Characterization of the Humoral Immune Response Elicited Against a Prophylactic Vaccine Composed of Recombinant Envelope Glycoproteins from Hepatitis C Virus

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Virology

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ABSTRACT

A global prophylactic vaccine for Hepatitis C Virus (HCV) remains elusive. The diversity of the virus is a major hurdle; a successful vaccine will need to protect against all 7 HCV genotypes (gt). Our lab is developing a vaccine comprising the two envelope glycoproteins of HCV (E1E2). An effective recombinant E1E2 (rE1E2) vaccine would likely work through generating broadly neutralizing antibodies (nAbs) binding conserved regions on E1E2 critical for entry. A rE1E2 vaccine derived from a single gt1a strain (HCV-1) has been shown to elicit broadly nAbs in guinea pigs, chimpanzees, goats, and healthy human volunteers. Epitope mapping of anti-E1E2 antibodies present within antisera from goats/humans immunized with HCV-1 rE1E2 was conducted through competition studies with monoclonal broadly nAbs targeting various epitopes within E1E2. Antisera were shown to compete with the binding of all broadly nAbs tested and competed especially well with broadly nAbs blocking the interaction between E2 and the major cell entry receptor CD81. Goats immunized with soluble E2 (sE2) derived from either a gt1a (HCV-1) or a gt2a strain (J6) were found to have strong strain-specific nAbs (E2 antisera were not tested for cross-neutralization). HCV-1 rE1E2, HCV-1 sE2, and J6 sE2 antisera were investigated to determine the mechanisms of neutralization. Synchronized time-of-addition experiments revealed that these vaccines elicited nAbs possessing kinetics of neutralization indicating a role early in entry – prior to/at the CD81 post-binding step. Further experiments showed HCV-1 rE1E2 and HCV-1 sE2 but not J6 sE2 goat antisera directly blocked the E2-CD81 interaction. As well, HCV-1 rE1E2 and HCV-1 sE2 antisera directly blocked the E2-SRB1 interaction (J6 sE2 antisera were not tested). These results suggest that immunizing with HCV envelope glycoproteins elicits nAbs that act at pre-binding and/or early post-binding

steps by inhibiting HCV interaction with HSPG, SRB1, and/or CD81. These results support the use of an E1E2 vaccine to induce cross-genotype neutralization. Modified rE1E2 antigens were created to improve vaccine production efficiency, immunoreactivity, and immunogenicity. While improving vaccine production was successful, modifying the antigen was not successful for improving the vaccine in the context of immune responses. However, valuable assays for rational vaccine design were gained and there are other possibilities for generating an optimized vaccine.

PREFACE

Chapter 3 of this thesis contains work published in the following journal articles:

Law, J.L., Chen, C., **Wong, J.A.,** Hockman, D., Santer, D.M., Frey, S.E., Belshe, R.B., Wakita, T., Bukh, J., Jones, C.T., Rice, C.M., Abrignani, S., Tyrrell, D.L., and Houghton, M. **2013.** A hepatitis C virus (HCV) vaccine comprising envelope glycoproteins gpE1/gpE2 derived from a single isolate elicits broad cross-genotype neutralizing antibodies in humans. ***PLoS ONE*** *8(3):e59776*.

I was responsible for the optimizations of protocols for neutralization assays. J.L. Law and M. Houghton were responsible for concept formation. J.L. Law was responsible for manuscript composition. M. Houghton was responsible for manuscript edits. J.L. Law and C. Chen were responsible for the bulk of the experimental data.

Wong, J.A., Bhat, R., Hockman, D., Logan, M., Chen, C., Levin, A., Frey, S.E., Belshe, R.B., Tyrrell, D.L., Law, J.L.M., and Houghton, M. **2014.** Recombinant Hepatitis C Virus Envelope Glycoprotein Vaccine Elicits Antibodies Targeting Multiple Epitopes on the Envelope Glycoproteins Associated with Broad Cross-Neutralization. ***J. Virol.*** *88(24): 14278-14288*.

I was responsible for the majority of the experimental data and manuscript composition. M. Houghton, J.L. Law, and I were responsible for concept formation. M. Houghton and J.L. Law assisted with manuscript edits. Generation of rE1E2 was performed by M. Logan. Virus generation and neutralization assays were performed by J.L. Law, D. Hockman, J. Johnson, A. Dahiya, C. Chen, and me.

Chapter 4 of this thesis contains work in preparation to be published:

Wong, J.A., Hockman, D., Logan, M., Chen, C., Frey, S.E., Belshe, R.B., Law, J.L., and Houghton, M. Vaccination with HCV envelope glycoproteins induces antibodies that prevent the interaction of E1E2 with critical cell entry receptors. In preparation.

I was responsible for the majority of the experimental data and manuscript composition. M. Houghton, J.L. Law, and I were responsible for concept formation. M. Houghton and J.L. Law assisted with manuscript edits. D. Hockman was responsible for some of the neutralization assay data. Generation of rE1E2 was performed by Michael Logan. Soluble E2 was provided by J. Marcotrigiano (NIH).

Chapter 5 of this these contains work published in the following journal articles

Logan, M., Law, J.L., **Wong, J.A.**, Hockman, D., Landi, A., Chen, C., Crawford, K., Kundu, J., Baldwin, L., Johnson, J., Dahiya, A., LaChance, G., Marcotrigiano, J., Law, M., Foung, S., Tyrrell, D.L., and Houghton, M. **2016.** Native Folding of a Recombinant gpE1/gpE2 Heterodimer Vaccine Antigen from a Precursor Protein Fused with Fc IgG. ***J. Virol.*** *91(1): e01552-16*.

I was responsible for the E1E2 ELISAs, mAb binding ELISAs, and competition ELISAs. M. Logan and M. Houghton were responsible for concept formation. M. Logan was responsible for manuscript composition. Virus generation, neutralization assays, and animal work were performed by J.L. Law, D. Hockman, J. Johnson, and C. Chen. Generation of rE1E2 antigens, biochemical assays, and E2 ELISAs were performed by M. Logan.

Law, J.L., Logan, M., **Wong, J.A.**, Kundu, J., Hockman, D., Landi, A., Chen, C., Crawford, K., Wininger, M., Johnson, J., Prince, C.M., Dudek, E., Mehta, N., Tyrrell, D.L., and Houghton, M. **2018**. Role of the E2 Hypervariable Region (HVR1) in the Immunogenicity of a Recombinant HCV Vaccine. ***J. Virol.*** *92(11): e02141-17.*

I was responsible for the E1E2 ELISAs, mAb binding ELISAs, and competition ELISAs. J.L. Law and M. Houghton were responsible for concept formation. J.L. Law was responsible for manuscript composition. Virus generation, neutralization assays, and animal work were performed by J.L. Law, D. Hockman, J. Johnson, and C. Chen. Generation of rE1E2 antigens, biochemical assays, and E2 ELISAs were performed by M. Logan.

DEDICATION

This thesis is dedicated to my wife, Nicole Wong, my parents, Jeremy and Teresa Wong, and my sister, Lyndsey Wong.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Michael Houghton. With his track record and highly distinguished career, it can hardly be surprising that he has been an incredible supervisor. He has taught me how to be a disciplined scientist at the everyday scale and truly see the big picture. He has been relentlessly encouraging and positive and demonstrated the impact a great scientist, humanist, and leader can have in health sciences. I was very fortunate to have him as a supervisor.

I would also like to thank Dr. John Lok Man Law for being an incredibly patient and caring mentor. If he could mentor me through my PhD, I dare say he could mentor anyone to do anything. I am also grateful to everyone else in the Houghton and Tyrrell labs for being such great resources and all around wonderful people to work with and learn from. I am thankful for the members of my supervisory committee Drs. David Evans, David Marchant, Luis Schang, D. Lorne Tyrrell, and Michael Houghton for their wise advice and guidance.

My wife, my parents, and my sister have been incredible sources of loving support and I owe so much to them. My friends Wafa Kamal, Adam Maunder, and Lindsay Canham have always rooted for my success. The squid has always supported me.

I have received generous financial support from the Canadian Liver Foundation, a non-profit that does wonderful work and advocacy for those afflicted with liver diseases and their families. The Canadian Network on Hepatitis C provided financial support in addition to a highly valuable program where I was shown the power of a very broad multidisciplinary approach to a problem and met many interesting people trying to solve the problems HCV causes in different ways.

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LIST OF SYMBOLS

>: greater than
<: less than
≥: greater or equal than
≤: less or equal than
%: percentage
°C: degrees in Celsius
g: gram
mg: milligram
µg: microgram
g: relative centrifugal force
h: hours
IC_{50}: 50% inhibitory concentration
IC_{90}: 90% inhibitory concentration
kDa: kilodaltons
L: litre
mL: millilitre
µL: microlitre
m: mass
M: moles
mM: millimoles
µM: micromoles
n: number of samples
nm: nanometer
OD: optical density
rpm: revolutions per minute
RT: room temperature
s: seconds
ms: milliseconds
µs: microseconds
t: timepoint
$TCID_{50}$: 50% tissue culture infectious dose
v: volume

LIST OF ABBREVIATIONS

5' UTR: 5' untranslated region

aa: amino acids
ACGFP: *Aequorea coerulescens* green fluorescent protein
Ad6: human adenovirus serotype 6
ALP: alkaline phosphatase
apoB: apolipoprotein B
apoC-1: apolipoprotein C-1
apoE: apolipoprotein E
AU: asymmetrical unit

BafA1: balifomycin A1
BiP: binding immunoglobulin protein
BVDV: bovine viral diarrhea virus

Cre: cyclization recombinase
CCAC: Canadian Council on Animal Care
CD81: cluster of differentiation 81
CD81bs: CD81 binding sites
c-di-AMP: cyclic dinucleotide adenosine monophosphate
ChAd3: chimpanzee adenovirus serotype 3
CLDN1: claudin-1
CLDN6: claudin-6
CLDN9: claudin-9
CME: clathrin-mediated endocytosis
ConA: concanamycin A
C-terminal: carboxyl-terminus

DAA: direct-acting antiviral
DMEM: Dulbecco's Modified Eagle Medium
DMSO: dimethyl sulfoxide
DTT: dithiothreitol

E1: HCV envelope glycoprotein 1
E2: HCV envelope glycoprotein 2
EDTA: ethylenediaminetetraacetic acid
EGCG: epigallocatechin gallate
EGFR: epidermal growth factor receptor
EIA: enzyme immunoassay
ELISA: enzyme-linked immunosorbent assay
EndoH: endoglycosidase H
EPgV: equine pegivirus
EphA2: ephrine receptor A2

FBS: fetal bovine serum
Fc-d: Fc-tag derived
FFU: focus forming unit
FP: fusion peptide
GAG: glycosaminoglycan
GFP: green fluorescent protein
GNA: *Galanthus nivalis* lectin antigen
GnTI: N-acetylglucosaminyltransferase I
gt: genotype

HAP: hydroxyapatite
HCV: hepatitis C virus
HCVcc: cell culture derived HCV
HCVpp: HCV pseudoparticles
HCVser: patient serum derived HCV
HDL: high density lipoprotein
HEK293T: human embryonic kidney 293T cells
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV: human immunodeficiency virus
HRP: horseradish phosphatase
HSPG: heparan sulfate proteoglycans
HRV3C: human rhinovirus protease 3C
HVR1: hypervariable region 1
HVR2: hypervariable region 2

IACUC: Institutional Animal Care and Use Committee
IgVR: intergenotypic variable region
IRES: internal ribosomal entry site

KLH: keyhole limpet hemocyanin

LDL: low density lipoprotein
LDLR: low density lipoprotein receptor
LPA: lymphoproliferation assay

mAb: monoclonal antibody
miRNA: microRNA
MLV: murine leukemia virus
MPER: membrane proximal external region
MPLA: monophosphoryl lipid A
MVA: modified vaccinia Ankara

nAb: neutralizing antibody
NCR: non-coding region
n.d.: not determined

NPC1L1: Niemann-Pick C1-like 1 cholesterol uptake receptor
NPHV: nonhuman hepaciviruses
NS3: HCV nonstructural protein 3
NS4: HCV nonstructural protein 4
NS5: HCV nonstructural protein 5
NS5B-SL3: NS5B stem-loop 3 *cis*-acting replication element
N-terminal: amino-terminus

OCLN: occludin

pAb: polyclonal antibodies
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PFA: paraformaldehyde
PP: PreScission protease
PWID: people who inject drugs

qRT-PCR: quantitative real time-polymerase chain reaction

rE1E2: recombinant E1E2 protein
RHV: rodent hepaciviruses

SDS: sodium dodecyl sulfate
sE2: soluble E2 protein
SRB1: scavenger receptor class B, type 1
STAT: signal transducer and activator of transcription
STING: stimulator of interferon genes

TfR1: transferrin receptor 1
TMD: transmembrane domain
tPA: tissue plasminogen activator

VLDL: very low density lipoprotein
VSVG: vesicular stomatitis virus G protein

WT: wild type

1 CHAPTER I: INTRODUCTION

1.1 HCV disease and global health burden

1.1.1 HCV disease

Hepatitis C was initially known as non-A non-B hepatitis and it was determined to be a small enveloped virus that was responsible for the majority of the cases of infectious hepatitis acquired parenterally. Using an exhaustive panning approach involving cDNA expression libraries from infected patients and radiolabeled patient antibodies made difficult by the absence of PCR, the causative agent was isolated and identified as Hepatitis C virus (HCV) in 1989 (Choo et al. 1989; Houghton 2009). This discovery also led to the first accurate EIA for the screening of circulating antibodies directed against HCV antigen (Kuo et al. 1989; Miyamura et al. 1990).

The *Hepacivirus* genus includes 14 species (Hepacivirus A-N); the genus is under the family *Flaviviridae* which includes the genera *Flavivirus*, *Pestivirus*, and *Pegivirus* (Smith et al. 2016). HCV was organized into 6 major genotypes with numerous subtypes within each genotype (Robertson et al. 1998). Later, an updated analysis of all available sequences confirms the organization of 6 major genotypes but a new sequence was tentatively assigned as genotype 7, subgenotype 7a (Nakano et al. 2012).

Hepatitis C is a very serious global health problem with an estimated ~70-150 million infected people worldwide and an estimated 1.7 million new infections a year (WHO, n.d.; The Polaris Observatory HCV Collaborators 2017). Particularly vulnerable are PWIDs, and the infection incidence in two cohorts of young adult drug users studied in the United States was found to be between 8-25% (Page et al. 2009; Mehta et al. 2011). In fact, new injectors are the most at risk at rapidly acquiring the disease, with some estimates that 25% will be infected within the first 2 years of using injection drugs (Hagan et al. 2008). Upon infection, the individual may either spontaneously clear the virus (14-46%), fail to clear and progress from acute infection – the first 6 months of infection – to chronicity (54-86%), or develop a severe case of fulminant hepatitis (<1%). A chronic HCV infection can eventually lead to cirrhosis of the liver (~15-51% of chronically infected patients) which can lead to hepatocellular carcinoma

(1-5%/year of cirrhotic patients) or hepatic decompensation (3-6%/year of cirrhotic patients), both of which combined with extrahepatic manifestations of the disease, can lead to death (2-4%/year of cirrhotic patients) (**Figure 1.1**). In fact, liver damage from chronic HCV infection is the leading cause for liver transplantation (Lavanchy 2009).

