Investigation of Host Factors Required for Lymphocytic Choriomeningitis Virus Entry

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Abstract

Lymphocytic choriomeningitis virus (LCMV) is an old-world arenavirus capable of causing severe diseases in humans. Despite the extensive use of LCMV in studying immune responses to viral infection, very little is known about its entry pathway. As an enveloped virus, the main determinant of LCMV entry is the viral glycoprotein, which allows for the fusion of the viral membrane with that of the target cell, upon specific triggers. While the exact triggers for LCMV GP are currently unknown, low pH and interaction with a yet to be identified host-encoded receptor are likely involved in the activation of the GP fusion activity. This thesis finds that a triad of histidine residues on LCMV's GP is absolutely critical for infection. Since mutation of the histidine triad had no effect on GP synthesis and did not completely abrogate its ability to bind to cells, our data suggest that the histidine triad are important for a step after virus internalization, potentially allowing low pH sensing. In addition, through the use of engineered soluble GPs, pulldown experiments, and mass spectrometry, various LCMV receptor candidates were identified. These candidates were further validated in order to identify crucial host proteins involved in LCMV entry. This study finds that LCMV GP interacts strongly with the Neuropilin proteins NRP1 and NRP2, and these cellular proteins may play a role in LCMV's entry pathway during infection.

Acknowledgements

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List of Abbreviations

Abbreviation	Term
CD44	Cluster of Differentiation 44
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTL	Cytotoxic T Lymphocyte
DMEM	Dulbecco’s Modified Eagle’s Medium
Dox	Doxycycline
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
GP	Glycoprotein
gRNA	Guide Ribonucleic Acid
H mut	LCMV virus with a mutated histidine triad in its GP

HA	Hemagglutinin
HIV	Human immunodeficiency virus
HM	Homogenization
HRP	Horse Radish Peroxidase
JSU	Jaagsiekte sheep retrovirus SU protein
LCMV	Lymphocytic choriomeningitis virus
LFV	Lassa fever virus
Luc	Luciferase
MHC	Major Histocompatibility Complex
MLV	Murine Leukaemia Virus
NRP	Neuropilin
NRP1	Neuropilin-1
NRP2	Neuropilin-2
NW	New World
OW	Old World
PBS	Phosphate-Buffered Saline
Pen/strep/glu	Penicillin-Streptomycin-Glutamine
RBD	Receptor Binding Domain
sE	Soluble ectodomain
SKI-1/S1P	Subtilase subtillsin kevin isoenzyme-1/site 1 protease
SSP	Stable Signal Peptide
Tfr1	Transferrin Receptor 1
VSV	Vesicular stomatitis virus
wt	Wild type
α-DG	α-Dystroglycan

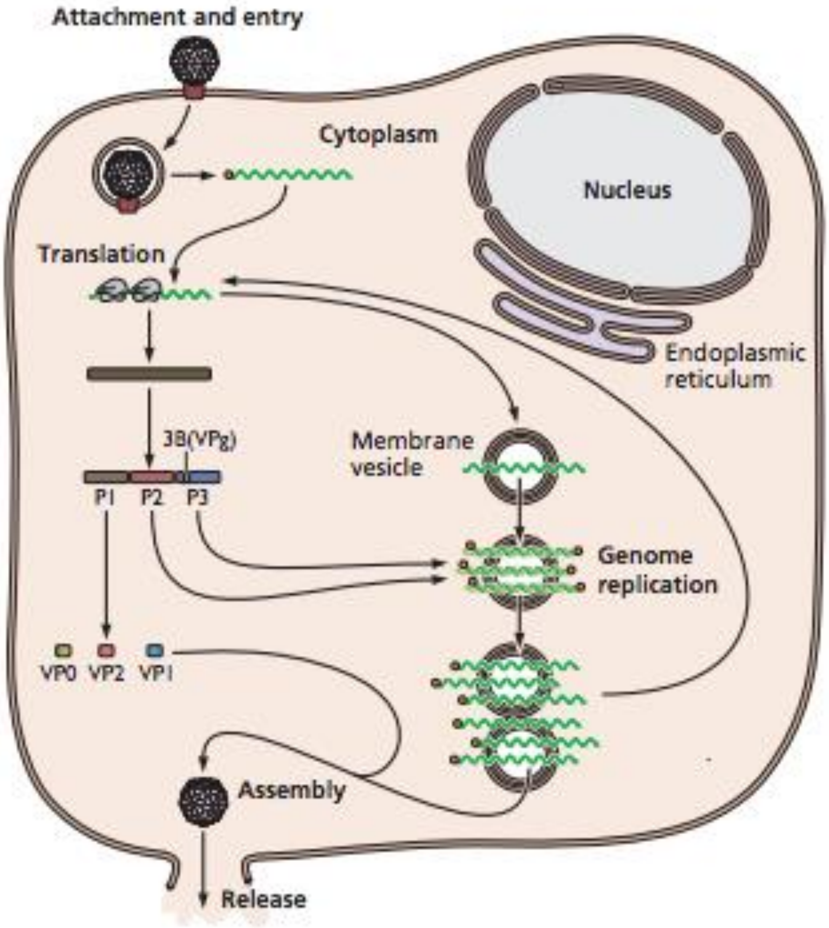
Introduction

Background

Viral Life Cycle and Entry

Viruses are submicroscopic intracellular parasites that are highly dependent on the host cell machinery for replication and production of progeny virions[1,2]. To be able to replicate, a virus must first attach to and enter a target cell[3]. After entry, viruses translate their genomes to produce the proteins that are required to produce subsequent infectious virions[4]. Once prepared, the genome is replicated many times to produce the payload for the new viruses[5]. After replication of all relevant viral genetic material, the new viruses are assembled in the viral producer cell with the components made from the translated viral genome[6]. Finally, these new viruses are released from the producer cell whereby they infect subsequent target cells, starting the lifecycle over again[7] (Figure 1).

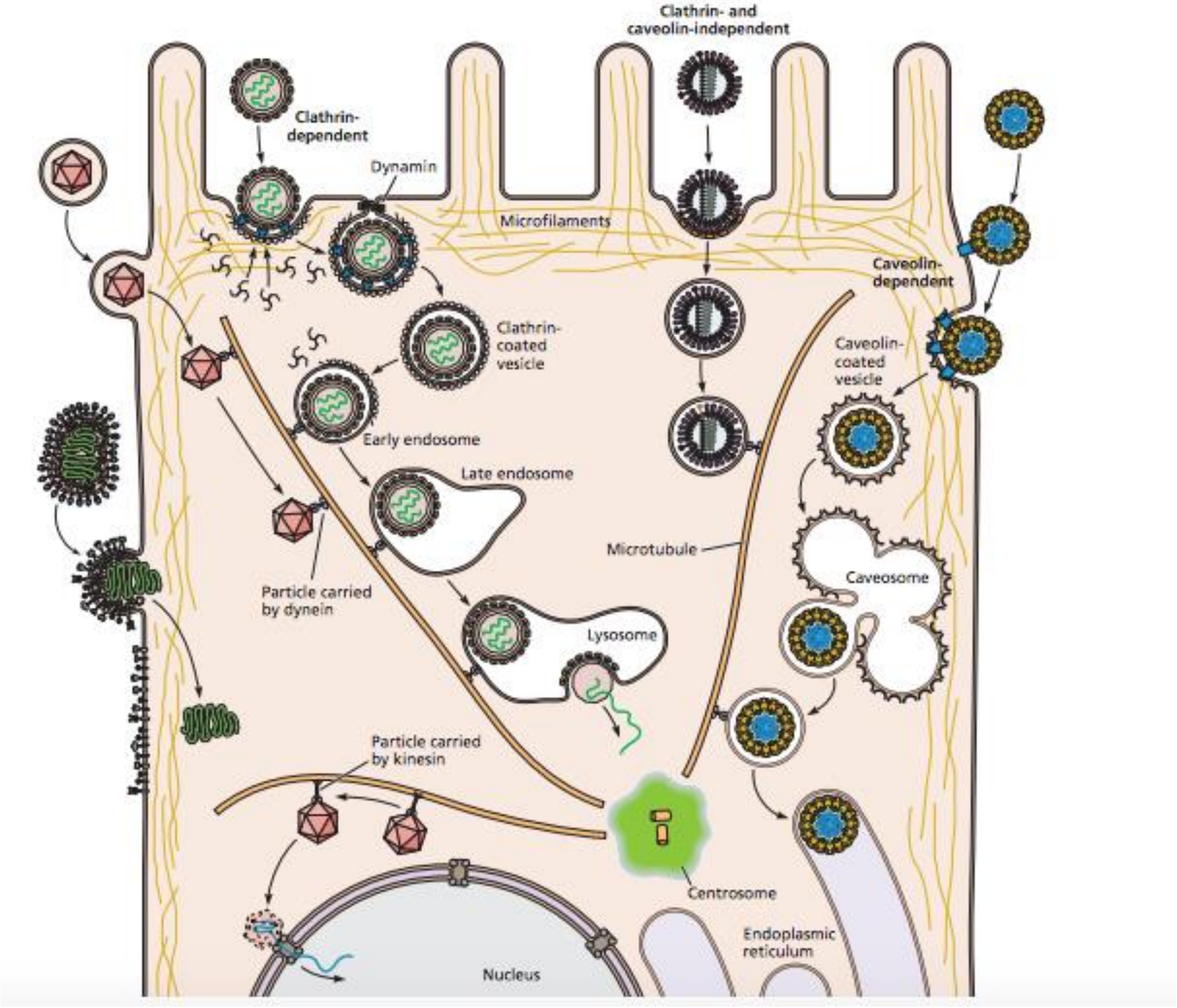
Figure 1. The Viral Lifecycle. Diagram showing the five main components of the viral life cycle: attachment and entry, translation, replication, assembly, and release. The cycle of poliovirus is shown as an example to represent all viruses. Reproduced from Flint et al., 2015.


Attachment and entry
Cytoplasm
Nucleus
Translation
Endoplasmic reticulum
Membrane vesicle
3B(VPg)
P1
P2
P3
Genome replication
VP0
VP2
VP1
Assembly
Release

Though all these stages of the life cycle are equally important for viral proliferation, virologists tend to focus on one of the steps, with this thesis concentrating on attachment and entry. Entry is a crucial component to the viral life cycle, as a virus can only proliferate if it is able to penetrate a host cell. Without efficient entry into a cell, a virus poses limited threat to its host.

Entry begins after random interactions between a viral particle and a cell which is susceptible to the invading virus[8]. After contact with the surface of the cell, the virion will bind to a receptor located on the surface of the cell, initiating the entry mechanism[9]. Though some enveloped viruses, such as measles, are capable of directly entering their host via the cell surface through fusion of the viral membrane with the host plasma membrane, several viruses require an internalization step into intracellular vesicles before membrane fusion can occur[10]. Cells often uptake viruses in similar ways to other macromolecules, usually through receptor-mediated endocytosis[9]. This internalization step can occur through a variety of mechanisms including: clathrin-coated pits, caveolin/raft mediated, clathrin-independent, and macropinocytosis[9] (Figure 2).

Figure 2. Methods of Entry by a Virus into a Cell. Diagram illustrating some of the various types of attachment and entry that viruses can use to gain access to the cytoplasm of their target cell. Reproduced from Flint et al., 2015.


Clathrin- and caveolin-independent
Clathrin-dependent
Dynamin
Microfilaments
Caveolin-dependent
Clathrin-coated vesicle
Caveolin-coated vesicle
Early endosome
Late endosome
Microtubule
Caveosome
Particle carried by dynein
Lysosome
Particle carried by kinesin
Centrosome
Endoplasmic reticulum
Nucleus

Some viruses utilize clathrin-coated pits to enter the cell, invaginating the plasma membrane, whereby the pit pinches off to form a vesicle, which rapidly fuses with other vesicles near the surface of the cell, forming endosomes[11]. The endosome will then continue along the internalization pathway, gradually acidifying from the mildly acidic environment of pH 6.5-6.0, to the lower pH environment of 5.0-6.0 found in the late endosome/lysosome[12].

Caveolin is a pathway which requires cholesterol to operate properly—as it is a major component of lipid rafts—and can be broken up into three subsets: caveolin 1-containing caveolae, dynamin 2-dependent/noncaveolar/raft-mediated, and dynamin-independent/noncaveolar/raft-mediated endocytosis[13–15].

Viral entry is further complicated by the fact that viruses do not always enter by discrete pathways. As an illustration, herpes simplex virus is able to entry the cell by three different routes, and influenza A can utilize both clathrin-dependent, and clathrin-independent pathways[16–18]. A virus' tropism is increased if it is able to exploit a variety of entry mechanisms that work with different cell types[19]. An example of such a scenario is with poliovirus, whereby the use of different entry pathways depends on if the virus is infecting neuronal or non-neuronal cells[19]. At the completion of the entry pathway occurs membrane fusion, allowing for the release of the viral genetic material into the host cell.

Viral Surface Proteins Required for Infection

The entry mechanism of enveloped viruses is dictated by viral proteins, termed glycoproteins due to their extensive glycosylation, which protrude from the viral membrane[15,20,21]. The roles of these glycoproteins are to interact with host-encoded attachment and entry receptors and/or to mediate viral membrane fusion[22]. While all these roles can be mediated by a single viral glycoprotein, some large complex viruses, such as herpes virus, that encode multiple different viral glycoproteins, will have glycoproteins important for attachment and a glycoprotein which specific function is membrane fusion[23,20].

During the attachment stage, it is possible for a virus to bind an initial receptor in order to initiate the endocytosis process, only to later switch to a different receptor to trigger a fusion event[22]. As an example, Porcine reproductive and respiratory syndrome virus interacts with both heparin sulfate and sialoadhesin during the attachment process, but neither of these attachment receptors are involved in triggering of fusion between the viral glycoprotein and its receptor, pSn. and host membranes[22].

Additionally, some glycoproteins, termed viral fusion proteins, are designed to be primed for large-scale conformational changes upon specific triggers eventually leading to fusion between the viral envelope and the host membrane[24]. While the types of triggers are diverse and virus-specific, the interaction between the viral glycoprotein and its cellular receptor is often the turnkey event in viral entry,

Viral Fusion & Fusion Proteins

Membrane fusion is a critical event for the entry of enveloped viruses. In order to allow for the virus to gain entry into the host cell, there must be fusion between the viral membrane and host membrane[25]. The fusion of two membranes implicate involves local changes in the membrane structure and requires an input of energy. This energy barrier for membrane fusion implies that spontaneous fusion is unlikely.[25] In order to overcome this energy barrier, enveloped viruses have evolved a viral membrane fusion protein which, through extensive conformational changes, is capable of overcoming the barrier and allowing membrane fusion[25].

This fusion protein is usually referred to as the virus' glycoprotein, though the name can vary depending on the family of virus. For example, the viral fusion glycoprotein is known as hemagglutinin for influenza viruses, and spike protein for the coronaviruses[26,27]. The virus this thesis investigates—Lymphocytic choriomeningitis virus—simply terms the protein its glycoprotein. Though the end result is the same, there are three different classes of viral glycoproteins, each with differences in structure, form, and mechanism of action[25]. These three classes are I, II, and III, represented by the prototypical virus of each class: Influenza, Dengue fever virus, and Vesicular stomatitis virus (VSV), respectively[28–31]. Class I viral fusion proteins are the best characterized of all the different glycoproteins[25]. These fusion proteins are made up of two different subunits—GP1 and GP2—which must be proteolytically cleaved from a precursor into their individual functional units in order to be active[25]. The GP1 subunit contains the receptor binding domain (RBD), and the GP2 subunit contains the fusion peptide[25]. At the cell surface or during the endocytic pathway, either in response to co-receptor binding or environmental cues (such as low pH), the GP1/GP2 trimer will undergo a conformational rearrangement, with the end result being separation of GP1 from GP2, which are held together through different forces[2532,33]. How GP1 and GP2 are held together depends on the virus, for example it is a disulfide bond in Influenza's HA1 and HA2, and through non-covalent interactions in the case of HIV's GP120 and GP41[32,33]. The displacement of GP1 frees the fusion peptide in GP2 to extend and insert itself into the target membrane, forming the extended intermediated, and eventually its collapse leading to the creation of a fusion pore[25]. The glycoprotein found on influenza is the prototypical class I viral fusion protein, and it is known as the hemagglutinin protein, broken down into its two subunits: HA1 and HA2[25]. Influenza responds to low pH in the endosome as the primary trigger leading to conformational change likely as a result of protonation of residues on its fusion protein near the buried fusion peptide[25].

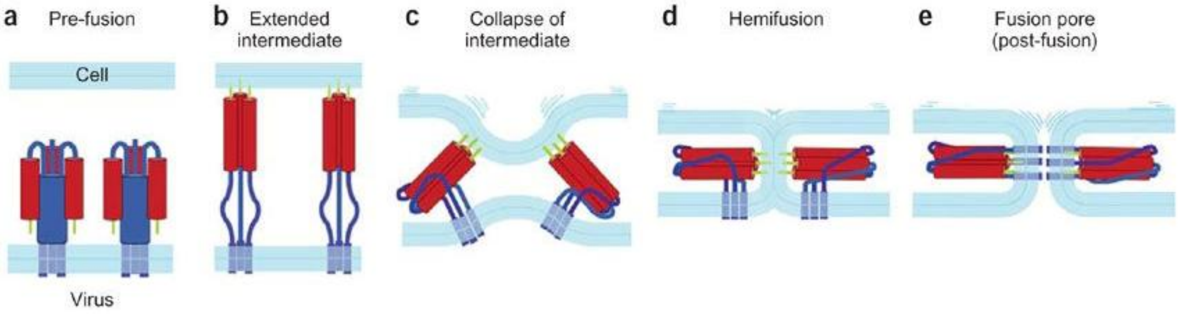
Class II proteins are found on viruses in the flavivirus family, including the human pathogens Yellow fever virus, Dengue fever virus, and Tick-borne encephalitis virus[25]. These viruses have two envelope proteins—M and E—with E displaying both receptor-binding and fusogenic capabilities, and M being the proteolytic subunit of the precursor prM, the form incorporated in the envelope of immature viral particles[25]. However, the terms can change for these two proteins depending on the virus. This maturation process is dictated by cleavage of the precursor by the enzyme furin during assembly, as the virus is budding from the endoplasmic reticulum[25]. This cleavage step of prM to M is critical in order to prime the virus for low pH-induced membrane fusion[25,34]. The E protein exists as a dimer and contains the ectodomain which is made up of domain I , II, and III as a distinct subdomain[35]. The C-terminal end of the sE chain is an immunoglobulin-like domain which is posited to have viral attachment functions[25]. As the virus moves through the endocytic pathway, lowered pH leads to disassociation of the dimers, letting the individual subunits swing outward[36]. The exposed fusion loops insert themselves into the target membrane, which re-clusters the subunits from a dimer into a trimeric formation[36]. These trimeric intermediates then collapse after rotation of domain III of the E protein, leading to the post-fusion conformation, and a resulting fusion pore (Figure 3)[25,36].

Class III glycoproteins contain features unique to them, with the main characteristic being that, unlike class I and II, the low pH-induced conformational change of the fusion protein appears to be reversible[37]. Virions which have been inactivated by extended periods of time at low pH can be reactivated by raising the pH of the environment the virus is located in[25,37]. The classic example of a class III glycoprotein is that harbored by Vesicular stomatitis virus (VSV), termed the G protein[25]. Structural representations show that two hydrophobic loops on the G protein each link a pair of antiparallel B-strands, and lie next to each other at the tip of an elongated domain[25]. Two separate domains form the active two-part fusion machinery, and similar to the flavivirus glycoprotein, there is a zipping alongside the fusion domains in the fusion-ready low-pH conformation[25].

