CTT ATG AGC ATA ATC TCA ACT TTC CAC TTG TC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>137 ISTF -&gt; VSSL</td>
<td>FP</td>
<td>GCT CTT ATG AGC ATA GTT TCG AGC CTA CAC TTG TCC ATC CCC AAC TTC AAT CAG TAT G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>145 PNFQN -&gt; RGNSN</td>
<td>FP</td>
<td>CAC TTG TCC ATC AGA GGG AAC TCC AAC TAT GAG GCA ATG AGC TGC GAT TTT AAT GGG G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>151 EAM -&gt; KAV</td>
<td>FP</td>
<td>CTG CAC ACT AAT ACC GTT ATT AAA ATC GCA GCT CAT TG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>159 G-&gt; N</td>
<td>FP</td>
<td>GAG CTG CGA TTT TAA TAA CGG TAT TAG TGT GCA GTA CAA CCT GAG TCA CAG C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>163 SVQ -&gt; TIQ</td>
<td>FP</td>
<td>TGG GGG AAA GAT TAC TAT TCA GTA CAA CCT GAG TCA CAG CTA TGC TG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>169 SHSY -&gt; TFSN</td>
<td>FP</td>
<td>GCA GTA CAA CCT GAC ATT CTC AAA TGC TGG GGA TGC AGC CAA CCA TTG TG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>174 GDA -&gt; QSA</td>
<td>FP</td>
<td>GTC ACA GCT ATG CTC AAT CAG CCA ACC ATT GTG GTA CTA TTG C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>177 ANHCG -&gt; QSQCR</td>
<td>FP</td>
<td>TGC TGG GGA TGC ACA GAG CCA GTG TAG AAC TGT TGC AAA TGG TGT GTT ACA GAC TTT TAT GAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>Type</td>
<td>Sequences</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>-----------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>183 VANG -&gt; PRGR</td>
<td>FP</td>
<td>GGG ATG GAC AAG TGG AAA GTT GAG ATT GCA TCC CCA GCA TAG CTG TGA CTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>CCA TTG TGG TAC TT TTAGG TAG AGT GTT ACA GAC TTT TAT GAG GAT GGC T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>189 QTF -&gt; DMF</td>
<td>FP</td>
<td>GCA AAT GGT GTG TTA GAT ATG TTT ATG AGG ATG GCT TGG GGT GGG AG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>CAT CCT CAT AAA AGT TTG TAA CAC ACC ATT TGC AAG AGT ACC ACA ATG G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>192 MRM -&gt; RT</td>
<td>FP</td>
<td>GTT ACA GAC TTT TCG TAC TGC TTG GGG TGG GAG CTA CAT TG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>CCA CCCCAAAGC GATA CGA AAA GTC TGT AAG ACA CCA TTTC GACA GTA CAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>196 WGGS -&gt; PGGK</td>
<td>FP</td>
<td>ATG AGG ATG GCT TTT GGT GGG AAG TAC ATT GCT TTT GAC TCG GCC CGT GG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>AGC AAT GTA CTT CCC ACC AAA AGC CAT CCA CAT AAA AGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>201 IAL -&gt; MRS</td>
<td>FP</td>
<td>GGT GGG AGC TAC AUG CGT TCT GAC TCA GGC CGT GCC AAG TGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>CTG AGT CAG AAC GCA TGT AGC TCC CAC CCC AAG CCA TC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GWGWTG insertion</td>
<td>FP</td>
<td>CTA CAT TGC TCT TGG TTG GGG ATG GAC TGG AAG TAC AGC TGT GGA AAC TGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>ACC AAG AGC AAT GTA GCT CCC ACC CCA AGC CAT CTC CAT AAA AG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>204 DSGRGN -&gt; SDGKTT</td>
<td>FP</td>
<td>CTA CAT TGC TCT TTT GAC TGG CAA AAG TAC ATG GGA CTG TAT TAT GAC TAG TTA TC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>CCA TGT AGT TTT GCC ATC AGA AAG AGC AAT GTA GCT CCC ACC CCA AG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>225 TTWED -&gt; RTWEN</td>
<td>FP</td>
<td>GAT ATT CCA AAA TCG TAG ATG TTT TCC GAT GAC ATC TCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>GCA GTG ATT TTC CCA GGT AGC ATT TTT GAG TAT CAG ATA TGG ATA ACT AGT CAT AAT ACA GTC C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>232 QFSR -&gt; TYAG</td>
<td>FP</td>
<td>GGG AAG ATC ACT GCA CAT ATG CAG GTC CAT CTC CCA TCG GTT ATC TGG GG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>CCG AGT GGA GTG GCC CCA TAT GTG CAG TGA TCT TCC CAG GTT GTA TTT TGG ATT ATC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward Primer (FP)</td>
<td>Reverse Primer (RP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>237 SPIGYLG -&gt; FGMSRIL</td>
<td>CTC GAG ACC ATT TGG TAT GTC TAG AAT TCT CCT CCT CTC ACA AAG GAC TAG AGA TAT TTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTG AGA GGA GGA GAA TTC TAG ACA TAC CAA ATG GTC TCG AGA ATT GGC AGT GAT CTT CC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>245 LSQR -&gt; SGEK</td>
<td>TAT CTC GGG CTC TCC CAA GAG AAG ACT AGA GAT ATT TAT ATT AGT AGA AGA TTG CTA GGA AC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATA AAT ATC TCT AGT CTT CTC TTG GGA GAG CCC GAG ATA ACC GAT GGG AG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 RDIYIS -&gt; KFFT</td>
<td>TCT CAC AAA GGA CTA AGT TCT CTA CTA GAA GAT TGC TAG GCA CAT TCA CAT GGA CAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCC TAG CAA TCT TCT AGT CTT AGT CCT TTG TGA GAG GAG CCC GAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>259 L -&gt; A</td>
<td>TAG TAG AAG ATT GGC TGG CAC ATT CAC ATG GAC ACT GTG AGA TTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGT GAA TGT GCA TTT TAG TTC AGC AGC AAG AAT CAT CCA CCT GGT CAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>282 R -&gt; K</td>
<td>GGA TAT TGT CTG ACC AAA TGG ATG CTA ATT GAG GCT GAA CTA AAA TGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAA TTA GCA TCC ATT TGG TCA GAC AAT ATC CCC CTG GTG TGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>285 LIE -&gt; ILA</td>
<td>ACC AGG TGG ATG ATT CTT GCT GCT GAA CTA AAA TGC TTC GGG AAC ACA GC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCA TTT TAG TCC AGG AGC AAG AAT CAT CCA CCT GGT CAG ACA ATA TCC CC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>302 NEK -&gt; NVN</td>
<td>GGC AAA ATG TAA TGT TAA CCA TGA TGA GGA ATT TTG TGA CAT GCT GAG G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCG GGA ACA CAG CTG TGG CAA AAT GTA ATG TTA ACC ATG ATG AGG AA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>315 FDFNKQ -&gt; IDYNKA</td>
<td>ATG CTG AGG CTG ATT GAC TAT AAC AAA GCT GCC ATT CAA AGG TTG AAA GCT GAA GCA CAA AT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCT TTG AAT GGC AGC TTT GTT ATA GTC AAT CAG CCT CAG CAT GTC ACA AAA TTC CTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>323 IQRL -&gt; LSKF</td>
<td>CAA CAA ACA AGC CTT GAG TAA GTT CAA AGC TGA AGC ACA AAT GAG CAT TCA GTT GAT C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTG CTT CAG CTT AGC ACT TAC TCA AGG CTT GTT TGT TGA AGT CAA ACA GCC TCA G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>328 AEAQMSIQ -&gt; EDVESALH</td>
<td>CAA AGG TTG AAA GAG GAC GTA GAA TCT GCC TTG CAC TTG ATC AAC AAA GCA GTA AAT GCT TTG ATA AAT GAC C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>337 INKA -&gt; FKTT</td>
<td>FP</td>
<td>ATG AGC ATT CAG TTA TTC AAA ACA ACA GTG AAT GCT TTG GTA AAT GAC CAA CTT ATA ATG AAG AAC CAT C</td>
<td>RP</td>
<td>CAC TGT TGT TTT GAA TAA CTG AAT GCT CAT TTG TGC TTC AGC TTT CAA C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>343 ALIN -&gt; SLIS</td>
<td>FP</td>
<td>CAA AGC AGT AAA TAG TTT GAT AAG TGA CCA ACT TAT AAT GAA GAA CCA TCT ACG G</td>
<td>RP</td>
<td>CAT TAT AAG TTG GTC ACT TAT CAA ACT ATT TAC TGC TTT GTT GAT CAA CTG AAT GCT CAT TTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>350 IMK -&gt; LMR</td>
<td>FP</td>
<td>AAA TGA CCA ACT TCT TAT GCG TAA CCA TCT ACG GGA CAT CAT GGG AAT TCC</td>
<td>RP</td>
<td>CCG TAG ATG GTT ACG CAT AAG AAG TTG GTC ATT TAT CAA CAT ATT TAC TGC TTT TGT G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>358 IMGI -&gt; LMGV</td>
<td>FP</td>
<td>CAT CTA CGG GAC CTT ATG GGA GTC ATT CCA TAC TGT AAT TAC AGC AAG TTT TGG AAC ACT GGG</td>
<td>RP</td>
<td>ACA GTA TGG AAT GAC CTC CAT AAG GTC CCC TAG ATG GTT CTT CAT TAT AAG TTG G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>369 Y -&gt; F</td>
<td>FP</td>
<td>TGT AAT TAC AGC AAG TTT TGG TAC CTC AAC AAC ACA ACT GGG</td>
<td>RP</td>
<td>GTT GAG GTA CCA AAA CTT GCT GTA ATT ACA GTA TGG AAT CTG GAT G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>373 NHTT -&gt; EHAK</td>
<td>FP</td>
<td>GTA TTG GTA CCT CGA ACA TGC AAA GAC TGG GAG AAC ATC ACT GCC CAA ATG TTG TTT GAC AAT GCC TGG GAG ACC</td>
<td>RP</td>
<td>CTT TGC ATG TTC GAG GTA CCA ATA CTT GCT GTA ATT ACA GTA TG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>379 RTSL -&gt; ETSV</td>
<td>FP</td>
<td>CAC ACA ACT ACT GGG GAA ACA TCA GTT CCC AAA TGT TTG CTT GTA TCA AAT GGT TCA TAC</td>
<td>RP</td>
<td>CCA ACA TTT GGG AAC TGA TGT TTC CCC AGT AGT TGT GTT GAT GTA CCA A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>389 S -&gt; T</td>
<td>FP</td>
<td>TGT TGG CTT GTA ACC AAT GGT TCA TAC TTG AAC GAG ACC CAC TTT TC</td>
<td>RP</td>
<td>GTA TGA ACC ATT GTT TAC AAG CCA ACA TTT GGG CAG TGA TGT TC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>402 DIEQQ -&gt; QIEQE</td>
<td>FP</td>
<td>CCC ACT TTT CTG ATC AAA TTG AAC AAG AAG CTG ACA ATG TCA AGG TGA TGT TAC AGA AAG GGG</td>
<td>RP</td>
<td>CAT ATT GTC AGC TCC TTT TCC AAT TTG ATC AGA AAA GTG GGT CTC GGT GTA ACA TGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>416 QKEYME -&gt; RKDYIK</td>
<td>FP</td>
<td>CAC TGA GAT GTT AAG GAA GGA TTA CAT AAA GAG GCA GGG GAA GAC ACC ATT GGG</td>
<td>RP</td>
<td>CTT TAT GTA ATC CTT CCT TAA CAT CTC AGT GAT CAT ATT GTC AGC TTG TTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>425 K -&gt; S</td>
<td>FP</td>
<td>GAG AGG CAG GGG AGC ACA CCA TTG GTT CTA GTT GAC CTC TTT G</td>
<td>RP</td>
<td>ACC CAA TGG TGT GCT CCC CTG CTC CAT ATA CTC CTT C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Legend:**
- **FP:** Forward Primer
- **RP:** Reverse Primer
- **Sequence:** DNA sequence in uppercase letters
<table>
<thead>
<tr>
<th>Sequence</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>429 GLV -&gt; ALM</td>
<td>FP: GAA GAC ACC ATT GGC ACT AAU GGA CCT CTT TGT GTT CAG TAC AAG TTT CTA TC</td>
<td>RP: CAC AAA GAG GTC CAT TAG TGC CAA TGG TGT CTT CCC CTG CCT CTC</td>
</tr>
<tr>
<td>434 FVF -&gt; LMF</td>
<td>FP: CTA GTT GAC CTC CTT AUG TTC AGT ACA AGT TTC TAT CTT ATT AGC ATC TCC CTT CAC</td>
<td>RP: ACT TGT ACT GAA CAT AAG GAG GTC AAC TAG ACC CAA TGG TGT CTT C</td>
</tr>
<tr>
<td>440 FYLI -&gt; AYLV</td>
<td>FP: GTT CAG TAC AAG TGC TTA TCT TGT TAT CTT CCT TCA CCT AGT CAA AAT ACC AAC TCA TAG</td>
<td>RP: GTG AAG GAA GAT AAG ATA AGC ACT TGT ACT GAA CAC AAA GAG GTC AAC TAG AC</td>
</tr>
<tr>
<td>459 VGK -&gt; KGG</td>
<td>FP: ACT CAT AGG CAT ATT AAA GGC GCC TCG TGT CCC AAA CCT CAC AGA TTG AAT C</td>
<td>RP: GTT TGG GAC AGC AGC CGC CTT TAA TAT GCC TAT GAG TTG GTA TTT TGA CTA GGT GAA G</td>
</tr>
<tr>
<td>470 NHM -&gt; TNK</td>
<td>FP: CAC AGA TTG ACT AAT AAG GGC ATT TGT TCC TGT GGA CTC TAC AAA C</td>
<td>RP: GGA ACA AAT GCC CTT ATT AGT CAA TCT GTG AGG TTT GGG ACA CGA CTT G</td>
</tr>
<tr>
<td>479 LYKQ -&gt; AFKV</td>
<td>FP: TGT TCC TGT GGA GCT TTT AAA GTG CCT GGT GTG CCT GTG AAA TGG AAG AG</td>
<td>RP: AGG CAC ACC AGG CAC TTT AAA AGC TCC ACA GGA ACA AAT GCC CAT ATG ATT CAA TC</td>
</tr>
<tr>
<td>486 PVK -&gt; KTV</td>
<td>FP: CAG CCT GGT GTG AAA ACC GTG TGG AAG AGA TGA</td>
<td>RP: TCA TCT CTT CCA CAC GGT TTT CAC AGG CAC ACC AGG CTG TTT GTA G</td>
</tr>
</tbody>
</table>

Table 2. LCMV-LASV short segment mutants primers.
Primer sequences were designed to introduce the mutations listed to the left of each primer pair. The primers are named according to the LASV sequence being substituted and the LCMV sequence that replaced it.
Figure 17. LCMV pseudoviruses are not neutralized by mAb 8.9F but are neutralized by 12.1F. Pseudoviruses bearing the LCMV glycoprotein were titrated in TZM-bl cells following incubation with mAb 8.9F or 12.1F.
Figure 18. Flow cytometry data for short sequence mutants.
293T cells were transfected with the various mutants listed in (TABLE) and stained with mAb 8.9F and 12.1F. The histogram shows the relative mAb 8.9F intensity levels of each mutant. Those marked with a single asterisk (*) denote mutants that had a positive 12.1F signal. Mutants with two asterisks (**) were unreactive with mAb 12.1F. GP1 mutants are listed in the first chart (A) and GP2 mutants are listed in the second (B).
Figure 19. mAb 8.9F unreactive short sequence mutants produce cleaved glycoprotein.