Until recently the standard-of-care for treatment was a 24-week course of pegylated interferon-α and ribavirin, which was moderately effective with around 80-90% cure rates for genotypes 2 and 3 but only 50% for genotypes 1 and 4. In addition, patients commonly experienced severe side effects including depression, nausea, and flu-like symptoms such as headaches (Patel and McHutchison 2004). This has in particular limited the uptake of treatment by the PWID population (Wiessing et al. 2014; Alavi et al. 2014). The great deal of focus on developing HCV direct-acting antivirals (DAAs) has paid off first in the form of effective DAAs directed against the NS3-NS4A protease that provided 65-75% cure rates against genotype 1 (Poordad et al. 2011; Jacobson et al. 2011). While the first set of NS3-NS4A protease inhibitors were only effective against genotype 1, a second generation of NS3-NS4A protease inhibitors have broader effectiveness (Bartenschlager, Lohmann, and Penin 2013). There is now a set of "miracle drugs" that target NS5A and NS5B that can be used in an interferon-free regimen that provides >90% cure rates in all genotypes (D'Ambrosio et al. 2017). These show potent and broad pangenotypic activity, with a high barrier to viral escape by mutation, especially when provided together in an interferon-free regimen (Powdrill et al. 2010; Sarrazin et al. 2017). DAAs appear to be an effective treatment to reduce progression of liver disease, reduce all-cause mortality in people with advanced liver disease, and prevent transmission in PWID populations (Grebely and Dore 2011; van der Meer et al. 2014; Martin, Vickerman, et al. 2013).

1.2 HCV structure

The HCV genome is a single-stranded RNA that is 9.6kb in length and encodes one single open reading frame that is translated to produce a polyprotein precursor that is cleaved co- and post-translationally by both viral and cellular proteases into 10 gene products. The N-terminal region encodes the structural proteins, which make up the viral particle. This includes the core protein (C) that forms the capsid and two envelope glycoproteins (E1 and E2) that mediate binding to human hepatocytes, interact with entry receptors, and are responsible for

fusion between the viral envelope and the endosomal membrane. The C-terminal region encodes the non-structural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. These are not incorporated into viral particles but are important for HCV replication and assembly of new virions through mediating RNA synthesis, post-translation processing, assembly, and suppression of innate host defense mechanisms. The genome is depicted in **Figure 1.2** (Lindenbach and Rice 2013).

The HCV virion has a lipid membrane bi-layer, is 40-80nm in diameter, heterogenous in its morphology, and lacks distinctive symmetry or surface features apart from an overall smooth surface (Catanese, Uryu, et al. 2013). Compared to other enveloped RNA viruses, HCV particles also have unusually low and varied buoyant densities, which can be lower than <1.10g/mL (Lindenbach et al. 2005). This low buoyant density is due to interactions of the virus particle with serum lipoproteins which form lipo-viral particles (LVPs). The associated apolipoproteins include apoA-I, apoB-48, apoB-100, apoC-1, and apoE (Thomssen et al. 1992; Kono et al. 2003; Felmlee et al. 2010; Diaz et al. 2006). The association with lipoproteins forms a particle resembling either a VLDL or LDL particle with cholesteryl esters accounting for almost half of the total HCV lipids (Merz et al. 2011). As opposed to serum LVPs, HCVcc tend to interact mainly with apoE instead of apoB – however, the use of human sera instead of FBS as a media supplement allows the generation of HCVcc that better represent serum LVPs (Steenbergen et al. 2013). It is thought that the nature of the HCV serum particle as an LVP could facilitate viral entry via interactions between apolipoproteins and attachment factors and entry receptors such as syndecans, HSPG, and SRB1, as well as evasion from the humoral immune response (Barth et al. 2003; Lefèvre et al. 2014; Shi, Jiang, and Luo 2013; Scarselli et al. 2002; Thi et al. 2012). This evasion has been shown to be due to enhanced infectivity due to increased interactions with attachment factors as well as masking of important neutralizing antibody epitopes (Bankwitz et al. 2017; Fauvelle et al. 2016). Apolipoproteins have been shown to be required for intracellular assembly and viral entry in the HCVcc cell culture model. It has been shown that apoliprotein B and E, the highest expressed exchangeable apolipoprotein molecules in the Huh-7 cell line, serve redundant critical roles in the formation of infectious HCV particles – cells lacking both apolipoprotein B and E were unable to produce infectious HCV virions (Fukuhara et al. 2014). This dysfunction was rescued by the exogenous expression of a variety of exchangeable apolipoproteins and even by expression of the amphipathic α-helices found in exchangeable

lipoproteins. Further research clarified that the absence of apolipoproteins leads to a reduced release of core protein from infected cells, and indicated apoE acts after capsid envelopment but prior to secretion (Hueging et al. 2014). As well, anti-apoE antibodies have shown potent neutralizing activity, indicating apoE on the surface of infectious virions is necessary to facilitate entry (Chang et al. 2007; Jiang et al. 2012). After incubation of secreted apoE from non-HCV infected cells and HCV virions from PHHs or Huh-7.5 cells, the virions have been shown to possess enhanced infectivity up to 8-fold across all genotypes and decreased sensitivity to neutralizing antibodies targeting E1 and/or E2 up to 10-fold (Bankwitz et al. 2017). Interestingly, the infection enhancement appeared to be independent of HVR1 and SRB1 but dependent on HSPGs. This indicated that HCV virions undergo apoE-dependent maturation to enhance infectivity and immune evasion. Adding to the importance of apolipoproteins in the HCV viral life cycle, apoC1 association with HCV has been shown to promote virus-cell membrane fusion (Dreux et al. 2007).

The variance in buoyant density has shown to influence infectivity and this can be explained by how the buoyant density affects interaction with nAbs and entry receptors – this will be further detailed below (Thi et al. 2012). The structure of the HCV virion is depicted in **Figure 1.3A**,**B**. It isn't well understood what the LVP looks like and exactly how the lipid components are interacting with the HCV virion. One possibility is that virions are transiently interacting with the lipoprotein components (**Figure 1.3A**) and the other possibility is that the LVP exists as a hybrid particle with a shared envelope (**Figure 1.3B**).

1.3 Models for HCV Infection

1.3.1 Models for in vitro infection

Culturing HCV *in vitro* or *in vivo* was very difficult in the early days of research as the only species HCV could infect were humans and chimpanzees and it wasn't possible to culture any chimpanzee or human-derived strains in cell lines. The first milestone for cell culture models to study HCV was the isolation of a human hepatoma cell line that could replicate the HCV genome but that wasn't capable of generating infectious virions (Lohmann et al. 1999; Blight, Kolykhalov, and Rice 2000). The next milestone was the development of HCV pseudoparticles (HCVpp), which involved pseudotyping the HCV envelope glycoproteins E1 and E2 on

lentiviral or retroviral particles. HCVpp can largely replicate the entry of HCV into the hepatocyte but not replication or egress (Bartosch, Dubuisson, and Cosset 2003). Infectivity of HCVpp can be heavily dependent on the strain of the envelope glycoproteins used for pseudotyping and some strains of E1E2 will create defunct HCVpp but most strains can be successfully pseudotyped. In fact, we now have representative HCVpp derived from the 7 major HCV genotypes (Meunier et al. 2005). Finally, after many failed attempts to do so, Dr. Takaji Wakita and colleagues discovered a genotype 2a isolate (JFH-1) from a patient with fulminant hepatitis that could grow efficiently in tissue culture (Wakita et al. 2005; Zhong et al. 2005). It was found that the regions required for this strain to grow efficiently were the nonstructural regions (NS3-NS5B) so it was determined the Core-NS2 regions could be replaced with those of other strains in order to produce cell culture derived HCV particles (HCVcc) that can progress through the entire life cycle but structurally resembles different strains (Gottwein et al. 2009). Similar to the HCVpp model, replacing the Core-NS2 regions can affect the infectivity and some strains can create successful chimeras while others cannot. This ability to structurally represent various strains is very critical to developing vaccines that are aimed at generating cross-neutralizing antibodies, as the antisera elicited against a vaccine can be effectively tested for the breadth of response. The *in vitro* models useful for studying HCV are outlined in **Figure 1.4**. As for cell cultures, the most common cell line for *in vitro* work is the Huh-7.5 human hepatoma cell line. Primary human hepatocytes are difficult to work with and in short supply but can offer a more relevant model physiologically.

While HCV has been seen to be able to be cultivated in other cells such as primary T-cells, it can only be efficiently grown in hepatocytes (Sarhan et al. 2012). Apart from the fact the full array of entry receptors and cofactors necessary or conducive to entry are found on hepatocytes but not on other cell types, there are other factors contributing to the hepatotropism of HCV. The miRNA mir-122 is a liver-specific miRNA that has been shown to bind the 5' UTR of the HCV genome and contributes to the efficient replication of HCV (Jopling et al. 2005). In fact, expression of miR-122 in HepG2 hepatoma cells that do not efficiently sustain the HCV life cycle along with human CD81 allows for efficient growth (Narbus et al. 2011). The hepatocyte is unique in the content of lipids, apolipoproteins, and cholesterol found within the cell and HCV virions incorporate these molecules during assembly and maturation. Blocking cholesterol and sphingolipids production in infected cells or depleting cholesterol and sphingolipids from

extracellular virions was shown to have severely deleterious effects on maturation and infectivity of produced virions (Aizaki et al. 2008). As detailed further in **Section 1.5**, apolipoproteins present in the HCV virion make a significant contribution in enhancing viral entry and evasion from neutralizing antibodies. However, apolipoproteins further contribute to the hepatotropic nature of HCV through enabling efficient replication and the formation, maturation, and secretion of viral particles (Fukuhara et al. 2014, 2017; Meunier, Russell, Engle, et al. 2008; Jiang and Luo 2009; Lee et al. 2014). In addition, it was found that the cytosolic protein SEC14L2 was able to enhance HCV RNA replication, even allowing viruses present in patient sera to replicate in SEC14L2-expressing Huh-7.5 cells (Saeed et al. 2015). SEC14L2 is found in high expression in only breast, prostate, and liver tissues, adding to the tropism of HCV (Wang et al. 2009).

1.3.2 Animal models for HCV propagation in vivo

Chimpanzees are currently the only species apart from humans that support a robust and full life cycle infection of HCV. However, chimpanzees are prohibitively expensive and the use of chimpanzees in health research is currently being heavily restricted due to ethical concerns. Tupaias (Chinese tree shrew) and pigtail macaques appear to maintain at least transient infection and research is currently undergoing to establish these animals as midsize animal models for HCV infection (Sourisseau, Goldman, et al. 2013; Vercauteren, de Jong, and Meuleman 2014; MacArthur 2012; Tong et al. 2011; Amako et al. 2010).

Work on rodent models has been more promising and these have been useful for studying HCV drug and vaccine development. The chimeric humanized liver mouse model involves replacing a mouse liver with a human liver. This requires a mouse strain that lacks B-cell, T-cell, and NK-cell mediated responses and encodes a gene that can induce damage to murine hepatocytes such as an overexpressed urokinase plasminogen activator transgene that is transcribed from the albumin promoter (Alb-uPA; (Mercer et al. 2001)). Transplanted human liver cells repopulate the mouse liver as its own cells die resulting in mice with a human liver compartment. These mice can be infected with any HCV strain, other human hepatotropic viruses, and *Plasmodium falciparum* (Dandri et al. 2001; Morosan et al. 2006; Sacci et al. 2006). This allows for a very relevant model especially when studying patient isolates. Importantly, this

model has been used to study virus neutralization by various monoclonal and polyclonal antibodies as well as vaccine antisera (Akazawa et al. 2013; Law et al. 2008; Meuleman et al. 2011; Desombere et al. 2016). The major drawback of this model is the lack of an intact immune system impairing the ability to actively immunize and test the protection afforded by vaccines. This issue is being tackled by developing an immunocompetent mouse model that can support HCV infection and its life cycle. Both virus entry and replication are restricted in murine hepatocytes and it's been shown the two factors critically restricting entry are the second extracellular loops of murine CD81 and OCLN, while it's been shown that the SRB1 and CLDN1 murine orthologues are sufficient for HCV entry (Ploss et al. 2009; Dorner et al. 2013, 2011). This led to the development of an immunocompetent mouse expressing human CD81 and OCLN through adenoviral gene delivery or by transgenic expression and these were shown to support HCV entry. These mice also expressed a luciferase gene that became activated after the Cre recombinase expressing HCV successfully entered the hepatocyte. While the result was a relatively weak signal, it was shown these mice could be immunized with a vaccine candidate and then directly challenged with infectious virus (Dorner et al. 2011). However, viral RNA replication is not efficient and can only recapitulate entry reliably. Studies have shown that PKR- and IRF3-mediated immune responses restrict HCV replication in murine hepatocytes (Chang et al. 2006; Lin et al. 2010). Based on these findings, it was found that a mouse that has a dysfunctional innate immune response (for example a $STAT^{-/-}$ mouse) that is also expressing human entry receptors can support the full life cycle of HCV (Dorner et al. 2013). This widens the applications of this promising small animal model. Much work is necessary to boost infectivity in these mice, as signals resulting from infection are still quite low. The small animal *in vivo* models useful for studying HCV are outlined in **Figure 1.5C**,**D** and **Figure 1.6A-C**.

By passaging HCV through mouse hepatocytes, researchers discovered adaptive mutations in E1 and E2 that allowed the virus to use murine CD81 and OCLN (Bitzegeio et al. 2010). Furthermore, this mouse-adapted virus was able to infect, replicate, and produce new infectious viral particles in immortalized mouse liver cells with defective innate immunity (Frentzen et al. 2014). Recent work showed this mouse-adapted virus could enter mouse hepatocytes *in vivo*, but could not establish persistent infection even in mice with blunted innate immunity (Schaewen et al. 2016). This is outlined in **Figure 1.5B**.

1.3.3 Animal models for HCV-like viruses' propagation in vivo

There are several HCV-related viruses that infect species such as horses, dogs, bats, wild mice, rats, and non-human primates like tamarins and marmosets (**Figure 1.5A**). The first viruses identified to be "HCV-like" viruses were the GB viruses. There are four GB viruses: GBV-A, GBV-B, GBV-C, and GBV-D. While all four viruses all have positive sense ssRNA genomes with somewhat similar organization and predicted protein structures as HCV, GBV-A, GBV-C, and GBV-D have been classified under the genus *Pegivirus* and they are not hepatotropic viruses (Stapleton et al. 2011; Smith et al. 2016). On the other hand, GBV-B is classified under the Hepacivirus genus, has a 25-30% homology to HCV, and can cause acute but limiting hepatitis in tamarin monkeys (Stapleton et al. 2011; Takikawa et al. 2010; Smith et al. 2016). It has been shown that GBV-B can cause a chronic HCV-like disease in marmosets, a promising finding for finding more relevant models of HCV infection in non-human primates (Iwasaki et al. 2011).

There is a group of viruses now identified as NPHV (non-primate hepaciviruses) organized under the *Hepacivirus* genus that have been found to infect horses and dogs – although it appears that horses are the natural hosts (Burbelo et al. 2012; Kapoor et al. 2011). The chronicity rate of NPHV seems to be low (~20-40%), but it does appear that NPHV can persist in horses, at least up to 6 months (Pfaender et al. 2015; Lyons et al. 2014). Infection leads to mildly elevated liver enzymes, infiltration of lymphocytes into the liver, mild hepatitis, and high amounts of viral RNA and negative strand RNA (indicator of viral replication) in the liver, supporting that NPHV are hepatotropic viruses (Pfaender et al. 2015). Interesting, not only is NPHV is the genetically closest relative of HCV (~60% similarity), the number and position of cysteine residues in E2 suggest the tertiary structure of NPHV E2 and HCV E2 may be quite similar (Kapoor et al. 2011; Scheel, Simmonds, and Kapoor 2015). There has also been a recent discovery of a novel equine pegivirus (EPgV) that is linked to liver disease in horses (Chandriani et al. 2013; Kapoor et al. 2013).