Despite the vast differences in the structures and architectures of glycoproteins between different viruses and families, they all mediate fusion in a similar way; they all mediate membrane fusion by undergoing extensive conformational changes, from a metastable pre-fusion conformation to a highly stable post-fusion conformation[25]. While the overall pre-fusion conformation differs depending on the class of viral fusion protein, they all exist as a trimer of hairpins in their post-fusion state[25]. This post-fusion conformational state is the most thermodynamically preferable, but, as mentioned, there is a kinetic energy barrier preventing the virus from achieving this state[25]. This puts the virus pre-fusion meta-stable state, stable enough to protect the integrity of its structure, but capable of achieving a more stable state which would result in fusion[25]. Therefore, the glycoprotein conformational change, from a high energy metastable state to a lower energy highly stable state liberates the free energy require for membrane fusion[25]. This conformational change usually occurs after binding of the glycoprotein with its receptor, though in some cases environmental cues, such as low pH, may be enough[25].

This conformational change extends the subunits of the glycoprotein towards the target membrane, and creates contact between the target cellular membrane and the fusion peptide of the glycoprotein[25]. Generally, the fusion peptide will be located at the C-terminal fragment of the larger glycoprotein, with the N-terminal receptor-binding domain fragment being removed, prior to initiation of the fusion event[25]. After the fusion peptide has extended and interacted with the target membrane, it next collapses, distorting the two bilayers into a ‘nipple-like’ conformation[38]. Distortion of these membranes is what lowers the energy barrier between the layers, allowing for the formation of a hemifusion stalk[25]. After the stalk is formed, it opens up to create a transient fusion pore, which causes an irreversible folding of the protein, resulting in a permanently open fusion pore[25]. It is through this pore that the viral genetic material enters the cytoplasm of the cell, commencing the next step in the viral life cycle (Figure 3).

Figure 3. Steps in Viral Glycoprotein Fusion. Diagram representing the steps involved in fusion between a viral glycoprotein and its target membrane. (A) The glycoprotein is in its pre-fusion confirmation, not yet having gone through the fusion process. (B) Next, the protein opens up and extends its fusion loop into the target membrane. (C) The extended intermediate collapses, a C-terminal segment of the protein folds back along the outside of its trimeric core. (D) Once the collapse of the intermediate has proceeded to the point where the bilayers of the glycoprotein and target membrane are in contact, a hemifusion stalk is formed. (E) The hemifused layers open up into a fusion pore, and the C-terminal ectodomain segment zips up to snap the refolded trimer into its post-fusion confirmation, preventing the resealing of the pore. Repurposed from Harrison, 2015.


a
Pre-fusion
Cell
Virus
b
Extended intermediate
c
Collapse of intermediate
d
Hemifusion
e
Fusion pore (post-fusion)

The fusion process is mediated entirely by the glycoprotein on the surface of the virus. In order for a virus' glycoprotein to be functional, there are two main events which must take place: priming and activation[25]. The priming step is usually the result of proteolytic cleavage of either the glycoprotein or an associated protein, and may take place during viral assembly, budding, or conversely during entry in the endosomal compartments[25]. After priming, the fusion protein is in its metastable state and an energy barrier must be overcome to transit to the post-fusion conformation[25]. Often, this barrier is lowered by rearrangement of the protein, a process known as activation, which can occur in response to binding to receptors and/or low pH[25]. At the end of this activation step, the free energy liberated by the rearrangement of the glycoprotein allows for fusion between the viral and target membranes[25].

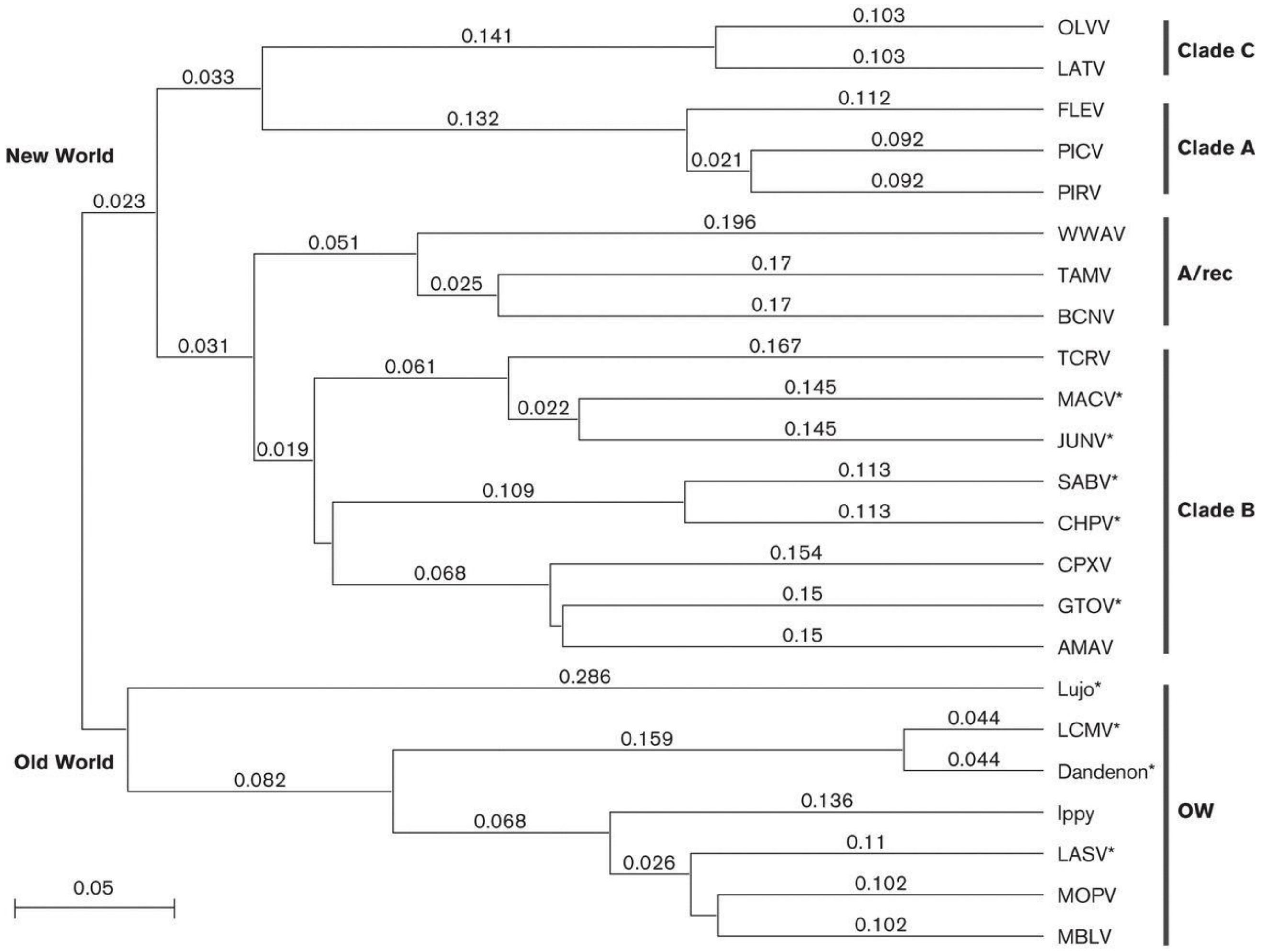
Fusion events remain the central component of viral entry for enveloped viruses. Molecules or ligands which target different steps of fusion (either the glycoproteins themselves, their receptors, or other components) can be potent therapies, as these factors are generally conserved between all species of a virus[25,39]. It has been shown in the clinic that small molecules, such as 5M038 and 5M030, are able to block the fusion event of HIV, thus rendering the virus unable to properly replicate, preventing productive infection[40]. Therefore, understanding a virus' glycoprotein and elucidating the entry receptor are very important, as it can lead to the identification of key targets for antiviral therapies[25].

Arenaviruses

Arenaviruses are viruses belonging to the family *Arenaviridae*[41]. Members of this family are enveloped, contain a bi-segmented, negative-sense RNA genome, and are non-lytic in their lifecycle[41]. Member of this family can be split into two different groups: Old

World (OW) and New World (NW)[41]. One of the ways the two groups are broken up is by geographic distribution, with the OW viruses usually being found in Africa, and the NW often located in South America[42]. The NW phylogeny can further be broken down into three clades—A, B, and C[42]. There are many examples of viruses in this family, with many being human pathogens. Members of the OW arenaviruses include: Mobala virus, Mopeia virus, Lassa fever virus, Ippy virus, Dandenon virus, Lymphocytic choriomeningitis virus (LCMV), and Lujo virus[42]. Members NW arenaviruses include: Amapari virus, Guanarito virus, Cupixi virus, Chapare virus, Sabia virus, Junin virus, Machupo virus, Tacaribe virus, Bear Canyon virus, Tamiami virus, White Water Arroyo virus, Pirital virus, Pichinde virus, Flexal virus, Latino virus, and Oliveros virus[42] (Figure 4).

Figure 4. Phylogenic Tree of Arenaviruses. Multiple sequence alignment of the complete GPC protein sequences from different arenaviruses by clustal w analysis. A phylogenetic tree was generated using the unweighted pair group method with arithmetic mean, using MacVector 12.6.0. Horizontal distances represent protein differences: bar, 0.05 amino acid changes per site. Arenavirus GPCs included are: Oliveros virus (OLVV) (GenBank accession no. YP_001649210), Latino virus (LATV) (Q8B121), Flexal virus (FLEV) virus (YP_001936019), Pichinde virus (PICV) (ABU39904), Pirital virus (PIRV) (YP025080), White Water Arroyo virus (WWAV) (Q911P0), Tamiami virus (TAMV) (Q8AYY5), Bear Canyon virus (BCNV) (A0PJ25), Tacaribe virus (TCRV) (P18141), Machupo virus (MACV) (NP899212), Junín virus (JUNV) (P26313), Sabia virus (SABV) (Q90037), Chapare virus (CHPV) (B2C4J0), Cupixi virus (CPXV) (Q8B115), Guanarito virus (GTOV) (Q8AYW1), Amapari virus (AMAV) (YP_001649208), Lujo virus (YP 002929490), lymphocytic choriomeningitis virus (LCMV) (AAX49341), Dandenon virus (ABY20729), Ippy virus (Q27YE4), Lassa virus (LASV) (P08669), Mopeia virus (MOPV) (P19240) and Mobala virus (MBLV) (Q2A069). OW, Old World arenaviruses. New World arenaviruses are divided into clades A, B and C, as well as the A/rec recombinant lineage. Pathogenic arenaviruses are indicated by an asterisk. Reproduced from McLay, Liang, & Ly, 2014.


0.103
OLVV
0.141
0.103
LATV
Clade C
0.033
0.112
FLEV
0.132
0.092
PICV
Clade A
New World
0.021
0.092
PIRV
0.023
0.196
WWAV
0.051
0.17
TAMV
A/rec
0.025
0.17
BCNV
0.031
0.167
TCRV
0.061
0.145
MACV*
0.022
0.145
JUNV*
0.019
0.113
SABV*
0.109
0.113
CHPV*
Clade B
0.154
CPXV
0.068
0.15
GTOV*
0.15
AMAV
0.286
Lujo*
0.044
LCMV*
0.159
Old World
0.044
Dandenon*
0.082
0.136
Ippy
OW
0.068
0.11
LASV*
0.026
0.05
0.102
MOPV
0.102
MBLV

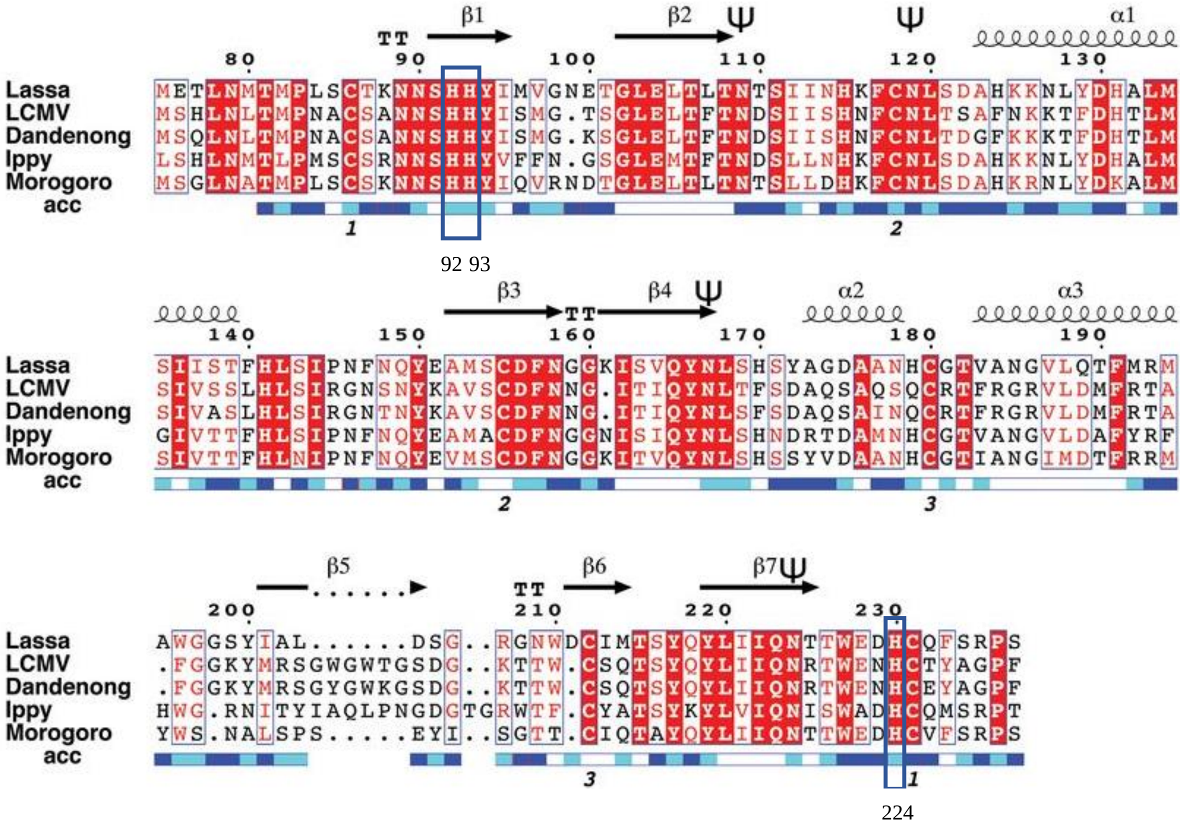
Not all of these viruses are pathogenic to humans, but one unifying characteristic between both the OW and NW, is all arenaviruses capable of human infection have a member of the rodent order as their reservoir[42]. The virus can be spread from rodents to their offspring through vertical transmission, and to humans by horizontal transmission[42]. Infections from arenaviruses tend to result in either hemorrhagic fever, as is the case with Lassa fever virus, or neurological disease, such as from LCMV[41].

Other than geographic separation, a key phylogeny difference between the OW and NW arenaviruses is the utilization of different surface receptors for entry[41]. The OW viruses primarily use α-dystroglycan (α-DG) for entry, especially LCMV, Lassa fever virus, Mopeia virus, and Mobala virus[41]. Interestingly, α-DG has also been shown to be utilized by some NW arenaviruses, primarily those in Clade C—Latino virus, and Oliveros virus[41]. Conversely, Transferrin receptor 1 (Tfr1) has been implicated as a primary receptor for members of the NW group of arenaviruses[41]. Experiments taking otherwise non-susceptible cells and expressing Tfr1 in them were able to allow for successful infection by the NW viruses Junin virus, Machupo virus, and Guanarito virus, but not Lassa fever virus, a member of the OW group[41]. It is important to note that these receptors, α-DG and Tfr1, are involved in endocytosis of the virus into the cell, but are not the entry receptors which are responsible for membrane fusion and entry of the virus into the cell. For some of these viruses, such as Lassa fever virus, their entry receptors are known (LAMP1), but others, such as LCMV, continue to be elusive[43].

For the most part, the entry process between arenaviruses is conserved among all groups[41]. These viruses harbour a class I viral fusion protein, known as the glycoprotein (GP), which is proteolytically cleaved into two active subunits, GP1 with the receptor-binding domain, and GP2 containing the fusion peptide[41]. In order for these viruses to be fusion-competent, a low pH environment is required, which coincides with the fact that arenaviruses are late-penetrating[41]. Late-penetrating viruses are viruses that delay penetration into the cytosol of host cells until after they have trafficked in vesicles deep in the cytoplasm[44].

In addition to the myriad of unique characteristics seen with arenaviruses, studies have illustrated other features of the glycoproteins of some arenaviruses which indicate potentially novel mechanisms for environmental sensing[43]. The ability for a virus to sense the environment that it is in is a key evolutionary advantage that allows it to have a greater chance of successful replication, thus proliferating. Because arenaviruses harbor a class I viral fusion protein, the activation step is irreversible, and should the fusion machinery become prematurely activated, the virus would be inactivated. Likewise, if a glycoprotein were to successfully fuse to a target membrane, but that cell was non-permissive, that infection would be considered abortive, and replication of the viral genome could not take place. This makes fusing at the right point, in a cell which can support replication, a key component to viral survival and proliferation. Different viruses use different mechanisms to ensure that this happens correctly, but a recent study by Cohen-Dvashi et al. has identified the ways in which Lassa fever virus carry out this process (2015). Lassa fever virus is an OW arenavirus which uses α-DG as its primary attachment receptor, but it is not sufficient to allow for viral entry[43]. Some of these arenaviruses, Lassa fever virus included, need an additional receptor to cause the conformational change of the glycoprotein which allows for membrane fusion[43]. Its entry receptor has been identified as LAMP1, and it switches from using attachment factors to exploiting this protein in a pH-dependent manner[45]. The mechanism by which it senses this drop in pH has been identified to be a cluster of histidine residues found in the GP1 subunit of the virus' glycoprotein[43]. These histidine residues are capable of being protonated and act like a pH probe, causing a conformational change which allows for binding to its entry receptor and subsequent fusion of the membranes, and entry into the cell[43]. It has been identified that these histidine residues are conserved on other arenaviruses—including the prototypical OW arenavirus LCMV—yet their role in these other viruses has not yet been validated[43] (Figure 5).

Figure 5. Arenavirus Glycoprotein Sequence Alignments. Multiple-sequence alignment of GP1s from Lassa virus, lymphocytic choriomeningitis virus (LCMV), Dandenong virus, Ippy virus, and Morogoro virus OW arenaviruses, showing the secondary structure elements as observed in the crystal structure of $GP1_{LASV}$. The numbering of the amino acids is based on the sequence of the Lassa virus glycoprotein precursor. Fully conserved residues are highlighted with a red background, and partially conserved residues are shown in red. A blue-to-white scale bar below the sequences indicates solvent accessibility (exposed to buried). Numbers below the sequences designate the locations of the disulfide bridges. The symbol 'Ψ' marks glycosylated asparagine residues. The LCMV histidine triad is highlighted with a blue box with the residue number labelled below. This graphical representation was generated by ESPript (25) (http://espript.ibcp.fr). Reproduced from Cohen-Dvashi, Cohen, Israel, & Diskin, 2015.