293T cells were transfected with the three short sequence mutations identified in (FIGURE) and whole cell lysate was run on a western blot. Blots were probed with a GP2 mix. An antibody against the ribosomal S3 protein was used as a loading control.
Figure 20. Immunofluorescent images of mAb 8.9F-negative short segment mutants
Transfected cells were air-dried on glass slides before staining with the specified antibodies. DAPI counterstain was used to highlight the nucleus.
<p>| <strong>S121T</strong> | <strong>FP</strong> | CAA ATT TTG CAA TCT GAC CGA TGC CCA CAA AAA GAA CCT CTA TGA CC  |
|           | <strong>RP</strong> | CTT TTT GTG GGC ATC GTT CAG ATT GCA AAA TTT GTG ATT AAT AAT GCT CGT GTT G  |
| <strong>D122S</strong> | <strong>FP</strong> | AAA TTT TGC AAT CTG TCT TCT GCC CAC AAA AAG AAC CTC TAT GAC CAC  |
|           | <strong>RP</strong> | GTT CTT TTT GTG GGC AGA AGA CAG ATT GCA AAA TTT GTG ATT AAT AAT GCT CGT G  |
| <strong>H124F</strong> | <strong>FP</strong> | AAT CTG TCT GAT GCC TTC AAA AAG AAC CTC TAT GAC CAC GCT CTT ATG AG  |
|           | <strong>RP</strong> | GTC ATA GAG GTT CTT TTT GAA GCC ATC AGA CAG ATT GCA AAA TTT GTG ATT AAT AAT G  |
| <strong>K125N</strong> | <strong>FP</strong> | GTC TGA TGC CCA CAA TAA GAC CTT CTA TGA CCA CGC TCT TAT GAG  |
|           | <strong>RP</strong> | GGT CAT AGA GGT TCT TAT TGT GGG CAT CAG ACA GAT TGC AAA ATT TGT G  |
| <strong>P145R</strong> | <strong>FP</strong> | TTT CCA CTT GTC CAT CAG AAA CTT CAA TCA GTA TGA GGC AAT GAG CTG C  |
|           | <strong>RP</strong> | CTC ATA CTT ATG GAA GTT TCT GAT GGA CAA GTG GAA AGT TGA GAT TAT GCT C  |
| <strong>N146G</strong> | <strong>FP</strong> | ACT TGT CCA TCC CCG GTT TCA ATC AGT ATG AGG CAA TGA GCT GGC  |
|           | <strong>RP</strong> | CCT CAT ACT GAT TGA ACC CGG GGA TGG ACA AGT GGA AAG TTG AG  |
| <strong>P147N</strong> | <strong>FP</strong> | GTC CAT CCC CAA CAA CAA TCA GTA TGA GGC AAT GAG CTT CGA TTT TAA TG  |
|           | <strong>RP</strong> | ATT GCC TCA TAC TGA TTG TTG TTG AAA GTT GAG  |
| <strong>N148S</strong> | <strong>FP</strong> | TCC ATC CCC AAC TTC TCC CAG TAT GAG GCA ATG AGC TGC GAT TTT AAT G  |
|           | <strong>RP</strong> | CAT TGC CTC ATA CTG GGA GAA GTT GGG GAT GGA CAA GTG GAA AG  |
| <strong>Q149N</strong> | <strong>FP</strong> | CAT CCC CAA CTT CAA TAA CTA TGA GGC AAT GAG CTG CTA TAA TGG G  |
|           | <strong>RP</strong> | GCT CAT TGC CTC ATA GTT ATT GAA GTT GGG GAT GGA CAA GTG GAA AG  |
| <strong>R250K</strong> | <strong>FP</strong> | CTC TCA CAA AGG ACT AAG GAT ATT TAT ATT AGT AGA AGA TGG CTA GAC ACA TCC AC  |
|           | <strong>RP</strong> | CTT CTA CTA ATA TAA ATA TCC TTA GTC CTT TGT GAG AGG AGC CGG AG  |
| <strong>Y253F</strong> | <strong>FP</strong> | CAA AGG ACT AGA GAT ATT TTC ATT AGT AGA AGA TGG CTA GGC ACA TCC ACA TGG  |
|           | <strong>RP</strong> | TAG CAA TCT TCT ACT AAT GAA AAT ATC TCT AGT CCT TTG TGA GAG GAG CC  |</p>
<table>
<thead>
<tr>
<th><strong>Table 3. Primers used for single amino acid substitutions.</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>A series of primers were created to introduce single base substitutions into the full-length LASV glycoprotein. These single substitution mutants were designed based on the mAb 8.9F-unreactive segment mutants previously identified.</td>
</tr>
</tbody>
</table>
Figure 21. H124F, P145R, and F147N mutations prevent mAb 8.9F surface recognition on transfected cells.
293T cells were transfected with the mutants listed in (TABLE) and assayed for reactivity against mAb 8.9F and 12.1F. Of these mutants, H124F, P145R, and F147N had no detectable signal. The S255T mutation eliminated both mAb 8.9F and 12.1F recognition.
Figure 22. Immunofluorescent images of mAb 8.9F-unreactive mutants.
Antibody binding of single amino acid mutants was visualized via indirect immunofluorescence. Cells were stained with mAb 8.9F or 12.1F and nuclei were counterstained using DAPI.
Figure 23. Slot blot of mAb 8.9F unreactive pseudoviruses.

Pseudoviruses were produced in 293T cells and purified before being immobilized on nitrocellulose using the Bio-Rad Slot Blot apparatus. The antibodies used for staining are listed on the left of the figure. Monoclonal antibodies 12.1F and 13.4E are GP1 and GP2-specific antibodies respectively. The A32 anti-HIV antibody served as a negative control.
Figure 24. Neutralization assay of H124, P145R, and F147N pseudoviruses with mAb 8.9F and 12.1F.

Mutant pseudoviruses bearing mAb 8.9F unreactive glycoproteins were tested for neutralization by mAb 8.9F and 12.1F in TZM-bl cell assay. Antibodies were diluted in half-log steps. Results show the data from three independent experiments.
<table>
<thead>
<tr>
<th>Primer</th>
<th>FP</th>
<th>RP</th>
</tr>
</thead>
<tbody>
<tr>
<td>T81A</td>
<td>GGA GAC ACT CAA TAT GCC TAT GCC TCT CTC CTG CAC AAA GAA CAA C</td>
<td>CAG GAG AGA GGC ATA GCC ATA TTG AGT GTC TCC ATG TTT AGT TCC AGA G</td>
</tr>
<tr>
<td>S91A</td>
<td>CTG CAC AAA GAA CAA CGC TCA TCA TTA TAT AAT GGT GGG CAA TGA GAC AGG</td>
<td>CAC CAT TAT ATA ATG AGC GTC TGT TTG TGT GCA GGA GAG AGG C</td>
</tr>
<tr>
<td>T101A</td>
<td>GTG GGC AAT GAG GCT GGA CTA GAA CTG ACC TTG ACC AAC AC</td>
<td>GGT CAG TTC TAG TCC AGC CTC ATT GCC CAC CAT TAT ATA ATG ACT G</td>
</tr>
<tr>
<td>S111A</td>
<td>CCT TGA CCA ACA CGG CTA TTA TTA ATC ACA AAT TTT GCA ATC TGT CTG ATC</td>
<td>GCA AAA TTT GTG ATT AAT AAT AGC CGT GTT GGT CAA GGT CAG TTC TAG TC</td>
</tr>
<tr>
<td>S121A</td>
<td>CAA ATT TTG CAA TCT GCC TGA TGC CCA CAA AAA GAA CTA TGA CC</td>
<td>CTT TTT GTG GCC ATC AGC CAG ATT GCA AAA TTT GTG ATT AAT AAT GCT CGT G</td>
</tr>
<tr>
<td>S169A</td>
<td>GTG CAG TAC AAC CTG GCT CAC AGC TAT GCT GGG GAT GCA GC</td>
<td>CCA GCA TAG CTG TGA GCC AGG TTG TAC TGC ACA CTA ATC TTT CCC</td>
</tr>
<tr>
<td>T226A</td>
<td>CTG ATA ATC CAA AAT ACA GCT TGG GAA GAT CAC TGC CAA TTC TCG AG</td>
<td>GCA GTG ATC TTC CCA AGC TGT ATT TTG GAT TAT CAG ATA TTG ATA ACT AGT CAT AAT ACA GTC</td>
</tr>
</tbody>
</table>

**Table 4.** Primer sequences for serine/threonine to alanine mutations.
Figure 25. T81A and S169A eliminate mAb 8.9F surface binding.

293T cells were transfected with plasmids expressing full-length Lassa virus glycoprotein containing point mutations to sites of N-glycan attachment. Signal strength of individual mutants was standardized to the wild-type mAb 8.9F signal.
**Figure 26. Slot blot of S/T -> A mutations probed with mAb 8.9F and 12.1F.**

Pseudoviruses were affixed to nitrocellulose using the Bio-Rad Slot Blot apparatus to test for reactivity with neutralizing antibodies mAb 8.9F and 12.1F. Black asterisks represent where pseudovirus was attached. Red asterisks indicate mutants that produced a mAb 8.9F signal on flow cytometry analysis.

<table>
<thead>
<tr>
<th></th>
<th>8.9F</th>
<th>12.1F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>T81A</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>S91A</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>T101A</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>S111A</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>S121A</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>S169A</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>T226A</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>LCMV</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>wt control</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3

Effect of mAb 8.9F-abrogating Mutations on Glycoprotein Processing and Pseudovirus Function

Specific Aim 3.1
Introduction

This chapter will investigate the functional effects of the mAb 8.9F non-binding mutations (H124F, P145R, and F147N) that were identified in Chapter 2. Specifically, we will investigate how these mutations affect glycoprotein processing and pseudovirus infectivity. In order to understand the effects of these mutations within the context of current research, a review of the viral mechanisms for humoral immune escape and the resulting impact on viral fitness will be provided.

Viruses have developed several general mechanisms by which to avoid destruction by the humoral immune system. These include modulation or mimicry of Fc receptors, inhibition of Fc-mediated complement destruction, and antigenic variation. As a means to avoid downstream Fc-signaling, herpes simplex virus type 1 (HSV-1) encodes two proteins, gE and gI, that are capable of binding IgG. When expressed on the surface of infected cells, gE reduces antibody-dependent cellular cytotoxicity by binding the Fc portion of the antibody following Fab-mediated antigen recognition. The gE and gI proteins of HSV-1 can also form a secreted, soluble heterodimer that is capable of binding one IgG molecule per dimer. In addition to blocking the Fc portion of antibodies, viruses may also take advantage of the cellular Fc receptors. In the case of HIV-1, opsonized viruses are capable of infecting CD4+ T cells via the Fc receptor when they pass germinal centers containing the virus bound to follicular dendritic cells. Virus-induced signaling of Fc receptors has also been found to play a role in the pathogenesis of measles virus infection. Studies have demonstrated that measles virus nucleoprotein activates the
FcγRII receptor. This receptor provides negative regulation of B cell antibody production and antagonizes B-cell receptor signaling.

In addition to disrupting Fc-mediated internalization and signaling pathways, viruses have also developed methods for preventing lysis and opsonization by complement. In the case of HIV-1, the budding virions are able to concentrate the compliment control proteins CD55 and CD59 on their envelope. As a result, when HIV-1 virus was generated in cell lines containing a high concentration of compliment control proteins, the virus showed resistance to antibody-mediated lysis by compliment. Vaccinia virus is also capable of regulating the antibody-mediated classical complement pathway. Studies have shown the vaccinia complement-control protein is capable of binding C3b and C4b and serving as a cofactor in compliment degradation. When the gene encoding this protein was deleted, vaccinia virus became susceptible to complement-mediated neutralization on in vitro testing. Both these viruses provide examples of situations in which strong antibody responses may be insufficient to eliminate viral infections.