In addition, there are a several hepaciviruses and pegiviruses similar to HCV found to infect diverse hosts including bats, rats, voles, wild mice, and non-human primates (Lauck et al. 2013; Quan et al. 2013; Drexler et al. 2013; Kapoor et al. 2013; Firth et al. 2014; Epstein et al. 2010). Liver inflammation, and high concentrations of viral RNA and the presence of negative

strand RNA in rats and bank voles suggested rodent hepaciviruses (RHV) have hepatotropism (Drexler et al. 2013; Firth et al. 2014).

It remains to be seen how applicable these HCV-like viruses could be to *in vivo* research as there are many hurdles. Although pegiviruses and non-HCV hepaciviruses are closely related to HCV phylogenetically and in genetic organization, these are still divergent viruses (at least 40% difference at amino acid level) which diminishes the relevance these viruses have to HCV. For the vast majority of these cases, the infection does not closely resemble HCV disease and does not result in persistent infection causing hepatitis. Furthermore, many of these hosts are difficult and costly to maintain in a laboratory setting – this is true for horses, which are the best host for NPHV, the closest relative to HCV. Despite these drawbacks, HCV research *in vivo* is heavily limited and learning more about relevant viruses and their models may prove to be very beneficial.

1.4 E1E2 structure

E1 and E2 are type I transmembrane proteins of approximately 190aa (31kDa) and 363aa (70kDa) respectively, and both contain a C-terminal hydrophobic region which serves as a lipid membrane anchor (Lavie, Goffard, and Dubuisson 2007). Expression in animal cell cultures leads to the retention of these proteins within the lumen of the ER as immature and anchored gpE1/gpE2 heterodimer complexes. These proteins can be truncated to remove the C-terminal hydrophobic regions to facilitate extracellular secretion of the soluble ectodomains (sE1 and sE2). It was found that while sE2 is stable on its own, sE2 is required to be co-expressed with sE1 to stabilize sE1 (Michalak et al. 1997). E1 and E2 are modified post-translationally with many N-linked glycosylations and intramolecular disulfide bonds (Michalak et al. 1997; Dubuisson and Rice 1996). In fact, the glycan shield makes up nearly half of the mass of the glycoproteins (Goffard et al. 2005).

X-ray crystallography is the gold standard for determining the atomic structure of a protein. However, this is a technically challenging methodology that is easily complicated by protein misfolding and aggregates as it requires the protein to pack uniformly to create a crystal. The folding of E1 and E2 require the host ER chaperone calnexin, while the ER chaperones BiP and calreticulin lead to nonproductive folding pathways (Dubuisson and Rice 1996; Choukhi et al.

1998). Thus, overexpression of these recombinant proteins tends to lead to misfolded disulfide-linked aggregates (Dubuisson and Rice 1996). Complicating X-ray crystallography, the E2 protein contains many disordered regions that do not pack well (Kong et al. 2013; Khan, Miller, and Marcotrigiano 2015).

Initial studies on the structure of E2 in respect to disulfide bond formation and threading the E2 polypeptide chain onto the structure of the fusion protein of other flaviviruses and alphaviruses proposed that the E2 protein would be a class II fusion protein with an extended conformation similar in structure as other Flaviviruses (Krey et al. 2010). After deleting the hypervariable regions of E2, two research groups independently determined the crystal structure of core region of E2 (E2 core) and found very similar results (Khan et al. 2014; Kong et al. 2013). This work showed that the E2 core was a non-extended globular protein constrained by disulfide bridges. This structure exists as two major protein sheets: one comprising a front layer and an immunoglobulin-like fold domain forming a central β-sandwich (common amongst fusion proteins), which contains the CD81 binding surface, and a back sheet with a unique structure (**Figure 1.7A-C**). The structure of E1 has only been partially resolved with the N-terminal portion (aa1-79) of E1 having been crystallized and its structure resolved (termed nE1). While there is much lacking in the structure, it did show an unexpected and novel structure similar to what was found with E2. E1 appears to be arranged as a disulfide linked, domain-swapped homodimer (**Figure 1.8A**,**B**). nE1 is composed of a single long α-helix sandwiched by two and three antiparallel β-strands. Interestingly, it has a fold similar to the steroidogenic acute regulatory protein-related transfer domain which binds to hydrophobic ligands like sterol and lipid molecules. Notably, the HCV particle is associated with apolipoproteins, providing a possible mechanism in how apolipoproteins associate with the HCV particle (Omari et al. 2014).

Interestingly, studies using biochemical assays, biophysical assays, and computational predictions of the heterodimer and high-order structure have suggested that E1E2 may form a trimer at the surface of HCV and that this could possibly be further assembled into a pentamer, with 12 pentamers predicted to form the virion (Freedman et al. 2017; Falson et al. 2015; Castelli et al. 2017).

1.5 HCV viral entry

1.5.1 Viral particle attachment

HCV particles are transported in the bloodstream and become in contact with hepatocytes after crossing the fenestrated endothelium of the liver sinusoids. Once the virions are in the space of Disse where the basolateral membranes of hepatocytes are present, the process of HCV entry begins with early binding of HCV virions on attachment receptors and factors. These receptors and factors include HSPG, syndecan-1, syndecan-4, and SRB1, and the contribution of each of these to binding is dependent on the density of the virion (Barth et al. 2003; Lefèvre et al. 2014; Shi, Jiang, and Luo 2013; Scarselli et al. 2002; Thi et al. 2012). At first, it was the envelope glycoproteins that were thought to be primarily mediating early interaction with HSPG and SRB1 (Barth et al. 2003; Scarselli et al. 2002), but now it's currently believed it is primarily virus-associated apoE that mediates these interactions (Jiang et al. 2012; Thi et al. 2012). As well, there is likely an interaction between the lipoproteins and the LDL receptor at the early phase of HCV entry – however, there is evidence that this leads to a nonproductive entry pathway (Agnello et al. 1999; Albecka et al. 2012). The process of HCV viral entry is depicted in **Figure 1.9**.

HSPG are in a class of molecules known as glycosaminoglycans (GAGs), large proteoglycans composed of heparan sulfate polysaccharide chains attached to a core protein located on the surface of most animal cells. The HSPG core proteins include the membrane-spanning syndecans, the lycosylphosphatidylinositol-linked glypicans, the basement membrane proteoglycan perlecan, and agrin (Carlsson et al. 2008; Kokenyesi and Bernfield 1994; Shi, Jiang, and Luo 2013). Their role in the context of the cell involves binding a variety of signaling molecules and modulating their functions (Esko and Selleck 2002). They also serve as attachment factors for many viruses (particularly enveloped viruses) during entry. These viruses include *Flaviviridae* family members Dengue virus (Chen et al. 1997), classical swine fever virus (Hulst et al. 2001), and tick-borne encephalitis virus (Mandl et al. 2001), as well as human papilloma virus (Giroglou et al. 2001), human herpesvirus 8 (Birkmann et al. 2001), and herpes simplex virus 1 (Shukla et al. 1999). It has been shown that soluble E2, apoE, and HCVcc (both intracellular and extracellular) can bind HSPG and kinetic studies using heparin as an entry inhibitor showed that HSPG was critical in the earliest steps of entry (Koutsoudakis et al. 2006;

Jiang et al. 2012; Barth et al. 2003). Determining the exact determinants of the interaction between apoE, E2, and HSPG has been difficult. However, the removal of HVR1 seems to abrogate binding between sE2 and heparin (Barth et al. 2006). Interestingly, HCVpp binding is not prevented by heparin, indicating that perhaps it is still apoE that is the prime determinant of attachment to HSPG and that HCVpp does not interact with HSPG for entry (Callens et al. 2005). A synthetic peptide derived from the receptor binding domain of apoE was able to block HCVcc infection, indicating this region is critical (Jiang et al. 2012). It has also been shown that siRNA-mediated knockdown of the membrane-spanning syndecans 1 and 4 (Syndecan-1 and Syndecan-4) reduced HCV attachment, while this was not the case with siRNA-mediated knockdown of other HSPG core proteins. As well, ectopic expression of Syndecan-1 and Syndecan-4 were capable of completely restoring HCV attachment and there is evidence they are likely interacting with apoE (Lefèvre et al. 2014; Shi, Jiang, and Luo 2013).

The LDL receptor is responsible for the capture and clearance of LDL from the bloodstream. It does this by internalizing LDL through clathrin-dependent endocytosis and then the LDL is delivered to the early endosome for clearance. The LDL receptor has been shown to interact with both apoB-100 as well as apoE and this provides support for the involvement of this receptor in the HCV entry cycle (Hussain, Dudley K. Strickland, and Bakillah 1999; Innerarity 2002). Indeed, patient serum-derived HCV particles could bind to LDL receptor-expressing cell lines, whereas sE2 could not and the interaction between LVPs and LDL receptor could be inhibited with a soluble human-LDL peptide (Agnello et al. 1999; Molina et al. 2007). HCVcc infection can be inhibited by VLDL, anti-apoE antibodies, and LDL receptor downregulation, consistent with apoE and the LDL receptor interaction facilitating entry (Hishiki et al. 2010; Owen et al. 2009). However, as stated above, there is strong evidence that interaction with the LDL receptor during the entry process may lead to a nonproductive entry pathway and it is very possible that downregulation of the LDL receptor could be inhibiting infection through mechanisms unrelated to entry such as assembly (Albecka et al. 2012).

SRB1 is important in lipid metabolism and it binds VLDL, LDL, and HDL (Thi et al. 2012). It was discovered that downregulation of SRB1 and anti-SRB1 antibodies could block both HCVpp and HCVcc infection and in addition, sE2 was shown to bind SRB1 directly (Scarselli et al. 2002; Zeisel et al. 2007; Bartosch, Vitelli, et al. 2003; Lavillette, Tarr, et al. 2005). It was determined that sE2 interaction with SRB1 was likely being mediated by HVR1

and the SRB1 residues involved in this interaction have been identified (Bankwitz et al. 2010; Bartosch, Vitelli, et al. 2003; Thi et al. 2012; Catanese et al. 2010). Deletion of HVR1 did not fully block entry which indicated that either other attachment factors are still partially sufficient or that SRB1 is necessary but interacts with other components in the LVP (Bankwitz et al. 2010). Specifically, HVR-1 deleted viruses have a lower dependence on SRB1 – these viruses are less sensitive to anti-SRB1 antibodies and SRB1 receptor silencing by siRNA (Prentoe et al. 2014; Bankwitz et al. 2010). Further research discovered that HCV utilizes SRB1 in multiple roles and this contributed to the confusion over how both HVR1 and SRB1 were involved in entry (Thi et al. 2012). While usage can depend on the lipoprotein makeup of the LVP, the "attachment" role mostly involves the interaction between apoE in the HCVcc LVP (or potentially apoB in the serum HCV LVP) and this is HVR-1 independent. Fitting with this evidence, sensitivity to neutralization by anti-apoE antibodies was unaffected by deletion of HVR1 from HCVcc viruses expressing the envelope proteins of strains from genotypes 1a, 2a, and 3a (Prentoe et al. 2014). This function of the SRB1 receptor is thought to not be relevant for HCVpp which do not contain apolipoproteins. The second function, "access", is the function that is the most critical of the three roles (this applies for HCVpp, HCVcc, and patient serum derived HCV (HCVser) entry) and does not require direct interaction with either apoE or E2. This function is reliant on the lipid transfer activity and it appears this role is critical in transitioning from virus attachment/capture to the rest of the entry steps. It likely mediates this by modifying the lipoprotein profile of the viral particles and enriching the cellular membrane with cholesterol. These actions could be contributing towards providing an optimal membrane environment for HCV entry and aid in the localization and movement of the other HCV receptors and at the same time modifying the HCV particle so that it can interact with the other HCV receptors. This "access" function can be blocked by inhibitors that prevent lipid transfer (Syder et al. 2011). There is another function, "enhancement", that does require the direct E2-SRB1 interaction and the presence of HDL as well as the lipid-transfer function of SRB1. These allow for an enhancement of HCV infection (both HCVpp and HCVcc) and the mechanism of this enhancement is not fully understood but this interaction between E2, SRB1, HDL, along with the lipid transfer function in a close manner could be facilitate receptor movement, interactions, and fusion (Voisset et al. 2005, 2006; Dreux et al. 2006; Thi et al. 2012). It is suspected that this "enhancement" function may be due to the lipid transfer function transferring apoC-1 (a major component of HDL) from HDL to HCV

which requires E2-SRB1 interaction mediated by HVR1 and it has already been shown that presence of apoC-1 can enhance fusion (Meunier et al. 2005; Dreux et al. 2007). It appears this "enhancement" function could be another contributor to viral evasion through an accelerated or more efficient entry process and this function is what seems to predominantly separate WT and HVR1-deleted viruses in the context of viral entry (Dreux et al. 2006; Voisset et al. 2006; Thi et al. 2012).

E1 associates with apolipoproteins but not E2 (Mazumdar et al. 2011). However, this was contradicted by evidence that apolipoprotein E interacts with E2 but not E1 (Lee et al. 2014). E1 is critical for attachment to CD36 which is necessary for entry (Cheng et al. 2016).

1.5.2 Post-binding events

CD81 is a tetraspanin adaptor cell surface molecule expressed on a great number of cell types. It possesses two extracellular domains, a small and a large extracellular loop (respectively, SEL and LEL) anchored to the cell membrane through four transmembrane domains (van Spriel and Figdor 2010). CD81 was the first HCV entry receptor to be identified by testing binding of sE2 to a mouse cell line expressing a human cDNA library (Pileri et al. 1998). E2 interacts with CD81 through the LEL and this interaction has been resolved structurally using cryo-EM (Kong et al. 2013; Khan et al. 2014; Drummer, Wilson, and Poumbourios 2002). The CD81 binding site on E2 is detailed in **Figure 1.10**. The residues that are critical for CD81 interaction are indicated on the crystal structure of E2 resolved in (Kong et al. 2013). These residues are located in 4 main places: CD81 binding loop/β-sandwich, E2 Epitope I (aa412-423) within the front layer, E2 Epitope II (aa434-446) also within the front layer, and a few residues located in the back layer. (Owsianka et al. 2006; Keck et al. 2012). CD81 downregulation and anti-CD81 antibodies can block infection by HCVser, HCVcc, and HCVpp (Wakita et al. 2005; Zhong et al. 2005; Bartosch, Vitelli, et al. 2003; Lavillette, Tarr, et al. 2005; Hsu et al. 2003; Bartosch, Bukh, et al. 2003; Molina et al. 2008; Kapadia et al. 2007). Interestingly, soluble CD81-LEL has no effect on HCVser infection and is only moderately effective against HCVcc (Molina et al. 2008). This suggests HCVcc and HCVser do not bind soluble CD81-LEL very effectively. Indeed, soluble CD81-LEL conjugated to sepharose beads was unable to precipitate virus from infected patient plasma (Wünschmann et al. 2000). In contrast, HCVpp can bind CD81 and soluble CD81-LEL

can potently block HCVpp infection (Bartosch, Dubuisson, and Cosset 2003; Hsu et al. 2003; Douam et al. 2014). As HCVpp does not contain the apolipoprotein content that HCVser and HCVcc do, this suggests the apolipoprotein content may prevent E2-CD81 interactions and that there is a step mediated likely by receptors that take part in HCV entry prior to CD81 (likely SRB1) that lead to the early particle rearrangements mentioned above that are required to modulate the lipids associated with the HCV virion to facilitate interaction with CD81. Consistent with the idea that CD81 is needed at a post-binding step, anti-CD81 antibodies are still just as capable at neutralizing entry after virions are already bound to the cell (Zeisel et al. 2007). A similar trend is seen with the anti-SRB1 antibody used in the paper – presumably, this antibody is blocking one of the "post-binding" functions of SRB1 and not the function involved in attachment.