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Lassa      METLNMTMPLSCTKNNSHHYIMVGNETGLELTLTNTSIINHKFCNLSDAHKKNLYDHALM
LCMV       MSHLNLTMPNACSANNSHHYISMG.TSGLELTFTNDSIISHNFCNLTSAFNKKTFDHTLM
Dandenong  MSQLNLTMPNACSANNSHHYISMG.KSGLELTFTNDSIISHNFCNLTDGFKKKTFDHTLM
Ippy       LSHLNMTLPMSCSRNNSHHYVFFN.GSGLEMTFTNDSLLNHKFCNLSDAHKKNLYDHALM
Morogoro   MSGLNATMPLSCSKNNSHHYIQVRNDTGLELTLTNTSLLDHKFCNLSDAHKRNLYDKALM
  acc

Lassa      SIISTFHLSIPNFNQYEAMSCDFNGGKISVQYNLSHSYAGDAANHCGTVANGVLQTFMRM
LCMV       SIVSSLHLSIRGNSNYKAVSCDFNNG.ITIQYNLTFSDAQSAQSQCRTFRGRVLDMFRTA
Dandenong  SIVASLHLSIRGNTNYKAVSCDFNNG.ITIQYNLSFSDAQSAINQCRTFRGRVLDMFRTA
Ippy       GIVTTFHLSIPNFNQYEAMACDFNGGNISIQYNLSHNDRTDAMNHCGTVANGVLDAFYRF
Morogoro   SIVTTFHLNIPNFNQYEVMSCDFNGGKITVQYNLSHSSYVDAANHCGTIANGIMDTFRRM
  acc

Lassa      AWGGSYIAL......DSG..RGNWDCIMTSYQYLIIQNTTWEDHCQFSRPS
LCMV       .FGGKYMRSGWGWTGSDG..KTTW.CSQTSYQYLIIQNRTWENHCTYAGPF
Dandenong  .FGGKYMRSGYGWKGSDG..KTTW.CSQTSYQYLIIQNRTWENHCEYAGPF
Ippy       HWG.RNITYIAQLPNGDGTGRWTF.CYATSYKYLVIQNISWADHCQMSRPT
Morogoro   YWS.NALSPS.....EYI..SGTT.CIQTAYQYLIIQNTTWEDHCVFSRPS
  acc
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The Arenavirus Signal Peptide

The arenaviruses contain a unique feature of their GP known as the Stable Signal Peptide (SSP), which is retained after cleavage as an essential part of their fusion protein[41]. Normally, the total length of signal peptides is around 15-30 residues, but certain peptides, such as those harbored by arenaviruses, have signal peptides much greater than this, including up to 65 residues, as found in Lassa fever virus[46]. Interestingly, it has been found that the enzyme used to cleave the precursor glycoprotein of certain arenaviruses (GP-C), subtilase subtilisin kexin isoenzyme-1/site 1 protease (SKI-1/S1P), is also involved in cleavage of the signal peptide of these viruses, and that this cleavage is likely necessary for maturation of the virus[46,47]. This findings add credibility to the hypothesis that the signal peptide of arenaviruses is involved in more than just the basic sorting and membrane integration function seen with other signal peptides[46]. This hypothesis is supported by the observations that in other arenaviruses, such as Junin virus, its signal peptide plays a key role in pH-dependent membrane fusion of the virus during entry. Given that arenaviruses are late-penetrating viruses dependent on low pH during their entry process, it is possible that the signal peptide plays the same role in other members of this family, or perhaps even more yet-to-be understood functions[48].

LCMV

Lymphocytic choriomeningitis virus (LCMV) is an enveloped Old World arenavirus, with a negative-strand RNA genome, ambisense coding strategy, and a class I viral glycoprotein[49,50]. LCMV is a human pathogen, often causing infection with no symptoms, but in some cases, individuals infected with the virus can experience neurotropic symptoms including aseptic meningitis or fatal encephalitis[51]. LCMV is also capable of causing severe flu-like symptoms, and can in some cases, be fatal[51].

Tropism

Studies have shown that different LCMV strains can have different tropisms, and that overall these can be divided into two groups[49]. The first relies heavily on binding to α-dystroglycan (α-DG) for entry, and the second exhibits a low affinity to α-DG, and a reduced dependence on this protein during entry[49]. LCMV viruses relying on α-DG tend to cause a persistent infection, an ability to infect cells within the white pulp of the spleen, and a subsequent loss of virus-specific Cytotoxic T Lymphocyte (CTL) response[49]. Conversely, the LCMV viruses less reliant on α-DG binding replicate more in the red pulp of the spleen, and infection is often rapidly resolved[49].

Reservoir

Similar to other arenaviruses, the natural reservoir for LCMV is rodents, with the virus being transmitted through contact with bodily fluids or inhaling aerosolized particles of excretes[52]. One study investigated the prevalence of LCMV virus in Spain, and found that within mice studied (*Mus musculus*), the virus was prevalent in 11.7% of them[52]. LCMV is also an important teratogen, with infection during pregnancy being associated with birth defects and spontaneous abortions[53]. LCMV is capable of infecting an individual persistently, and there is evidence of reactivation of the infection in an affected individual during times of hypoxia, including during a heart attack, stroke, or ischemia[54].

Pathogenesis

It was first observed in 1936 that LCMV is capable of persistently infecting mammalian cells without being cleared by the immune system, resulting in a different form of pathogenesis than had been seen at the time[55]. Additionally, not all organisms infected with LCMV display symptoms[56]. Disease effect and lethality mainly arise as a result of tissue damage caused by cytotoxic T cells[56]. These T cells recognize a complex of LCMV epitope peptides on MHC Class I molecules at the surface of the virally infected cell, leading them to attempt to clear the infected cells[56]. This effort often leads to the tissues in the host organism being damaged[56]. Given that there are more than 30 strains of LCMV that have been isolated, there is great fluctuation between the symptoms and prognosis of an individual infected with LCMV, which is primarily determined by the strain the individual is infected with, and the host MHC Class I haplotype[56].

History

In addition to its role as a human pathogen, LCMV is also widely used in the field of immunology as a model organism[57]. LCMV has been used in some of the most fundamental studies elucidating mechanism of how the immune system works, and has contributed to research that has later gone on to win three separate Nobel prizes[57]. Some of the findings which have used LCMV integrally have been the Major Histocompatibility Complex restriction, T cell memory, persistent infections, T cell exhaustion, and how immune pathology affects disease[57]. Despite its importance as both a human pathogen, and model organism, LCMV's entry pathway has been poorly characterized, and its entry receptor continues to be unknown.

Entry Pathway

Work has been done to characterize LCMV's entry pathway. Initial attachment of the virus to the cell is mediated through binding to α-DG, independent of cholesterol[58]. After initial attachment however, internalization occurs through the use of cholesterol, and is clathrin and caveolin-independent[58]. Additionally, LCMV does not require dynamin or actin for entry, which is anathema to the new world arenaviruses, namely Junin virus[58]. During the entry process, LCMV appears to enter through non-coated vesicles, which progress towards a lower pH state[58,59]. Studies have indicated that this dependence on cholesterol is only relevant during the entry stage of infection, and is not needed during later steps[58]. Additionally, the cholesterol requirement appears to be conserved with other members of the arenavirus family, namely Lassa fever virus[58]. LCMV's entry also seems to be independent of macropinocytosis since actin skeletons—an important component of macropinocytosis—seem to be undisturbed throughout the LCMV entry process[58]. It is known that exposure of the virus to low pH is important, but it is unknown if low pH is critical to trigger GP conformational change or for binding to a yet-unknown receptor protein[60].

Hypothesis

Similarly to lassa fever virus, lymphocytic choriomeningitis virus contains a triad of histidine residues in its glycoprotein. I hypothesize that this triad is important for viral entry and required for binding to a yet-to-be discovered entry receptor.

Problem

This thesis attempts to better understand, and characterize, the entry pathway of LCMV. As mentioned, LCMV remains a human pathogen for which there is no cure or treatment, yet in some cases, although rare, is central in causing human suffering. Additionally, LCMV continues to be used as a tool to uncover more and more about the complicated inner workings of the immune system. Despite LCMV being an important virus, very little is known about its entry pathway. Included in this is a lack of understanding of its glycoprotein, environmental sensing ability, host factors required for entry, and entry receptor. Additionally, conflicting literature exists surrounding which proteins are truly both involved and essential in LCMV's entry pathway.

Identification of factors required by LCMV for entry is important for a variety of reasons. Firstly, from a clinical perspective, targeting receptors with small molecules or biological drugs can yield high rates of protection from the virus for patients, as receptor utilization tends to be a conserved feature of viral entry. From a research perspective, understanding how LCMV gains entry into a cell is critical to furthering the understanding of its lifecycle and infection pattern. This, in turn, would have great benefits for those studying the virus to develop models, as it completes gaps in understanding which have previously hampered further understanding of immune pathways.

Approach

The approach to elucidate more about the virus' entry begins by looking at its glycoprotein, the key protein involved in mediating entry. This thesis wanted to examine what the outcome of mutation of the conserved histidine triad was, and if it shared any similar results to previously reported literature by a related virus—Lassa fever virus. After understanding more about the glycoprotein itself, and the effect on infection was after mutation of these conserved histidine residues, this thesis moves on to understanding the crucial proteins required for binding of the receptor binding domain, and triggering a fusion event. These results show several possible proteins involved in LCMV entry.

Objectives

- Investigate the purpose of the histidine triad on LCMV’s glycoprotein by studying the effect on infection after mutation of the histidines into tyrosines.
- Identify entry receptor candidates using soluble glycoprotein and mass spectrometry analysis.
- Validate the candidate receptors using binding assays.

Materials & Methods

Viral Glycoprotein Constructs

Addition of N- and C-termini tags on LCMV GP

A FLAG tag was added to the N-terminus of the wt LCMV glycoprotein using pCAGGs LCMV (We strain) GP (kind gift of Dr. James Cunningham, Brigham and Women's Hospital) as a template, by creating two fragments through PCR with primers A and B, and C and D (Table 1). These fragments were joined together by PCR using primers C and D. An HA tag was added to the C-terminus of the wt GP with primers a and b (Table 1). These different inserts were placed into the pCAGGs vector with restriction enzymes NsiI and NheI. DH5α bacteria were then transformed and plated on LB Agar plates containing Ampicillin (100µg/ml). Colonies were picked, screened and the constructs were sequenced.

Mutation of LCMV GP

Overlapping PCR was performed to mutate the three histidine residues found in both the generated FLAG and HA tagged wt glycoproteins to tyrosine residues (H92Y, H93Y, and H224Y). Three individual fragments of the glycoprotein were generated with primers 1 & 2, 3 & 4, and 5 & 6 (Table 1). These fragments were then joined together with primers 1 & 6, and put into the pCAGGs vector with restriction enzymes NsiI and NheI. DH5α bacteria were then transformed and plated on LB Agar plates containing Ampicillin (100µg/ml). Colonies were picked, screened and the constructs were sequenced.

Generation of a soluble LCMV GP1

A soluble GP1 protein was generated by using Gibson assembly to clone the GP1 (receptor-binding domain) of both the wt and histidine mutated glycoproteins in frame with the Fc region of human IgG into the pCSI vector (kind gift of Dr. Shan-Lu Liu, The Ohio

State University). The GP1 region was amplified by PCR using primer i & ii and the pCSI vector containing the hIgG-Fc was amplified using primers iii & iv (Table 1). Gel purified fragments were combined at a 1:1 ratio (100ng total), and mixed with the Gibson assembly reaction solution (0.2 M Tris-HCl, 0.4 M $MgCl_2$, 20 mM dNTP mix, 0.2 M DTT, 5% w/v PEG-8000, 20 mM NAD, 10U/uL T5 exonuclease, 2U/uL Phusion polymerase, 40U/uL Taq ligase) for 1 hour at 72°C. DH5α bacteria were then transformed and plated on LB Agar plates containing Ampicillin (100µg/ml). Colonies were picked, screened and the constructs were sequenced.

Plasmid preparation for transfections

All constructs were verified by Sanger sequencing. Plasmid DNAs were amplified and purified (Qiagen), and DNA concentrations were measured by spectrophotometer using Nanodrop (ThermoFisher).

Table 1: Oligo Sequences Used in the Creation and Mutation of Viral Glycoprotein Constructs. Table containing the oligo sequences used in the creation of constructs for viral and protein production. Oligo code is referenced in the text to refer to the specified primer and sequence listed in the table. Oligo names listed are the names used when ordering the primers.

Construct	Oligo Code	Oligo Name	Oligo Sequence
FLAG-tagged LCMV	A	Lower_FLAGLCMVGP	GTCATCGTCTTTGTAGTCGCCACAGGACCTACC
	B	Upper_FlagLCMVGP	GACTACAAAGACGATGACGACAAGATGTACGGCCTTAATG
	C	Upper_LCMVGP_NsiI	CTGAATGCATGCCACCATGGGTCAGATTGTG
	D	Lower_LCMVGP_notag	GACTGCTAGCTCAGCGTCTTTTCCAGATAGTTTTTACACC
HA-tagged LCMV	a	Upper_LCMVGP_NsiI	CTGAATGCATGCCACCATGGGTCAGATTGTG
	b	Lower_LCMVGP_HA (NheI)	GACTGCTAGCTCAAGCGTAATCTGGAACATCGTATGGGTAGCGTCTTTTCCAGATAG
LCMV H mutants	1	Upper_LCMV_58C	CTGAATGCATGCCACCATGGG
	2	LowerLCMVGPHH/YY	CCCATACTGATGTAGTAATAAGAGTTGTTGACTG
	3	UpperLCMVGPHH/YY	CAGTCAACAACTCTTATTACTACATCAGTATGGG
	4	LowerLCMVGP_H230Y	CCTGCATATCTACAGTAGTTTTCCCAAGTC
	5	UpperLCMVGP_H30Y	GACTTGGGAAAACTACTGTAGATATGCAGG
	6	Lower_LCMV_notag_58C	GACTGCTAGCTCAGCGTCTTTTC
LCMV GP1	i	IgGvector_forward	GTCGACGGCCTAGGATATCGATACGTAG
	ii	Gibson_IgGvector_Rev	GCGGCCGCGGGTACAATT
	iii	Gibson_LCMVWe_GP1_Fwd	GGAATTGTACCCGCGGCCGCACCGCCATGGGTCAGATTG
	iv	Gibson_LCMVWe_GP1_RevTm68	GGCTCTACGTATCGATATCCTAGGCCGTCGACGAGAAACTTTGTCTTTTCCTGAG

Cell Culture

Cells and media

HT1080, Vero and 293T cells were obtained from ATCC and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Wisent) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich) and Penicillin-Streptomycin-Glutamine (pen/strep/glu, 10,000U/mL, Gibco), at 37°C, 5% CO_2, and 100% relative humidity.

IgG-depleted FBS was generated by incubating the FBS with pre-washed protein G beads (ExAlpha) at a 50:1 ratio (DMEM: bead slurry) overnight with rotation at 4°C. The beads were then removed by centrifugation (2,000rpm, 5min, 4°C) and the supernatant was sterilised by filtration using a 0.22μm filter (VWR). The resulting IgG depleted FBS was used to prepare the soluble protein production medium (DMEM, 5% IgG-depleted FBS, pen/strep/glu).

Virus Production and purification

To generate murine leukemia virus (MLV) pseudotypes, 293T cells were co-transfected, using the JetPrime transfection reagent (Polyplus transfection), with plasmids encoding MLV gag-pol genes, a retroviral vector encoding the Beta galactosidase (LacZ) or firefly luciferase (Luc) reporter gene, and plasmids encoding the VSV G (all plasmids were kind gifts of Dr. James Cunningham, Brigham and Women's Hospital) or the different LCMV GPs. Media was replaced 24 hours post-transfection and collected at 48, 72, and 96 hours post-transfection.

Collected culture media containing viruses were spun down at 1,500rpm for 5 minutes to remove cellular debris and the supernatant was filtered through a 0.45um syringe filter (VWR). Viruses in the supernatant were then pelleted by ultracentrifugation

(20,000rpm, 1h30, 4°C) over a 20% sucrose cushion. Pelleted viruses were then resuspended in 300uL of PBS, aliquoted, and stored at -80°C.

Infections

Purified viruses or supernatants containing viruses were added to cells (50% confluence) in media containing 8μg/mL polybrene. Media was replaced 24 hours post-infection and infection measured 96 hours post-infection.

For infection with MLV pseudotypes encoding LacZ, infected cells were fixed for 15 min at room temperature with formalin, washed three times with PBS and incubated overnight at 37°C in LacZ stain solution (2mM $MgCl_2$, 5mM potassium ferrocyanide, 5mM potassium ferricyanide (Sigma), 50 μg/mL X-Gal (ThermoFisher)). Blue foci were counted and infectious titer calculated (number of foci/volume of virus or supernatant used = FFU/mL).

For infection with MLV pseudotypes encoding Luc, infected cells were washed with PBS and incubated for 1min on a shaker with 1:1 DMEM without phenol red, and Beta-Glo substrate (Promega) followed by an incubation in the dark at room temperature for 30 minutes. Luminescence was then measured using a plate reader according to the manufacturer's specifications, with an integration time of 0.20 seconds (BioTek Instruments, Synergy Neo2).

Protein production

293T cells seeded on 15-cm dishes were transfected with the soluble LCMV GP constructs or a plasmid encoding the Jaagsiekte sheep retrovirus SU protein fused with human IgG (JSU, kind gift of Dr. Shan-Lu Liu, Ohio State University) using the JetPrime transfection reagent according to the manufacturer's protocol. Twenty-four hours post-transfection, the media was replaced with soluble protein production medium. Culture supernatants containing the proteins were collected at 48, 72, and 96 hours post-transfection. Collected media was spun down at 2,000rpm for 5 minutes at 4°C and supernatants were filtered using 0.45µm filters to further remove cells and cell debris.

To purify the protein, disposable columns, 1cm in diameter and holding 5mL of volume (ThermoFisher) were packed with 750uL of Protein G beads, followed by a wash with 15mL of PBS. Filtered supernatants containing the proteins were applied unto the column using a peristaltic pump. The flow rate was adjusted so that there was a continual flow of media through the column with flow-through media re-applied for a period of 24 hours. The column was then washed with PBS (20 times the bead volume). Beads with bound proteins were either used as is for pull down assays (see sections below) or the proteins were eluted using 1M Glycine pH3.0 and collected in 120ul fractions. Each fraction was neutralized with 15µl 1M Tris/HCl pH 8.0 and analyzed for the presence of the protein by Western Blot. Fractions containing the highest amount of GP-Fc were combined and final protein concentrations were determined by SDS-PAGE with BSA standards and staining with SYPRO Ruby (ThermoFisher) according to the manufacturer’s protocol.

Creation of NRP1, NRP2, CD44 and Tfr1 deficient cell lines

The NRP1, NRP2, CD44, and Tfr1 knockout cells were generated by first producing lentiviral vectors via transfection of 293T with plasmids encoding pCWCas9 (gift from Eric Lander & David Sabatini, Addgene plasmid # 50661) (Wang et al., 2014), psPAX2, and VSV-G in a 4:3:1 ratio. The lentiviral vectors were then used to infect HT1080 cells, followed by puromycin selection, to obtain a doxycycline-inducible Cas9 population.

Two gRNAs were used to target the genes for NRP1, NRP2, CD44, and Tfr1 (Table 2). For the NRP1/NRP2 double knockouts, one gRNA for each gene was used alternatively to create two separate double knockouts, increasing the likelihood of success (Table 2). gRNAs were designed by choosing exons from the gene of interest and running them through a CRISPR guide design resource (Feng Zhang Lab, http://crispr.mit.edu/).

The gRNAs were cloned into the lenti sgRNA(MS2)_zeo backbone (gift from Feng Zhang, Addgene plasmid # 61427) using pDonor_sU6 (gift from Andrea Ventura, Addgene plasmid # 69351) using a previously published method[61]. Lentiviruses encoding dual gRNAs were produced in 293T cells as above and used to infect the dox-inducible Cas9 HT1080 cells. Following zeocin selection, Cas9 expression was induced as needed.

Table 2: Sequence of Guide RNAs Used in Knockout Cell Creation

Gene Target	Sequence of gRNA 1	Sequence of gRNA 2
CD44	CTACAGCATCTCTCGGACGG	AAGGGCACGTGGTGATTCCC
Tfr1	TTTCCACCATCTCGGTCATC	AAGGAGACTGTCCCTCTGAC
NRP1	AGGTCGAATCCGATCCTCAT	GAGAGGATTCCTACCGAGAG
NRP2	ACTTTATCGAGATTCGGGAT	GGACTGCAAGTACGATTGGC
NRP1 G1/NRP2 G2	AGGTCGAATCCGATCCTCAT	GGACTGCAAGTACGATTGGC
NRP1 G2/NRP2 G1	GAGAGGATTCCTACCGAGAG	ACTTTATCGAGATTCGGGAT

Induction of Guide RNA Expression

On Day 0, the 60,000 cells are seeded into 6-well plates. Induction is accomplished at the time of seeding by adding a 1000X stock of Doxycycline (Sigma-Aldrich) for a final concentration of 1ug/mL. The plate is left in the incubator under standard conditions and allowed to induce for 96 hours post-seeding. After four days of incubation, the cells were detached, counted and diluted to seed 6.875x10^3 cells into a 96-well opaque plate. Infection was performed as described above.