While Fc receptor blocking and inhibition of classical complement pathways provide a means for some viruses to cope with humoral immune responses, neither of these mechanisms appears to play a critical role in the ability of arenaviruses such as Lassa virus to avoid neutralization. In part, this is due to the relatively low number of genes encoded by RNA viruses relative to their DNA counterparts. Also, the immunological escape mechanisms described above are understood to aid in establishing latency. As Lassa virus is an acute infection, it is unlikely that the virus will adapt these strategies.
For Lassa virus and other small RNA viruses, antigenic variation provides a key adaptive advantage against the host immune system. Antigenic variation refers to the process by which spontaneous genomic mutations allow a virus escape host immune mechanisms. As a result, a new viral population containing the mutation is established. Mutations readily arise during arenavirus replication due to the low fidelity and lack of proofreading capabilities of the RNA-dependent RNA polymerase. Mutations of the surface glycoproteins are particularly important in avoiding neutralization. While the majority of these mutations will result in the formation of defective particles, some will retain their infectivity while escaping antibody recognition. This new viral population will then expand to replace the susceptible population. During this time, the host immune response will stimulate affinity maturation of other B-cell lineages and produce a new set of neutralizing antibodies. The interplay between viral mutations and host immune adaptations is an endless, dynamic process.

Studies on hepatitis C (HVC) treatments provide a well-documented example of the dynamic relationship between viruses and neutralizing antibodies. In a recent study, researchers examined the viral adaptations caused by incubating HCV with increasing concentrations of different antibodies targeting the E2 subunit. While two of these antibodies CBH-2 and HC-11, reacted with a similar epitope, the escape mutations that developed in response to treatment varied depending on the antibody used. For CBH-2 escape mutations, these changes did not affect viral fitness whereas the HC-11 escape mutations had compromised binding to the cellular receptor for the virus. When testing the third antibody, HC-1,
researchers were unable to recover any escape mutants. Following epitope mapping, researchers found that the residues necessary for HC-1 antibody binding overlapped with the residues used in cell binding. In addition to antibodies with similar epitopes causing different viral mutations, antibody concentration may also direct viral neutralization escape. When HCV was grown in the presence of antibody HC 33.1, unique escape pathways were noticed at different antibody concentrations. At higher mAb concentrations, mutations appeared that reduced virus infectivity but reverted back to the native phenotype once the antibody was removed. For the viruses grown in a low concentration of HC 33.1, variants exhibited stable mutations that improved in vitro fitness and reduced antibody binding. Studies have also identified several glycan deletions on the envelope of HCV that increase receptor binding and neutralizing antibody recognition. These N-linked glycans may act as a mechanism for obscuring neutralizing epitopes that play a critical in virus function. Taken together, these results suggest that simply identifying an antibody epitope is insufficient for understanding its therapeutic potential. Additional steps must be taken to understand how the virus may escape neutralization and how the mutations affect viral fitness.

While antigenic variation may seem a never-ending battle for the humoral immune system, two recent studies of HIV broadly neutralizing antibodies (bNAb) suggest a successful vaccination strategy is not impossible. These studies could provide a new methodology for approaching vaccine design. In the first study, researchers performed a longitudinal study characterizing the viral populations of two patients who produced a PGT121-like bNAb. At the time of initial diagnosis, the
HIV strain infecting these patients lacked the N332 glycan critical in PGT121 recognition. Within six months of infection some quasispecies had mutated to include a glycosylation site at that position. And by 15 months when a bNAb similar to PGT121 was first detected, all quasispecies within the host tested positive for the mutation. Using longitudinal sequencing data from HIV infected individuals, researchers found this antigenic shift was not uncommon. These early results suggest that immune pressure can influence pathogens to expose epitopes that elicit bNAbs. Even more impressive were recent findings by Gao, et. al. in which two separate B-cell lineages cooperated to direct the antigenic changes of HIV towards producing a bNAb epitope. In their proposed mechanism, a helper B-cell line selected D-loop mutants that, while escaping its own neutralizing antibodies, would produce an epitope more easily recognized by a bNAb B-cell lineage and thus promote affinity maturation and expansion. Based on these results, potential vaccines could be designed to guide the humoral immune system through the necessary epitope intermediates resulting in a robust expression of bNAbs. Antigenic variation of viruses should not be viewed merely as a mechanism of immune escape, but also as an exploitable trait.

Thus far we have covered how viruses escape neutralizing antibodies by directly altering the sensitive epitope. In addition, antigenic sites on viruses may stimulate the production of non-neutralizing antibodies than can disrupt the binding of neutralizing antibodies (Figure 27). The figure by Nicasio, et. al. summarizes the mechanisms for non-neutralizing antibody inhibition as well as those mechanisms discussed earlier in the chapter. Two main mechanisms for
this antibody-mediated interference have been proposed. In the first mechanism, the interfering antibody binds an epitope that sterically hinders neutralizing antibody binding while in the other binding of the interfering antibody induces a structural change in the neutralizing epitope. During HCV infection, it has been determined that non-neutralizing antibodies are produced against a closely located epitope of the E2 subunit where neutralizing antibodies bind. When sera from chronically infected monkeys was tested for neutralization, non-neutralizing antibodies appeared to interfere with nAb binding. If these non-neutralizing antibodies were depleted, serum neutralization potency was increased. Similar observations have been made in the case of influenza virus where an early non-neutralizing antibodies response correlated with severe disease. The role of non-neutralizing antibodies in influenza infection is of particular concern as multiple infections with different strains may occur over a lifetime. If non-neutralizing antibodies epitopes are maintained among different strains, this would promote the early production of non-neutralizing antibodies and complicate the development of strain-specific neutralizing antibodies.

In summary, the antigenic changes resulting from pressures of humoral immunity have a wide impact on viral fitness, antibody development, and host survival. By improving our understanding of this complicated interplay of factors, we will be able to develop better vaccines that not only potently neutralize, but also prevent the generation escape mutants. While we do not have a robust understanding of the escape mutations generated by Lassa virus during natural infection, the three mutations we identified in Chapter 2 do provide us with a small
window into investigating the impact of neutralization-resistant mutations on viral fitness. In this chapter we will examine how the H124F, P145R, and F147N mutations affect glycoprotein processing and pseudovirus function.

**Methods**

**Cell Culture**

See General Materials and Methods

**Western blot**

Protein lysate from cells and pseudoviruses were analyzed by western blot as described in the General Materials and Methods section. A mix of mAb 4.1F, 13.4E, and 24.6C antibodies at 2µg/ml was used to probe for GPC/GP2 cleavage. This was followed by incubation with IRDye 680RD Goat anti-Human IgG secondary diluted 1:10,000. mAb 183, a murine antibody against p24 was used as a loading control with pseudovirus samples. IRDye 800CW Goat anti-Mouse IgG diluted 1:10,000 was used for secondary staining. Ribosomal S3 protein was used as the loading control in experiments with cell extract. A rabbit anti-human S3 antibody diluted 1:1,000 followed by IRDye 800CW Goat anti-human antibody diluted 1:10,000 were used for detection.

**Flow Cytometry**

The basic protocol we used for analyzing cells via flow cytometry is described in the General Materials and Methods section. In this chapter we measured cell-surface expressed glycoprotein and total glycoprotein. For the latter experiment, cells were permeabilized by incubation in 100µL of 0.1% Triton X
before addition of the primary antibody. The primary antibodies used for these experiments were mAb 8.9F, 12.1F, or a mix of GP2 antibodies composed of mAb 4.1F, 13.4E, and 24.6C. The secondary antibody used for these experiments was a FITC-conjugated Goat anti-Human IgG Fc antibody diluted 1:500.

**Indirect Immunofluorescence**

See General Materials and Methods

**Pseudovirus purification and titration**

See General Materials and Methods. Pseudovirus titration was done in triplicate and repeated three times.

**Cell binding assay**

Pseudoviruses were generated and purified as described in the General Materials and Methods section. Vero E6 cells were seeded in 6-well plates and grown to 80% confluency in DMEM+10% FBS. Media was removed and cells were washed twice with cold PBS. 100uL of concentrated pseudovirus was diluted in 400uL PBS and then added to the cells. Pseudovirus was incubated while rocking the cells on ice for 1 hour. Following incubation, cells were washed with cold PBS on ice to remove any unbound virus. Cells were then lysed with RIPA buffer. Following cell lysis, the standard western blot protocol was followed. A mix of GP2 antibodies (4.1F, 13.4E, 24.6C) diluted to 2ug/ml was used as the primary probe to test the western blot for viral proteins.

**Statistical Analysis**

Data analysis was performed using the Prism 6 software (GraphPad Software Inc., La Jolla, CA). The threshold for significance was set at 95% (p < 0.05) for all
experiments. Comparisons between different glycoprotein mutants relative to the wild type were performed using a ordinary one-way ANOVA. Virus titration and antibody neutralization experiments were done in triplicate and repeated at least twice.

**Results**

**H124F, P145R, and F147N mutations do not compromise glycoprotein processing**

To begin our investigation, we first assessed glycoprotein precursor cleavage by measuring the strength of the GPC and GP2 bands from the lysate of cells transfected with mAb 8.9F unreactive glycoprotein. The percent cleavage was measured by dividing the GP2 signal by the GPC+GP2 signal. The H124F mutation maintained the highest level of cleavage relative to the control at 91.3%. Mutations P145R and F147N cleaved GPC at 69.9 and 83.6%, respectively. Based on these results, mutations that affect mAb 8.9F binding do not have a major effect on protein cleavage. In addition to the total protein produced by these mutations, we measured surface glycoprotein levels to test if there were any effects on protein trafficking to the membrane. When we measured transfected cells that were stained with the GP2 antibody mix, we found that mAb 8.9F-abrogating mutations did not significantly inhibit surface expression. Two mutants, H124F and P145R had higher surface signals than the control while the expression level of F147N was 96% of the wild type (Figure 28). Based on these findings, we concluded that these mutations
eliminating mAb 8.9F binding are not critical to the cleavage or surface transport of Lassa virus glycoprotein.

**Short segment mutant 250 RDIYIS is capable of cleaving GPC while S255T inhibits glycoprotein production.**

When researching mAb 8.9F non-binding mutants, we identified region 250 RDIYIS as a potential antibody binding site (Figure 18). However, when single mutations within this region were made, we discovered the S255T mutation completely prevented binding of mAb 8.9F and 12.1F, which was being used as a marker for correct protein folding. To investigate the cause of this observation, we performed western blotting and immunofluorescence experiments. When comparing S255T to the 250 RDIYIS mutant, no GPC or GP2 was detectable for the S255T mutant (Figure 30). As expected, immunofluorescent images confirmed flow cytometry results (Figure 31).

**H124F, P145R, and F147N result in the formation of noninfectious pseudoviruses**

While glycoprotein processing and surface transport is maintained with mutations, we also investigated how these mutations could impact the formation of infectious pseudoviruses. In order to test how mutated glycoprotein is integrated into pseudoviruses, we ran purified samples on western blot and probed with GP2 mAbs. On analysis, H124F mutants had the strongest GP2 signal, and the signal of F147N also appeared to be slightly stronger than the control. The P145R mutant had
a lower signal relative to the control. Despite differences in GP2 integration, pseudoviruses with mAb 8.9F-abrogating mutations are able to integrate glycoprotein into the budding pseudovirion (Figure 32). Following glycoprotein integration, we measured the ability of these pseudoviruses to bind its cellular receptor, alpha-dystroglycan, in Vero E6 cells. Results from cell binding experiments show a faint, but still detectable GP2 signal for each of the mutants (Figure 33). Due to the poor signal strength for both the mutants and controls, no quantitative analysis was performed. Finally, we titrated pseudoviruses in TZM-bl cells. While all viruses produced a detectable signal, the relative infectivity of these particles was much lower than the wild-type virus. Pseudoviruses H124F, P145R, F147N were 20%, 2%, and 5% as infectious as the wild type, respectively. Based on these results, the mutations we have previously identified in Chapter 2 as abrogating mAb 8.9F binding also appear to have a profound impact on the formation of infectious pseudoviruses. Of these mutations, P145F has the lowest pseudovirus expression of GP2 in addition to the poorest titer.

We also evaluated pseudovirus infectivity for single base mutations within the 121 SDAHK and 145 PNFNQ regions that did not abrogate mAb 8.9F binding. These results showed that D122S and N148S mutations also reduced pseudovirus infectivity to the levels at or below H124F. S121T and K125N mutations lowered infectivity to approximately 50% of wild-type pseudovirus. Finally, the infectivity of N146G and Q149N particles were at or above the levels of the wild type. As a result of these observations, we conclude that a low pseudovirus titer does not necessarily guarantee poor mAb 8.9F binding. We also conclude that while H124F, P145R, and
F147N maintain the ability to produce pseudovirions and bind the cellular receptor for Lassa virus, these mutations profoundly compromise infectivity.

**Discussion**

Understanding the adaptations of viral antigens when placed under selective pressure by antibodies is a key facet of effective vaccine design. By discovering which antigenic changes are favored as well as their impact on viral fitness, researchers will be able to determine the correct antibody composition for prophylactic treatments and improve monitoring of vaccinated populations for escape mutants. To that end, this chapter sought to simulate adaptations that Lassa virus glycoprotein could obtain during natural infection if it were under pressure from mAb 8.9F. These adaptations were tested by examining how the changes impacted key steps in glycoprotein processing, pseudovirus formation, and, most importantly, infectivity.