HCV binding to CD81 leads to multiple signaling pathways (involving MAPK, PI3K/AKT, and RhoGTPase family members) which allows for the diffusion of HCV-CD81 complexes towards sites of viral particle internalization, possibly at the tight junctions located at the apical membrane (Harris et al. 2010; Brazzoli et al. 2008; Harris et al. 2008; Farquhar et al. 2012; Liu et al. 2012). The host factors EGFR and EphA2, receptor tyrosine kinases, have been shown to be critical in virus-CD81 complex lateral diffusion and viral entry. Inhibitors to these factors potently impair viral entry and consistently, ligands of these receptors such as EGF can enhance HCV infection (Lupberger et al. 2011; Brazzoli et al. 2008). As well, HCV binding to CD81 induces the activation of EGFR (Diao et al. 2012). Further study has indicated that EGFR promotes entry through activation of the GTPase HRas which promotes CD81 lateral diffusion and the formation of CD81-CLDN1 heterodimers which allows for a trimeric complex of HCV-CD81-CLDN1 – it is this trimeric complex that can be seen being internalized together in imaging studies (Diao et al. 2012; Coller et al. 2009; Zona et al. 2013). There is evidence that even though E2 is sufficient for SRB1 and CD81 interaction, the presence of E1 is capable of modulating those interactions, although whether the presence of E1 is interacting with the receptors itself or affecting the conformation of E2 is currently unknown (Douam et al. 2014; Russell et al. 2009). It has been shown that cysteine residues within E1 influence E1E2 heterodimerization and interaction of E2 with CD81 (Wahid et al. 2013).

The tight junction proteins CLDN1 and OCLN were discovered by screening a cDNA library derived from permissive Huh-7.5 cells into the non-permissive HEK293T and NIH3T3

cell lines (stably expressing SRB1 and CD81) and determining what would allow virus entry (Ploss et al. 2009; Evans et al. 2007). As opposed to SRB1 and CD81, which are mostly enriched at the basolateral membrane, these molecules are expressed at the apical membrane – although there is evidence CLDN1 is present at the basolateral membrane in polarized hepatocytes complexed with CD81 (Harris et al. 2010).

CLDN1 is composed of two extracellular domains, extracellular loop 1 and 2 (EL1 and EL2) anchored to the cell membrane through four transmembrane domains (Gunzel and Fromm 2012). Downregulation of CLDN1 in Huh-7.5 has shown to potently inhibit HCVpp and HCVcc entry and the critical residues of CLDN1 necessary for HCV entry were found to lie in EL1 (Evans et al. 2007). A polyclonal antisera directed against CLDN1 was shown to block infection by neutralizing E2-CD81-CLDN1 associations – consistently, the antisera showed kinetics of neutralization either concurrent or shortly after that of an anti-CD81 antibody (Krieger et al. 2010; Sourisseau, Goldman, et al. 2013). Importantly, the localization of CLDN1 into tight junctions was critical for HCV infection and HCV colocalizes with CD81-CLDN1 complexes close to the intercellular junctions, suggesting entry does indeed occur at the tight junctions (Yang et al. 2008; Liu et al. 2009; Harris et al. 2010, 2008; Farquhar et al. 2012). However, potentially due to the lack of a relevant *in vitro* model for infection in regards to cell polarity, receptor complexes can still be seen at basolateral intercellular junctions and appear to be endocytosed at the basolateral membrane under imaging techniques (Harris et al. 2010; Coller et al. 2009). While the interaction of the complex in relation to the tight junctions is not fully clear, the formation of the CD81-CLDN1 complexes following CD81 activation and lateral diffusion does promote HCV association with the complex that allows for internalization. Although it was initially thought that the envelope glycoproteins do not interact with CLDN1 directly, it was determined that the E1E2 heterodimer in the context of HCVpp was able to bind CLDN1, while sE2 could not. The nature of this interaction – whether it is an epitope formed by E1 and E2 together or by one of the two proteins (with the other protein stabilizing the interaction surface) – remains to be determined (Douam et al. 2014). Interestingly, in cells lacking Claudin-1, Claudin-6 (CLDN6) and Claudin-9 (CLDN9) could rescue HCV entry (Meertens et al. 2008; Zheng et al. 2007). In addition, alternate Claudin usage seems to be variable between HCV strains. When CLDN1 and CLDN6 were co-expressed in human hepatoma cells, presence of anti-CLDN1 mAbs led to viral escape by usage of CLDN6 (Haid et al. 2013). Antibody therapy

using an antibody targeting CLDN1 appears to be promising – a humanized anti-CLDN1 mAb was capable of preventing infection by diverse strains of HCVpp, including DAA-resistant strains, into primary human hepatocytes (PHH) and could cure HCV infection in chronically infected human-liver chimeric mice (Colpitts et al. 2018). In PHH cell cultures from 12 different donors, CLDN6 and CLDN9 expression levels were very low and these proteins did not facilitate escape from the humanized anti-CLDN1 mAb, indicating the concern over HCV virions entering in the presence of anti-CLDN1 mAbs during therapy may be unnecessary.

OCLN also has two extracellular loops, EL1 and EL2 and it is the EL2 that seems to be critical for HCVpp entry (Ploss et al. 2009). Although the role of OCLN in entry is even less well understood than CLDN1, neutralization kinetic experiments using an antibody against Flag-tagged OCLN showed that this antibody was potently neutralizing for HCVpp and HCVcc temporally after anti-CLDN1 but before endosomal acidification and fusion (Sourisseau, Goldman, et al. 2013). As OCLN is restricted to the tight junctions in polarized cells, it supports the model that HCV-CD81-CLDN1 require the tight junctions in some fashion for internalization (Harris et al. 2010). A recent imaging study using a three-dimensional polarized hepatoma system has confirmed that HCV virions colocalize with the early entry receptors (SRB1, CD81, and EGFR) at the basolateral membrane and then accumulates at the tight junctions in an actin-dependent manner (Baktash et al. 2018). They then associate with CLDN and then OCLN and this leads to an EGFR-dependent internalization.

1.5.3 Virus Endocytosis and Fusion

HCV is internalized through clathrin-mediated endocytosis and then HCV-receptor complexes migrate toward Rab5a-containing endosomal compartments (Farquhar et al. 2012; Coller et al. 2009; Meertens, Bertaux, and Dragic 2006). TfR1 is a transmembrane protein involved in the iron uptake from the bloodstream into intracellular compartments and downregulation of TfR1 or anti-TfR1 antibodies can inhibit both HCVpp and HCVcc infection. However, the role of TfR1 in entry is unknown – it is only known that anti-TfR1 antibodies act at a timepoint after the action of anti-CD81. Silencing of the protein required for TfR1 internalization was also capable of inhibiting infection, indicating that it may play a role in virus internalization (Martin and Uprichard 2013). As well, the NPC1L1 has also been identified as

another HCV entry factor, and knockdown of NPC1L1 or treatment with the specific inhibitor ezetimibe could block infection. NPC1L1 localizes to the apical, canalicular surface of polarized hepatocytes and has the function of re-uptake of cholesterol from bile and can be internalized to endosomal compartments. Neutralization kinetic experiments with ezetimibe indicated that NPC1L1 is active prior to endosomal acidification and while its role is unknown, it's suspected that it occurs around the time of internalization and involves cholesterol uptake. Consistently, there was a correlation between the cholesterol content of viral particles and their dependence on NPC1L1 during entry (Sainz Jr et al. 2012; Jia, Betters, and Yu 2011).

It was discovered that HCV could transmit from cell-to-cell without leaving the cell and that this was independent of the classical entry and internalization pathways and it is believed this allows another method of viral evasion of neutralizing antibodies (Timpe et al. 2008). SRB1, CLDN1, and OCLN have been found to be important for cell-to-cell transmission and it was shown that anti-SRB1 antibodies could block cell-to-cell transmission (Timpe et al. 2008; Witteveldt et al. 2009; Brimacombe et al. 2011; Catanese, Loureiro, et al. 2013). It is currently unclear if CD81 is necessary, as some studies have shown that it is dispensable for cell-to-cell transmission while others have shown that it does have a role (Witteveldt et al. 2009; Brimacombe et al. 2011; Catanese, Loureiro, et al. 2013). TfR1 has also shown to have less of a role in cell-to-cell transmission while NPC1L1 is critical (Martin and Uprichard 2013; Barretto et al. 2014). Interestingly, cell-to-cell transmission is the main route of spread for DAA-resistant HCV mutants and leads to viral persistence in the cell culture model in the presence of DAAs (Xiao et al. 2014). Combining inhibitors targeting viral entry and DAAs led to rapid clearance of the virus. While cell-free entry is obviously critical for the initiation of infection, persistence and spread of chronic infection may be in part due to cell-to-cell transmission (Timpe et al. 2008). Therefore, this method of transmission may need to be an important consideration in the eradication of HCV due to the potential escape of virus from anti-E1E2 neutralizing antibodies and DAAs. Controlling this route of transmission could increase the susceptibility of the virus to interventions and prevent viral spread.

Escape from the endosomal membrane via fusion is a multistep process involving a conformational change of the fusion protein(s), insertion of the fusion peptide into the host membrane, lipid mixing of external membrane leaflets (hemifusion), and full fusion of the two membranes, which leads to the release of the HCV RNA into the cytosol (**Figure 1.11**). Apart

from that, viral fusion in general has a great amount of diversity in the different fusion protein structures, conformational changes, and cellular fusion sites (Li and Modis 2014). For HCV, after clathrin-mediated endocytosis and trafficking to Rab5a endosomes, fusion is initialized by endosomal acidification (Meertens, Bertaux, and Dragic 2006). This triggers the fusion protein(s) conformational changes that lead to fusion. Consistently, bafilomycin A1 and concanamycin which block endosomal acidification, are also capable of blocking HCV infection (Meertens, Bertaux, and Dragic 2006; Sharma et al. 2011). Interestingly, it seems that HCVcc engagement with CD81 may prime the HCVcc virion for fusion, as HCVcc virions are relatively resistant to low pH (Sharma et al. 2011).

The mechanisms of HCV fusion are still very poorly understood and limited by the lack of understanding of the precise structure of E1E2. The fact that the HCV fusion process includes priming steps dependent on receptor interaction, viral particle rearrangement, and a low-pH trigger highlights that this process requires several host factors and is a very complex process. As mentioned above, apoC1 associated with the viral particle has been shown to modulate membrane fusion (Dreux et al. 2007). It is clear that HCV E2 is not a class II fusion protein and it is currently unknown where the fusion peptide is and if it resides in E1, E2, or is formed from the interaction of the two proteins together, although analysis of both proteins have identified a few candidates (Lavillette et al. 2007; Drummer, Boo, and Poumbourios 2007). It is likely that E1 and/or E2 represent a new class of fusion protein. Of note, it was discovered that a peptide from the E2 Stem Domain that contains a membrane-proximal heptad repeat sequence was capable of potently and specifically inhibiting fusion, indicating that this may be the region responsible for the fusion peptide (Chi et al. 2016). The pestivirus BVDV has similarities to HCV as it harbours two envelope glycoproteins E1 and E2 which are of similar size as HCV E1 and E2. As well, it was also thought to belong to a novel class of fusion proteins. Upon crystallization of BVDV E2, it was determined it has an extended structure lacking a fusion loop and was poorly responsive to low pH, making it unlikely to be a fusion protein (El Omari et al. 2013; Li et al. 2013). It is currently proposed that BVDV E2 binds entry receptors and upon low pH, undergoes conformational changes that allow the putative fusion protein E1 to initiate fusion. It is possible but currently unknown if this model could be accurate for HCV E1 and E2. However, as HCV E2 is a globular protein dissimilar to BVDV E2 and E2 core does not appear

to be responsive to low pH, HCV may have an entry and fusion process quite different from BVDV (Khan et al. 2014).

1.6 Difficulties of an HCV vaccine

The largest hurdle for HCV vaccine development is the aforementioned extremely high diversity which even exceeds that of HIV (Torres-Cornejo and Lauer 2017). Many factors contribute to the diversity of HCV: an error-prone RNA dependent RNA polymerase that lacks proof-reading activity, resulting in a mutation rate of 2.5×10^{-5} mutations per nucleotide per genome replication (Ribeiro et al. 2012); a high replication rate leading to the production of an estimated 10^{12} new HCV virions in the infected liver every day (Neumann et al. 1998), the long-lived nature of infected cells and the low turnover rate of replication complexes (Ribeiro et al. 2012), and the frequent transmission of the virus via blood transfusion, nosocomial transmission and injection drug use during the 20th century. As a result of this high diversity, HCV exists as a quasispecies (a group of viruses related by similar mutations competing within an environment undergoing selection pressures) within an infected individual. This leads to issues concerning vaccine-elicited immune responses (both humoral and cell-mediated) as the large number of closely related viruses mutated at different positions affords HCV the ability to evade the antiviral response. A response against HCV needs to be capable of clearing the majority of virions targeting highly conserved critical epitopes which could lead to the presence of only resistant strains that are less well-adapted and deficient in propagation.

In addition to these hurdles, HCV has several other mechanisms to evade vaccine-elicited antibodies (**Figure 1.12A-F**). These include the HVR1 in the N-terminus of E2 that may act as an immunological decoy to mask a conserved hydrophobic region that is necessary for entry (detailed further below), N-linked glycans associated with E1 and E2 that are capable of masking the proteins from nAbs (detailed further below), lipids associated with the HCV particle that are capable of masking E1E2 from nAbs, cell-to-cell transmission, and the presence of non-neutralizing antibodies that may be able to interfere with the binding of neutralizing antibodies (Cashman, Marsden, and Dustin 2014).

1.7 HCV Immunity

1.7.1 Re-infection

Chimpanzees and humans that spontaneously cleared a primary infection were more likely to clear it upon the second infection, with a lower peak viremia, and a shorter infection course – these data supported the viability of an effective prophylactic vaccine (Lanford et al. 2004; Bassett et al. 2001; Prince et al. 2005; Page et al. 2009). In one study, of the 25% human patients that cleared the infection the first time, 83% cleared it upon their second time (Osburn et al. 2014). However, while some studies showed that this prevention of infection extended to cross-genotype protection (Lanford et al. 2004; Osburn et al. 2014; Page et al. 2009; Bassett et al. 2001), others showed cross-genotype protection could be very limited (Prince et al. 2005). A recent study that used a much larger sample size (n=452) than the aforementioned studies showed that patients that spontaneously cleared their primary infection were 49% less likely to clear a secondary infection if it was a heterologous strain (Islam et al. 2017).

Discouraging evidence came from the fact that chimpanzees and humans can still be reinfected. However, reinfection in the patients that cleared the second infection was associated both with broadened cellular immune responses and the generation of cross-reactive antibodies. While 60% of the individuals who cleared the second infection possessed cross-reactive antibodies, none of the individuals who did not clear the second infection did (Osburn et al. 2010). In an older study, it was found that HBV-positive HCV-negative patients undergoing OLT and being treated by a preparation of polyclonal immunoglobulins against HBV sAg – a preparation thought to contain anti-HCV antibodies – were less likely to be infected with HCV (Feray 1998).