At the time of seeding for infection, some cells were lysed and analysis of knockdown efficiency by immunoblot.

Pulldown Experiments & Proteomics

Immunoblotting

SDS-PAGE precast gels (Bio-Rad) were setup in an electrophoresis tank (Bio-Rad) and the tank filled with 1X running buffer (10X running buffer diluted with water, made up of 25mM Tris base, 190mM glycine, 0.1% SDS). Protein samples were resolved and then transferred to a PVDF membrane (10X transfer buffer, made up of 25mM and 190mM glycine, diluted with water and 20% methanol)

After transfer, the membrane was blocked in 5% milk in TBST (20mM Tris pH 7.5, 150mM NaCl, 0.1% Tween 20), and put in primary antibody overnight at 4°C. The following day, the membrane was incubated in secondary antibody coupled to horseradish peroxidase (HRP) and imaged using a Bio-Rad Chemidoc imager according to the manufacturer's recommendations. In order to image, either Clarity (Bio-Rad) or Clarity Max (Bio-Rad) ECL substrate was used with the HRP-linked secondary antibodies according to the manufacturer's protocols.

Receptor Pulldown

Four maxi plates of HT1080 cells were grown to confluency. After aspirating the media, the cells were thoroughly washed with 1X PBS. The PBS was then aspirated and 1.5mL of homogenization (HM) buffer (250mM sucrose, 1mM EDTA, 10mM HEPES buffer, pH adjusted to 7.0) was added to each of the plates. The cells were put at 4°C and allowed to incubate with the buffer for 10 minutes. Cells were then scrapped, collected and pooled to make approximately 6mL of cells. These cells were then added to a dounce homogenizer and were mechanically disrupted by 20 pumps each of pestles (loose and tight). Afterwards, a 50uL sample was taken to be checked for cell viability by Trypan blue (Bio-Rad). The douncing process was repeated until cell viability was under 25%. The homogenate was then aliquoted to Eppendorf tubes and centrifuged at 13,000 RPM for 5 minutes at 4°C. The supernatant HM buffer was removed, and 500uL of fresh HM buffer was added to wash the cells, which were spun down again. The supernatant was removed, and all of the membrane debris and nuclei were re-suspended in 1mL of HM buffer. The resuspended membranes and nuclei were then aliquoted out into four different tubes, each labeled with the different lysis buffer condition that is going to be used. These tubes were spun down again, and the supernatant removed. The conditions were of Triton (50mM Tris-Hcl, 150mM NaCl, 1% Triton X-100, 5mM EDTA, plus protease inhibitor cocktail (Cell Signaling technology) and CHAPS (150mM NaCl, 50mM HEPES, 0.1% CHAPS, plus protease inhibitor cocktail) as the two different lysis buffers, each at either pH 7.0 or 5.5. The samples were incubated for 15 minutes, vortexing occasionally, and spun down at 12,000 RPM for 15 minutes at 4°C. The supernatants were then split into three different new tubes (one for each bait protein). 75uL of beads-bait complexes were added to the tubes and incubated overnight at 4°C with rotation.

The following day, the tubes were spun down at 4°C for 2 minutes at 2000 RPM. The flow-through was removed and transferred to a new, properly marked tube, and stored at -80°C. The beads were then washed three times with their respective lysis buffer. 100uL of 1X denaturing buffer (10X denaturing buffer [NEB Biosciences] diluted with water) was then added to the beads and samples were then boiled for 10 minutes. Samples were spun down and supernatants were transferred to a new tube. The beads were also saved and frozen at -80°C. 20uL of 6X loading buffer with 5% 2-mercaptoethanol was added to each elution tube, and the samples were further boiled for 5 minutes.

60uL of each sample was then loaded onto a 20x20-cm, 8%, 1.0mm, 15-well SDS-PAGE gel, and run for 6 hours at 200 volts at 4°C. At the completion of the run, the gel was silver stained (Pierce) according to the manufacturer's protocol. Bands of interest were excised and sent for analysis by mass spectrometry.

Mass Spectrometry Analysis

Mass spectrometry analysis was completed by the Ottawa Hospital Research Institute's proteomic Mass Spectrometry Service. After in-gel trypsin digestion, 40% of the digest was injected (see Figure 7 for details) for liquid chromatography/mass spectrometry analysis.

For the analysis, the "CID_20_v4" method was used. The mascot parameter was set to "Human CID 13C1". The resulting data was exported to Scaffold for display and analysis.

Figure 6. Supplemental Details Used in LC-MS During Pulldown Experiment for Protein Identification. List of specifications used during the mass spectrometry run during the final pulldown experiment used to generate results for this thesis. The specifications can be generalized for

	Hardware
HPLC	Dionex Ultimate 3000 RSLC nano (Thermo Scientific)
Spectrometer	Orbitrap Fusion Lumos (Thermo Scientific)
Source	"Flex" Nanospray (Thermo Scientific)
Software	Xcalibur 4.0.27.10 (Thermo Scientific); Fusion Lumos 2.1 Build 1565 (Thermo Scientific); DCMSLink 2.14 Build 3818 (Thermo Scientific)
	HPLC Conditions
Solvent A	0.1% FA
Solvent B	0.1% FA
Flow rate	300 nanolitres per minute
Gradient	5% - 36% B over 15 minutes
Precolumn	C18 PepMap100 (Thermo Scientific) 300 µm i.d. x 5 mm
Column	C18 2µ, 100 Å Acclaim PepMap RSLC (Thermo Scientific) 75 µm i.d. x 15 cm
	Tandem MS Conditions
Design	Data Dependent Acquisition of MS^2 spectra from most abundant ions in MS
Dynamic Exclusion	2 counts within 10 s triggers exclusion for 60 s
MS	
Survey Scan	m/z 400-1500 at 120,000 resolution
Target AGC	4.0e5
Internal Calibration	Yes (Easy-IC)
MS^2	
Charge State	Required, +2 to +7
Isolation width	1.6
Activation method	CID
NCE	35
Q	0.25
Max injection time	35 ms
Other	Inject Ions for all available parallelizable time: enabled
	Mascot[1] Parameters
Search type	MS/MS Ion search
MS tolerance	±5 ppm
MS/MS tolerance	±0.6 Da
Enzyme	Trypsin
Mass values	Monoisotopic; 13C k=1
Instrument type	ESI-TRAP
Fixed modifications	Carbamidomethylation (C)
Variable modifications	Acetyl (Protein N-term), Gln->pyro-Glu (N-term Q), Oxidation (M)

Analysis of Protein Binding by Flow Cytometry

293T cells were seeded into a 12-well plate. 24 hours after, each well was transfected with a plasmid encoding the protein to be examined. 24 hours post-transfection, the cells were detached from the plate with 200uL PBS+5mM EDTA and re-suspended to 1mL with 800uL PBS with 2% FBS. 100,000 cells were transferred to a FACS tube on ice, and the total volume was increased to 100uL, with a corresponding amount of PBS/2%FBS. The soluble protein was added to each experimental tube and incubated at 4°C for 1 hour. The tubes were spun down at 1500 RPM for 5 minutes in a pre-chilled centrifuge at 4°C. The supernatant was decanted, and the cells were re-suspended with 500uL PBS/2%FBS. They were then spun down once more, the supernatant decanted, and the cells re-suspended in 500uL PBS/2%FBS. 1.0uL of secondary antibody (anti-hIgGFc coupled with FITC) (abcam) was added to each tube and incubated for 1 hour at 4°C. Cells were then spun down and washed with 500uL PBS/2%FBS, as mentioned, and re-suspended in a final volume of 250uL PBS/2%FBS. Cells were then analyzed for soluble protein binding by flow cytometry (BD LSRFortessa).

Receptor Candidate Validation through Co-Immunoprecipitation

On Day 0, 500,000 293T cells were plated into a 12-well plate. 24 hours post-seeding, the cells were transfected with a combination of the virus' glycoprotein harbouring a FLAG tag, and the protein to be studied. 24 hours post-transfection, the cells were taken by suspending in 1mL in PBS, and transferred into 500uL aliquots in an appropriate number of aliquots for the conditions being studied. The cells were spun down at 1500 RPM for 5 minutes, and the supernatant PBS removed. The cells were then lysed in 500uL of Triton or CHAPS lysis buffer (at different pHs) with 100X protease inhibitor, incubated on ice for 10 minutes, and spun down at 13,000 RPM for 10 minutes, at 4°C. Anti-FLAG agarose beads (Sigma-Aldrich, A2220) were prepared and washed three times in the appropriate lysis buffer, and 50uL of beads were added to a new Eppendorf tube. 400uL of the pre-cleared supernatant was then transferred directly into the appropriate beads-containing tube. The tubes were then put on a rotator in a cold room overnight, and the left-over input saved at -80°C.

The following day, the tubes were removed from the cold room, and spun down at 2000 RPM for 2 minutes to pellet the beads. The flow through was removed, transferred to a new tube, and frozen at -80°C. The beads were then washed three times with 500uL of their respective lysis buffers. At the last wash the beads were pelleted, and the supernatant was removed and discarded. 100uL of 1X denaturing buffer (10X denaturing buffer diluted with water) was added to the beads, and the samples were boiled at 98°C for 10 minutes. The tubes were spun down one final time at 2000 RPM for 2 minutes, and 100uL of the buffer was removed and transferred to a new tube. 20uL of 6X LSB with 5% 2-mercaptoethanol was added to the denaturing buffer, and the sample was boiled again for 5 minutes.

15uL of sample was then loaded onto an 8% acrylamide SDS-PAGE gel and a standard western blotting technique was performed. After blocking, the blot was incubated with the primary antibody of the protein being studied, and the blot was imaged the next day to see if the protein was present in the lane where there was co-incubation between the viral glycoprotein and candidate receptor. After imaging, the blot was stripped using stripping buffer (ThermoFisher), and re-blocked for 1 hour. The blot was re-incubated with primary anti-FLAG antibody and incubated overnight in order to measure the amount and presence of bait protein in the appropriate lanes. Similarly, the input was run on gel in the exact same manner in order to identify the amount of protein originally being incubated, with the blot being stripped and measured for GAPDH as a loading control.

Results

A Conserved Histidine Triad is Important for LCMV Infection

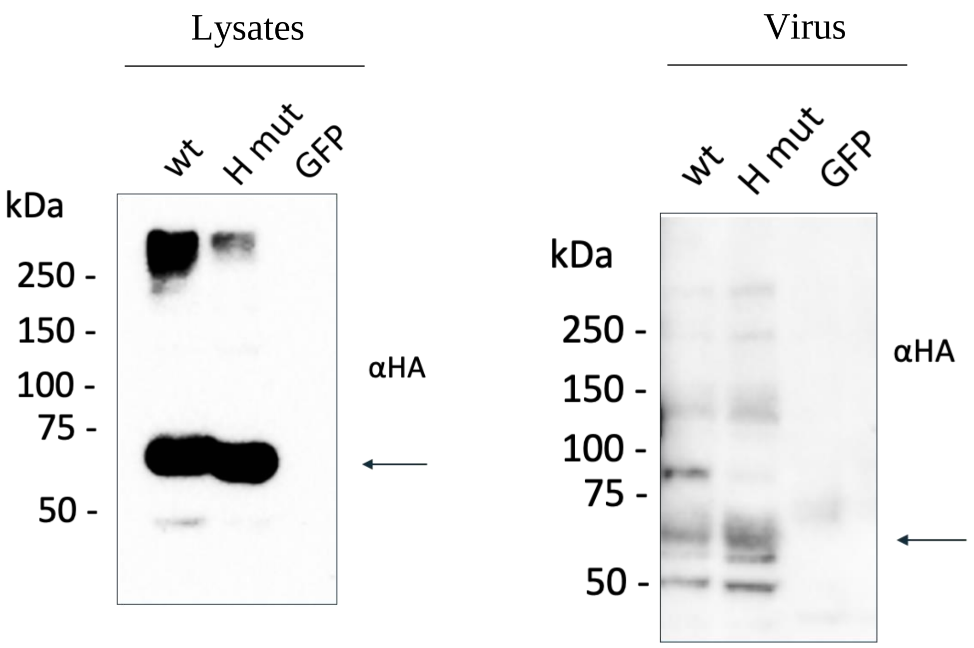
Previous studies on influenza virus have shown that histidines in HA are implicated in the activation of the fusion activity of HA[62]. In this case, histidine residues, which have a pKa of approximately 6.0, are important sensors of physiological changes in the endosomes as they can be protonated in this acidic environment[62]. A histidine triad in lassa fever virus GP has also been shown to be important to allow acidic pH-dependent binding to its cellular receptor[43]. To investigate whether the histidine triad found in LCMV GP is important for LCMV entry, a mutated version of the LCMV GP was created which changed the residues to tyrosines (H92Y, H93Y, and H224Y). Similar to histidines, tyrosines are polar amino acids, however, these cannot be protonated at pH values found in endosomes or lysosomes. Epitope tags, HA or FLAG, were also added at the N- or C-terminus for detection. The wt and mutated LCMV GP were then expressed in cells and used to pseudotype murine leukemia virus to assess the GP ability to mediate viral entry.

We found that the mutation did not have a significant effect on expression in cells (Fig.7A). Indeed, both the wt and H mutant were well expressed in viral producer cells and expected cleavage products were detected. In addition, both GPs were incorporated at similar level into the viral particles. These results indicated that the mutation did not have a dramatic effect on the overall structure of GP as the H mutant was well expresses, processed, and incorporated into viruses.

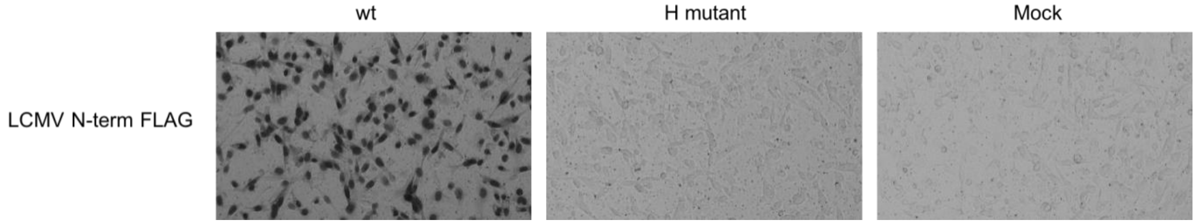
However, although the GPs were incorporated into the virions, we observed, using the infection assays, a dramatic decrease in the ability of the H mutated LCMV GP to mediate viral entry when compared to the wt virus (Figure 7B-C).

Figure 7. Infection of wt and H mutant LCMV Virus. Western blots were performed on the viral producer cells and virions themselves to test for expression of the HA tag of the glycoprotein, which was confirmed to be present in both the wt and H mutant glycoproteins (A). Pseudotyped LCMV virus harbouring either the wt or H mutant glycoprotein was infected with the results measured by either the LacZ staining with X-Gal (B), or Beta-Glo assay (C). A complete drop in infectious titer was seen in the virus with the mutated glycoprotein, compared to the wt virus, in both assays.

A

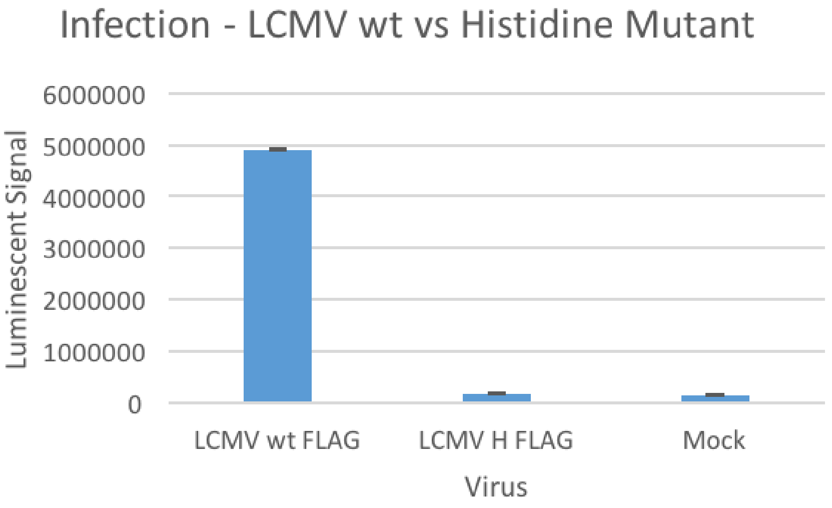


B



Virus	Titer - wt	Titer – H mutant
N-terminal FLAG	1.04 x 10^6 FFU/mL	<2 FFU/mL
C-terminal HA	5.55 x 10^4 FFU/mL	<2 FFU/mL
Mock	<2 FFU/mL	<2 FFU/mL

C



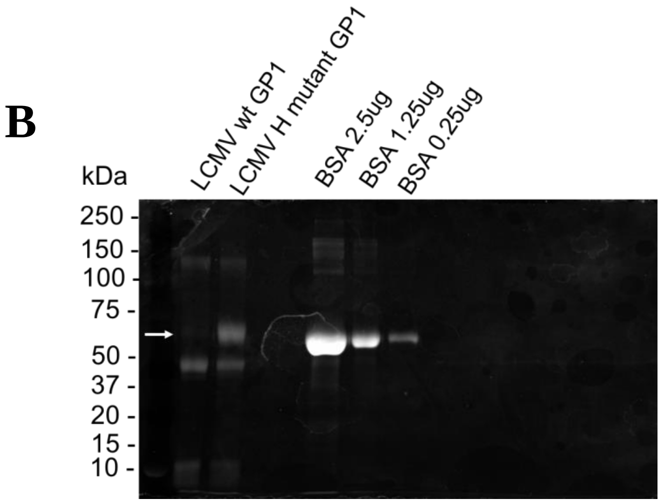
Indeed, MLV vectors encoding LacZ and pseudotyped with the wt tagged LCMV GP exhibited high levels of transduction (Figure 7C). In contrast, the viruses harboring the H mutated LCMV GPs were completely unable to infect the target cells, and resulted in a level of LacZ activity similar to the mock infected condition. To confirm these infection results and investigate if the viruses harboring the H mutant LCMV were completely inactive, infection was measured by a LacZ staining assay with X-gal (Figure 7C). Using this assay, we were unable to detect a single infectious virion, while viruses harboring the wt tagged LCMV GPs exhibited titers above 10^4 FFU/mL. These results indicate that the histidine triad, while not required for expression, processing, and incorporation onto viral particles, is absolutely required for infectivity. This histidine triad could be important for the GP conformational changes required for membrane fusion or, similarly to the lassa fever virus mechanism of entry, could be required for binding to a yet-to-be identified receptor.