We found that mutations capable of escaping mAb 8.9F neutralization can still properly traffic the glycoprotein and form stable pseudoviruses; however, the particles are severely limited in their infectivity. Of the mutations we identified, P145R seems the least likely to provide a suitable escape route. In addition to an extremely low titer, the P145R mutation resulted in the lowest amount of cleaved GP2 protein, which is a requirement for pseudovirus formation. The low levels of GP2 are also reflected in weak signal of purified P145R on western blot. The H124F mutation seems to provide a more preferential escape mutant in regards to minimizing the impact on pseudovirus composition and function. H124F cleaved the
precursor glycoprotein at nearly the same levels as wild type. Also, H124F
glycoprotein appeared to be incorporated into budding pseudoviruses at a higher
level than the wild-type pseudovirus. Despite the high level of incorporation, H124F
only produced a mildly infectious virus. This observation may be due to the
mutation disrupting interactions between subunits thus preventing the formation of
fusogenic trimer. In regards to our finding that the S255T mutation did not produce
detectable glycoprotein, it may be the case that a frameshift error occurred during
PCR mutagenesis. In order to clarify these results, we will repeat S255T
mutagenesis as well as reevaluate the primer design. It is possible that while
expanding the plasmid stock, a random mutation was inserted that eliminated
protein translation. It will also be necessary to created additional single mutants of
the 250 RDYIS region that lack residues 251 and 252. These amino acids were
deleted in the short segment mutant, but included in the single amino acid
substitution mutants.

Several additional experiments should be undertaken in order to better
understand the structural and functional implications of mAb 8.9F-abrogating
mutations. The most important experiment would be to passage the virus under
BSL-4 conditions with low concentrations of mAb 8.9F to gain better insight into
how the virus glycoprotein would adapt to antibody selection pressures in vitro.
Escape mutants generated from those experiments could be evaluated in a manner
similar to the methods we used in this chapter. Additionally, escape mutants could
be serially passaged without antibody pressures to see if the virus would revert
back to its wild-type sequence. This would help us determine if mAb 8.9F-
abrogation mutations are stable within the viral genome. As we have demonstrated that our mAb 8.9F-abrogating mutations still retain some infectious activity, a more thorough investigation of how these mutations affect receptor interactions is warranted. As our cell-binding experiment proved relatively inconclusive, using a more precise measure of receptor-ligand binding such as surface plasmon resonance (SPR) would be helpful. SPR is able to measure changes in the angle of polarized light reflected from a surface bound with protein. In our case, the surface would be coated with the alpha-dystroglycan receptor and pseudovirus-purified glycoprotein could be added. The results would give insight into whether mAb 8.9F-abrogating mutations affected receptor binding or played a larger role in disrupting the function of GP2-mediated fusion. Developing a recombinant glycoprotein construct able to form stable trimers of the extracellular domain would also be helpful in understanding how these mutations affect receptor binding. Generation of this construct could also aid us in our attempt to map mAb 8.9F binding by X-ray crystallography. This procedure has been effective in the mapping of HIV antibodies with complex epitopes. To measure how the GP1 mutations we identified affected GP2 function, we could perform experiments measuring GP2-mediated cell fusion. For this experiment, cells would be transfected with the full-length glycoprotein and allowed to express the glycoprotein for 24hrs. Cells media would be replaced with an isotonic buffer of pH 5.0 to trigger GP2-mediated cell fusion. Giemsa staining would be used to visualize and count the number of nuclei per fused cell. By using these techniques, we would gain a more robust and clinically applicable understanding of how mAb 8.9F escape mutations affect viral fitness.
A. Escape mechanisms from humoral response against surface viral proteins

Figure 27. Schematic representation of viral antigenic escape methods. Figure adapted from 184.
Figure 28. Mutations abrogating mAb 8.9F binding do not significantly affect GPC cleavage.
293T cells were transfected with plasmids encoding 8.9F unreactive full-length glycoprotein or the wild-type glycoprotein. Cell lysate was run on western blot and probed with GP2 antibodies. The percent cleavage was calculated by densitometric analysis with Image Studio.
Figure 29. Surface transport of GP1 for mAb 8.9F-unreactive mutants is comparable to wild type.
293T cells were transfected with plasmids encoding the full-length glycoprotein as well as mAb 8.9F unreactive mutants. Transfected cells were washed and surface stained with a GP2 antibody mix. The GP2 signal levels were measured via flow cytometry.
Figure 30. The S255T mutation produces no detectable GPC or GP2 signal.
293T cells were transfected with plasmids encoding full-length glycoprotein including single mutations of the 250 RDIYIS region. Western blotting using GP2 mAb probes assessed glycoprotein cleavage. Ribosomal S3 protein was used as a loading control.
Figure 31. Immunofluorescent images of 250 RDIYIS single mutations. 293T cells were transfected with plasmids encoding full-length glycoprotein with single mutations of the 250 RDIYIS region. Cells were washed and air-dried to glass slides. The primary antibodies used for staining are listed to the left of the figure.
Figure 32. Mutations eliminating mAb 8.9F binding do not prevent glycoprotein incorporation into pseudoviruses.

293T cells were transfected to produce pseudoviruses for each of the mAb 8.9F abrogating mutations. Pseudoviruses were purified on a sucrose gradient and resuspended in lysis buffer before probing on western blot. Viral glycoprotein incorporation was assessed with a GP2 mAb mix. Levels of p24 were used as a loading control for pseudoviruses.
Figure 33. Pseudoviruses with mAb 8.9F-abrogating mutations are able to bind Vero E6 cells.

293T cells were transfected to produce pseudoviruses for each of the mAb 8.9F abrogating mutations. Pseudoviruses were purified and incubated with Vero E6 cells at 4°C for one hour to allow cell binding. Unbound virus was removed by PBS wash and cells with bound virus were lysed and analyzed on western blot.
Figure 34. Mutations affecting mAb 8.9F binding reduce pseudovirus infectivity.
293T cells were transfected to produce pseudoviruses containing single amino acid mutations from the 121 SDAHK and 145 PNFNQ regions. Pseudovirus infectivity was quantitated via TZM-bl infection assay. Results show the average infectivity from three experiments.
Chapter 4
Role of N-linked Glycosylation in Lassa Virus Glycoprotein Processing and Function

Specific Aim 3.2
**Introduction**

N-linked glycosylation (NLG) sites of viral glycoproteins (GP) have been shown to influence protein processing, receptor binding, and modulation of host immune responses \(^\text{191,192}\). In this chapter we wanted to examine specifically how the N-linked glycosylation sites found in the GP1 receptor binding subunit of Lassa virus affect glycoprotein processing and infectivity. In order to place our research in context of the current work being done in this field, we will briefly review key studies examining the impact of glycosylation on viral fitness. In addition, we will cover the current glycan research being done on Lassa virus and related arenaviruses.

N-linked glycosylation is a host process of post-translational protein modification that has been found to occur in both animal and insect cells. The basic principles of this process involve attachment of a core glycan structure to the amide nitrogen of asparagine found in the conserved Asn-X-Ser/Thr motif \(^\text{193}\). The glycan core consists of a conserved trimannosyl-chitobiose structure that is further modified by the attachment of additional mannose and glucose sugars. This glycan structure is synthesized on a dolichol carrier lipid found in the ER. Following synthesis, the entire branched-chain glycan is transferred via an oligosaccharyltransferase to the protein as it is being translated \(^\text{194}\). As the glycoprotein transitions from the ER to the cis-Golgi, glycosidases may remove attached glucose molecules in order to promote proper folding. Once inside the Golgi network, the glycoprotein can be modified by a number of glycosyltransferases and glycosidases to create one of the three major types of N-
linked glycans: high-mannose, complex, or hybrid. As its name suggest, high-mannose glycans are those composed exclusively of mannose bases. As these glycans are synthesized early in the post-translational processing pathway, protein folding is thought to protect the sugars from further modification by glycosidases. Complex glycans refer to attached oligosaccharides that contain a number of various glycans including galactose, fucose, or N-acetyl glucosamine. Hybrid glycans are those that share characteristics of both high-mannose and complex N-glycans. In addition to different sugar residues, the number of terminal chains in the attached glycan may vary, ranging from two to four branches. 

While the N-linked glycosylation pathway normally functions in the alteration of host proteins, many different viruses have been found to co-opt these enzymatic processes. Glycosylation of viral proteins has been demonstrated to have a role in glycoprotein trafficking, attachment, and pathogenicity. One widely studied example is the hemagglutinin (HA) subunit of the influenza virus. HA functions in cell receptor binding and cell-virus membrane fusion. Possible locations of HA glycosylation range from five to eleven sites depending on the strain. Correct glycosylation of HA is important as elimination of N-linked glycosylation sites has been demonstrated to inhibit proper protein folding. In addition to influencing correct protein folding, glycosylation can also have an effect on the function of the HA molecule. In order for HA to bind its sialic acid receptor, HA must first be cleaved by host proteases. However, if a glycan is located too close to the cleavage site, HA is unable to be processed and this results in abolished infectivity. Glycosylation sites in HA have also been demonstrated to modulate the strength of receptor
binding. When pseudoparticles lacking two NLG sites from the receptor binding region of HA were generated, the resulting mutants were unable to be cleaved from the sialic acid of erythrocytes by the viral neuraminidase due to the strength of the receptor interaction\textsuperscript{199}. As a result, these mutant viruses showed a decrease in titer due to the poor release of budding particles\textsuperscript{200}. Interestingly, the deficiency in virus release could be overcome when the neuraminidase gene was swapped to a more enzymatically active subtype\textsuperscript{200}. These results suggest that changes in N-linked glycosylation sites can impact viral fitness and reassortment.

N-linked glycosylation of envelope proteins has also been demonstrated to affect West Nile virus assembly and infectivity. WNV contains two distinct lineages (L1 and L2) with the L1 lineage being linked to more severe disease and neuroinvasiveness. The envelope of WNV contains two proteins, the premembrane (prM) and envelope (E). The prM subunit contains one glycosylation site that is shared between the lineages while the L1 strain has a unique glycosylation site in its E subunit. The glycosylation site on the envelope protein has been demonstrated to play a major role in the neuroinvasiveness of the L1 strain versus the L2 strain. When four strains of WNV isolated from patients in 1999 were used to experimentally infect mice, the two strains containing the N-linked glycosylation site resulted in lethal infection while the mice infected with the non-glycosylated E protein had lower mortality. Differences in mortality between the two groups were abolished when mice were infected by intracerebral injection. These findings suggest that the glycosylation site played a direct role in allowing the virus to enter the CNS\textsuperscript{201}. The effect of prM and E glycosylation on viral particle production and
infectivity was examined using a reverse-genetics system. In this study, researchers found that the elimination of the NLG site in prM reduced the number of sub-viral particles released and lowered the infectivity of reporter particles. The most interesting result was seen when the NLG site of the E protein in the lineage II virus was removed. When tested in mammalian cells, viruses containing glycosylated E protein resulted in a slight decrease in infectivity relative to the non-glycosylated form. However, when tested in C6/36 insect cells, virus containing a glycosylated E protein was 30-times less efficient at infection. These results suggest glycosylation may have a variable impact on viral replication in different host systems. This finding is of particular interest as Lassa virus also replicates in different host systems albeit both are mammals.

Viral glycoproteins may alter their surface N-linked glycans to escape immune pressures and hide sensitive epitopes. This is particularly well characterized with the HIV virus. The surface envelope protein gp120 in HIV contains between 18 and 33 potential N-linked glycosylation sites with the average being 25. In addition, these potential sites are frequently glycosylated with as much as half the mass of gp120 being the result of polysaccharides. Studies examining how the HIV virus adapts to the host during the course of infection have led to the proposal of a ‘glycan shield’ that acts to mask neutralizing epitopes while maintaining the receptor-binding function of the glycoprotein. The glycan shield was first proposed by Wei, et. al. when studying the escape patterns of the virus in recently infected patients. They found that the escape mutations were relatively sparse, did not include known neutralizing epitopes, and involved N-linked
glycosylation sites. Additionally, when pseudoviruses containing the naturally occurring mutations were assessed in neutralization assays, researchers found that the changes in N-linked glycosylation patterns conferred resistance to autologous and epitope-specific neutralizing antibodies. These results suggest that a rapidly adapting rearrangement of glycosylation patterns aid in escape from host antibodies. The theory of a glycan shield to protect against neutralizing antibodies was further supported by research on the V1/V2 region of HIV gp120. The V1/V2 region is believed to act as a shield that protects the receptor-binding region. In this study, potential N-linked glycosylation sites were found to accumulate in the V1/V2 region of HIV over the two-year period examined following infection. When the V1/V2 sequences from chronically infected patients were tested in a neutralization assay, researchers found these mutant strains showed reduced neutralization sensitivity without seriously affecting receptor interactions. In addition to masking epitopes, some N-linked glycosylation sites may compose neutralizing epitopes. Such is the case for the epitope recognized by the broadly neutralizing antibody 2G12. This antibody was determined to recognize an epitope composed of mainly high mannose N-glycans. This epitope is highly conserved through HIV-1 strains and these glycans are likely to play an important role in viral fitness.

Viral glycosylation patterns also modulate innate immune pathways. This is primarily believed to occur through interactions of the virus glycoprotein with C-type lectin receptors (CLRs). CLR stimulation can down-regulate proinflammatory cytokine release, which in turn prevents dendritic cell maturation and promotes
continued infectivity. For example, WNV E protein glycosylation has been shown to modify host responses in a glycan-dependent manner. When testing the neuroinvasive, glycosylated E protein, researchers found that the release of proinflammatory cytokines IL-1B and TNF-a was increased relative to the non-glycosylated form. Immune responses to WNV infection are also dependent on host-specific glycosyltransferases. When the virus is generated in insect cells, the E protein contains a larger number of high-mannose glycans in relation to virus generated in humans. When the viruses are used to infect plasmacytoid dendritic cells, the insect-generated virus causes lower INF-a expression. The high-mannose glycosylation pattern of insect-derived WNV has also been implicated in reducing cell signaling responses to double-stranded RNA in the cytoplasm. In summary, the glycosylation of viral envelope proteins serve a multitude of purposes. Not only can glycosylation patterns affect the formation of stable and infectious virions, but glycosylation patterns may also arise as to modulate immune responses and escape antibody recognition.