1.7.2 Cell-mediated response to HCV

Strong and early HCV-specific CD4+ and CD8+ T cell and humoral responses are associated with spontaneous clearance of acute HCV infection (Osburn et al. 2010; Dowd et al. 2009; Osburn et al. 2014; Gerlach et al. 1999). Spontaneous clearance is also associated with seroconversion leading to potently neutralizing anti-HCV responses (Cashman, Marsden, and Dustin 2014). As well, the CD4+ and CD8+ T cell responses are persistent and do not diminish

until the viral load is under control. In cases of chronicity, the antibodies tend to be poorly neutralizing until after the infection progresses to chronicity and by that time the antibodies lag behind the mutation rate of the virus and the CD4+ and CD8+ T cell responses diminish prior to control of the infection which leads to a rebound in viremia **(Figure 1.13A,B)**.

A blunted CD4+ T-cell response associated with chronic HCV was shown to be due to both anergy (loss of both IFN-γ secretion and proliferation) and exhaustion (loss of T-cell proliferation followed by a rapid decline in IFN-γ producing cells) (Ulsenheimer et al. 2003). Studies using antibody-mediated depletion in chimpanzees challenged with HCV show that the lack of either the CD4+ and CD8+ memory T cell lymphocyte populations resulted in chronic infection even when the other immune populations were intact (Shoukry et al. 2003; Grakoui et al. 2003). Indeed, spontaneously resolved infections in humans are associated with early, strong, and persistent CD4+ T cells responsive to a broad range of HCV antigens (Wiesch et al. 2005; Day et al. 2002; Pape et al. 1999; Diepolder et al. 1995). Interestingly, a new study characterizing the CD4+ T-cell responses of 31 patients with acute HCV responses found that early and broad CD4+ T-cell responses were raised in all 31 patients regardless of clinical outcome, albeit those progressing to chronic infection had CD4+ T-cells that were non-proliferating in the standard LPA. However, these broad CD4+ T-cell responses undergo rapid exhaustion and deletion in the majority of patients (Wiesch et al. 2012). A similar result was also seen when comparing the CD8+ T cell response between patients that either spontaneously cleared the infection or progressed to chronic infection (Lechner et al. 2000; Cox et al. 2005; Urbani et al. 2006; Cooper et al. 1999). HCV escapes the CD8+ T-cell immune response through viral escape as well as promoting T cell exhaustion and this knowledge is being used to generate better options for treatments. In one case, chimpanzees chronically infected with HCV were treated with an antibody inhibitor against the inhibitory receptor programmed cell death 1 (PD-1), a cell surface receptor that is a major contributor towards CD8+ T cell exhaustion in cases of HCV persistence. The chimpanzee with a history of a broad T-cell response to multiple HCV proteins exhibited lowered viremia while the other chimpanzees with narrower T-cell responses showed no difference in viremia. This evidence indicates that a T-cell based therapeutic vaccine to broaden the anti-HCV cell-mediated response along with immunotherapy to block mechanisms of T-cell exhaustion may be an effective method to reduce viremia in chronically infected patients (Fuller et al. 2013). Despite a great deal of study, there is still much unknown

on what functional and phenotypic properties of CD4+ and CD8+ T cell responses correlate to clearance and that will be an important goal towards vaccine development.

1.7.3 Humoral response to HCV

Early induction of cross-reactive neutralizing antibodies is strongly correlated with the spontaneous clearance of HCV (Dowd et al. 2009; Osburn et al. 2010, 2014; Pestka et al. 2007). In cases that lead to chronicity, strong and broad cross-neutralizing antibodies may develop, but this is a slow development, and by the time they develop it appears that the antibodies can no longer control viremia, probably because the diversity of the virus has outgrown the neutralizing response (Wang, Keck, and Foung 2011; Logvinoff et al. 2004; Pestka et al. 2007; Osburn et al. 2010, 2014). Specifically in one study, the majority of chronically infected patients exhibited cross-neutralizing activity against viruses carrying the envelope proteins from genotypes 1a, 1b, and 2a – however these responses arose late in the chronic phase of infection (Logvinoff et al. 2004). When examined closer with one patient's antisera (Patient H), it was found autologous nAbs developed within 7 weeks, while heterologous nAbs only developed after 33 weeks. An increase in the neutralization activity and a detection of an anti-E1E2 IgG ELISA titer also occurred at 33 weeks. Other studies have shown a similar delay in anti-E1E2 ELISA titers during the acute phase of infection (Chen et al. 1999).

1.8 Requirement for an HCV vaccine

While emerging DAAs are being hailed as "miracle drugs", many problems persist in the control of HCV disease. Exorbitant costs are showing to be prohibitive even in countries with high amount of resources and therefore the issues will be an even greater concern in countries with less access to medical resources (Callaway 2014). Indeed, over 80% of the total people infected with HCV live in low- or middle-income countries (Hanafiah, K et al. 2013). While much can and is being done by limiting transmission networks amongst PWIDs, including needle and syringe exchanges, counselling, education, and opiate substitution therapy, the screen and treat approach is unlikely to lead to full control of the disease and a prophylactic vaccine for HCV remains a crucial requirement (Liang 2013; Thomas 2013; Martin, Hickman, et al. 2013).

The vaccine approach avoids the more complicated logistics of wide-spread screening, gaining second contact with infected individuals, and ensuring adherence to antiviral regimens. And while companies supplying DAAs are severely cutting prices for the low-income countries, poor accessibility to blood screening means HCV infection is more likely to be diagnosed at a later stage where DAAs will not be able to reverse established liver disease, especially HCC. Importantly, reinfection is possible after curing using DAAs in both chimpanzees and humans (Callendret et al. 2014; Sarrazin et al. 2017; Midgard et al. 2016). Indeed, a vaccine approach was primarily responsible for the global eradication of the only two infectious diseases to be fully eradicated, smallpox and rinderpest.

Sterilizing immunity – preventing the acute and chronic phases of HCV infection – would be ideal. However, since the acute phase of HCV is typically asymptomatic and has very low morbidity and mortality, it is not necessary for an effective HCV prophylactic vaccine (Lauer and Walker 2001). Instead, the goal should be to prevent chronic infection (Houghton 2011) (**Figure 1.14A**,**B**). There have been studies clearly proving the importance of virus-specific cellular mediated immunity in control over infection and furthermore, chimpanzees were shown to eradicate infection despite poor anti-HCV antibody responses (Shoukry et al. 2003; Grakoui et al. 2003; Cooper et al. 1999; Logvinoff et al. 2004). Indeed, some researchers in the field believe that a vaccine focused on eliciting a strong primarily cellular mediated immune response is more likely to be effective than a vaccine focused on eliciting a primarily humoral immune response due to the fact that despite a high diversity in the nonstructural proteins of HCV, there is less variability than in the envelope glycoproteins (Liang 2013).

Several research groups have been interested in a prophylactic vaccine consisting of the HCV envelope glycoproteins. Most of these projects have been interested in generating a soluble E2 (sE2) vaccine due to the higher immunogenicity of E2, better understanding of E2 in the process of viral entry, and easier generation and purification – E2 is secreted extracellularly and results in fewer aggregates than recombinant E1E2 (rE1E2). However, as E1 is wholly necessary for viral entry, it likely plays a critical role in entry that could be blocked by antibodies – indeed, there are a few anti-E1 neutralizing antibodies and a soluble E1 vaccine was able to protect chimpanzees against HCV challenge while a HVR-1 deleted soluble E2 vaccine could not (Verstrepen et al. 2011). As well, E1 has been shown to modulate E2 interactions with SRB1 and CD81 and an E1E2 vaccine is more likely to resemble the surface of the HCV virion (Douam et

al. 2014; Russell et al. 2009). One of the major goals of the Houghton Lab is to generate an effective prophylactic rE1E2 vaccine. This kind of vaccine would hopefully elicit a strong broadly-neutralizing response early in infection – a response that has been shown to correlate with spontaneous clearance (Osburn et al. 2014; Pestka et al. 2007). Early research in this field concluded that anti-E1E2 antibody response would largely be strain-specific and there would be little cross-genotype protection. A prototypical rE1E2 vaccine derived from the gt1a strain HCV-1 has shown to be effective at protecting chimpanzees from chronic infection following both homologous and heterologous genotype 1a challenge, indicating that despite the many mechanisms HCV possesses to evade the humoral immune response, a vaccine directed towards generating a humoral response could still be very promising – although genotypes can be highly varied, even a regional HCV prophylactic vaccine could be successful at significantly lowering the disease burden (Houghton 2011). Promisingly, the antisera from the chimpanzees showed cross-neutralizing activity against HCVcc exhibiting the envelope glycoproteins from diverse strains, indicating even a vaccine representing a single strain may afford very broad protection (Meunier et al. 2011). Similar results were also seen in guinea pigs and mice (Stamataki et al. 2007). These promising data led to a Phase I clinical trial for safety and immunogenicity performed in healthy, HCV-negative human volunteers. These volunteers exhibited strong anti-E1E2 ELISA titers, CD4+ T cell responses, and capacity for inhibiting the interaction between sE2 and CD81 (Frey et al. 2010).

Soluble E2 vaccines still hold potential to be effective HCV vaccine immunogens. Simultaneous deletion of the HVR1, HVR2, and IgVR from the E2 reveals an E2 core protein that has accelerated binding to CD81 relative to wild-type E2 protein (McCaffrey et al. 2016). This E2 core protein is present in monomer as well as high-molecular-weight form. The high-molecular-weight form appears to be promising, eliciting stronger and broader immunogenicity and neutralizing responses in vaccinated mice relative to either the monomer form of the E2 core protein or wild-type E2 protein (Vietheer et al. 2017). Another approach is taken by generating E2 in *Drosophila* S2 insect cells and generating a trivalent vaccine constituting E2 from three different strains from genotypes 1a, 1b, and 3a. The immunogenicity and neutralizing responses in vaccinated mice were stronger and broader across the different clades of HCV than the monovalent vaccine comprising the E2 from a genotype 1a strain.

One promising T-cell vaccine is constructed of two adenoviral vectors based on two rare adenoviral serotypes (Ad6 and ChAd3) expressing the nonstructural proteins NS3-NS5B from a genotype 1b strain which elicited strong and durable CD4+ and CD8+ T cell responses in a Phase I clinical trial in humans (Barnes et al. 2012). This vaccine did not show greater spontaneous clearance than controls: 4/5 vaccinated chimpanzees cleared the virus and 3/5 unvaccinated chimpanzees cleared the virus in the same time-frame. However, average peak viremia was over 100-fold lower in the vaccinated chimpanzees relative to the unvaccinated chimpanzees (Folgori et al. 2006). A similar vaccine using the same NS3-NS5B from the genotype 1b strain but using the ChAd3 and MVA vectors is being pursued in a Phase II clinical trial with high-risk HCV-negative young PWIDs. This same vaccine was also tested in genotype 1 chronically infected patients to determine if this vaccine could be used as a therapeutic vaccine. However it did not seem to be able to overturn T-cell exhaustion and did not lower viral load (Kelly et al. 2016).

One particularly innovative attempt at an HCV vaccine is to express the E1E2 heterodimer in edible lettuce (*Lactuca sativa*) using *Agrobacterium*-mediated transient expression technology – an approach using plant-based delivery systems to lower costs and simplify implementation (Clarke et al. 2017).

An interesting approach to a therapeutic vaccine to clear chronically infected patients was utilizing self-adjuvanting lipopeptides for dendritic cell immunotherapy. These lipopeptides contained two components: peptides containing strong CD8+ T-cell epitopes derived from HCV sequences and dipalmitoyl-*S*-glyceryl cysteine lipid, which is a ligand for Toll-like receptor 2 on dendritic cells. However, phase I clinical trials showed that this approach failed to reduce viral loads in patient volunteers and did not stably enhance CD8+ T-cell responses.

1.9 Support for neutralizing antibodies for preventing the development of a chronic infection

Historically, most prophylactic viral vaccines – importantly, subunit vaccines – have been designed with the focus on eliciting antibodies that can neutralize viral entry. There has been a great deal of doubt that an antibody response could be effective against highly variable

viruses such as HIV and HCV. And while T-cell responses appear to be critical for clearance, one study of a rare cohort of 7 people infected from a single-source outbreak and also had primary antibody failure showed that an antibody response was not necessary for control of the infection (Semmo et al. 2006). While T-cell mediated immunity is very important and the target of many new vaccine candidates, there is evidence that neutralizing antibodies targeting the envelope glycoproteins (E1 and E2) on the surface of the virion play a role in spontaneous clearance of infection by preventing cell entry. A summary of the possible methods the virus could enter and how it could be blocked by neutralizing antibodies is indicated in **Figure 1.15**.

Reports have shown that in two instances of single source outbreaks, while all patients at some point developed a robust neutralizing antibody response to the homologous HCVpp strain, an early robust neutralizing antibody response was tightly associated with clearance of the infection, while a late neutralizing antibody response was seen in patients who proceeded to develop a chronic infection (Pestka et al. 2007; Lavillette, Morice, et al. 2005). A more recent study has shown that an early broadly neutralizing antibody response against a panel of genotype 1 viruses *in vitro* was predictive of clearance in a cohort of prospectively monitored young injection drug users. Importantly, it was shown that the strongest predictor of clearance was the breadth of the neutralizing response – how many different strains that could be neutralized by more than 50% (Osburn et al. 2014). Given that HCV exists as a diverse quasispecies even early into infection, this makes intuitive sense and provides an immune correlate important to vaccine development. In addition, in a study on a unique patient who spontaneously cleared infection late in the chronic phase, it was found clearance was associated with the appearance of neutralizing antibodies and reversal of HCV-specific T-cell exhaustion. Immune correlates for protection are very important in vaccine development and especially for HCV, a pathogen with very limited preclinical models.

Monoclonal antibodies have also been shown to prevent HCV infection upon challenge in the humanized chimeric mouse model. (Desombere et al. 2016; Giang et al. 2012; Law et al. 2008). Although these challenges usually involved mAb pretreatment for three days prior to challenge, this indicates potential for a humoral immune response to control infection.

There has been much work done in studying cross-neutralizing patient sera and antisera from animals immunized with E1E2, with a particular focus on cross-neutralizing monoclonal antibodies (mAbs) isolated from the patients and animals (reviewed in (Sautto et al. 2013)). This

has resulted in an extensive collection of mAbs with well-characterized epitopes and neutralizing activity. Both patient sera and mAbs have been shown to prevent chronic infection *in vivo* using the chimeric human liver SCID/uPa mouse model and chimpanzees (Law et al. 2008; Giang et al. 2012; Morin et al. 2012; Meuleman et al. 2011). Taken together, while cell-mediated immunity is protective, a prompt cross-neutralizing antibody response likely contributes to protection as well. Given the vast diversity of the virus, much of which is located within E1E2, an optimal global prophylactic vaccine may need to elicit antibodies capable of neutralizing the entry of highly variable strains.

A vaccine based on the recombinant envelope glycoproteins (rE1E2) from a single 1a strain (HCV-1) protected chimpanzees from a chronic infection following homologous and heterologous genotype 1a viral challenge (reviewed in (Houghton 2011)). Antisera from the immunized chimpanzees were shown to exhibit broad *in vitro* cross-neutralizing activity against all of the world's major global genotypes (Meunier et al. 2011). A phase I clinical trial was conducted in human volunteers with a similar antigen (Frey et al. 2010). This trial showed the prototypical recombinant vaccine was capable of eliciting strong anti-E1E2 ELISA titers and CD4+ T-cell responses, and in addition, the antisera from the individuals were capable of blocking the binding of soluble E2 to the large extracellular loop of CD81, indicating the possibility these antisera could block HCV entry.