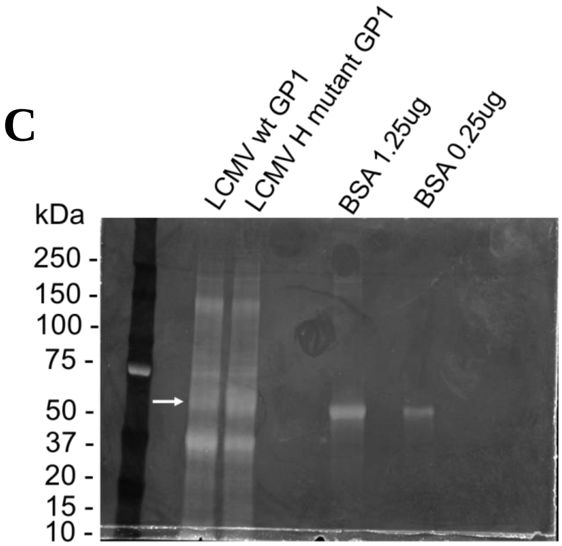
A Soluble LCMV GP1 Protein Can Be Expressed and is Binding-Competent

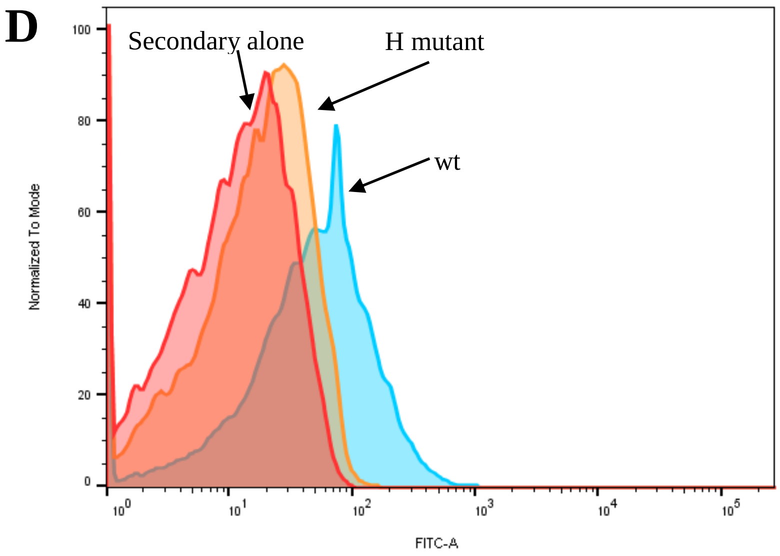
With the goal of identifying candidate LCMV receptors A soluble version of the GP1 subunit, which contains the receptor binding domain, fused to human IgG Fc was generated and produced in 293T cells. The initial construct had a non-native signal peptide found originally in the pCSI vector (Figure 8A).

Figure 8. Production of Soluble GP1 Protein. (A) Schematic of the constructed protein containing the GP1 domain alongside a hIgGFc domain, signal peptide, all cloned into a pCSI backbone. This cloning utilized a pCSI backbone containing a hIgGFc region and a signal peptide, where the GP1 domain was cloned in between. (B) SYPRO Ruby-stained gel of the production of GP1 soluble protein containing the original signal peptide, with a significant decrease in wt production compared to the H mutant construct. Arrow indicates band of protein. The protein is quantified vs BSA standards. (C) SYPRO Ruby-stained gel of the production of GP1 soluble protein containing the arenavirus signal peptide, with a significant increase in wt production compared to the original signal peptide. Arrow indicates band of protein. The protein is quantified vs BSA standards. (D) The wt and H mutant soluble protein tested for binding ability by flow cytometry to untransfected Vero cells. Data shows an increase in signal for the wt and H mutant constructs as compared to the secondary alone, indicating a positive ability of the protein to bind. Legend indicates the different conditions. Wt and H mutant constructs are shown vs the secondary antibody alone, to account for auto-fluorescence. Peaks are labelled to represent appropriate condition.


A
Signal Peptide
GP1
hIgGFc
pCSI


B
LCMV wt GP1
LCMV H mutant GP1
BSA 2.5ug
BSA 1.25ug
BSA 0.25ug
kDa
250
150
100
75
50
37
20
15
10


C
LCMV wt GP1
LCMV H mutant GP1
BSA 1.25ug
BSA 0.25ug
kDa
250
150
100
75
50
37
20
15
10


D
Secondary alone
H mutant
wt
Normalized To Mode
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
10^5
FITC-A

After production, the yield of the protein was tested by SYPRO Ruby gel against BSA standards and we obtained very low concentration of the wt protein. Intriguingly, the H mutant construct produced high yields (Figure 8B). We hypothesized that this may be caused by the use of the signal peptide found in the pCSI vector, which is different than the signal peptide found in LCMV's GP in the wild. To test this hypothesis, a new signal peptide which is derived from the peptide found in arenaviruses, herein known as the arenavirus signal peptide (AVSP), was cloned into the vector, replacing the existing signal peptide. This led to dramatically increased amounts of protein being produced, though the histidine mutant was always more highly expressed (Figure8C).

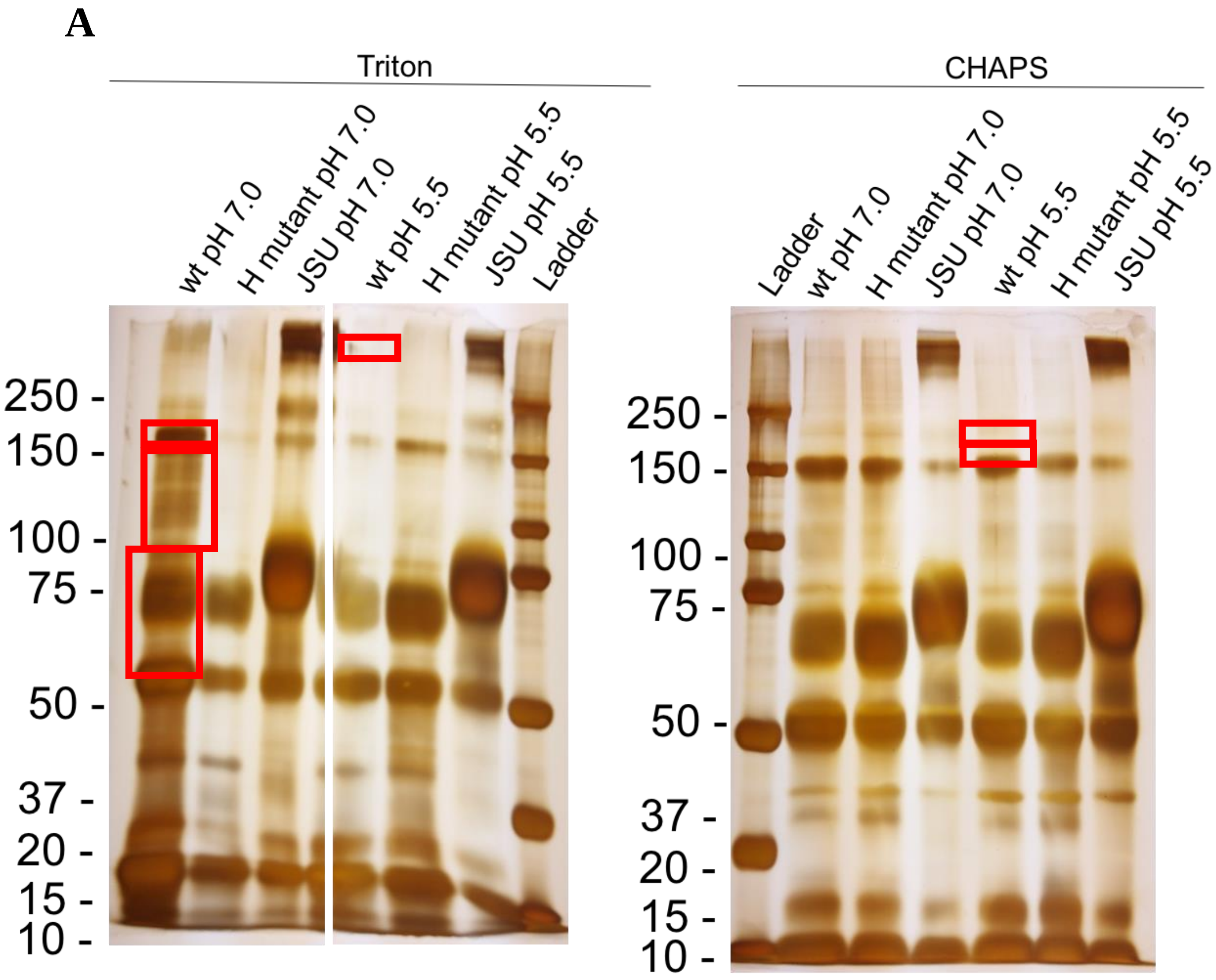
To assess whether the soluble GP1 constructs were functional, we next assessed the ability of the purified proteins to bind the surface of cells using flow cytometry. For these experiments, highly susceptible Vero cells were used as they are known to express several of the LCMV attachment factors[63]. Cells were incubated with the soluble proteins at 4°C and anti-human IgG coupled to FITC was used to detect protein cell surface binding by flow cytometry. We found that both the wt and H mutant proteins were able to bind to cells when compared to the cells only incubated with the secondary antibody alone (Figure 8D). These results indicate that the soluble GP1 constructs are able to bind to host proteins and suggest that these could be used as tools to isolate candidate entry receptor proteins.

Mass Spectrometry Analysis of Proteins Pulled Down with LCMV Bait Identified Multiple Potential Binding Partners

Since virus entry receptors are known to be proteins associated with membranes, the pull-downs experiments were performed using solubilized membranes extracts from susceptible cells. The human HT1080 cells were chosen as these are very susceptible to

LCMV infection, and therefore are presumed to express high levels of entry receptors[64]. Membranes were first isolated using a dounce homogenizer and membranes proteins were solubilized using two different detergents. In addition, nuclei were removed by differential centrifugation. To assess the importance of pH, solubilization was also performed at pH7.0 and 5.5. After performing the pulldown experiment, the proteins were run on gel and visualized using silver staining (Figure 9A). We found that the soluble proteins bound to several host proteins. As expected, the band pattern for the LCMV proteins was distinct from that obtained with the JSU protein. We also found that the intensity of the bands was dependent on the detergent used, which might be due to the increased ability of Triton X-100 to solubilize protein when compared to CHAPS. Finally, although difference could be observed, low pH did not lead to extensive changes in the band patterns for the wt LCMV GP1. Bands that were unique in the wt lanes at neutral or acidic pH were cut and sent to mass spectrometry for identification.

Figure 9. Pulldown Experiment Results. (A) Silver stained images of large-format gel run with eluted pulldown protein. Ran both Triton and CHAPS lysis buffers used, in pHs 7.0 and 5.5. Wt, H mutant, and JSU bait protein were used in each condition. Bands were extracted from wt conditions that were not present in the JSU control lane, and sent for identification through mass spectrometry. Extracted areas are indicated by red-bordered square on the gel. (B) Table containing the results from bands sent for identification. Results displayed are those narrowed down to transmembrane proteins isolated. Number of peptides quantified in each of the conditions are also displayed along with the protein name, accession number, and molecular weight. Highest probability candidates chosen to continue on with validation are highlighted.


A
Triton
wt pH 7.0
H mutant pH 7.0
JSU pH 7.0
wt pH 5.5
H mutant pH 5.5
JSU pH 5.5
Ladder
250 -
150 -
100 -
75 -
50 -
37 -
20 -
15 -
10 -
CHAPS
Ladder
wt pH 7.0
H mutant pH 7.0
JSU pH 7.0
wt pH 5.5
H mutant pH 5.5
JSU pH 5.5
250 -
150 -
100 -
75 -
50 -
37 -
20 -
15 -
10 -

B

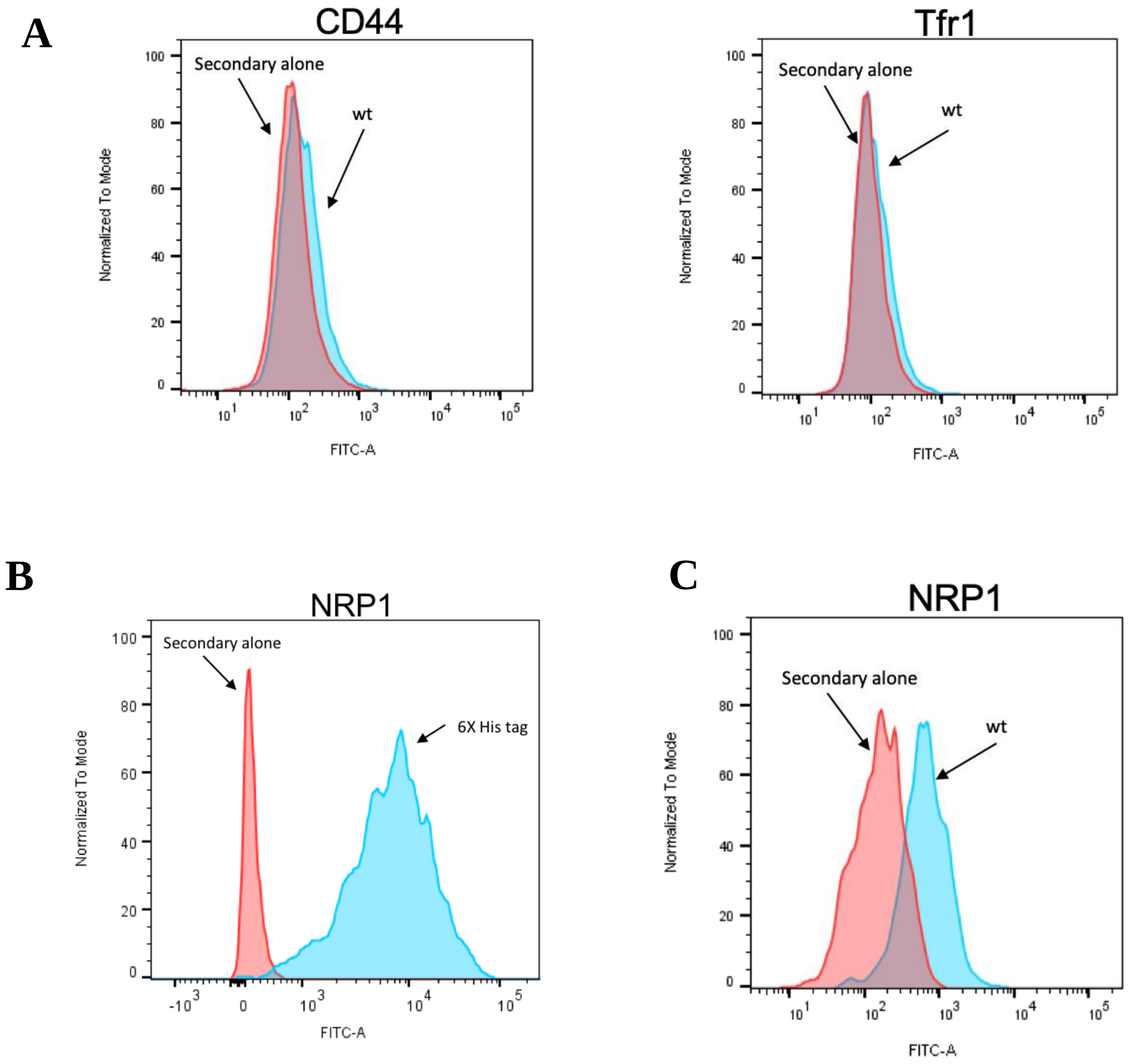
			Number of Peptides					
Protein Name	Accession #	Molecular Weight	Triton 7 wt 160 KDA	Triton 7 wt 100-150 KDA	Triton 7 wt 60-100 MKDA	Triton 5.5 wt 280 KDA	CHAPS 5.5 wt 240 KDA	CHAPS 5.5 wt 180 KDA
Neuropilin-1	NRP1_Human	103 kda	0	6	2	19	0	0
CD44 antigen	CD44_Human	82 kda	0	0	0	8	0	0
Transferrin receptor protein 1	TFR1_Human	85 kda	0	0	0	0	5	0
Cluster of AP-2 complex subunit beta	AP2B1_Human	105 kda	0	0	41	0	0	0
Neutral alpha-glucosidase AB	GANAB_Human	107 kda	0	0	11	2	3	1
ATP-dependent RNA helicase A	DHX9_Human	141 kda	3	6	2	3	0	3
Hornerin	HORN_Human	282 kda	14	0	0	0	0	0
Band 4.1-like protein 3	E4L13_Human	121 kda	0	18	0	0	0	0
Ubiquitin-associated protein 2-like	UBP2L_Human	115 kda	4	5	2	0	0	2
Fibronectin type III domain-containing protein 3B	FND3B_Human	133 kda	11	2	0	0	0	0
Ribosome-binding protein 1	RRBP1_Human	152 kda	0	10	5	0	0	0
Sodium/potassium-transporting ATPase subunit alpha-1	AT1A1_Human	113 kda	0	6	6	0	0	0
Chondroitin sulfate proteoglycan 4	CSPG4_Human	251 kda	0	0	0	8	0	0
Volume-regulated anion channel subunit LRRC8A	LRC8A_Human	94 kda	0	0	11	0	0	0
Inner nuclear membrane protein Man1	MAN1_Human	100 kda	0	0	10	0	0	0
Tissue-type plasminogen activator	TPA_Human	63 kda	0	1	8	0	0	0
Ribonucleases P/MRP protein subunit POP1	POP1_Human	115 kda	0	8	0	0	0	0
Demoglein-1	DSG1_Human	114 kda	0	0	1	4	0	0
Golgi integral membrane protein 4	GOLI4_Human	82 kda	0	7	0	0	0	0
Zinc finger RNA-binding protein	ZFR_Human	117 kda	0	5	2	0	0	0
Pre-B-cell leukemia transription factor-interacting protein 1	PBIP1_Human	92 kda	0	0	7	0	0	0
Chondroitin sulfate synthase 2	CHSS2_Human	85 kda	0	0	3	0	0	0
eIF-2-alpha kinase activator GCN1	GCN1_Human	293 kda	0	0	0	0	4	0
Xylosyltransferase 2	XYLT2_Human	97 kda	0	0	3	0	0	0
Syndecan-1	SDC1_Human	32 kda	0	0	0	3	0	0
Large proline-rich BAG6	BAG6_Human	119 kda	0	0	0	0	0	3
Aspartyl/asparaginyl beta-hydroxlyase	ASPH_Human	86 kda	0	38	14	0	0	0

Upon receipt of the list of protein candidates, the data was sorted to only focus on transmembrane proteins, and the proteins with the highest number of peptides and specificity were given the highest priority for validation (Figure 9B). Of the results, three proteins were deemed to be the most likely potential candidates: CD44, Tfr1, and NRP1 (Figure 9B). The next step was to begin validating these proteins as the entry receptor.

LCMV Binds Selectively to Surface-Expressed Neuropilin-1

The GP1 construct was used to test the cells over-expressing the candidate proteins to see if there was specific binding by the bait to any of the hits. For these experiments, 293T cells were chosen, as they have low susceptibility, and surface binding of LCMV GP is minimal on these cells. 293T cells were transfected with plasmid DNA encoding the cDNA of each candidate receptor. We did not observe increase LCMV GP binding to cells overexpressing CD44 or Tfr1 (Figure 10A). However, we found that overexpressing NRP1 led to a dramatic increase in LCMV GP1 binding to cells.

Figure 10. Testing of Receptor Candidates by Flow Cytometry. Plasmids expressing receptor candidates CD44, Tfr1, and NRP1 were transfected into 293T cells and incubated with the soluble protein to test the binding level of the wt protein to the candidates. It is seen that there is no specific binding to either CD44 or Tfr1 by either soluble protein, and that binding level is at the basal level previously seen in untransfected 293T cells (A). In order to test the NRP1 protein and ensure that it is being properly expressed to confirm this result, the cells transfected with NRP1 were incubated with a primary antibody against the 6X His tag contained within the plasmid. An appropriate secondary antibody coupled with FITC was then added and the cells ran by flow. The result shows strong expression of the NRP1 protein in the 293T cells (B). The soluble proteins both bind highly specifically to NRP1, much greater than either basal binding, or CD44 and Tfr1 (C).