In comparison to our knowledge of the effects of surface glycosylation on HIV and influenza, our understanding of how N-linked glycans modulate arenavirus glycoproteins is still in its early stages. The glycosylation sites in the GP2 proteins of arenavirus are relatively conserved at four sites while sites within the GP1 subunit may vary from six to eight. For Lassa virus, all seven sites in GP1 and the four sites in GP2 have been shown to be glycosylated. Some of these sites were found to be critical for glycoprotein cleavage while others were dispensable. A study examining the effect of the glycosidase EndoH on Lassa virus GP processing suggested that
GP1-GP2 cleavage is necessary for complex N-glycosylation\textsuperscript{162}. The importance of N-glycosylation at particular sites may vary according to species. A study by Bonhomme, et. al., found that NLG sites critical for glycoprotein cleavage in Lassa virus were not conserved at the analogous sites within LCMV\textsuperscript{209}.

At this point in time, no work has been done to examine how N-linked glycosylation may impact Lassa virus infectivity. However, research similar to this has been performed with LCMV virus\textsuperscript{210}. These studies found NLG sites to mediate infection through inhibition of glycoprotein processing or GP2-mediate fusion. In the first case, three non-infectious mutants were identified that were unable to cleave GPC. As other studies have demonstrated that glycoprotein cleavage is a necessary step in arenavirus assembly, this is a likely mechanism for the reduction of infectivity in these mutants\textsuperscript{21}. Based on data from the same study, two additional NLG mutants were identified that, while able to fully process GPC, did not form infectious virus. These mutants were assessed by DBT cell fusion assay and found to have poor fusogenic activity. Thus, N-glycans appear to affect GP2-mediated fusion. Virus fitness and tissue specificity are also influenced by the deletion of NLG sites. When LCMV viruses missing single glycosylation sites were used to infect macrophage and neuronal cell lines, researchers found five mutations with altered tropism and growth kinetics plus three mutants that spontaneously restored the NLG site during serial passage\textsuperscript{209}.

The purpose of this chapter is to examine how N-linked glycosylation affects the Lassa virus glycoprotein. Here we use the novel approach of creating two different glycoprotein mutants for each of the NLG sites identified in the GP1
subunit. Through this approach we will better separate the effects caused by amino acid substitution from those caused by N-glycan deletion. The results of these experiments will increase our understanding of the functional role of N-linked glycosylation in Lassa virus.

**Methods**

**Cell Culture**

See General Materials and Methods

**Design of N-glycosylation mutants**

We used the NetNGlyc prediction software as well as previous research identifying glycosylation sites in the Josiah glycoprotein to locate residues suitable for site-directed mutagenesis. In addition to the mutants designed for Chapter 2, we created additional N-glycan deletions by substituting the asparagine in the Asn-X-Ser/Thr motif with a glutamine residue. We included at least two base changes in the mutant codon as a way to safeguard against spontaneous mutations reverting the glycoprotein to its wild type amino acid sequence.

**Site-directed mutagenesis**

See General Materials and Methods section.

**Western blotting**

Protein lysate from cells and pseudoviruses were measured by western blot as described in the General Materials and Methods section. A mix of mAb 4.1F, 13.4E, and 24.6C antibodies at 2ug/ml was used to probe for GPC/GP2 cleavage. This was followed by incubation with IRDye 680RD Goat anti-Human IgG secondary
diluted 1:10,000. The p24 protein was used as a loading control with pseudovirus samples and measured via staining with mAb 183, a murine antibody to p24. IRDye 800CW Goat anti-Mouse IgG diluted 1:10,000 was used for secondary staining of mAb 183. Ribosomal S3 protein was used as the loading control in experiments with cell extract. Rabbit anti-human S3 antibody was used as a loading control for transfected cells. The antibody was diluted 1:1,000 followed by IRDye 800CW Goat anti-human antibody diluted 1:10,000 for western blot detection.

**Indirect Immunofluorescence**

See General Materials and Methods and Chapter 1 for the protocol. Antibodies used for staining are listed with the figure legend.

**Flow cytometry**

The basic protocol we used for analyzing cells via flow cytometry is described in the General Materials and Methods section. In this chapter we measured cell-surface expressed glycoprotein and total cell glycoprotein. For the latter experiment, cells were permeabilized by incubation in 100uL of 0.1% Triton X before addition of the primary antibody. The primary antibodies used for these experiments were mAb 8.9F, 12.1F, or a mix of GP2 antibodies composed of mAb 4.1F, 13.4E, and 24.6C. The secondary antibody used for these experiments was a FITC-conjugated Goat anti-Human IgG Fc antibody diluted 1:500.

**Pseudovirus purification and titration**

See General Materials and Methods for detailed protocols. Pseudovirus titration was done in triplicate and repeated twice.

**Slot blot**
See General Materials and Methods section.

**Statistical Analysis**

Data analysis was performed using the Prism 6 software (GraphPad Software Inc., La Jolla, CA). The threshold for significance was set at 95% (p < 0.05) for all experiments. Comparisons between different glycoprotein mutants relative to the wild type were performed using an ordinary one-way ANOVA. Virus titration experiments were done in triplicate and repeated at least twice. Measurements of GPC cleavage were performed three times. Comparisons between different mutations of the same glycosylation site were performed using student’s t-test.

**Results**

**Removal of N-linked glycosylation sites affects GPC cleavage**

In order to test how N-linked glycosylation sites affect GPC cleavage by the S1P/SKI-1 protease, we created full-length GPC constructs containing GP1 NLG site deletions and transfected them into 293T cells. 48 hours post-transfection the cells were lysed and the samples were run on western blot. As the mutations were all located in the GP1 region, we probed the blots with a mix of GP2 specific antibodies in order to avoid confounding errors that might occur from using GP1 mAbs. The blots were analyzed with Image Studio software in order to quantify total protein produced (GPC+GP2) and the cleavage ratio (GP2/ total GP). When examining the western blot, we found two mutations, N89Q and S169A, that did not produce a cleaved GP2 signal. The S169A GPC band also ran significantly lower than GPC bands from the other mutations (Figure 35). The values for total GP produced in these
mutants ranged from 83% to 106% as compared to the wild type (Figure 36). While the total GPC production did not vary widely between mutants, great variability was observed in the cleavage of Lassa virus GPC into its subunits. As expected, the N89Q and S169A mutations showed the lowest GP2 production. In addition, N109Q, N119Q, N167Q, T81A, and S91A all resulted in lower GP2 levels than found in the wild-type mutation (Figure 37). There were no mutations that significantly increased GPC cleavage above the activity of the wild type. Based on these results, we conclude that mutations of NLG sites play a diverse role in proteolytic cleavage with some sites, such as N89Q, being essential while mutations such as T101A show no difference as compared to the wild type. Glycosylation sites likely have virus and perhaps strain-specific effects on cleavage as evidenced by the observation that deletion of analogous NLG sites in LCMV do not eliminate glycoprotein cleavage 210.

**N-glycans present on GP1 affect surface transport**

As glycoprotein cleavage has been shown to be independent of glycoprotein transport to the surface of cells, we used flow cytometry and immunofluorescence to measure how NLG mutations affected GP2 surface expression. GP2 surface signals were detectable for all transfected mutants suggesting that single N-linked glycans in GP1 are not an absolute requirement for surface transport. The signal strength of the mutants ranged between 130% and 65% of the wild-type control (Figure 38). Thus, NLG sites may influence glycoprotein transport without abolishing it. Also, these results further confirmed the observation that GPC cleavage is unnecessary for surface transport. The surface signal for the N89Q mutant was very similar to
wild type despite the fact that cleaved GP2 was barely detectable for this mutant. In addition, the N169A mutation that produced a slightly smaller GPC product on western blot was detectable with a signal equal to 77% of the wild type. As we were able to detect S169A, this suggests that the truncation caused by this N-glycan mutation did not remove the GP2 epitopes used in detection. The results of immunofluorescent image analysis provided similar conclusions as the flow cytometry data except in the case of N89Q (Figure 39). When observed under the microscope, N89Q-transfected cells did not produce a visible signal.

**Deletion of N-linked glycosylation sites affects pseudovirus assembly**

To date, the role of NLG sites in Lassa virus assembly is unknown. In order to explore this relationship, we tested the effect that single NLG deletions in the GP1 region have on the ability of Lassa pseudoviruses to integrate GP2 into the budding pseudovirus. In order to test the impact of these substitutions, we generated pseudoviruses by co-transfection of 293T cells with the GPC plasmid and the SG3 backbone. Generated pseudoviruses were ultracentrifuged through a 30% sucrose gradient in order to remove any glycoprotein that was not virion associated. The pelleted pseudovirus was resuspended and run on a western blot probing for GP2 and p24. The p24 protein served as a loading control to confirm that cells were transfected. Analysis of the western blot identified three N→Q mutations (N89Q, N109Q, N119Q) and three S/T→A (T81A, S91A, and S169A) mutations without a detectable GP2 signal (Figure 40). The absence of a signal for N89Q and S169A was expected, as mutants unable to cleave GPC do not integrate glycoprotein into the
budding virus membrane. Of the mutations able to cleave GPC, N99Q, T101A, S121A, and N224Q produced GP2 signals that were greater than the wild type control (Figure 41). It is also worth mentioning that the viruses producing a weak or non-existent GP2 signal also had low p24 signals. This may be due to correctly processed GP trimers increasing the stability of budding particles. We also created a correlation plot between GP cleavage and GP2 pseudovirus incorporation in order to observe any potential relationships (Figure 42). Analysis of this chart shows that once cleavage drops below 80% of the control, the ability of GP2 to integrate into pseudoviruses is reduced dramatically. Based on these findings, we are able to conclude that N-linked glycosylation sites play a major role in the assembly of pseudovirus particles and that a minimum level of GP2 cleavage may be required for pseudovirus production.

**N-linked glycosylation modulates viral infectivity primarily by virus production**

To examine how deletion of GP1 NLG sites affects infectivity, we titrated purified pseudovirus in TZM-bl cells as described in the methods section. The removal of glycosylation sites led to the production of pseudoviruses that were more and less infectious than the wild type (Figure 43). Three N→Q mutations (N89Q, N109Q, N119Q) and three S/T→A (T81A, S91A, and S169A) mutations resulted in non-infectious particles while the T101A and S121A mutations produced pseudoviruses that were more infectious. When considering the mechanism by which NLG site deletions affect infectivity, we hypothesized that the changes were
due to differences in pseudovirus production. As we had already measured the GP2 levels in mutant pseudoviruses, we created a scatter plot to chart the relationship between the GP2 content of pseudoviruses and infectivity. There was a nearly linear relationship between GP2 signal strength and infectivity (Figure 44). We identified two outliers (N99Q and N224Q) on the graph that contained higher levels of GP2 in pseudoviruses without showing a concurrent increase in infectivity when compared to the wild type. Perhaps in these situations, the N-linked glycan is modulating a mechanism of the infective process such as receptor binding or fusogenicity.

In contrast to GP2 incorporation, GPC cleavage shows a more ambiguous correlation with pseudovirus infectivity (Figure 44). When the scatter plot is analyzed, we find that mutants able to cleave GPC at levels similar to the positive control can range in infectivity from over 100% to below 50%. When glycoprotein cleavage drops below 70% no infectious mutants are observed. As a result of our findings in this chapter, we propose the following model for the effect of N-glycans on Lassa virus infectivity. In order to produce infectious virus, a minimum level of glycoprotein cleavage is necessary to promote efficient pseudovirus formation. The formation of pseudoviruses is significantly affected by removal of glycosylation sites. Once incorporated into pseudoviruses, the levels of glycoprotein have a stochastic effect on infection that appears to be mostly independent of glycosylation. Thus it appears that glycosylation primarily affects infectivity though means associated with the integration of glycoprotein into pseudovirus and subsequent budding processes.
N-glycans modulate neutralizing antibody recognition

In addition to examining how glycosylation sites affect viral function, we also explored whether these sites could obscure or enhance neutralizing antibody epitopes. We chose to analyze two antibodies, mAb 8.9F and 12.1F that we previously characterized in Chapters 1 and 2. For these experiments, mutant pseudoviruses were tested via slot blot and cell-surface expressed glycoproteins were analyzed via flow cytometry. Of the NLG mutations that produced infectious pseudoviruses, we found mAb 8.9F signals on the slot blot tended to be weaker than the wild type except in the case of N79Q and T81A. Most mutants produced bands equal to wild type when the blot was stained with 12.1F. The exceptions to this included N89Q, S91A, and N167Q. There was also a general trend for mAb 12.1F to produce a stronger signal than mAb 8.9F for the pseudoviruses tested. This observation may be due to increased mAb 12.1F affinity or a larger number of mAb 12.1F antigenic sites on the pseudovirus surface. The p24 signals for all mutants were positive suggesting that 293T cells were successfully transfected with the plasmids (Figure 46). Results from mAb 8.9F surface staining varied depending on the N-glycan that was deleted. Three mutations, T81A, N89Q, and S169A, were undetectable. Removal of glycosylation sites generally produced a weak signal, except in the case of the N99Q, T101A, S111A, and N224Q mutations. These mutations were able to react with mAb 8.9F at levels similar to the wild type. (Figure 45)

Two mutants, T81A and N89Q, showed some discrepancy between the slot blot and flow cytometry data. It is possible that these mutants may produce
glycoprotein and very low levels and by ultracentrifugation the pseudovirus was concentrated to detectable levels. In contrast, N109Q, N119Q, and N167Q were undetectable on slot blot but produced a signal for mAb 8.9F when measured by flow cytometry. Slot blot results for these mutations were likely negative because of the impaired ability for these samples to form stable pseudovirus as demonstrated by Figure 40. Based on these experiments, we conclude that NLG sites may alter the affinity of mAb 8.9F and 12.1F for the glycoprotein but do not abolish binding. The N-glycan mutants that were negative on slot blot were a result of failed pseudovirus formation and do not allow us to draw any conclusions about how epitope recognition would be affected.