The evidence indicates that an effective prophylactic vaccine should elicit strong and broad responses from cell-mediated immunity and humoral immunity. In addition, a therapeutic vaccine should also possess these properties as well as reversal of T-cell exhaustion.

1.10 HCV neutralizing antibodies

All antibodies bind to a 3D structure produced by the protein they are binding. However, some antibodies can have an epitope that is sufficiently represented by a short peptide length ("linear epitope"). The remainder have a discontinuous epitope that cannot be sufficiently represented by a single peptide length ("conformational or discontinuous epitope"). The vast majority of characterized nAbs that target E1E2 have conformational epitopes. However, there are still nAbs that bind to linear epitopes and there are at least 4 of these linear epitopes: E1 Epitope I (E1 peptide aa313-327); E2 HVR1 (aa396-407); E2 Epitope I (aa412-423); and E2

Epitope II (aa434-446) (Meunier, Russell, Goossens, et al. 2008; Hsu et al. 2003; Owsianka et al. 2005; Keck et al. 2013, 2012). As covered above, viral entry includes binding, post-binding, internalization, fusion, and uncoating of the capsid steps. Neutralizing antibodies are capable of preventing the entry of viruses through inhibiting any of these steps potentially – whether it is by interfering directly with the region responsible with the particular step, inhibiting conformational changes that are necessary for the particular step, or otherwise preventing the interaction between the viral and cellular components (Aoki et al. 2009; Ku et al. 2015; Rossey et al. 2017; Sabo et al. 2011; Law et al. 2008; Nybakken et al. 2005). A summary of the antibodies that bind to E1E2 is depicted in **Figure 1.16**. This is not an exhaustive list of all the anti-HCV antibodies but does cover the majority and covers all the major neutralizing epitopes.

1.10.1 Anti-E1 Antibodies

Few antibodies that target E1 protein only have been characterized. A4 is a non-neutralizing antibody known to require aa197-207 in the E1 protein for binding, although whether it can bind this peptide directly has not been determined (Dubuisson et al. 1994). H-111 is an antibody capable of binding a peptide at the N-terminus of E1 (aa192-202) similar to A4 – however it is capable of inhibiting the binding of HCV-LPs to MOLT-4 cells and prevents the infection of HCV virions into Raji cells (Keck, Sung, et al. 2004). These are two older systems of detecting the inhibition of binding and infectivity and the newer generation of assays have not been used to further characterize H-111.

IGH520 and IGH526 are the only anti-E1 antibodies that have been found to have cross-neutralizing activity (Meunier, Russell, Goossens, et al. 2008). Both antibodies bind to the conserved region of aa313-327 in the E1 protein – this region is known as E1 Epitope I. These antibodies have been shown to be effective at the post-binding stage but not at the time of binding (Haberstroh et al. 2008).

1.10.2 Anti-E2 Antibodies

Many monoclonal antibodies with broad cross-neutralizing activity have been isolated and characterized (reviewed in (Sautto et al. 2013)). As stated above, the HVR1 region is one of

great interest as it appears to have a multifactorial influence. Despite its possible role as an immunological decoy, antibodies directed against HVR1 can be neutralizing – however, this seems to be strain-specific (Farci et al. 1996; Shimizu et al. 1996; Zibert, Schreier, and Roggendorf 1995; Kato et al. 1994). The nAbs 9/27 and H77.16 bind the C-terminal end of HVR1 and are capable of blocking the E2-SRB1 interaction to neutralize entry (Hsu et al. 2003; Sabo et al. 2011). The majority of anti-E2 antibodies recognize overlapping regions on the E2 protein: E2 amino acids (aa) 412-424, 433-447, 523-540, 613-616 contain residues critical for binding to CD81 (Keck et al. 2012; Owsianka et al. 2006) (**Figure 1.10**). The CD81 receptor binding site (CD81bs) on E2 is a conserved conformational structure that interacts with CD81 during entry and many cross-neutralizing antibodies bind various residues in the CD81bs (**Figure 1.10**, **Figure 1.16**). The aa412-424 region contains the E2 Epitope I and antibodies such as AP33, HC33.1, and HC33.4 are capable of blocking the interaction between E2 and CD81 and are broadly neutralizing (Owsianka et al. 2005; Potter et al. 2012; Keck et al. 2013). Interestingly, the nAb H77.39 directed against this region is capable of blocking both SRB1 and CD81 (Sabo et al. 2011). A group of antibodies that recognize aa433-447, which contains E2 Epitope II as well as a couple of residues in the aa613-616 region in the back layer of E2, are potently broadly cross-neutralizing and also block E2-CD81 interaction (Keck et al. 2012). Another group of nAbs are centered around the residues in the CD81 binding loop/β-sandwich aa523-540 but also can interact with other residues found in the other CD81 binding regions (Law et al. 2008).

1.10.3 Anti-E1E2 Antibodies

There are only two antibodies that have been found to target full-length E1E2 and both are nAbs. AR4A and AR5A target epitopes outside the CD81bs and do not inhibit interaction with CD81 (Giang et al. 2012). These mAbs require the native E1E2 heterodimer for binding and do not bind E1 alone, E2 alone, or denatured E1E2. These mAbs were isolated through an exhaustive-panning strategy of an antibody antigen-binding fragment (Fab) phage-display library generated from a patient chronically infected with HCV using the E1E2 heterodimer protein pre-blocked with previously isolated anti-E2 mAbs to obtain unique and rare mAbs. They are broadly cross-neutralizing but the exact binding modes are currently unknown. Alanine-scanning

mutagenesis has shown these antibodies share many critical binding residues at the N-terminus of E1 and the C-terminus of E2 which was surprising as AR4A and AR5A can bind E1E2 simultaneously without cross-competition. It is likely these shared residues are necessary for proper E1E2 folding that allows the epitopes of AR4A and AR5A to be presented for proper binding. Only D698 and D639 of E2 were uniquely critical for the binding of AR4A and AR5A, respectively. Interestingly, D698 is within the highly conserved membrane proximal external region (MPER) containing a heptad repeat sequence (aa675-699) (Drummer and Poumbourios 2004). A heptad repeat is a structural motif following a seven amino acid pattern of HPPHCPC, where H represents hydrophobic residues, P represents polar residues, and C represents charged residues (Chambers, Pringle, and Easton 1990). This region has been shown to be important for E1E2 heterodimerization and membrane fusion (Rychłowska et al. 2011; Chi et al. 2016; Drummer and Poumbourios 2004). A region consisting of aa687-703 has also been predicted to form an amphipathic α-helix partially embedded in lipid membrane (Albecka et al. 2011). It is possible mAbs binding this region could be preventing entry similar to how mAbs binding the HIV-1 MPER were shown to prevent entry by restricting movement of the helices required for viral entry (Song et al. 2009). Therefore, it is possible AR4A inhibits heterodimerization or fusion or both by binding to this region. There is still much to be discovered about how the epitopes of these mAbs are formed by E1E2 heterodimerization.

1.10.4 Infected patient anti-E1E2 antisera

Limited studies have been performed on sera from chronically infected patients and the mechanisms of neutralization. One study found that antibodies present in the sera of the patients were generally ineffective in blocking the binding step and the activity appeared to be occurring at a post-binding step coinciding similar to the SRB1 and CD81 interaction steps. As well, the sera showed an ability to block the mixing of HCVpp with liposomes in an *in vitro* fusion assay, indicating that antibodies present in the sera may be capable of directly preventing fusion (Haberstroh et al. 2008).

1.10.5 Role of HVR1 in humoral immunity

As stated above, HVR1 plays an important role in entry and appears to mediate interaction between E2 and SRB1 and is necessary for the "enhancement" function of SRB1. Fitting with this model, while HVR1 is not wholly necessary for entry, the deletion of HVR1 or preincubation with anti-HVR1 antibodies can dramatically reduce cell entry of HCVcc (Bankwitz et al. 2010; Scarselli et al. 2002; Bartosch, Vitelli, et al. 2003; Catanese et al. 2007; Thi et al. 2012; Flint, Maidens, et al. 1999; Forns et al. 2000). Pressure by neutralizing antibodies has led to envelope gene evolution, particularly in HVR1 (Liu et al. 2010; Weiner et al. 1992; von Hahn et al. 2007; Farci et al. 2000).

While HVR1 plays a role in HCV entry, there is evidence that it could be shielding or affecting the accessibility of more conserved neutralizing epitopes. This could make it an immunological decoy in two mechanisms, since 1) it is an immunologically dominant epitope that mutates rapidly meaning a significant portion of the immune response is being developed against a moving target and 2) it is inhibiting the action of antibodies directed against conserved neutralizing epitopes. Importantly, experiments with both HCVpp and HCVcc with either the HVR1 intact or deleted showed that a panel of neutralizing monoclonal antibodies were more effective against HVR1-deleted virus than wild type, supporting the idea of HVR1 as an immunological shield or affecting conserved epitopes directly or indirectly (Bankwitz et al. 2010; Prentoe et al. 2016). Interestingly, this not only included the mAbs targeting the disparate regions making up the CD81 binding site on E2, but also mAbs targeting epitopes found in E1, E2, and E1E2 not associated with the E2-CD81 interaction. The heightened sensitivity to neutralization of HVR1-deleted HCVcc mutants was also found when testing with chronically infected patient sera (Prentoe et al. 2011). Furthermore, when testing representative strains of HCVcc derived from six major genotypes, it was found that the strains lacking HVR1 showed not only enhanced neutralization across the genotypes, but also normalized the neutralizing activity between the strains relative to the wildtype strains, indicating that different sensitivities of neutralization of different strains could largely be a result of HVR1 (Prentoe et al. 2016). Fitting with the hypothesis that HVR1 exists as an immunological shield of neutralizing epitopes, deletion of HVR1 increases the ability of sE2 to bind to CD81 (Scarselli et al. 2002; Roccasecca et al. 2003). Of note, a neutralizing mAb targeting residues within HVR1 (aa406, 408, and 410) was capable of blocking binding by a panel of broadly cross-neutralizing mAbs targeting the

aa412-423 region to E2 and preventing neutralization (Keck et al. 2016; Sabo et al. 2011). Therefore, it's very possible that a deletion of HVR1 could provide a better vaccine antigen.

1.11 Role of N-glycosylation in humoral immunity

HCV E1E2 contained a total of 16 N-linked glycosylation sites – 5 in E1 and 11 in E2 – and most of them are conserved. Intracellular ER-resident E1E2 has mostly high-mannose type glycans but HCVcc E2 contains high-mannose type and complex glycans, indicating HCVcc E1E2 complexes are matured through the Golgi apparatus (Lavie, Goffard, and Dubuisson 2007). Site-directed mutagenesis studies have shown that the HCV glycans in E1 and E2 have multifunctional roles in proper E1E2 folding, viral particle secretion, entry, and shielding of neutralizing epitopes (Helle et al. 2010, 2007; Chen et al. 2014; Helle, Duverlie, and Dubuisson 2011; Goffard et al. 2005; Falkowska et al. 2007). In addition, some HCV glycans in E2 protect against antibody neutralization. The HCV glycan sites at E2N1, E2N2, E2N4, E2N6, and E2N11 reduce the sensitivity of HCVcc to neutralization, indicating these glycans could protect the binding of neutralizing antibodies binding the E2 protein. This indicated a possibility that the glycans could be deleted to produce a more optimal vaccine that could elicit a better response to those neutralizing epitopes. Indeed, E1N3, E2N1, and E2N6 seem to be good candidates as deletion by substitution does not seem to be deleterious to the normal folding of E1E2 and E2N1 and E2N6 are located within areas critical for E2-CD81 interaction – these areas contain epitopes targeted by broadly cross-neutralizing antibodies (Helle et al. 2010, 2007; Goffard et al. 2005).

Interestingly, mutation at the E2N1 glycosylation site from an asparagine to either serine or threonine at residue 417 in the E2 protein (N417S/T) can result in a glycan shift which changes the glycosylation site from N417 to N415. This was shown to disrupt the neutralizing activity of two neutralizing antibodies binding in this region (they bind to E2 Epitope I) but not by two other neutralizing antibodies that bind to the same region (Pantua et al. 2013). Structural studies co-crystallizing one of the two antibodies, still able to neutralize the virus after the glycan shift, along with the peptide 412-423 showed that antibody was capable of binding because it was able to disrupt the peptide's β-hairpin with the antibody's very long complementarity-determining region 3. In contrast, the other antibody that could not neutralize the glycan shift mutant was blocked by the glycan on N415 (Li et al. 2015).

1.12 Role of interfering non-neutralizing antibodies in humoral immunity

There are non-neutralizing antibodies capable of interfering with the neutralization of immune globulin preparations. It was determined from eluting the fractions of immune globulin preparations from chronically infected patients reactive to either the region aa412-aa426 (E2 Epitope I) or aa434-446 (E2 Epitope II) that the E2 Epitope II-reactive fraction could modestly block the neutralizing activity of the fraction directed against E2 Epitope I. Removing antibodies targeting a synthetic peptide comprising aa434-aa446 (E2 Epitope II) from immune globulin preparations from both chronically infected human patients and chimpanzees vaccinated with the HCV-1 rE1E2 vaccine resulted in the preparation having a somewhat higher neutralizing activity against homologous and heterologous strains of HCVpp and HCVcc (Zhang et al. 2009). Similar results were seen in the Phase I Clinical Trial performed in healthy human volunteers using the HCV-1 rE1E2 vaccine (Kachko et al. 2015).

This was investigated further by preparing and analyzing two peptide-mAb crystal structures (peptide aa412-426): one with a non-neutralizing mAb (mAb #8; interfering activity was not determined), and one with a broadly cross-neutralizing mAb (mAb#27). These data showed these two antibodies bound the peptide from opposing angles explaining this phenomenon and perhaps providing a possibility of an E2 Epitope II peptide that could be altered to preferentially elicit only broadly cross-neutralizing antibodies. Other studies using alanine-scanning mutagenesis have come to similar conclusions (Duan et al. 2012). In addition, a set of neutralizing antibodies to E2 Epitope II did not compete and in fact acted synergistically with E2 Epitope I-directed antibodies (Keck et al. 2013).

However, the exact mechanism of interfering non-neutralizing antibodies directed against E2 Epitope II competing with the neutralizing activity of antibodies directed against E2 Epitope I has not been investigated. Presumably E2 Epitope II-directed antibodies could be binding the E2 protein in a way to compete with the binding of E2 Epitope I-directed antibodies. This competition could be through a direct competition between antibodies binding epitopes overlapping with each other or more likely indirect through steric hindrance.

1.13 Objectives

The objectives of this body of work were to characterize the humoral immune responses elicited by an HCV vaccine comprising recombinant E1E2 heterodimer protein. The first objective was to determine the strength and breadth of neutralization elicited in vaccinated subjects (human and goats). The second objective was to characterize the epitopes in the E1E2 heterodimer that were targeted by antibodies present in the vaccinees' antisera. The third objective was to determine the mechanisms of neutralization of the antibodies present in the antisera. The Houghton lab is currently developing a second generation recombinant E1E2 vaccine formulation to introduce into clinical trials. The fourth objective was to interrogate novel recombinant E1E2 immunogen derivatives. The antigenicity of these immunogens and the epitopes targeted by antisera generated against these immunogens were investigated.