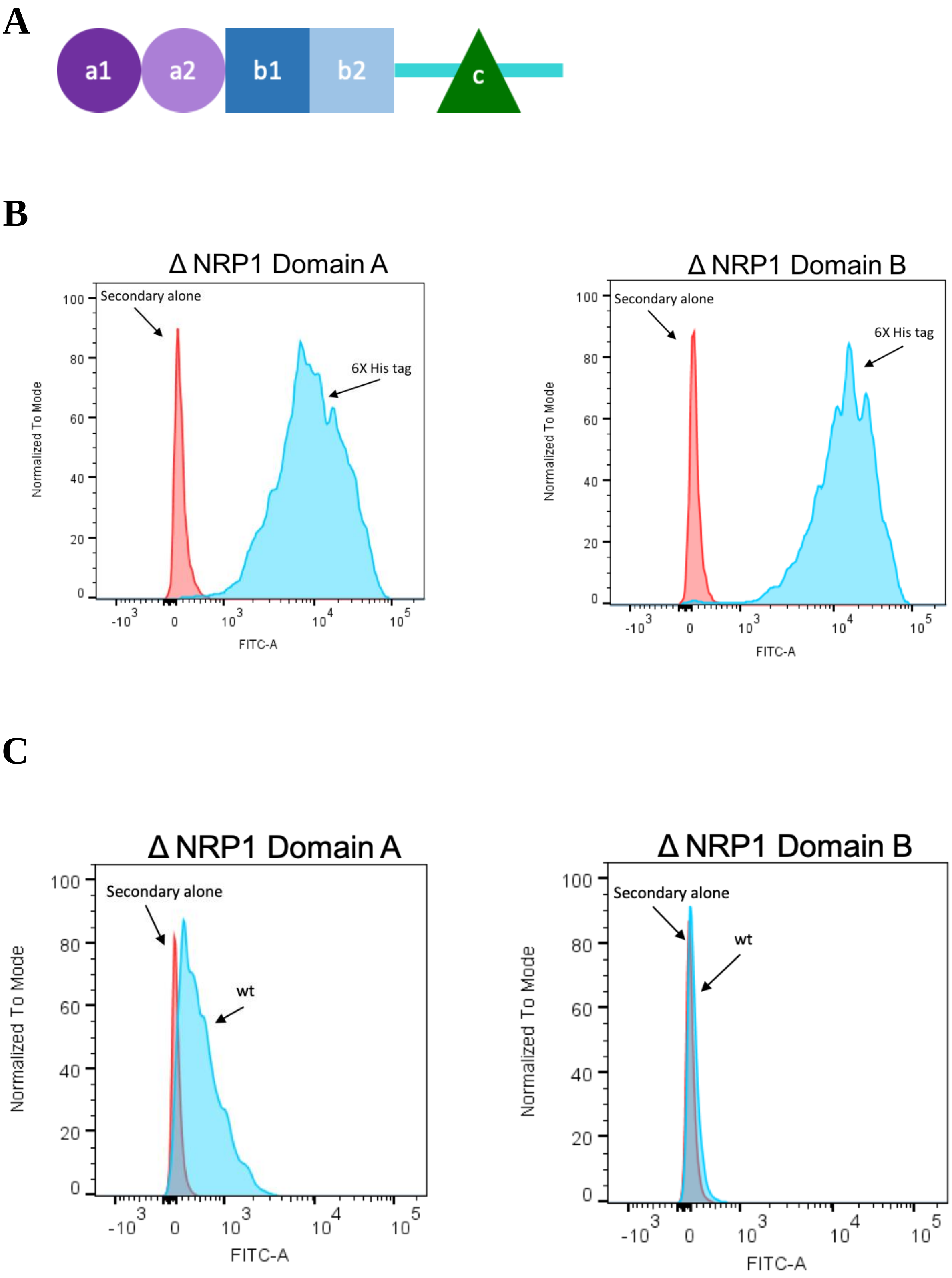

A
CD44
Secondary alone
wt
Normalized To Mode
0
20
40
60
80
100
10^1
10^2
10^3
10^4
10^5
FITC-A
Tfr1
Secondary alone
wt
Normalized To Mode
FITC-A
B
NRP1
Secondary alone
6X His tag
Normalized To Mode
-10^3
0
10^3
10^4
10^5
FITC-A
C
NRP1
Secondary alone
wt
Normalized To Mode
FITC-A

In order to rule out potential other reasons for the higher NRP1 binding, the protein was tested by flow cytometry to ensure its proper expression at the surface, using the 6X His tag contained within the plasmid. The protein was confirmed to be highly expressed at the surface of the cell and functioning properly (Figure 10B). This result indicates that the LCMV GP1 protein is specifically binding to NRP1 suggesting it could be a receptor for LCMV (Figure 10C).

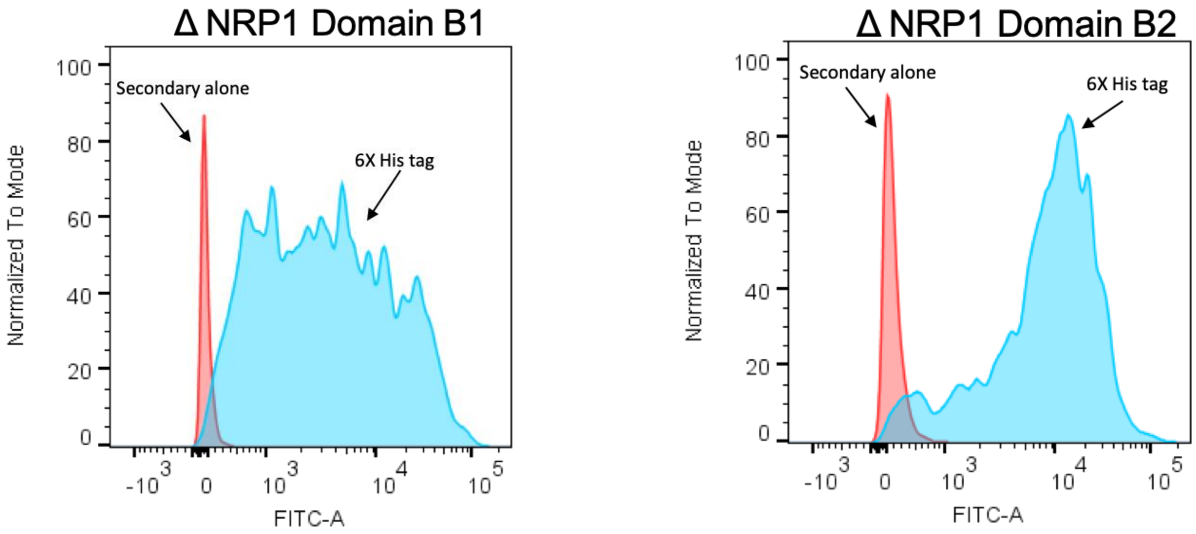
LCMV Binds Selectively to the B1 Domain of Neuropilin-1

The ectodomain of NRP1 can be divided in multiple subdomains. Since we observed specific binding of LCMV GP to NRP1, we next sought to identify the NRP1 binding region. We therefore generated deletion mutants of this protein, removing one at a time its two core ectodomains a and b (Figure 11A). The deletion mutants of NRP1 were then expressed in 293T cells and were tested for LCMV GP1 binding by flow cytometry. Both deletion mutants were well expressed at the cell surface as assessed by surface staining using anti-his tag antibodies (Figure 11B). Interestingly, we found that the soluble protein LCMV GP1 was able to bind cells expressing the construct lacking the a domain, but was unable to bind to those expressing NRP1 lacking the b domain. These experiments strongly suggest that LCMV GP is binding to the b domain of NRP1 (Figure 11C).

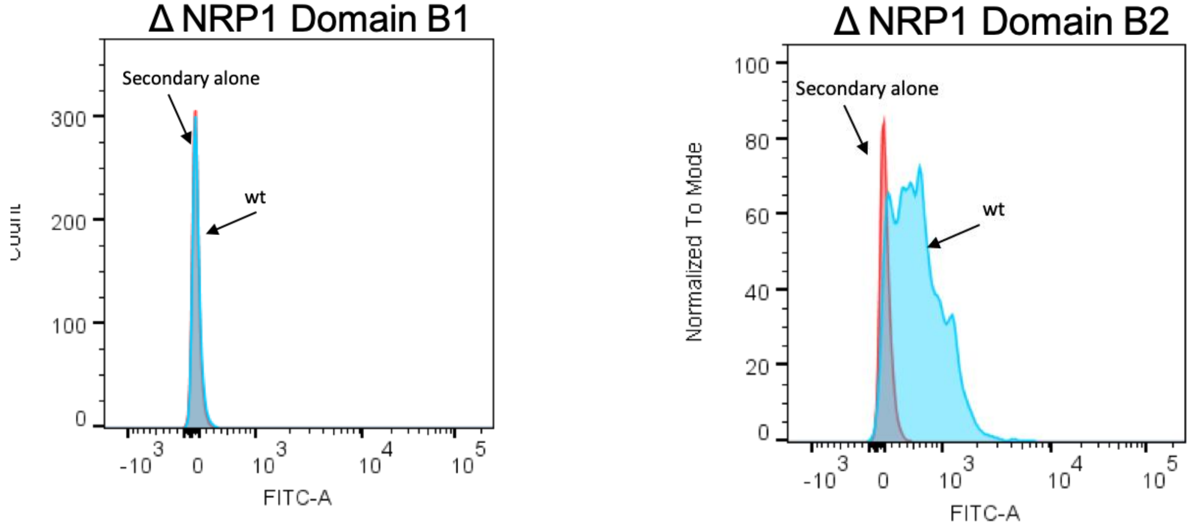
Figure 11. Testing of NRP1 Truncated Constructs by Flow Cytometry. A schematic structure of the ectodomains of the NRP protein (A). Plasmids expressing NRP1 with either it’s a or b domain deleted were transfected into 293T cells and incubated with the soluble protein to test the binding level of the wt protein to the candidates. In order to ensure the deletions didn’t affect the protein’s expression, the cells transfected with the deletion mutants were incubated with a primary antibody against the 6X His tag contained within the plasmid. An appropriate secondary antibody coupled with FITC was then incubated and the cells ran by flow. The result shows strong expression of both deletion mutants on the surface of the 293T cells (B). It is seen that the wt protein bind more specifically to the construct missing it’s a domain, than the plasmid with its b domain deleted, indicating that viral binding is to the b domain of NRP1 (C). To further narrow down the binding region, the b1 or b2 subdomains were removed from NRP1. The level of expression of the protein was first tested through it’s his tag, and showed that both of the new constructs are well expressed (D). The wt protein is able to bind much greater to the protein missing its b2 domain, indicating that the virus is binding to the b1 domain of NRP1 (E).


A
a1
a2
b1
b2
c
B
Δ NRP1 Domain A
Secondary alone
6X His tag
Normalized To Mode
FITC-A
Δ NRP1 Domain B
Secondary alone
6X His tag
Normalized To Mode
FITC-A
C
Δ NRP1 Domain A
Secondary alone
wt
Normalized To Mode
FITC-A
Δ NRP1 Domain B
Secondary alone
wt
Normalized To Mode
FITC-A
0
20
40
60
80
100
-10^3
0
10^3
10^4
10^5

D


Δ NRP1 Domain B1
Normalized To Mode
100
80
60
40
20
0
Secondary alone
6X His tag
-10^3
0
10^3
10^4
10^5
FITC-A
Δ NRP1 Domain B2
Normalized To Mode
100
80
60
40
20
0
Secondary alone
6X His tag
-10^3
0
10^3
10^4
10^5
FITC-A

E

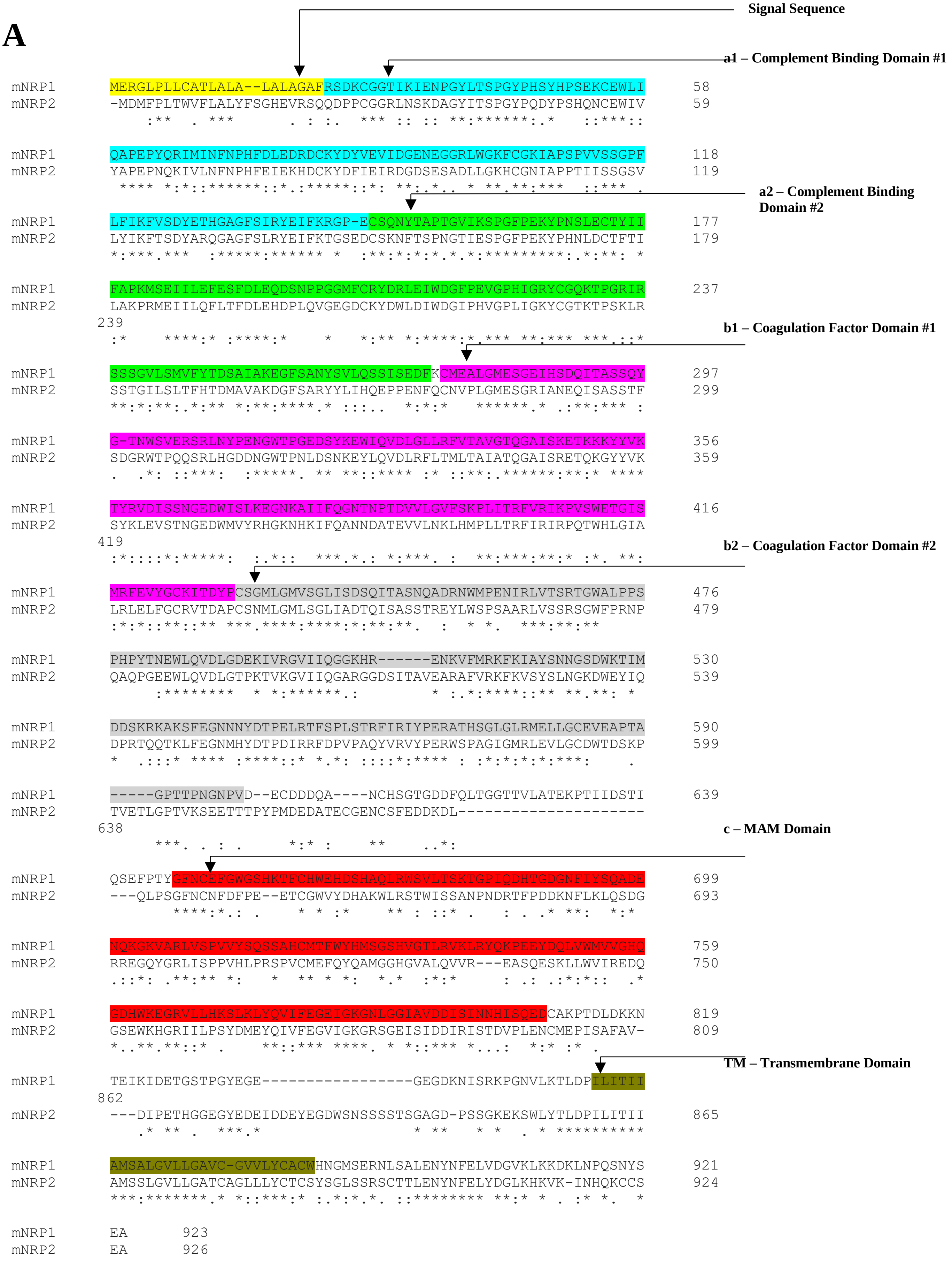

Δ NRP1 Domain B1
300
200
100
0
Secondary alone
wt
-10^3
0
10^3
10^4
10^5
FITC-A
Δ NRP1 Domain B2
Normalized To Mode
100
80
60
40
20
0
Secondary alone
wt
-10^3
0
10^3
10^4
10^5
FITC-A

The b domain can be further divided into two subdomains, b1 and b2. To narrow down the potential binding domain of the virus to NRP1, two further deletion mutants of NRP1 constructs lacking either the b1 or b2 domain were generated and tested by flow cytometry. Again, both deletion mutants were well expressed at the surface of cells as observed by surface staining using anti-his antibody (Figure 11D). When using the LCMV GP1 proteins, we found strong binding to the Δb2 construct, indicating that the LCMV GP1 protein binds to the b1 domain of NRP1 (Figure 11E). In contrast, there was no discernable binding using the NRP1 construct lacking the b1 domain (Figure 11E). These results strongly indicate that the LCMV GP1 specifically binds to the b1 domain of NRP1 (Figure 11E).

LCMV Can Also Bind Selectively to Neuropilin-2

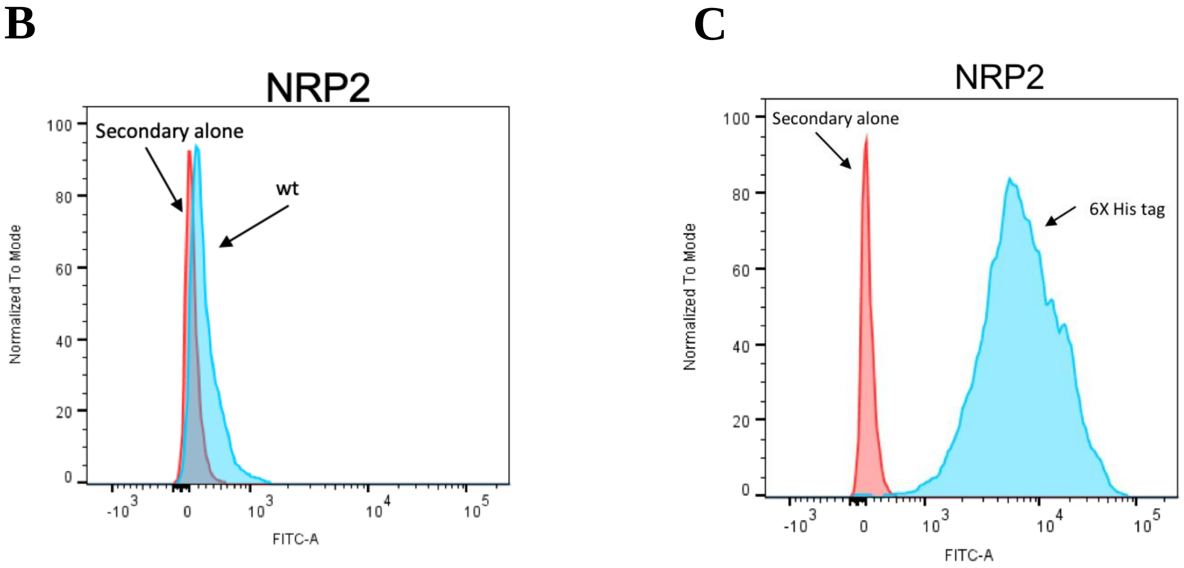
NRP2 is a homolog of NRP1, both belonging to the same family of Neuropilin proteins. Interestingly, NRP2 is also involved in viral entry as a co-receptor for another old-world arenavirus, Lujo virus[65]. Therefore, it was hypothesized that it may play a role in the entry of LCMV as well. These suspicions were increased when a sequence alignment between the two proteins were performed and it was revealed how similar NRP1 and NRP2 are to each other (Figure 12A).