**Deletions of N-linked glycosylation sites by asparagine and serine/threonine substitution methods show a differential effect on Lassa glycoprotein**

Previous studies examining the role of N-linked glycosylation sites in arenavirus glycoproteins eliminated the glycosylation motif by substituting serine (S) or threonine (T) for alanine (A). As alanine shares similar biochemical properties to serine and threonine, it is generally assumed that alanine substitutions should not significantly impact the overall protein structure or function. In other studies examining N-glycans, researchers removed the glycosylation site by substituting asparagine (N) with glutamine (Q). As this mutation only adds one carbon atom to the amino acid side chain, a minimal impact on the overall structure of the protein should be observed. However, studies have found that the amino acid substitutions chosen to delete a N-glycan attachment site
can obfuscate the role of the glycan itself\textsuperscript{211,212}. In order to better separate the effects of amino acid substitution from glycan elimination, we created two mutations for each N-glycosylation site. Each pair of mutants was analyzed for statistically significant differences in their ability to cleave GPC and infect cells. In cases where both mutations generated similar results, it is likely to assume that amino acid substitutions did not affect the results and that any changes were a result of the N-glycan. In situations where one mutation led to negative results, it was assumed the amino acid change and not the missing glycan was responsible. In analyzing pairs of N-glycan mutants for glycoprotein cleavage, we found the glycosylation sites at positions 99 and 224 were similar to one another with a 95% confidence interval (Figure 47). While not statistically significant, mutations eliminating the glycosylation site at 119 had similar GPC processing values. For the other mutant pairs, at least one mutation showed mild activity in each case. There were no pairs that both eliminated cleavage. When comparing pseudovirus infectivity, we found the N99 and N224 pairs were both infective. Also the N\textsubscript{⇒} Q and S/T\textsubscript{⇒} A mutations at each site did not significantly differ from one another (Figure 48). In comparison to the wild type, the elimination of the N99 glycan did not affect infectivity and the N224 glycan had a very small effect. The only pair that had no infectious activity was at position 89. Two positions, 109 and 119, had pairs with one mutation comparable to the wild type and the other relatively low. In these situations, we assume that the glycan was not critical for infection. By comparing different mutations of the same N-glycosylation site, we were able to differentiate between the effects of swapping amino acids and removing glycans on GPC.
processing and infectivity. Our results showed that the glycosylation sites at positions 99 and 224 are minimally important while site 89 may impact infectivity.

Discussion

N-linked glycosylation is an important post-translational process that expands the functional repertoire of proteins by the addition of specific glycan residues. In addition to the role NLGs play in cellular functions, viral proteins have adapted to utilize these molecules. In particular, the viral proteins embedded in the surface of enveloped viruses have been found to be heavily glycosylated and in some situations account for over half the mass of the surface glycoprotein. The effects that glycosylation may have on viral proteins are diverse and range from influencing correct folding in the Golgi to mediating infectivity to modulating host immune responses. The impact of glycosylation on HIV gp120 and influenza virus HA structure and function has been well characterized. However, very little work has been done on examining the NLG sites identified in the Lassa virus glycoprotein. Understanding the patterns and effects of glycosylation for the Lassa virus GP can help improve monitoring for high-risk mutations as well as provide virologists with a better understanding of the processes involved in GP receptor binding and virion formation.

In order to appropriately study the effects of glycosylation on the LASV GP, we created two separate mutants for each of the possible NLG sites identified in the GP1 subunit. We chose to focus on the GP1 subunit, as it would allow us to use the monoclonal antibodies we have identified against GP2 to probe for the glycoprotein.
Another reason for selecting the GP1 region for mutational analysis over the GP2 region is due to the variability of GP1 glycosylation sites in arenaviruses. While the GP2 region contains 4 conserved NLG sites (excluding Old World viruses LCMV and Dandenong virus) the GP1 ranges from 6 to 8 sites and are more widely dispersed within the protein. Due to the higher variability of NLG sites in GP1, we hypothesize that we will be more likely to come across novel effects of glycosylation in Lassa virus through this approach. When designing the mutational primers for this study, we took an approach that differed from previous work on arenavirus glycosylation. In previously published studies examining glycosylation, authors would delete the NLG sites by substituting the first or third amino acid of the Asn-X-Ser/Thr motif with a structurally similar base. However, none of these authors acknowledged that the observed effects may be due either in part or wholly to the amino acid substitution and not the removal of the N-glycosylation site. Based on our results from Chapter 2 where LCMV amino acids were substituted into the LASV GPC, we strongly believe insertion of similar bases does not guarantee structural homology or similar reactivity. Our study attempted to address the issue by creating two separate mutations, either N->Q or S/T->A, for each NLG site in GP1. The substituted bases were selected based on their similar biochemical properties to the amino acids they replaced. By examining the mutant pairs for each site of glycan attachment, a more reliable conclusion could be made as to whether the effects of the mutation were due to removal of the glycan. If both of the results for a mutation were negative or similarly different in comparison to the wild type, then it was assumed that the N-glycan might have influenced the function being measured.
However, if one mutant was positive and another negative, then it was assumed that the negative result was a cause of the amino acid substitution and not due to absence of the glycan. While this framework provides a better way to understand the effects of glycosylation in comparison to a single site mutation, there are still limitations that exist. For example, our results could be affected if a base substitution eliminating N-glycan function also resulted in a concurrent compensating mutation that restored function. Additional mutants eliminating GP1 N-glycan motifs would help to resolve situations where our results did not agree with one another.

In examining the functional effects N-glycans have on protein processing, we began our study by measuring the amount of glycoprotein produced. In terms of raw glycoprotein synthesis, eliminating NLG sites did not seem to have a major impact. While this does not confirm that the protein is in the correct native structure, it does suggest that there is no significant increase in cellular protein degradation pathways when these proteins are expressed. Mutations affecting glycoprotein cleavage were identified when cell extract was analyzed on western blot. Two mutations, N89Q and S169A, produced GP2 signals that were barely detectable on analysis. However, because the complimentary mutations, S91A and N167Q, were able to cleave the glycoprotein it seems reasonable to assume that the amino acid change was the factor influencing GPC cleavage. Our results of S/T->A mutations on glycoprotein cleavage differ greatly from the results published by Eichler, et. al. who found that mutations T81A, S91A, T101A, and S121A prevented cleavage. It is unclear why such dramatic differences would be observed. One
possible explanation may be due to the cell lines and expression vectors our study used. We used the Lassa Josiah strain GPC in an LM2 vector and transfected HEK 293T cells as opposed to Eichler et. al who transfected the Josiah GPC in the pCAGGS vector into Vero cells. Perhaps the cellular glycosylation mechanisms and transport pathways within these different cell types led to the disparate results. Our results also found that many of the mutants were able to cleave the glycoprotein at rates similar to the positive control. In the mutations that did result in lower GPC cleavage, the value did not drop below 40% when excluding the two cleavage-resistant mutations. Two sites, N99 and N224, were identified where the percent GPC cleaved did not differ in a statistically significant manner between mutations. For these two positions it is likely to assume that the N-glycan does not exhibit a meaningful effect in the production or proteolytic cleavage of GPC.

We also found that N-glycosylation sites in GP1 are not a requirement for glycoprotein surface transport. All generated N-glycan mutants produced detectable surface signals and ranged between 130% and 65% of the wild-type control. In addition, we confirmed that GPC cell surface transport is not abolished by poor GPC cleavage in Lassa virus. Earlier data demonstrated that when a small molecular inhibitor blocked GPC cleavage, transport of the uncleaved GPC to the cell surface still occurred. This observation was further supported by our data on the surface GP expression of the N89Q and S169A mutants. These mutations removed NLG sites and effectively prevented GPC cleavage, however, analysis by flow cytometry still detected glycoprotein on the surface of cells. Other researchers have demonstrated similar findings in regards to the surface transport of uncleaved glycoprotein.
Biotinylation of surface proteins could be used as an additional method for determining the amount of glycoprotein transported to the cell surface. In this method, surface proteins are conjugated to biotin and precipitated on streptavidin beads. The advantage of this approach over flow cytometry is that it allows for the visualization of the total surface-transported protein from a group of transfected cells as opposed to quantifying the signal strength of individual cells.

Our next goal was to investigate how changes in N-glycan sites would affect GP2 incorporation into pseudoviruses. We found six mutants, T81A, N89Q, S91A, N109Q, N119Q, N167Q, and S169A that significantly lowered (<20% of wild type) the amount of GP2 incorporated with two of the mutants, N89Q and S169A, producing no signal. It is likely to assume poor GP2 production, as determined by earlier experiments with these mutants, was the reason for the low GP2 incorporation levels. Once we began analyzing the mutant pairs of each individual NLG site, we discovered that absence of the N-glycan at sites 99 and 224 does not cause significant inhibition of GP2 incorporation. For the two mutations of the N99 glycan, both resulted in GP2 signals that were stronger than the wild type. At the N224 glycosylation site, the T226A incorporate GP2 at levels slightly below the wild type while the GP2 signal measured for N224Q were near 150% of the positive control. As the glycans at positions 109 and 119 had one mutant capable of forming pseudoparticles, we assume that these N-glycan positions are not essential in GP2 incorporation and virus budding. There is potential that the N-glycan attachment site at position 79 may reduce but not abrogate pseudovirus formation. Both mutations for this site caused GP2 to be incorporated at levels below 50%.
Additionally, both mutations produced strong GP2 signals on western blot suggesting that there was ample glycoprotein available for budding particles.

One major limitation and possible source of error in our pseudovirus experiments are our use of the HIV backbone plasmid SG3. The possibility exists that our approach has generated different results from what would be seen if live Lassa virus were used. While HIV-based pseudoparticles have been shown to closely mimic lab-adapted Lassa virus behavior in neutralization studies, there is little research on the similarities between the production and assembly of Lassa pseudovirus and wild-type virus. In particular, the Z protein of Lassa has been implicated as a major factor in organizing the glycoprotein spike \(^{214,215}\). While HIV does contain a homologous protein to Lassa Z protein called p17, it is unclear how the association with the GPC would function \(^{216}\). It may be the case that the interactions of the pseudovirus p17-GPC complex are weaker than the Z protein-GPC interactions of live virus. As a result, the pseudovirus may integrate GP2 into budding particles at a lower efficiency as compared to Lassa virus. Experiments using Lassa virus will need to be completed under BSL-4 conditions. An alternative approach to this technique would be to use a LCMV backbone for generating pseudovirus in hopes the more closely related Z protein would provide accurate results \(^{217}\).

After characterizing the pseudovirus composition, we measured how N-glycosylation site mutations affected infectivity. As expected, those mutations that poorly incorporated GP2 were minimally infective. If a mutation was able to integrate GP2 at levels 20% or greater as compared to the control, infectious
particles were detectible on TZM-bl cell assay. These results suggest that N-glycan mutations play a major role in mediating infectivity through pseudoparticle assembly. Interestingly we found two mutations, T101A and S121A, which produced pseudoviruses more infectious than the control. Glycans with functions similar to these have been found in HIV and influenza. They are proposed as a method to shield important and highly conserved functional motifs \textsuperscript{199}. Despite high infectivity, circulating viruses with these mutations may be quickly eliminated by host immune responses as a result of increased access to a neutralizing epitope. The elevated infectivity of T101A and S121A particles may be due to improved GP2 incorporation or from a structural change in the protein. Of these two hypotheses, the former seems more likely. Both of these mutations demonstrated GP2 levels greater than the control when measuring GP2 incorporation into pseudoviruses. Despite this evidence, studies examining the effect of T101A and S121A on receptor binding should still be performed. When examining the mutation pairs for each N-glycan site, we found site 89 to be the only one that may be essential in infectivity. For all other pairs, at least one mutation produced an infectious virus suggesting the noninfectious viruses were a result of the amino acid change. In addition, we found non-significant differences in infectivity between the mutant pairs at sites 99 and 224. These results suggest that the amino acid substitutions chosen did not have a differential effect on the data and that the changes observed were likely due to effects of the N-glycan.

NLG mutations may also have slight effects modulating receptor binding and GP2-mediated fusion with a resulting decrease in infectivity. Experiments
measuring these effects *in vitro* will be important in furthering our understanding. In Chapter 3 we described receptor-binding experiments performed by incubating mAb 8.9F-unreactive pseudoviruses with Vero E6 cells. Measuring fusogenicity may be another important experiment to perform with the N-glycan mutants. It is possible that glycan residues in GP1 affect GP2 fusion as studies have found intersubunit non-covalent interactions capable of reducing viral fusion. Effects of GP1 NLG sites on fusion can be measured via a DBT cell assay \(^{210}\). In this method, cells are transfected with the full-length glycoprotein and media is replaced with a low pH buffer to induce spontaneous fusion. Fusogenicity is quantified by measuring the nuclei per cell.

Our final goal for this chapter was to expand on data from Chapter 2 that examined how N-linked glycosylation sites might impact mAb 8.9F recognition. We previously determined that T81A and S169A might play a role in mAb 8.9F epitope binding. Those findings are reviewed in light of the new experiments we have done in this chapter. It is clear that S169A was negative on flow cytometry because the mutation prevented glycoprotein cleavage. As mAb 8.9F recognition requires processed glycoprotein trimers, any mutation that prevents GPC cleavage will also eliminate antibody binding. Thus, we conclude that while S169A is capable of eliminating mAb 8.9F binding, the mechanism is due to disruption of precursor cleavage and not epitope destruction. The case for T81A is similar in that it seems the mutation does not specifically eliminate the epitope but impacts the overall formation of the glycoprotein structure. The T81A mutation exhibits detectable GPC cleavage and surface transport, however the pseudoviruses produced are non-
infectious. We have previously shown that the mAb 8.9F epitope is very labile and easily disrupted. We hypothesize that an impairment of trimer formation, as indicated by non-infectious particles, prevented mAb 8.9F epitope recognition.