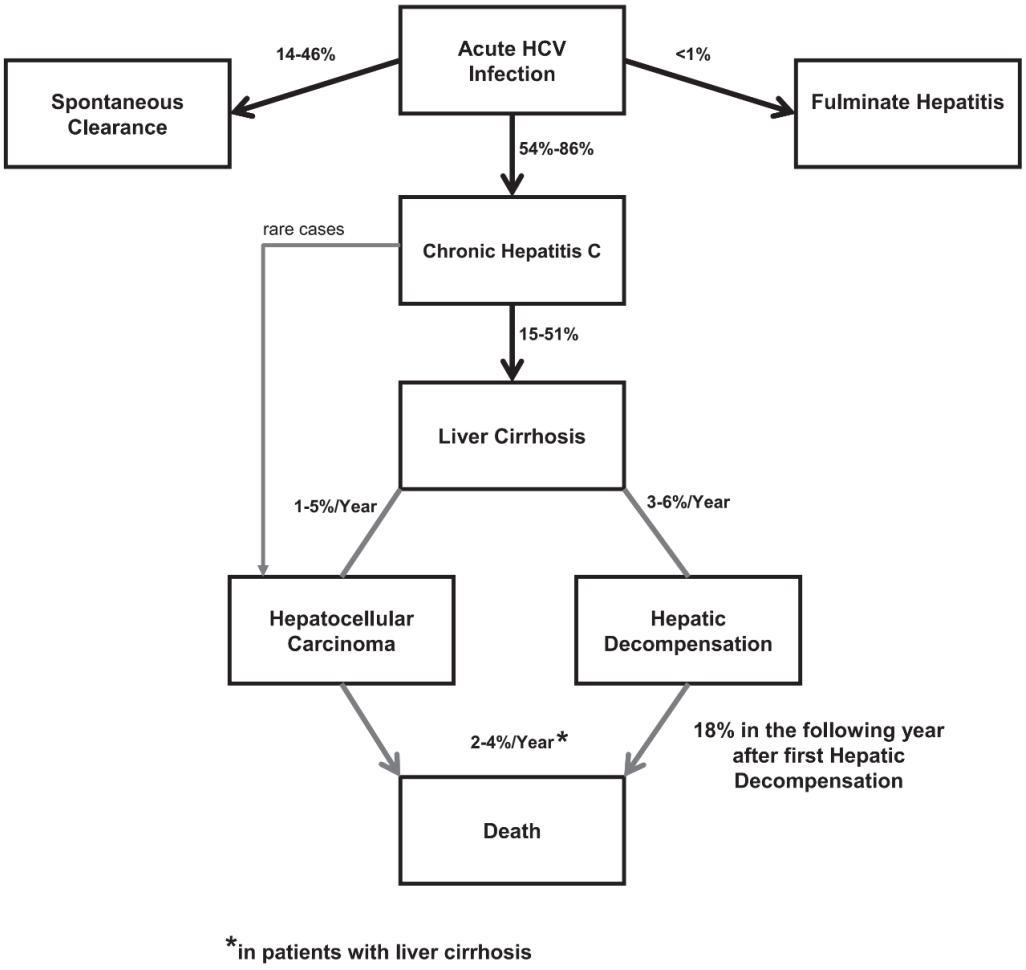


Figure 1.1 Natural History of HCV.
Infection with Hepatitis C Virus leads to serious issues (hepatocellular carcinoma and hepatic decompensation) that can lead to death. Figure taken from (Maasoumy and Wedemeyer 2012).

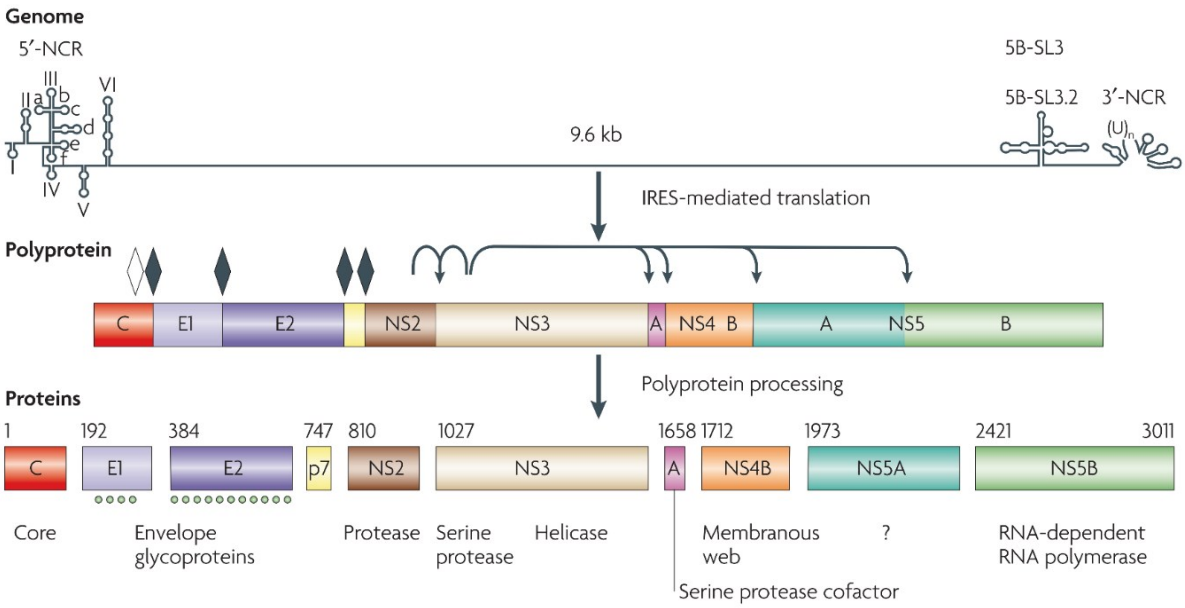


Figure 1.2 Genetic organization of HCV.
The HCV genome is a 9.6-kb positive-sense single stranded RNA with RNA secondary structures in the 5'-NCR, 3'NCR, core gene, and the NS5B-SL3. IRES-mediated translation of the RNA leads to a polyprotein precursor that is processed by both viral and cellular proteases co- and post-translationally into the mature structural and nonstructural proteins. Amino-acid numbering is indicated above each protein. Numbering is based on the genotype 1a H strain (GenBank accession number AF009606). Solid diamonds above the polyprotein indicate cleavage sites by the endoplasmic reticulum signal peptidase and open diamonds indicate further C-terminal processing of the core protein by the signal peptide peptidase. Arrows indicate cleavages by the NS2-3 and NS3-4A viral proteases. Open dots below the E1 and E2 proteins indicate N-linked glycosylation sites. Figure taken from (Moradpour, Penin, and Rice 2007).

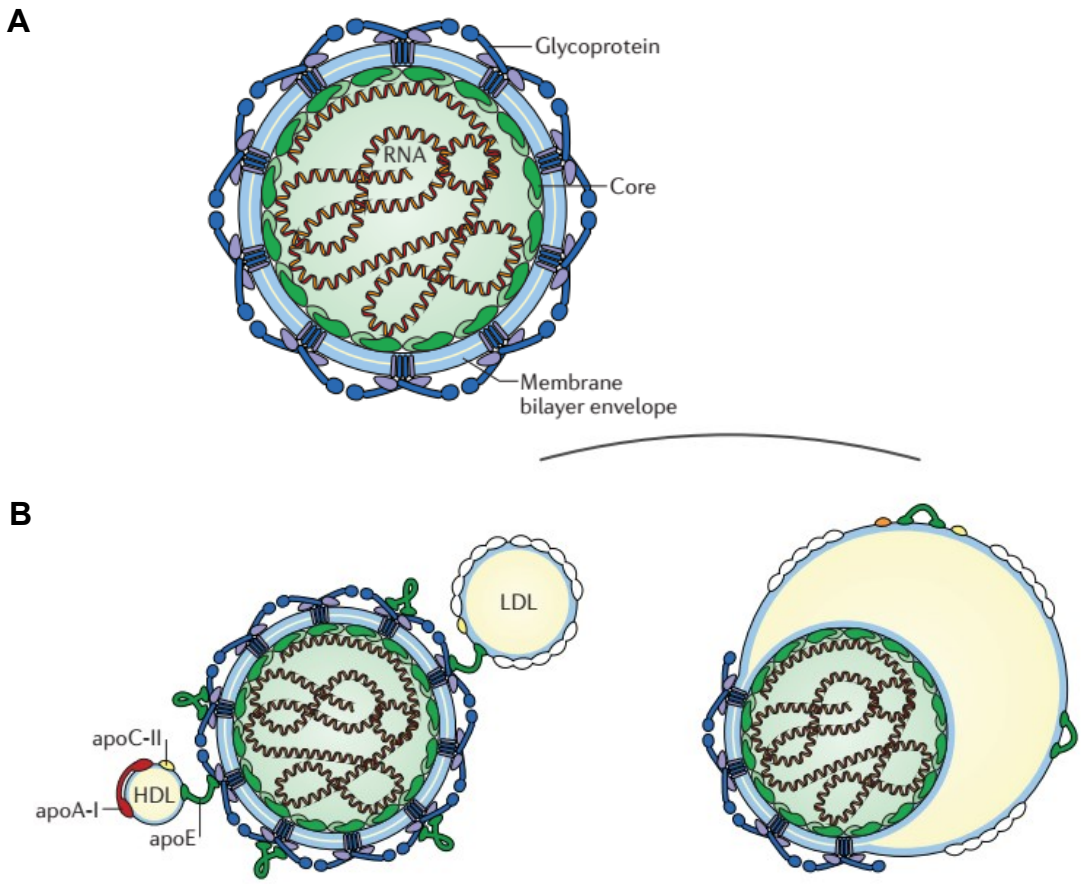


Figure 1.3 Structure of HCV virions.
(A) Depiction of the HCV particle without any associated lipoproteins. Shown is the RNA enclosed by the Core protein making up the capsid, altogether forming the nucleocapsid. The nucleocapsid is surrounded by a membrane bilayer envelope studded with the E1 and E2 glycoproteins. (B) The HCV particle depicted in **Figure 1.3A** is associated with host lipoproteins (namely LDL, apoA, apoB, apoE, and apoC) to form a lipoviral particle (LVP). It is currently not understood exactly what the LVP looks like. On the left is one proposed model that has HCV particles and the serum lipoprotein components transiently interacting. On the right is a model that has the HCV particle integrated and sharing the envelope with an LDL particle. Figure taken from (Lindenbach and Rice 2013).

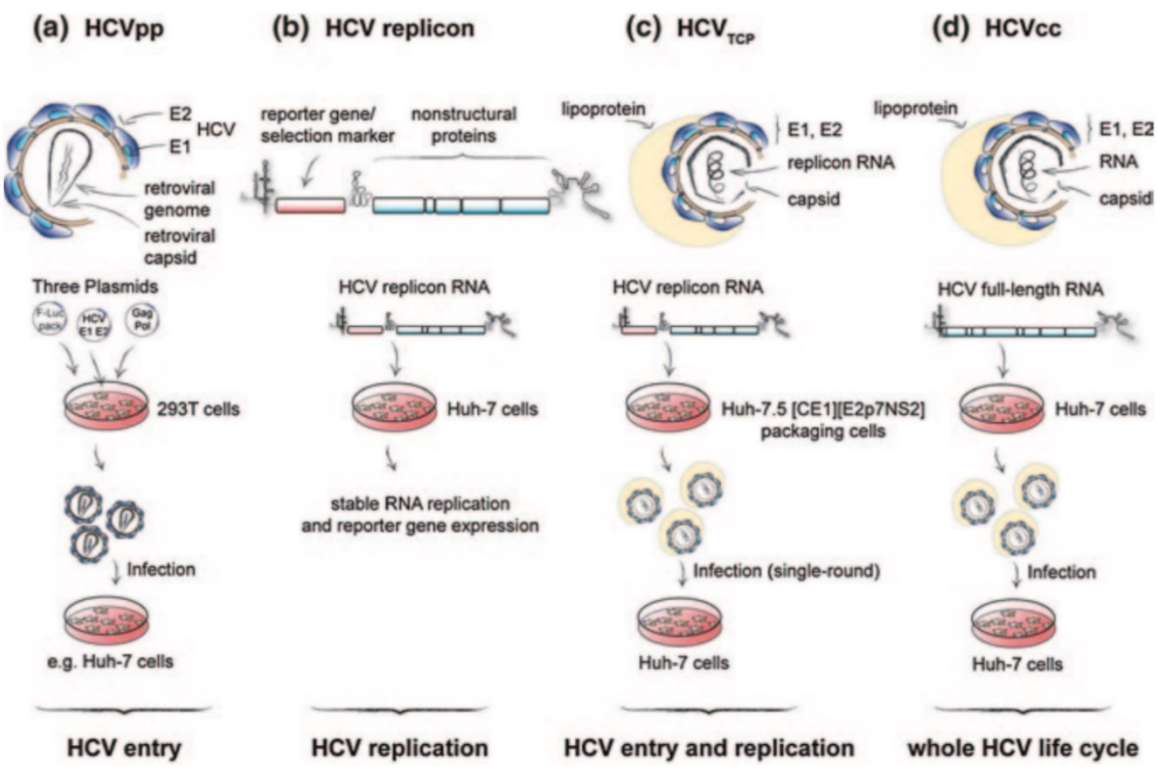

(a) HCVpp
(b) HCV replicon
(c) HCV$_{TCP}$
(d) HCVcc
E2
E1
HCV
retroviral genome
retroviral capsid
reporter gene/ selection marker
nonstructural proteins
lipoprotein
E1, E2
replicon RNA
capsid
RNA
Three Plasmids
F-Luc pack
HCV E1 E2
Gag Pol
293T cells
Infection
e.g. Huh-7 cells
HCV replicon RNA
Huh-7 cells
stable RNA replication and reporter gene expression
Huh-7.5 [CE1][E2p7NS2] packaging cells
Infection (single-round)
HCV full-length RNA
HCV entry
HCV replication
HCV entry and replication
whole HCV life cycle

Figure 1.4 Model systems for studying HCV *in vitro*.
(A) HCVpp can be used to specifically interrogate the entry process. They are generated by transfecting a HEK293T cell with three plasmids encoding (1) retroviral or lentiviral gag/pol, (2) a retroviral/lentiviral vector coding for a reporter gene, usually a luciferase gene, and (3) a retroviral/lentiviral vector encoding HCV E1 and E2. This generates a retroviral or lentiviral particle that encodes a reporter gene displaying the HCV envelope glycoproteins that can be used in detecting the intracellular signal arising from successful infection. These particles enter in a manner very similar to the HCV particle (with differences arising from the lack of lipoprotein association). (B) The HCV replicon can be used to specifically monitor HCV replication. A reporter gene or selection marker is placed upstream of the genome (NS3-NS5B are the minimal components necessary. The second IRES upstream of the genome allows for translation of the genome. The replication of the genome is measured by the expression of the reporter gene. (C) HCV trans-complemented particles (HCV_{TCP}) are used to limit the HCV life cycle to just HCV entry and replication. They are created by transfection of subgenomic replicon RNA encoding only the nonstructural proteins into "packaging cell lines" that stably express the lacking structural proteins (these are provided in *trans*). This allows for the generation of particles that fully resemble HCVcc except that they contain the subgenomic replicon RNA, so they are not capable of producing new virions. (D) HCVcc are capable of the full life cycle of HCV and are based on the nonstructural proteins being from a genotype 2a isolate that is capable of efficiently growing in cell culture (although the structural proteins can be from any strain. They are generated by transfecting the full viral genome into permissive human hepatocytes. Translation and RNA replication produces viral particles that are capable of infecting new hepatocytes. Figure taken from (Steinmann and Pietschmann 2013).