Figure 12. Investigation of NRP2 for Potential LCMV Binding. Sequence alignment showing the similarity between the two Neuropilin proteins: NRP1 and NRP2. These sequences are mouse NRP, the same as the constructs used in the lab (A). A flow binding experiment was then performed with the wt construct incubated with cells expressing the NRP2 protein. The result was binding of the wt soluble protein specifically to NRP2, greater than the basal level binding usually seen (B). The expression of NRP2 on the surface of the cell was measured to ensure proper incorporation through measuring the 6X His tag contained within the protein. The result was strong expression of the protein on the surface of the 293T cells (C).



```
mNRP1           MERGLPLLCATLALA--LALAGAFRSDKCGGTIKIENPGYLTSPGYPHSYHPSEKCEWLI      58
mNRP2           -MDMFPLTWVFLALYFSGHEVRSQQDPPCGGRLNSKDAGYITSPGYPQDYPSHQNCEWIV      59
                    :**  . ***      . : :.  *** :: :: **:******:.*   ::***::

mNRP1           QAPEPYQRIMINFNPHFDLEDRDCKYDYVEVIDGENEGGRLWGKFCGKIAPSPVVSSGPF      118
mNRP2           YAPEPNQKIVLNFNPHFEIEKHDCKYDFIEIRDGDSESADLLGKHCGNIAPPTIISSGSV      119
                 **** *:*::******::*.:*****::*: **:.*.. * **.**:***  ::*** .

mNRP1           LFIKFVSDYETHGAGFSIRYEIFKRGP-ECSQNYTAPTGVIKSPGFPEKYPNSLECTYII      177
mNRP2           LYIKFTSDYARQGAGFSLRYEIFKTGSEDCSKNFTSPNGTIESPGFPEKYPHNLDCTFTI      179
                *:***.***  :*****:****** *  :**:*:*:*.*.*:*********:.*:**: *

mNRP1           FAPKMSEIILEFESFDLEQDSNPPGGMFCRYDRLEIWDGFPEVGPHIGRYCGQKTPGRIR      237
mNRP2           LAKPRMEIILQFLTFDLEHDPLQVGEGDCKYDWLDIWDGIPHVGPLIGKYCGTKTPSKLR
              239
                :*    ****:* :****:*    *   *:** *:****:*.*** **:*** ***.::*

mNRP1           SSSGVLSMVFYTDSAIAKEGFSANYSVLQSSISEDFKCMEALGMESGEIHSDQITASSQY      297
mNRP2           SSTGILSLTFHTDMAVAKDGFSARYYLIHQEPPENFQCNVPLGMESGRIANEQISASSTF      299
                **:*:**:.*:** *:**:****.* :::..  *:*:*   ******.* .:**:*** :

mNRP1           G-TNWSVERSRLNYPENGWTPGEDSYKEWIQVDLGLLRFVTAVGTQGAISKETKKKYYVK      356
mNRP2           SDGRWTPQQSRLHGDDNGWTPNLDSNKEYLQVDLRFLTMLTAIATQGAISRETQKGYYVK      359
                .  .*: ::***:  :*****. ** **::**** :* ::**:.******:**:* ****

mNRP1           TYRVDISSNGEDWISLKEGNKAIIFQGNTNPTDVVLGVFSKPLITRFVRIKPVSWETGIS      416
mNRP2           SYKLEVSTNGEDWMVYRHGKNHKIFQANNDATEVVLNKLHMPLLTRFIRIRPQTWHLGIA
              419
                :*::::*:*****:  :.*::  ***.*.: *:***. :  **:***:**:* :*. **:

mNRP1           MRFEVYGCKITDYPCSGMLGMVSGLISDSQITASNQADRNWMPENIRLVTSRTGWALPPS      476
mNRP2           LRLELFGCRVTDAPCSNMLGMLSGLIADTQISASSTREYLWSPSAARLVSSRSGWFPRNP      479
                :*:*::**::** ***.****:****:*:**:**.  :  * *.  ***:**:**

mNRP1           PHPYTNEWLQVDLGDEKIVRGVIIQGGKHR------ENKVFMRKFKIAYSNNGSDWKTIM      530
mNRP2           QAQPGEEWLQVDLGTPKTVKGVIIQGARGGDSITAVEARAFVRKFKVSYSLNGKDWEYIQ      539
                     :********  * *:******.:        * :.*:****::** **.**: *

mNRP1           DDSKRKAKSFEGNNNYDTPELRTFSPLSTRFIRIYPERATHSGLGLRMELLGCEVEAPTA      590
mNRP2           DPRTQQTKLFEGNMHYDTPDIRRFDPVPAQYVRVYPERWSPAGIGMRLEVLGCDWTDSKP      599
                *  .:::* **** :****::* *.*: ::::*:**** : :*:*:*:*:***:    .

mNRP1           -----GPTTPNGNPVD--ECDDDQA----NCHSGTGDDFQLTGGTTVLATEKPTIIDSTI      639
mNRP2           TVETLGPTVKSEETTTPYPMDEDATECGENCSFEDDKDL---------------------
              638
                     ***. . : .     *:* :    **    ..*:

mNRP1           QSEFPTYGFNCEFGWGSHKTFCHWEHDSHAQLRWSVLTSKTGPIQDHTGDGNFIYSQADE      699
mNRP2           ---QLPSGFNCNFDFPE--ETCGWVYDHAKWLRSTWISSANPNDRTFPDDKNFLKLQSDG      693
                       ****:*.: .    * * :*    ** : ::* .   : . .* **:  *:*

mNRP1           NQKGKVARLVSPVVYSQSSAHCMTFWYHMSGSHVGTLRVKLRYQKPEEYDQLVWMVVGHQ      759
mNRP2           RREGQYGRLISPPVHLPRSPVCMEFQYQAMGGHGVALQVVR---EASQESKLLWVIREDQ      750
                .::*: .**:** *:  *  ** * *:  *.*  :*:*     : .: .:*:*::  .*

mNRP1           GDHWKEGRVLLHKSLKLYQVIFEGEIGKGNLGGIAVDDISINNHISQEDCAKPTDLDKKN      819
mNRP2           GSEWKHGRIILPSYDMEYQIVFEGVIGKGRSGEISIDDIRISTDVPLENCMEPISAFAV-      809
                *..**.**::* .    **::*** ****. * *::*** *...:  *:* :* .

mNRP1           TEIKIDETGSTPGYEGE-----------------GEGDKNISRKPGNVLKTLDPILITII
              862
mNRP2           ---DIPETHGGEGYEDEIDDEYEGDWSNSSSSTSGAGD-PSSGKEKSWLYTLDPILITII      865
                   .* **  .  ***.*                 * **   * *  . * **********

mNRP1           AMSALGVLLGAVC-GVVLYCACWHNGMSERNLSALENYNFELVDGVKLKKDKLNPQSNYS      921
mNRP2           AMSSLGVLLGATCAGLLLYCTCSYSGLSSRSCTTLENYNFELYDGLKHKVK-INHQKCCS      924
                ***:*******.* *::***:* :.*:*.*. ::******** **:* * . :* *.  *

mNRP1           EA       923
mNRP2           EA       926
```

* *


B
C
NRP2
Secondary alone
wt
Normalized To Mode
0
20
40
60
80
100
-10^3
0
10^3
10^4
10^5
FITC-A
NRP2
Secondary alone
6X His tag
Normalized To Mode
0
20
40
60
80
100
-10^3
0
10^3
10^4
10^5
FITC-A

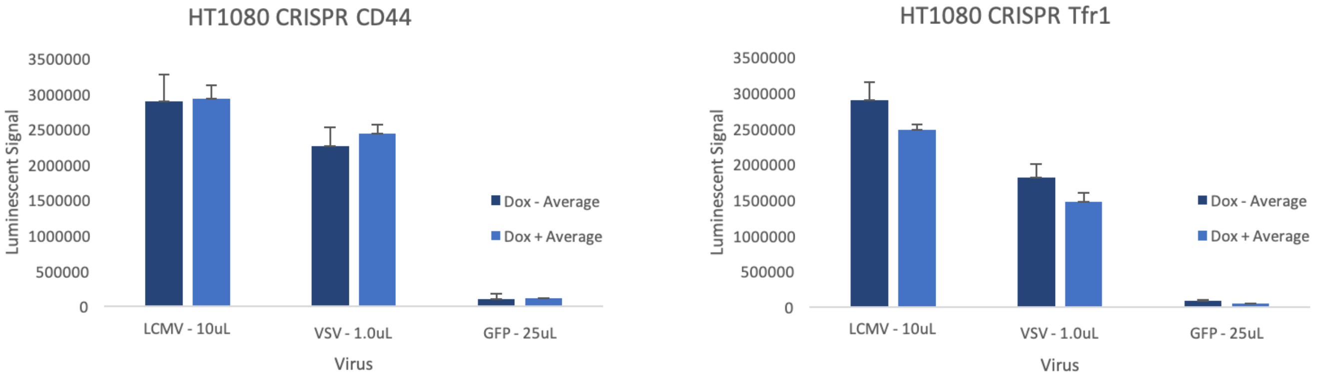
In order to validate whether NRP2 is involved in LCMV entry, we performed a flow binding experiment to test for specific binding of the LCMV soluble GP1 protein to NRP2. We found that NRP2 was well expressed at the surface of transfected 293T, using anti-his antibodies for surface binding (Figure 12C). Importantly, we observed a significant, although weaker, binding of LCMV GP1 to expressing cells (Figure 12B). This shows that LCMV GP is able to bind to both NRP2 and NRP1.

Reduction of Expression of NRP1 and NRP2 Reduces LCMV's Ability to Enter Cells

The binding experiments suggest that NRP1 and NRP2 could be LCMV entry receptors, which would then mean that those cellular proteins would be required for infection. To test this, we generated knockout cell lines of the different candidate receptors using our inducible Cas9 system. Cells were induced, plated, and then infected with LCMV and VSV pseudotypes. The later virus is a completely unrelated virus harbouring a class III viral fusion protein which would act as the control. The goal was to see a reduction in specific infection in the induced cells for LCMV, but not VSV. For the CD44 and Tfr1 knockout cells, there was no specific decrease in LCMV infection seen for the induced cells (Figure 13A).

Figure 13. Testing of Receptor Candidates through Infection of Knock Out Cell Lines. (A) Infection results as measured by the Beta-Glo assay for knockouts of CD44 and Tfr1 proteins. (B) Infection results as measured by the Beta-Glo assay for single knockouts of NRP1 and NRP2 proteins. (C) Infection results as measured by the Beta-Glo assay for double knockouts of NRP1 and NRP2 proteins. The double knockouts have two different versions, each with a swapped guide. In the first set of double knockouts, the NRP1 is targeted by guide 1, and NRP2 by guide 2. In the second set of double knockouts, NRP1 is targeted by guide 2, and NRP2 by guide 1.

A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VSV - 1.0uL
GFP - 25uL
Virus
Dox - Average
Dox + Average
HT1080 CRISPR Tfr1
Luminescent Signal
3500000
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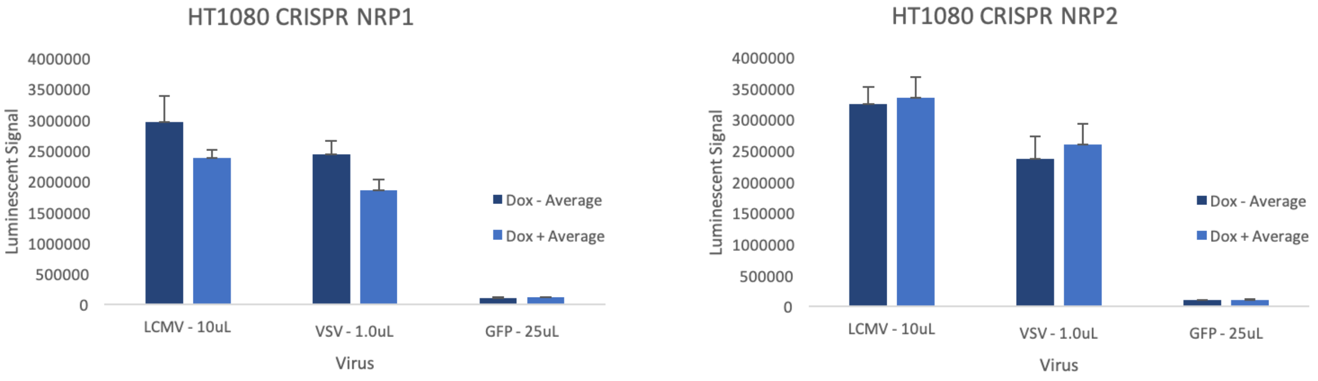
LCMV - 10uL
VSV - 1.0uL
GFP - 25uL
Virus
Dox - Average
Dox + Average

B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LCMV - 10uL
VSV - 1.0uL
GFP - 25uL
Virus
Dox - Average
Dox + Average
HT1080 CRISPR NRP2
Luminescent Signal
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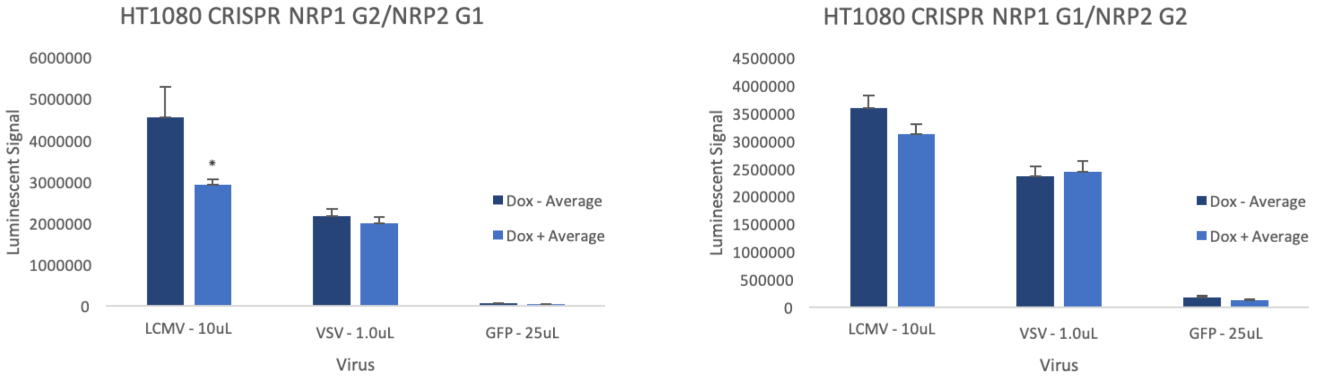
LCMV - 10uL
VSV - 1.0uL
GFP - 25uL
Virus
Dox - Average
Dox + Average

C


HT1080 CRISPR NRP1 G2/NRP2 G1
Luminescent Signal
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LCMV - 10uL
VSV - 1.0uL
GFP - 25uL
Virus
Dox - Average
Dox + Average
HT1080 CRISPR NRP1 G1/NRP2 G2
Luminescent Signal
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LCMV - 10uL
VSV - 1.0uL
GFP - 25uL
Virus
Dox - Average
Dox + Average

For the cells with guide RNAs targeting only NRP1 and NRP2, there did not appear to be a specific decrease of LCMV as well (Figure 13B). Interestingly, in one of the double targeted cell line (both NRP1 and NRP2), we observed a statistically significant decrease in infection for LCMV in the induced condition, with no effect on VSV (Figure 13C). This indicates that there is likely a role for NRP1, NRP2, or both in conjunction, during the infection process, potentially as entry receptors.

Discussion

Lymphocytic choriomeningitis virus is a global pathogen, and a virus of special interest to those who study the immune system and its function. The aim of this research project was to identify host proteins which bind to the LCMV GP, and what their potential role might be in the entry mechanism of the virus. It was found that a triad of histidine residues found on the virus' glycoprotein is highly implicated in its ability to penetrate cells. If these amino acids are mutated or changed, the virus appears to be unable to produce productive infection, which is likely the result of the loss of pH-sensing ability found in the late endosome. The virus utilizes proteins in order to traffic and enter the cell, and among these are the two Neuropilin proteins, NRP1 and NRP2. A soluble version of the virus' GP binds selectively to these two proteins with greater specificity than other potential binding partners. It is still unclear at this time whether the NRP1/2 proteins are merely necessary for infection, or if they are both necessary and sufficient. Further studies will attempt to identify the exact mechanism of fusion undertaken by the virus, and whether further steps or proteins are needed beyond these two.

To better understand the GP of LCMV, it was investigated before attempts to identify potential binding partners were undertaken. It had been known that the triad of histidine residues previously found to be important to LFV's entry pathway were conserved in LCMV, but little was known about what the role these residues played in the virus entry mechanism[43]. Furthermore, it was unclear what effect any mutation would have on the normal incorporation and function of LCMV GP. Thus, the first step was to make three mutations in the virus' GP, changing the histidine triad to tyrosine residues. Interestingly, it was found that there was no change in the ability of the GP to be produced and expressed in viral producer cells, and the GP was properly incorporated into the virions (Figure 8C). This suggested that the histidine triad did not have a structural role in the GP, and that they weren't specifically necessary to ensure that the GP was produced and incorporated in viral particles. What was extremely interesting however, was that when these properly incorporated pseudotyped viral particles were used during infection, no discernable penetration of susceptible target cells could be seen by multiple infection assays (Figure 8A-B). This lack of penetration suggests that the virus is unable to properly bind to its receptor to enter the target cells or that the triad is required for the GP final conformational change to mediate membrane fusion.

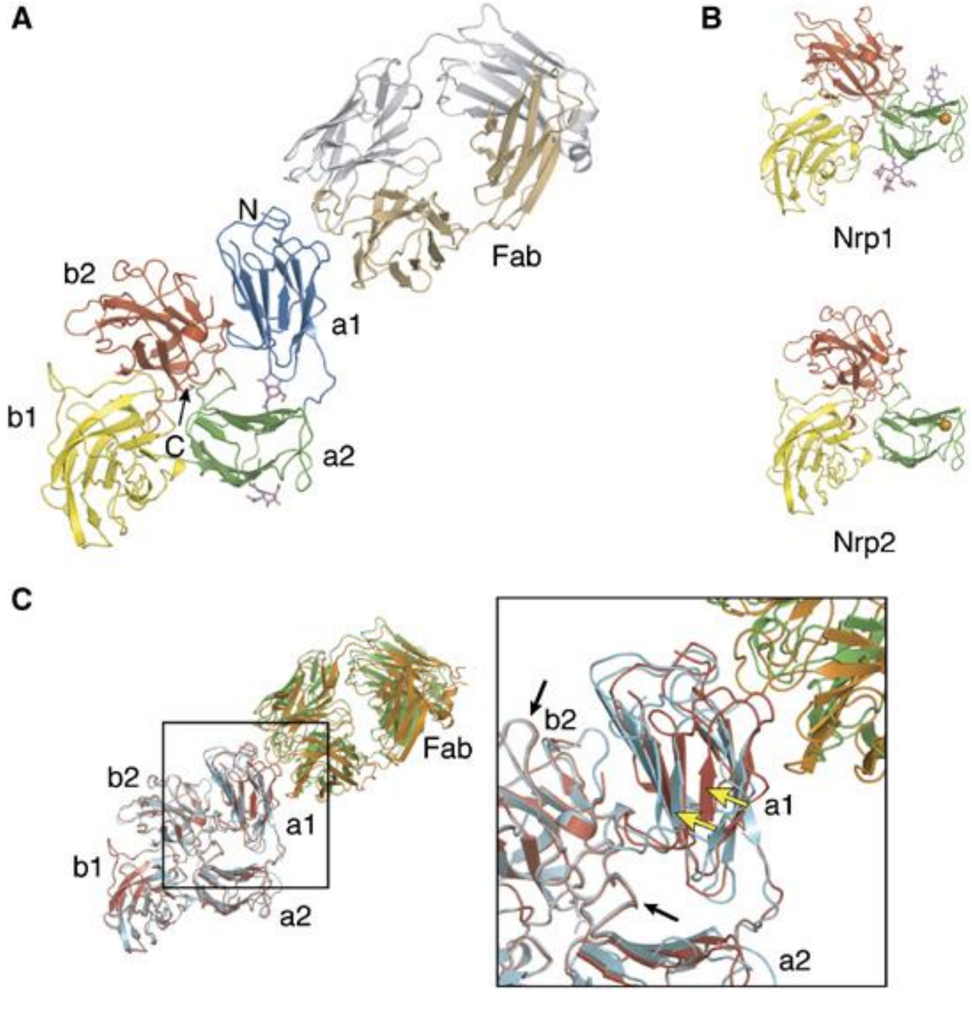
The first hypothesis is that the histidines are involved in environmental sensing, as per their role with Lassa fever virus, which requires a pH-dependent switch in order to transfer to its entry receptor, triggering LFV's fusion event[43,45]. In this model, the virus needs the histidine triad to become protonated, suggesting to the GP that it is in the late endosome, a suitable compartment for fusion, and priming the GP to bind to its receptor, initiating the fusion event and release of viral genes. A second hypothesis is that the pH acts specifically on the GP itself to cause a conformational change which primes the virus to bind to its entry receptor initiating the fusion event. In this scenario, unlike LFV which requires low pH to bind to LAMP1, pH alone would be sufficient to cause the LCMV GP conformational change[45]. Such a mechanism would mirror the actions of Influenza during its entry, which requires a low pH environment to cause the hemagglutinin protein to become primed and active for binding to sialic acid, thus initiating fusion with the endosomal membrane[66]. Future studies will be necessary to discern the exact role of the histidine triad, and why its functioning is so important for LCMV entry. In order to study this mechanism, it would be highly informative to look at binding between the wt and H mut GP in both neutral and acidic conditions. A complication with this approach is that many of the conventional ways to study binding, such as flow cytometry, are not effective when working with low pH conditions. To overcome this barrier, other techniques such as liposome fusion could be performed. This approach was successfully deployed to uncover the effects of low pH on the six-helix bundle in ASLV's Env protein, which had similar experimental constraints and objectives to the ones listed here[67].