When we analyzed the mutation pairs of each NLG site, we did not find sufficient evidence to suggest that glycosylation plays a direct role in mAb 8.9F epitope recognition. In both flow cytometry and slot blot, there was at least one mutant for each pair that was found to be positive. In regards to the differential effect of amino acid changes at the same glycosylation site, we found eliminating the glycan at position 99 to be the only site yielding similar results between mutants. We should note that these two mutations also showed similarities in terms of glycoprotein processing and GP2 incorporation. Our results suggest that when eliminating NLG sites, the amino acid chosen has the ability to cause wide phenotypic variations. For these reasons, we propose that future research examining glycosylation sites of arenaviruses include at least two mutations per glycosylated residue in order to avoid confounding data. Despite mostly negative results, the method we developed for examining N-glycans can easily be applied to other viral glycoproteins.

N-glycans play a critical role influencing the formation, infectivity, and immune escape of viruses. In this chapter, we set out to investigate how single N-linked glycosylation sites in the GP1 subunit of Lassa virus would impact viral fitness. We approached this problem by developing a series of two mutations for each site of glycan attachment. Our approach is superior to previous efforts as it expands the breadth of viral factors examined and probes the effects of different
mutations for a single N-glycan. In summary, we found mutations of N-glycan sites to have a broad effect on glycoprotein processing, formation of infectious pseudovirus, and antibody binding. However, these effects frequently appeared to be due to the impact of the amino acid substitution and not the absence of the glycan. There were only two NLG sites at 99 and 224 with mutant pairs that behaved similarly. In the case of these two sites, elimination of the N-glycan created mutants with properties similar to the wild type suggesting that the glycans at these two positions have little effect on glycoprotein function. Our data also showed that N-glycans may alter infectivity, but there is not enough conclusive evidence to suggest that any N-glycans are essential. Our correlation data suggests that deletion of GP1 N-glycans affected pseudovirus infectivity at stages during or before pseudovirus assembly. Finally, we found that none of the glycans in the GP1 region were essential to the epitope recognized by mAb 8.9F, an antibody we characterized in earlier chapters.
<table>
<thead>
<tr>
<th>N79Q</th>
<th>FP</th>
<th>CAT GGA GAC ACT CCA AAT GAC CAT GCC TCT CTC CTG CAC AAA G</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP</td>
<td></td>
<td>GAG GCA TGG TCA TTT GGA GTG TCT CCA TGT TTA GTT CCA GAG TCT G</td>
</tr>
<tr>
<td>N89Q</td>
<td>FP</td>
<td>CTC CGT CAC AAA GCA AAA CAG TCA TCA TTA TAT AAT GGT GGG CAA TG</td>
</tr>
<tr>
<td>RP</td>
<td></td>
<td>CAT TAT ATA ATG ATG ACT GTT TTG CTT TGT GCA GGA GAG AGG CAT GG</td>
</tr>
<tr>
<td>N99Q</td>
<td>FP</td>
<td>ATT ATA TAA TGG TGG GCC AAG AGA CAG GAC TAG AAC TGA CCT TGA C</td>
</tr>
<tr>
<td>RP</td>
<td></td>
<td>CTA GTC CGT TCT CTT GGC CCA CCA TTA TAT AAT GAT GAC TGT TGT TCT TTG</td>
</tr>
<tr>
<td>N109Q</td>
<td>FP</td>
<td>CTG ACC TTG ACC CAA ACG AGC ATT ATT AAT CAC AAA TTT TGC TCT C TCT G</td>
</tr>
<tr>
<td>RP</td>
<td></td>
<td>GTG ATT AAT AAT GCT CGT TTG GGT CAA GGT CAG TTC TAG TCC TGT CTC</td>
</tr>
<tr>
<td>N119Q</td>
<td>FP</td>
<td>ATT ATT AAT CAC AAA TTT TGC CAA CTG TCT GAT GCC CAC AAA AAG AAC CTC</td>
</tr>
<tr>
<td>RP</td>
<td></td>
<td>GGC ATC AGA CAG TTG GCA AAA TTT GTG ATT AAT AAT GCT CGT TTG GGT C</td>
</tr>
<tr>
<td>N167Q</td>
<td>FP</td>
<td>GAT TAG TGT GCA GTA CCA ACT GAG TCA CAG CTA TGC TGG GGA TG</td>
</tr>
<tr>
<td>RP</td>
<td></td>
<td>ATA GCT GTG ACT CAG TTG GTA CTG CAC ACT AAT CTT TCC CCC ATT AAA ATC</td>
</tr>
<tr>
<td>N224Q</td>
<td>FP</td>
<td>CAA TAT CTG ATA ATC CAA CAA ACA ACC TGG GAA GAT CAC TGC CAA TTC</td>
</tr>
<tr>
<td>RP</td>
<td></td>
<td>GAT CTT CCC AGG TTG TTT GTT GGA TTA TCA GAT ATT GAT AAC TAG TCA TAA TAC AGT C</td>
</tr>
</tbody>
</table>

Table 5. List of primers for asparagine to glutamine substitutions.
**Figure 35. Glycoprotein cleavage of N-glycosylation mutants.**

293T cells were transfected with plasmids encoding Lassa virus glycoprotein with mutations to N-linked glycosylation sites. 48 hours post-transfection cells were lysated and the protein extract was analyzed via western blot. The blots were probed with primary antibody mixes against the GP2 subunit which also recognized uncleaved GPC.
Figure 36. Measurement of GPC cleavage by N-glycosylation mutants.

In order to calculate the percent of cleaved glycoprotein, the strength of the individual bands on Figure xx were quantified using the Image Studio program. Percent cleavage was calculated by dividing the GP2 signal by the sum of the GP2 and GPC signal.
Figure 37. Measurement of total glycoprotein production by N-glycosylation mutants.

Measuring the density of the GPC and GP2 bands from figure xx and adding them together quantified the total glycoprotein produced by N-glycan mutants. The results are expressed as a percentage of wild type glycoprotein production.
Figure 38. Glycoprotein surface expression of N-glycosylation mutants.
Transport of the glycoprotein to the surface of transfected cells was measured by flow cytometry and staining with GP2 antibodies. Values are expressed as a percentage of wild type surface transport.
Figure 39. Surface immunofluorescent images of N-glycan mutants staining with mAb 8.9F.

Transfected 293T cells were stained by indirect immunofluorescence with mAb 8.9F followed by anti-human FITC antibody. DAPI counterstain was used to visualize cell nuclei.
Figure 40. Incorporation of GP2 in mutant pseudoviruses.

Incorporation of GP2 into the budding pseudovirions was measured by western blotting. Pseudoviruses were purified before isolation of protein. Blots were probed with a GP2 mix and an anti-p24 antibody that served as a loading control.
Figure 41. Densitometric analysis of Figure 40.

Signal strength of GP2 and p24 bands was measured with ImageStudio 6. Mean band intensity was calculated and subtracted from background. Signal strength was calculated relative to wild-type GP2 incorporation.
Figure 42. Correlation of glycoprotein cleavage to GP2 incorporation in pseudoviruses.

Scatter plots were generated using GraphPad Prism charting the percent of GP2 incorporation in pseudoviruses with the corresponding percent of GPC cleavage for the same mutation.
Figure 43. Titration of pseudoviruses with N-glycan mutations.

Pseudoviruses containing mutations at N-linked glycosylation sites were generated in 293T cells and titrated on TZM-bl cells. Infectivity was measured by luciferase activity and results were plotted in comparison to wild-type pseudovirus. Results show the average of three independent experiments.
Figure 44. Correlation plots of pseudovirus infectivity versus GP2 incorporation and glycoprotein cleavage.

Scatter plots were generated using GraphPad Prism to compare infectivity versus GP2 incorporation (A) or percent glycoprotein cleavage (B). The dotted line in Figure B indicates 80% glycoprotein cleavage.
Figure 45. mAb 8.9F and 12.1F surface staining of N-glycan mutants.
Transfected 293T cells were stained with mAb 8.9F and 12.1F to measure the effect of glycosylation sites on antibody recognition. The signal strength of antibody binding was measured by flow cytometry using indirect fluorescence. Results were standardized to wild type glycoprotein reactivity for each antibody.
Figure 46. Slot blot of pseudoviruses with N-glycan deletions.

Pseudoviruses were affixed to nitrocellulose using the Bio-Rad Slot Blot apparatus to test for reactivity with neutralizing antibodies mAb 8.9F and 12.1F. Samples for each antibody blot are arranged in the following order from top to bottom: Column 1- N79Q, N89Q, N99Q, N109Q, N119Q, N167Q, N224Q, wild type. Column 2- T81A, S91A, T101A, S111A, S121A, S169A, T226A, LCMV.
Figure 47. Paired data for GPC cleavage of mutations at individual N-glycosylation sites.

Data from Figure 36 was rearranged to group mutations for individual N-glycosylation sites together and student’s t-tests were used to compare the individual mutations to one another. Mutations that were statistically similar to one another (p<0.05) are noted above.
Figure 48. Paired infectivity data of mutations at individual N-glycosylation sites.

Data from Figure 43 was rearranged to group mutations for individual N-glycosylation sites together and student’s t-tests were used to compare the individual mutations to one another. Mutations that were statistically similar to one another (p<0.05) are noted above.
**Summary**

In the last 50 years over 335 emerging diseases have crossed into human populations, increasing the global burden of disease. Over 60% of these infections result from zoonotic transmission. Lassa virus, the causative agent of Lassa fever, is one such emerging zoonotic disease in West Africa. As the population in Lassa virus-endemic regions continues expanding, the disease burden and risk of the virus spreading to new regions will increase. To curb future infections, interventions in public health, diagnostic techniques, and therapeutic options must be developed and effectively implemented. Our lab has extensive experience in the isolation of therapeutic antibodies for HIV. We have applied our talents in this field towards examining the antibody response to Lassa virus in hopes of creating a suitable immunotherapy to protect against infection. During our screening process, we identified an antibody mAb 8.9F with unique neutralization characteristics. Here we report, for the first time, on a quaternary neutralizing antibody against the Lassa virus glycoprotein.

In Specific Aim 1, we set out to characterize mAb 8.9F as recognizing features consistent with a quaternary neutralizing epitope (QNE). QNEs have been identified in several other viral species including HIV, influenza A virus, and hepatitis C virus. The epitopes in the previously mentioned examples all display the QNE in the context of the native protein multimer. The degree of post-translational processing necessary for QNE recognition varies between examples with attached glycans occasionally playing a role. Our first indication that mAb 8.9F may recognize an epitope not present on the linear protein or secondary structure came when we
found mAb 8.9F was unreactive on ELISA experiments but effectively neutralized pseudovirus particles.

Once we determined that mAb 8.9F could react with the glycoprotein trimer in pseudoviruses, we probed deeper into those treatments that would destroy the epitope. In doing so, we found that incubation of transfected cells or pseudovirions with RIPA buffer, an ionic and reducing detergent, would eliminate the ability of mAb 8.9F to precipitate glycoprotein. RIPA treatment did not eliminate antigen binding when we tested mAb 12.1F, a neutralizing antibody recognizing a secondary structure epitope on GP1. These results suggest that the mAb 8.9F epitope is extremely labile and can be disrupted by detergents that separate the glycoprotein trimer complex. Additionally, we found that mAb 8.9F could visualize surface glycoprotein if transfected cells were allowed to air dry on glass slides. However, methanol and formaldehyde fixation techniques destroyed the epitope.

Once we had identified the conditions under which the mAb 8.9F epitope could be preserved, we went on to examine which glycoprotein subunits composed the epitope. Lassa virus glycoprotein is transcribed as a polyprotein containing stable signal peptide (SSP), glycoprotein 1 (GP1), and glycoprotein 2 (GP2) subunits. Host proteases cleave the precursor protein into its functional state thus allowing the formation surface trimers. We determined which of the subunits mAb 8.9F bound by immunoprecipitating transfected cells with the antibody before lysing the cells in RIPA buffer. Interestingly, RIPA pre-treatment eliminates the mAb 8.9F epitope but is not strong enough to disrupt the antibody-antigen complexes. The antibody-antigen complexes were purified on Protein A beads and analyzed via
western blot. Blots were probed with a mix of GP1-specific or GP2-specific antibody mixes that recognized linear epitopes. Unfortunately we have not isolated any antibodies to the SSP and were thus unable to determine if mAb 8.9F may bind that protein as well. Our results for this experiment showed that mAb 8.9F precipitated both GP1 and GP2 and each subunit produced a strong signal band. During these experiments we also noticed that mAb 12.1F pulled down a faint GP2 band. We hypothesized that this may be due to weak interactions between the GP1-GP2 complexes and not due to antibody binding of the subunit. These results let us to speculate that perhaps the bands we observed on the mAb 8.9F blot were not entirely due to antibody-glycoprotein binding. In order to test whether mAb 8.9F was directly interacting with each subunit, we incubated antibody-antigen complexes in sodium thiocyanate. This is a chaotropic agent that can destabilize non-covalent interactions between protein complexes. We reasoned that low concentrations of NaSCN would disrupt GP1-GP2 interactions while at higher concentrations the antigen would be released from the antibody being tested. Our results showed that at low concentrations of NaSCN, mAb 12.1F quickly released GP2 while GP1 remained bound at very high concentrations. In the case of mAb 8.9F the signal for each subunit began to decrease slightly after 1M NaSCN. As this was past the threshold of GP2 release in mAb 12.1F we assume mAb 8.9F directly interacted with each subunit. Additionally, GP1 and GP2 dissociated from mAb 8.9F at similar rates. This finding suggests that GP1 and GP2 were contributing equally to the mAb 8.9F epitope.
Finally, we wanted to determine if cleavage of the GP1-GP2 site was a requirement for mAb 8.9F recognition. To test this question, we cultured transfected cells with a recently identified small molecule inhibitor of the site-1 protease. After 48 hours, we measured mAb 8.9F surface reactivity. The results of this experiment showed that mAb 8.9F reactivity was reduced in a dose-dependent manner, thus suggesting that glycoprotein cleavage is critical for mAb 8.9F epitope recognition.

In Specific Aim 2, we sought to further examine the epitope structure of mAb 8.9F by identifying critical residues via site-directed mutagenesis. The overall approach for this aim used a ‘knock-out’ method where amino acids from the Lassa virus glycoprotein plasmid were replaced with the homologous sequences from lymphocytic choriomeningitis virus, a closely related arenavirus. Initially we substituted sequences of one to seven bases in length into the plasmid. This approach reduced the number of mutations we needed to design and allowed for the identification of regions that could be further probed. When we surveyed the knock out mutants for mAb 8.9F reactivity, we found many segments that did not react with the antibody. Unfortunately most of these regions also did not react with our control antibody, mAb 12.1F. Lack of 12.1F binding suggests the glycoprotein may have been inappropriately trafficked or formed a random coil. There is a small chance the mutation removed the mAb 12.1F epitope despite earlier data showing mAb 12.1F to be reactive with LCMV. Additional antibodies should be tested in order to clarify these results. Three mutations were found in the GP1 region that eliminated mAb 8.9F binding and retained mAb 12.1 reactivity, 121 SDAHK, 145 PNFNQ, and 250 RDIYS. Each of these three mutants produced the correct sized
band on western blot suggesting the mutations did not cause a truncation of GP2. While we did not identify any regions in GP2 that were solely unreactive with mAb 8.9F, our immunoprecipitation data leads us to assume that if we used a wider number of antibodies to look for surface expression we would identify regions of interest.

Next, we created a series of single amino acid substitution mutants for each of the three regions identified. This new set of mutants was again tested by flow cytometry for reactivity against mAb 8.9F and 12.1F. Results from this experiment identified H124F from the first region in addition to P145R and F147N from the second region as mAb 8.9F-unreactive mutants. Strangely, the 250 RDIYIS region contained one mutation, S255T, which eliminated mAb 8.9F and 12.1F binding while the other mutations reacted with both antibodies. When the single mutants of region 250 were tested on western blot, the S255T mutant did not show any band for GPC or GP2. Perhaps an error occurred during PCR mutagenesis resulting in a frameshift mutation that prevented protein expression.

Up to this point in our examination of amino acids that compose the mAb 8.9F epitope, we had only measured antibody reactivity with the glycoprotein as expressed on cells. In order to generate a more complete picture of how antibody reactivity is affected, we tested mutant pseudoviruses by slot blot and neutralization assay. In the slot blot experiment, we immobilized the mutant pseudoviruses on nitrocellulose and probed the blot with mAb 8.9F, 12.1F, and 13.4E. The latter two antibodies are GP1 and GP2 specific antibodies respectively. Mutant pseudoviruses retained reactivity with 12.1F and 13.4E while the mAb 8.9F blot was only positive
for the wild type pseudovirus. On neutralization assay, all pseudoviruses showed a
dose-dependent inhibition when incubated with mAb 12.1F but even very high
concentrations of mAb 8.9F were unable to neutralize the pseudoviruses bearing
point mutations. Taken together, these experiments provide compelling evidence
that H124, P145R, and F147 play a role in the epitope of mAb 8.9F or contribute to
its stability.

As previous studies have shown highly neutralizing antibodies with glycan-
based epitopes, we also explored the possibility of N-glycans composing a portion of
the mAb 8.9F epitope. To do so, we deleted single N-linked glycosylation sites within
GP1 by site-directed mutagenesis of the serine or threonine within the glycosylation
motif to an alanine. The resulting mutants were transfected into 293T cells and mAb
8.9F and 12.1F binding signals were measured by flow cytometry. Of these
mutations, T81A and S169A abrogated mAb 8.9F binding without eliminating 12.1F
reactivity. We wanted to confirm these results by slot blot; however, in this
experiment we found that T81A produced a signal against mAb 8.9F. It is unclear
why this is the case, but if T81A produces glycoprotein at a very low level then it
may be detectible when concentrated pseudovirus is measured. While the S169A
mutation seemed to affect the mAb 8.9F epitope, later experiments found this result
was due to the inhibition of glycoprotein cleavage and thus incorrect timer
assembly.

In our final Specific Aim, we wanted to examine how mutations that affected
8.9F reactivity impacted glycoprotein processing and pseudovirus function. We also
explored the impact of N-linked glycosylation on these factors. Understanding how
the glycoprotein and virus functions are affected by mAb 8.9F-abrogating mutations provides a hypothetical picture for the utility of mAb 8.9F as a therapeutic. If mutations that affect mAb 8.9F binding do not have a deleterious effect on viral fitness then it is likely that the virus would quickly escape antibody neutralization. The ideal antibody therapy would target an epitope for which escape mutants would have extremely compromised fitness.

To test the impact of H124F, P145R, and F147N mutations on glycoprotein function, we measured total GPC production, GPC cleavage, and transport of glycoprotein to the surface of transfected cells. While glycoprotein production and cleavage were slightly reduced, these mutations did not significantly inhibit the early processes of glycoprotein synthesis. The mutants had similar levels of detectable glycoprotein on the surface of transfected cells as compared to the wild type.

Our next goal was to examine how these changes affected the pseudovirus. First we measured the incorporation of GP1 into budding pseudoviruses by running purified particles on western blot. We found that not only did GP1 integrate into the membrane of budding pseudoviruses, but in the case of the H124F and F147N mutants the GP1 signal appeared to be stronger than the wild type. Our findings measuring receptor binding by mutant pseudoviruses closely mirrored these results with the same two mutants displaying increased cell receptor binding. We also wanted to determine how these mutations affected infectivity. When the pseudoviruses were titrated, we found an extremely compromised infectivity for all mutants. The relative infectivity ranged from 5% for H124F to less than 1% for
Thus mutations that abrogate mAb 8.9F binding do not seem to affect the early steps in viral replication or pseudovirus formation but do exhibit an extremely deleterious effect on infectivity. Unfortunately, Lassa virus is classified as a BSL-4 agent so we are unable to perform experiments examining whether adaptation via a compensatory glycoprotein mutation would occur during in vitro serial passaging of the live virus.

Our final aim in this study was to determine if deletion of N-linked glycosylation sites by N to Q and S/T to A substitutions showed different effects on glycoprotein processing and function. To test these questions we created a library of N to Q and S/T to A mutations for each possible site of N-glycan attachment within the GP1 subunit. NLG sites play a diverse role in protein processing, function, and antigenicity. We performed a series of experiments to test the impact of NLG mutations on each of these factors. Our first set of experiments looked at glycoprotein production and processing. Western blot data showed that all mutants produced glycoprotein at relatively similar levels and could successfully transport glycoprotein to the surface of cells. However, the mutants differed in their ability to produce cleaved GP2. Approximately half of the mutants had similar or greater cleavage activity while two mutants, N89Q and S169A, did not produce a visible GP2 band. When we measured the effect of NLG deletions on GP2 incorporation into pseudoviruses, a significantly larger number of mutants were found to have impaired function. Only four mutations retained activity similar to the wild type and seven had low to undetectable signals. The results for pseudovirus infectivity closely mirrored the GP2 incorporation data with the same mutants that showed strong
GP2 incorporation retaining their infectivity. Our last experiment was to examine
the impact of N-glycans on the antigenicity of mAb 8.9F and 12.1F. We examined
antibody binding to the surface of transfected cells and to pseudoviruses
immobilized on nitrocellulose. We used flow cytometry to measure surface signals
and found three mutants that were unreactive with both mAb 8.9F and 12.1F. None
of the mutants were negative for only one antibody. The same mutations that
allowed for the formation of infectious pseudoviruses also exhibited the greatest
antibody binding. Data from the slot blot experiment was less clear. The N89Q
mutation that was negative on flow and did not produce a GP2 signal when cellular
protein was run on western blot generated a positive signal against mAb 8.9F on
slot blot. This is likely due to an experimental error as our previous research has
clearly demonstrated that GPC cleavage is required for mAb 8.9F reactivity. Despite
this odd finding, the remainder of the data closely mirrors the flow cytometry
results.

Based on these experiments we are left with several important conclusions
about the function of NLG sites in the Lassa virus glycoprotein. First, it is clear that
the mutation chosen to delete the glycosylation site can have a significant impact on
the observed result. For several mutant pairs we found that one mutation could
abolish activity while another may still retain some function. In this situation, it is
likely that the amino acid substitution and not the deletion of the N-glycan caused
the negative result. If only one set of mutations, for example N to Q, were
synthesized this could lead to incorrect conclusions regarding the role of N-glycans.
Generating at least two mutations for each glycosylation site resulted in a better
understanding of glycan’s properties. We can also conclude that NLG sites are important in mAb 8.9F epitope recognition in so far as glycosylation sites regulate glycoprotein cleavage and thereby trimer formation. Without the formation of a stable protein trimer, a prerequisite of the mAb 8.9F epitope, it is impossible to state conclusively that those N-glycans compose part of the epitope. Additionally, our analysis of the correlation between glycoprotein processing and infectivity has yielded interesting results. Among the NLG mutants, we found a linear relationship between GP2 pseudovirus incorporation and infectivity. As all of the mutations lacked a NLG site, we concluded that removal of glycans in the GP1 region affect infectivity by a process at or before glycoprotein incorporation into pseudovirions. We also found that there seemed to be a minimum requirement for GPC cleavage in order to form infectious particles. When cleaved GP2 is produced at a rate less than 80% of the wild type, a steep decrease in infectivity occurs. While these results are preliminary and correlation-based, they do encourage further investigation into the stoichiometric relationship between glycoprotein processing and infectivity.
List of References

10. Meyer, B. J. & Southern, P. J. Concurrent sequence analysis of 5′ and 3′ RNA termini by intramolecular circularization reveals 5′ nontemplated bases and 3′ terminal heterogeneity for lymphocytic choriomeningitis virus mRNAs. *jvi.asm.org*
16. Wallat, G. D. *et al.* High-resolution structure of the N-terminal


18. Morin, B. et al. The N-Terminal Domain of the Arenavirus L Protein Is an RNA Endonuclease Essential in mRNA Transcription. journals.plos.org 6, e1001038


31. Burri, D. J. et al. The role of proteolytic processing and the stable signal peptide in expression of the Old World arenavirus envelope


47. Salazar-Bravo, J., Ruedas, L. A. & Yates, T. L. Mammalian reservoirs of


89. Spence, J. S., Melnik, L. I., Badani, H., Wimley, W. C. & Garry, R. F. Inhibition of Arenavirus Infection by a Glycoprotein-Derived Peptide with a Novel Mechanism. *jvi.asm.org*


105. Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5. vir.sgmjournals.org


112. Wei, X. *et al.* Emergence of resistant human immunodeficiency virus type


127. Flynn, N. M. *et al.* Placebo-controlled phase 3 trial of a recombinant


Chapter 12, Unit 12.10 (2011).


161. Gupta, R., Jung, E. & Brunak, S. NetNGlyc 1.0 Server. *cbs.dtu.dk*


173. Isaacs, S. N., Kotwal, G. J. & Moss, B. Vaccinia virus complement-control


188. ElHefnawi, M. *et al.* Identification of novel conserved functional motifs


220. Cleaveland, S., Laurenson, M. K. & Taylor, L. H. Diseases of humans and

Biography

Benjamin Thomas Bradley was born in Baton Rouge, Louisiana on September 25th, 1986 to Tom and Germaine Bradley. He graduated as valedictorian from Catholic High School of Baton Rouge in 2005. Upon graduation, Ben accepted an academic scholarship to attend Tulane University. On August 29th, 2005 Hurricane Katrina hit New Orleans. The flood damage shut down the university and led Ben to spend the Fall semester at Princeton University. Returning to New Orleans, he pursued a B.S. degree in Cellular and Molecular Biology. During this time, Ben worked in the lab of Dr. Bruce Bunnell where he gained his first experiences as a research scientist. His work focused on characterizing the therapeutic benefits of mesenchymal stem cells. During the spring of 2008, Ben was awarded an internship at GlaxoSmithKline in the Metabolic Pathways and Cardiovascular Therapeutic Area Unit. During his internship, he worked closely with Dr. Douglas Johns investigating small molecular regulators of endothelial function. Ben graduated summa cum laude from Tulane University in 2009. He also completed an Honor’s Thesis and became a member of the Phi Beta Kappa honor society. That fall, Ben joined the Physician Scientist Program at Tulane University. In 2013, Ben joined the lab of Dr. James Robinson where he completed his dissertation focusing on the antibody response to Lassa virus infection. Ben is an active cyclist and helped found the Tulane University Cycling Association. In 2014, the team established the Tulane Cycling Training Center. Also in 2014, he placed 7th in the Individual Time Trial at the USA Cycling Collegiate Nationals in Richmond, VA. In 2015, Ben won the Leader’s Jersey and Men’s A Omnium for the South Central conference.
CHARACTERIZATION OF A PUTATIVE QUATERNARY NEUTRALIZING EPITOPE ON THE LASSA VIRUS GLYCOPROTEIN

A DISSERTATION

SUBMITTED ON THE TWENTY-SECOND DAY OF APRIL 2015

TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF MEDICINE

OF TULANE UNIVERSITY

FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

BY

Benjamin T. Bradley

APPROVED:

Director, James E. Robinson, M.D.

Bruce A. Bunnell, Ph.D.

Robert F. Garry, Ph.D.

Cindy A. Morris, Ph.D.

John S. Schieffelin, M.D.