The mutations of the histidine residues go beyond just the implications of the loss of the virus' ability to infect. Results obtained during the attempt to identify binding proteins suggest a unique role of the GP's signal peptide. As mentioned, the stable signal peptide found on the LCMV GP is unusually long[46]. The traditional model of the arenavirus' GP signal peptide is that it interacts with and stabilizes the transmembrane region of GP2 during production, as has been studied in Lassa fever virus[3]. These results suggest that this is possibly not the case with LCMV. This is because attempts to produce a soluble version of a glycoprotein which lacked the transmembrane domain found in GP2 failed without the correct LCMV signal peptide, in the wt construct (Figure 9B). If this was indeed the only role of the SSP, no problem with production would be expected when using a different signal peptide for expression and secretion of the soluble protein of the producer cells. What is even more surprising is that when producing the same soluble protein which had a mutated histidine triad, the protein was consistently produced in high yields, regardless of which signal peptide was used (Figure 9B-C). These reports challenge the idea that the signal peptide is necessary to stabilize the protein, as the H mutant construct should show the same reduced secretion as the wt when it is missing its native signal peptide. One possible explanation is that the Golgi apparatus, in which the LCMV GP transits, is slightly acidic and could cause GP binding or activation. Thus, this suggests that there would have to be a mechanism to prevent the virus from bring protonated during assembly and maturation, which would result in premature binding to its entry receptor, and inactivation of GP. This would explain why the H mutated constructs are unaffected, as their loss of environmental sensing ability prevent their premature protonation, and thus they are successfully secreted without premature binding in the Golgi. This would mean that the SSP of LCMV has a critical role in preventing the protonation of wt GPs during the natural replication process. Other pH-dependent viruses have evolved similar strategies, such as the use of M2 and Influenza. During the replication process, Influenza uses its M2 protein to create an ion channel which raises the pH of the trans-Golgi network, to prevent premature protonation and conformational change of its HA protein, which would lead to the virus being inactive, and likely trapped in the viral producer cell[68,69]. This raises the startling hypothesis that the unusually long stable signal peptide of LCMV might be involved in the creation of a channel which acts as a mechanism to neutralize the low pH of the Golgi, much in the same way as M2 and Influenza.

Previous studies have shown the benefits to using a proteomics-based approach to identify receptor candidates used by a virus during entry. Researchers studying the entry of SARS and MERS both utilized a proteomic approach to identify their receptors, ACE2 and DPP4, respectively[70,71]. This thesis took a similar approach of these studies and created a soluble GP1 (receptor-binding domain) as a bait protein to be used in pulldown experiments, with candidate identification being accomplished through mass spectrometry. The drawback to using this approach, is that of the hits generated, there can be many non-specific proteins binding to the bait. While some of them are obvious contaminants (such as keratin), others can be truly binding to the GP, but are biologically irrelevant as they are cytosolic and could never be involved with entry or fusion in an in vivo model. Some of these non-specific hits could have been reduced with more stringent conditions, other detergents, more washing, or using a beads-alone control. As a way to reduce the background within this experiment, the results were filtered to only display candidates which contained a transmembrane domain, a necessity for a potential viral entry receptor. The next challenge was to try to identify which of these remaining hits is likely the correct receptor. While this may not be the best approach, it was decided to use previous literature to rank them based on likelihood and priority. This process left NRP1, CD44, and Tfr1 at the top of the list as the three most likely candidates to screen first (Figure 10B). In order to validate these candidates, other measures such as binding studies and knockouts were subsequently utilized to test the specificity and necessity of the candidate proteins. More recent studies have been using genetic approaches like CRISPR or haploid cell lines to generate genome-wide knockouts. While these are powerful approaches and have been used to identify key proteins involved in the entry process of viruses like Lujo or HIV, because LCMV can bind to NRP1 or NRP2, these other approaches would not have been able to identify the potential entry receptor the way that the pulldown experiments in this thesis were able to[72,65].

Tfr1, CD44, and NRP1 were the first three hits to be validated based on the informed likelihood of their authenticity as receptors, since all three of these proteins had connections to other arenaviruses. Tfr1 is the primary receptor often used by the New World arenaviruses, CD44 is related to CD63 likely used as a co-receptor by Lujo virus, and NRP1 is in the same family as NRP2 which is also a receptor for Lujo virus[42,65]. This hypothesis was confirmed when clear specific binding was seen by flow cytometry to NRP1, with no binding to either CD44 or Tfr1 (Figure 11A-B). The validity of this binding would be further confirmed if a specific binding region on NRP1 could be identified. The NRP proteins 1 and 2 contain four domains: a, b, c, and TM. The a and b domains can be further broken down into two subdomains each: a1 and a1, and b1 and b2, respectively. The a and b domains are extracellular so these were the ones tested for binding[73–75]. Using deletion mutants, it was found that the LCMV GP bound to the b1 domain of NRP1 (Figure 11E). Interestingly, the b1 domain of NRP1 and its associated protein NRP2 are structurally identical (Figure 14).

Figure 14. Overall Domain Architecture of Neuropilins. (A) Domain organization of Nrp2 (blue, a1; green, a2; yellow, b1; red, b2) in complex with the Fab fragment of anti-panNrpA (tan, heavy chain; gray, light chain). N-glycosylated residues are indicated in magenta. (B) Ribbon representation of the Nrp a2b1b2 structures; the orange spheres highlight a bound calcium ion. (C) Superposition of the Nrp2/Fab complex from two different crystal forms based on the a2b1b2 domains. Note the poor superposition of the a1 domains (yellow arrows) in comparison to the a2b1b2 region (black arrows). Structure figures were produced with PyMol (http://www.pymol.org). Taken from Appleton et al. 2007.


A
N
b2
a1
Fab
b1
C
a2
B
Nrp1
Nrp2
C
b2
Fab
b2
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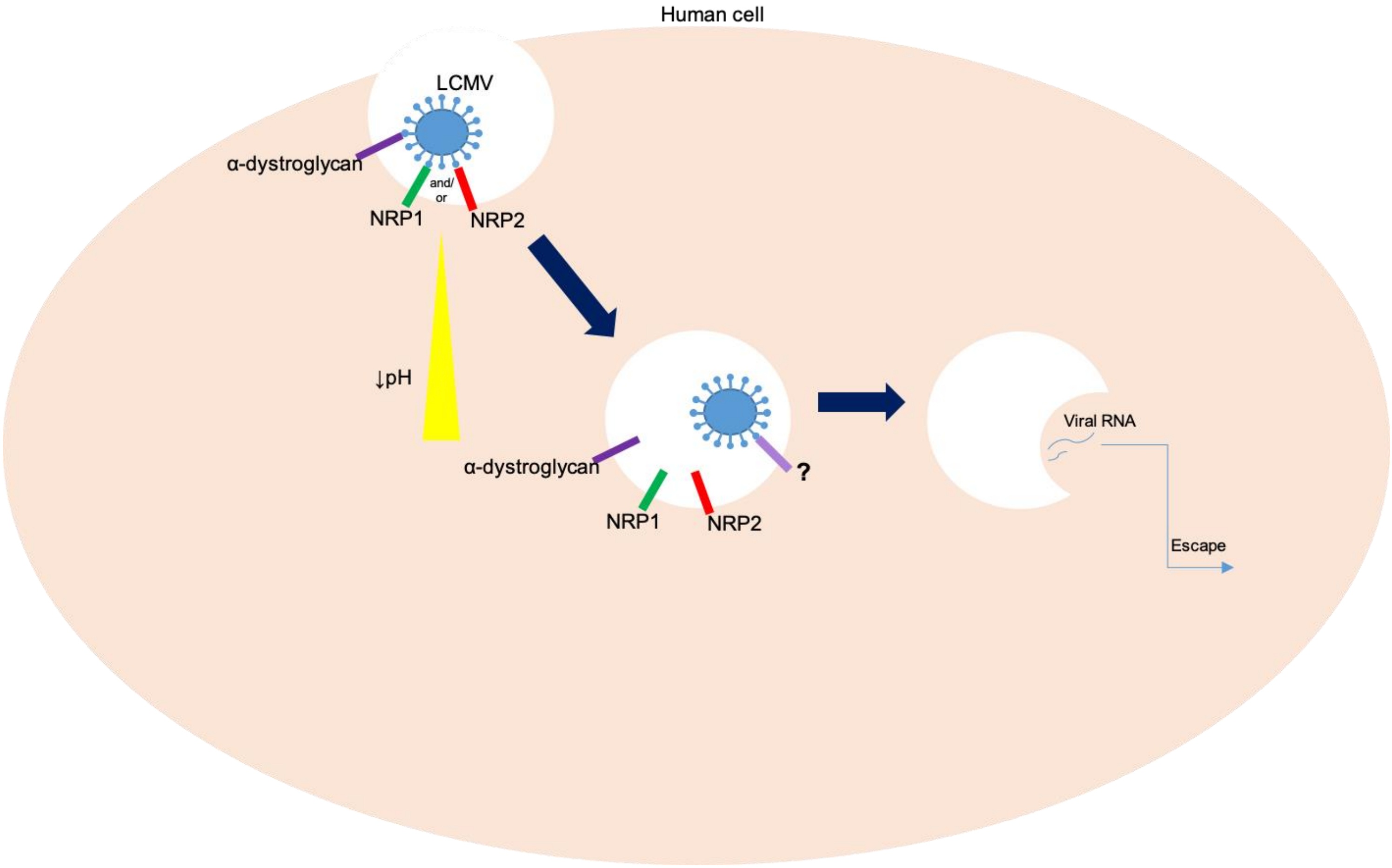
b1
a2
a1
a2

This suggested that LCMV could bind to NRP2 as well. Indeed, after testing the binding of the soluble protein to NRP2 by flow, it was seen that there was strong, specific binding. Taken together, this seems to indicate the startling conclusion that LCMV can use either NRP1 or NRP2 in the entry process. Future studies should identify the potential differences in affinity that LCMV has for either of these proteins, using techniques such as Surface Plasmon Resonance. This would help to identify whether LCMV has a preference for one NRP protein or the other, or if they can be used entirely in a first-come basis, indicating the likelihood of a truly redundant system. If this hypothesis is correct, it would allow the virus to increase its tropism and have a greater ability to infect a variety of cells.

If NRP1 or 2 can be used as receptors during entry, then it is likely that knocking out just one or the other would not be sufficient to block infection. Indeed, in a single knockout system when only NRP1 or 2 was targeted, there was no statistically significant decrease in LCMV infection seen (Figure 15B). However, in the cell line that targeted both NRP1 and NRP2 proteins, a statistically significant decrease in infection for LCMV alone was seen. While these results still need to be confirmed in cells that are complete knockouts, these preliminary results indicate that the virus may be able to use either NRP1 or 2 to accomplish its goals. This study could be further enhanced through the use of Western blotting techniques to quantify the decrease in protein expression for the knockdown cells. This could be particularly important for LCMV in the wild. In such a case, in its animal reservoir, LCMV is usually transmitted vertically which induces tolerance in the host, so the virus must be able to replicate in different organs in the body, including the thymus[76].

From these data and previous studies, we propose that LCMV first initiates endocytosis by binding to its primary attachment factor, α-dystroglycan, either in conjunction with this binding, or after, the glycoprotein on LCMV binds to the b1 domain of either NRP1 or NRP2 depending on which it interacts with first. In this case, the virus can use either protein and they bind competitively. After binding, the virus begins the endocytotic process, and is internalized and trafficked through to the late endosome. As the endosome matures towards a lysosome, the environmental pH drops, priming the virus' glycoprotein through the histidine triad contained within it. After protonation, the GP is able to bind to its intracelluar receptor. This may be a different region on NRP1 or NRP2 that the virus binds to after protonation, or may be a different protein entirely. After binding to its entry receptor, a fusion event occurs, and the viral RNA is able to escape into the cytoplasm, continuing the infection cycle (Figure 15). Future studies will be required to test this model further.

Figure 15. Proposed Model of LCMV Infection. Diagram showing a proposed model of LCMV's entry pathway. Inferences are made about potential entry strategies the virus uses. In this case either after, or in conjunction with, α-dystroglycan binding, LCMV binds NRP1 and/or NRP2 triggering endocytosis. The virus is then trafficked into the late endosome, lowering the pH of its environment. After the pH is lowered, LCMV then binds to an unidentified entry receptor, leading to fusion between the viral and host membranes. After fusion, the viral genome is able to enter into the cytoplasm.


Human cell
LCMV
α-dystroglycan
and/
or
NRP1
NRP2
↓pH
α-dystroglycan
NRP1
NRP2
?
Viral RNA
Escape

Contributions of Collaborators

Dr. Marceline Côté

Dr. Cöté helped immensely with the cloning of the different constructs as well as providing support in troubleshooting and running experiments throughout the entire thesis. She also acted as my mentor and teacher, and was responsible for working to help optimize all assays and techniques.

Ms. Corina Warkentin

Ms. Warkentin performed all of the flow cytometry analyses. Upon receipt of the prepped samples, she operated the flow cytometer and other related instruments to obtain the data. After data collection, Ms. Warkentin analyzed the data using available software programs, and presented it neatly through the creation of figures incorporated into the results section of this thesis.

Ms. Yuxia Bo

Ms. Bo was responsible for the creation of the CRISPR knock-out cell lines using a method proprietary to the lab. In order to do this, she performed all of the relevant cloning, cell selection, and validation. She also performed preliminary experiments with the cells to ensure that they were inducing properly and capable of providing accurate data. In addition to the CRISPR, Ms. Bo also assisted with troubleshooting of my cloning, and assistance with various techniques such as infections, western blotting, and ancillary cloning.

Dr. Lawrence Puente

Dr. Puente was responsible for the preparation, running, and analysis of the mass spectrometry samples displayed in this thesis. After excision of fixed and stained bands of interest, the samples were sent to Dr. Puente who transformed them into results. These results were generated through the use of Scaffold software which in turn was accessible to the lab.

Mr. Joshua Brost

As an undergraduate volunteer, and later honours student in the lab, Mr. Brost contributed to the early work on LCMV, including the generation of the various constructs. He was involved with the cloning to create the wt and mutated LCMV virus, as well as the wt and mutated soluble protein. Mr. Brost was also involved in early pulldown experiments, and troubleshooting of the proteomic process, including a variety of approaches which were eventually abandoned.

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Curriculum Vitae

Education

Master of Science, Microbiology and Immunology (Candidate), University of Ottawa
2018

Currently enrolled in graduate studies in the University of Ottawa's Faculty of Medicine. Research is heavily translational focused on drug development and commercialization. Supervised by Dr. Marceline Côté, one of Canada's top virologists, and the Canada Research Chair in Molecular Virology and Antiviral Therapeutics. On track for graduation in 2018, expected grade of *magna cum laude*.

Honours Bachelor of Health Science, University of Ottawa, *Cum Laude*
2016

Received an honours bachelor degree from the University of Ottawa's Health Science program, graduating *cum laude*. As one of its first in the country, this program takes a heavily interdisciplinary approach to give students' a wide and rigorous background in health, science, business, and economics. This program took an international standpoint to give a global perspective on the interplay between the numerous factors affecting health and health policy.

Experience

Researcher, University of Ottawa/Ottawa Hospital Research Institute Jan 2014 – Present

- Scientific researcher in multiple laboratories throughout undergraduate and graduate education.
- Started by researching the relationship between certain types of enzyme activity and cholesterol. Later transitioned to investigating and testing small-molecule therapies for highly pathogenic emerging viruses, such as Ebola and Marburg. Graduate research has taken the form of understanding viral entry into cells, and identifying host receptors involved in pathogenicity.
- Experience in research has given a strong critical thinking and analytical ability. Able to breakdown and understand complex data sets and understand a wide variety of scientific and research concepts.

Advisor, Sparkfest/Inventure June 2017 – Present

- Youth entrepreneurship non-profit start-up. Organization founded by secondary school students to build entrepreneur capabilities of students in the National Capital Region, through an annual conference, and ongoing networking platform.
- Acting as an advisor, primarily in business development. Mentoring leaders of the organization to identify and approach key businesses that could act as productive sponsors. Successfully secured partnerships, sponsorships, and the commitment of resources from companies like InvestOttawa, Shopify, and Google.

Founder & Consultant, Attica Consulting November 2015 – Present

- In order to get experience with lead generation, self-marketing, and consulting, founded Attica Consulting. Attica is an Ottawa-based research consulting company focused on health, healthcare, and science management.

- Successes include: assisting US-based pharma startup with connecting with the right professionals to enhance the R&D process of their products, providing technology policy recommendations to Standing Committees of the Senate of Canada, and successfully overseeing the expansion of a medium-sized business into new product lines and distribution channels (online) which provided a new diverse revenue stream for the business.
- Experiences creating and promoting a business, as well as building partnerships gave a strong passion towards business development and consulting.

Sales & Marketing Associate/Strategy Consultant, Tomlin Group May 2016 – December 2016

- Leveraged analytical thinking skills to obtain a leadership position in a medium-sized manufacturing company producing luxury home living products, in order to gain business experience and balance science skills.
- Placed in charge of assisting the VP Sales and Marketing to expand the business into an entirely new market segment.
- Successfully conducted market research and discovered both the most valuable products to the company's customers, as well as the best distribution channel to distribute these products.
- Successfully launched the new products with an online distribution focus, and optimized marketing and sales strategies to draw new customers to the company. Communication lines and contract negotiations were successfully created with representatives from large multinationals such as Molson-Coors.

Volunteer Experience

- Let’s Talk Science
    - Worked to improve science literacy of secondary school students by doing in-class outreach activities and lessons.
- Canadian Ski Patrol System
    - Volunteer primary medical provider and situation responder on ski hills and at special events.
    - Responsibilities include ensuring the safety of all patrons on the ski hill, and providing primary medical care to injured skiers.
    - Youngest Ski Patroller in Ontario
- Toronto Police Service Marine Unit – Community Outreach Rescue Program
    - Used lifeguarding and lifesaving skills to teach rescue techniques to underprivileged, inner-city kids in Toronto.
- University of Ottawa Foot Patrol
    - Volunteered throughout undergraduate as a Foot Patroller, providing safe walks to members of the university community.
    - Focused on teamwork and interpersonal communication in order to provide a professional and safe walking experience.
- Students Helping Orphans in Africa with Relief (S.O.A.R.)
    - Student ambassador responsible for organizing fundraising and awareness events throughout high schools in the Greater Toronto Area.

    - Through a partnership with the Stephen Lewis Foundation, worked to raise awareness about the orphans left behind by the HIV/AIDS crisis raging throughout Sub-Saharan Africa.

Publications

Conference Presentations

- Ebola Virus Requires Host Signaling for Entry (2015)
- Flibanserin: The Effects on an Intervention on the Symptoms of Hypoactive Sexual Desire Disorder in Pre-Menopausal Women (2016)
- Developing Tools to Track Viruses During Entry (2017)
- A conserved role for a histidine triad in old-world arenavirus glycoprotein during viral entry (2018)

Featured Articles/Columns (Paid)

- Gould Maule, G. (2016, January 7). It's time to regulate prescription drug prices. *The Ottawa Citizen*.
- Gould Maule, G. (2017, February 22). Gut feelings no substitute for data. *The Winnipeg Free Press*

University Class Lectures

- "Factors and Complications in Prescription Drug Pricing" January 15, 2016. – University of Ottawa
- "International Health and Disease: Can Industry Play a Role?" November 22, 2016 – University of Ottawa
- "*Contagion*: The Realities of Controlling Pandemics in a Fictionalized Model" March 14, 2017 – University of Ottawa
- "Laboratory Research: Process and Outcomes" November 1, 2017 – Carleton University