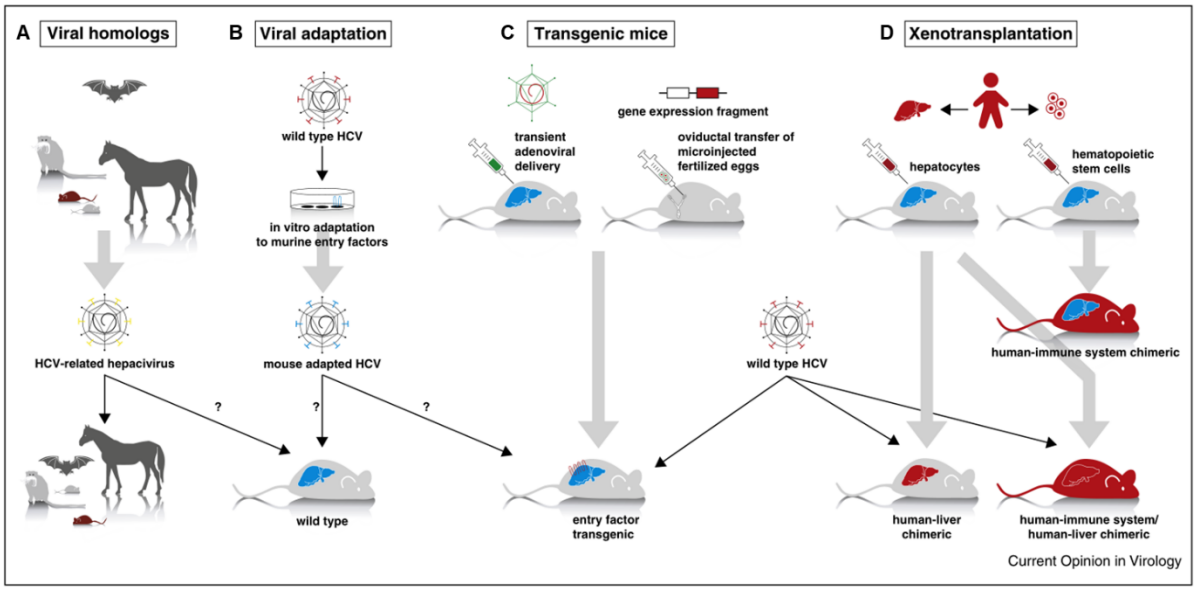


Figure 1.5 Model systems for studying HCV with animal models *in vivo*.
(A) HCV-related viruses that infect non-human species such as wild mice, rats, tamarins, marmosets, bats, and horses. These infections can be studied in the natural hosts and may eventually be studied in immune competent laboratory inbred mice. (B) HCV passaged in mouse hepatocytes has led to a mouse-adapted HCV virus capable of utilizing mouse entry receptors CD81 and OCLN, although it remains to be seen if there will be a mouse-adapted HCV virus that can establish persistent infection in mice. (C) Transgenic mice expressing human entry receptors (CD81 and OCLN) can support infection by non-adapted virus. (D) Chimeric human liver mouse models require repopulation of the mouse liver by human hepatocytes. This requires the mouse to be immunodeficient unless HLA-compatible hematopoietic stem cells are also transplanted resulting in a dually reconstituted mouse. Figure is taken from (Burm et al. 2018).

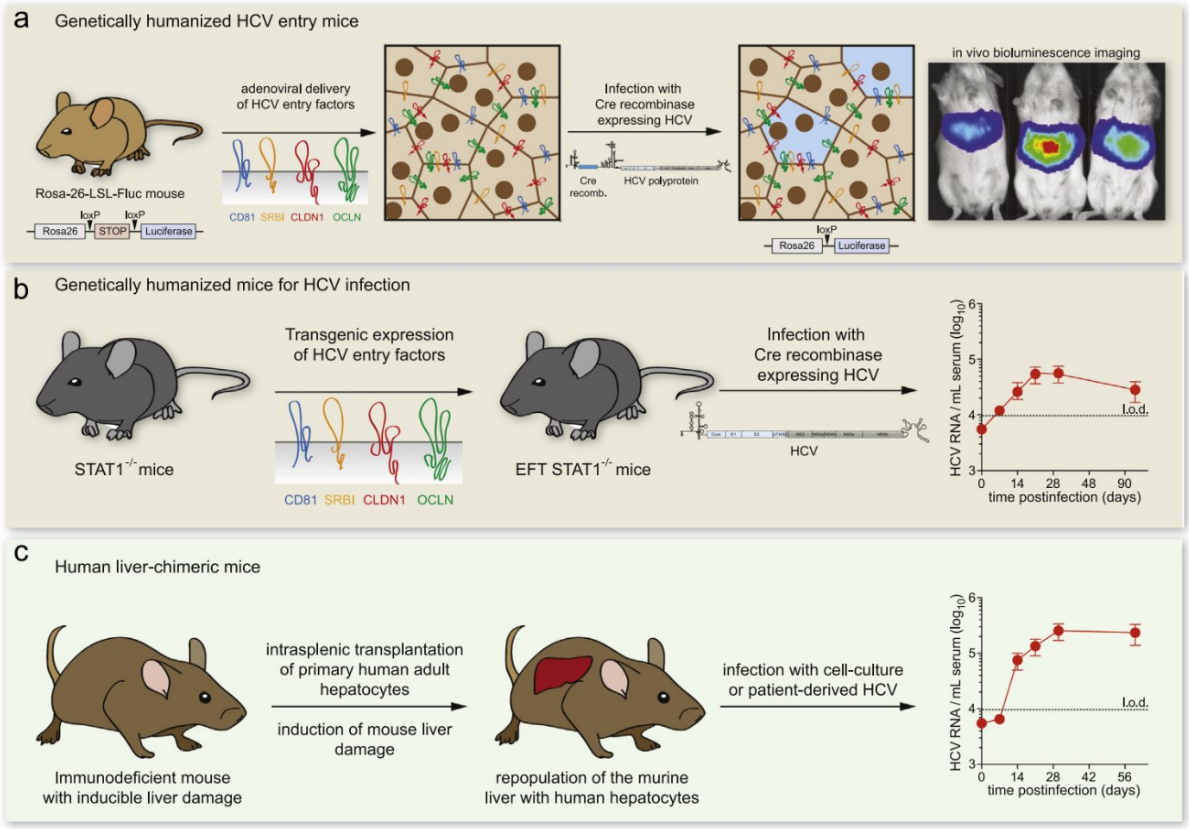


Figure 1.6 Model systems for studying HCV in mouse models *in vivo*.
(A) Rosa26Fluc luciferase reporter mice with intact immune systems are able to support HCV entry by transgenic or adenoviral expression of the human orthologs of HCV receptors (hSRB1, hCD81, hCLDN1, hCD81). An HCV strain containing Cre recombinase in the genome allows for Cre/loxP-based excision of the transcriptional stop cassette, which activates luciferase expression. This can be quantified by bioluminescence imaging *in vivo*. (B) Genetically humanized mice that can support the full HCV life cycle are gained by transgenically expressing the human orthologs of HCV receptors in STAT$^{-/-}$ mice. This allows for persistent HCV infection that can last for up to 90 days. (C) To generate a human liver chimeric mouse, mice with a knocked out immune system and inducible liver damage receive transplantation of primary human adult hepatocytes and mouse liver cell damage is induced to repopulate the murine liver with human hepatocytes which can then be infected by patient-derived HCV. Figure is taken from (Vercauteren, de Jong, and Meuleman 2014).

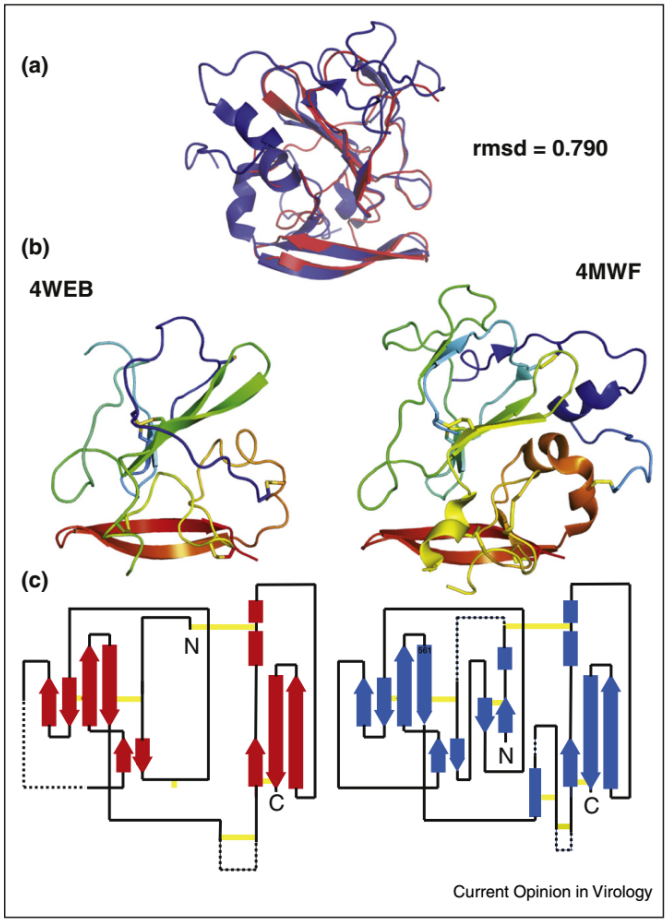


Figure 1.7 E2 core structures.
(A) Superposition of E2 core structures solved in (Kong et al. 2013) (PDB ID: 4WMF) and (Khan et al. 2014). (B) Cartoon diagram of the two individual core structures. (C) Topology diagram of the core domain. Secondary structure and disulfide bonds (yellow) are highlighted. Figure taken from (Khan, Miller, and Marcotrigiano 2015).

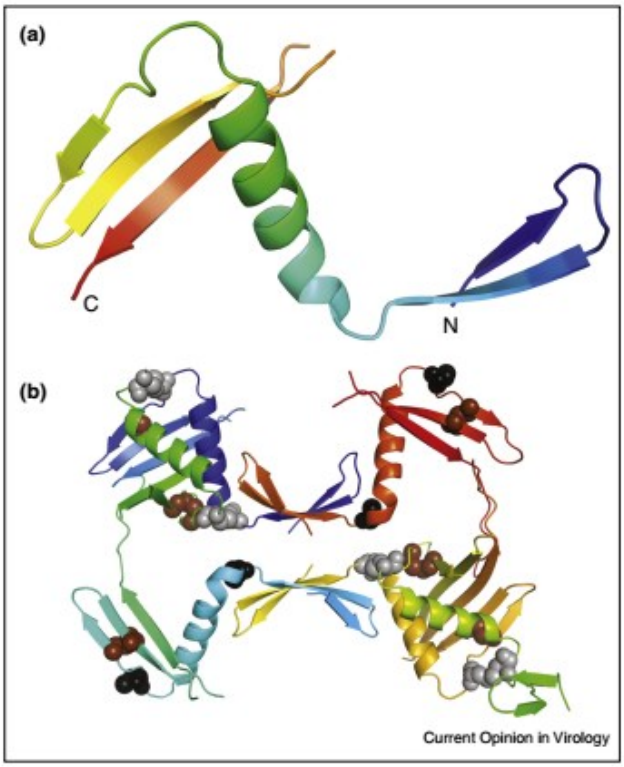


Figure 1.8 E1 crystal structure.
(A) Cartoon diagram of the partially resolved nE1 monomer (PDB ID: 4UOI) (Omari et al. 2014). It is coloured by rainbow from N- to C-terminus. (B) Cartoon diagram showing 6 monomers in asymmetric unit (AU). Disulfide bonds are shown as spheres coloured brown for intramolecular, gray for intermolecular with another molecule in the AU, and black for intermolecular between two AUs. Figure taken from (Khan, Miller, and Marcotrigiano 2015).

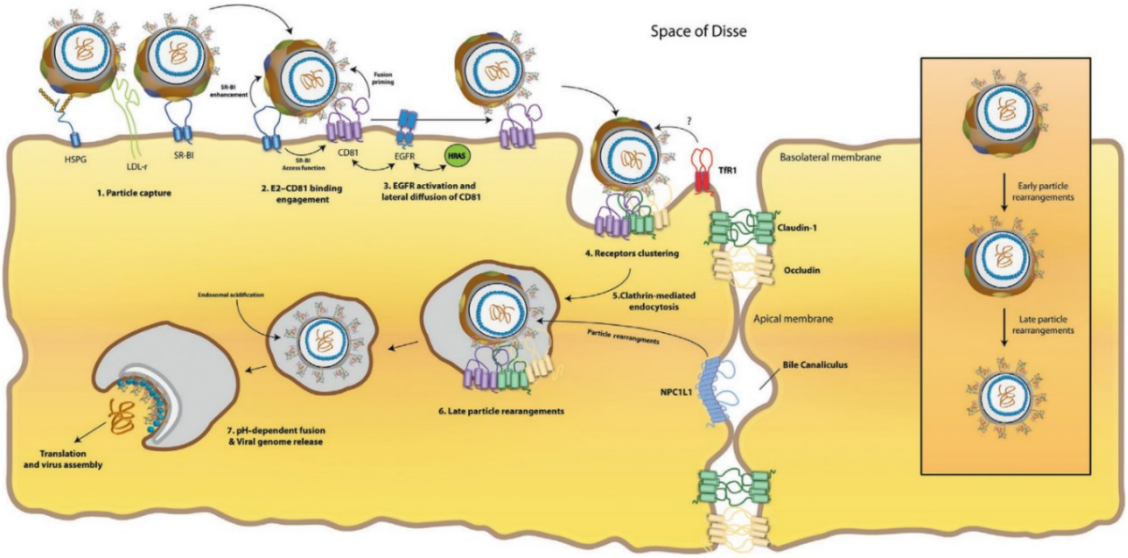


Figure 1.9 HCV entry into hepatocytes.
The hepatocytes are in yellow and the membrane is the brown border outlining the cells. The area at the top and bottom of the cell are the basolateral membrane and the area contacting adjacent cells is the apical membrane. The steps of HCV entry are identified from 1 to 7. Arrows indicate the directional pathways of the viral particle. Step 1: Initial binding involves the lipoviral particle attaching nonspecifically to HSPG, LDL-r, SRB1, and Syndecans (latter not shown). Step 2: This allows for post-binding interactions with other functions of SRB1 as well as CD81 which leads to the activation of EGFR signaling. Step 3: Activation of EGFR and HRas signalling induces the lateral diffusion of HCV-CD81 complexes toward the apical membrane. Step 4: The HCV-CD81 complexes form a trimer complex with Claudin-1, which likely involves each of the three components having direct interaction with the other two components. Step 5: Receptor clustering leads to internalization of the trimeric complex (as visualized by (Coller et al. 2009)) by clathrin-mediated endocytosis and although the function of Occludin is unclear, it is thought to facilitate this step. Step 6: Late particle rearrangements occur during the migration of the complex toward Rab5-endosomal compartments. Similar to Occludin, the function of NPC1L1 is a mystery but it is thought to facilitate the late particle rearrangements and the particle is primed for fusion. The role of TfR1 is unknown. Step 7: Endosomal acidification induces E1E2 refolding that exposes the fusion peptide (thought to be in E1). Insertion of the fusion peptide into the endosomal membrane induces E1E2 rearrangements that drive the membrane merging and the release of viral RNA into the cytosol. The different particle rearrangements that occur throughout entry are indicated at the right of the figure. Figure taken from (Douam, Lavillette, and Cosset 2015).

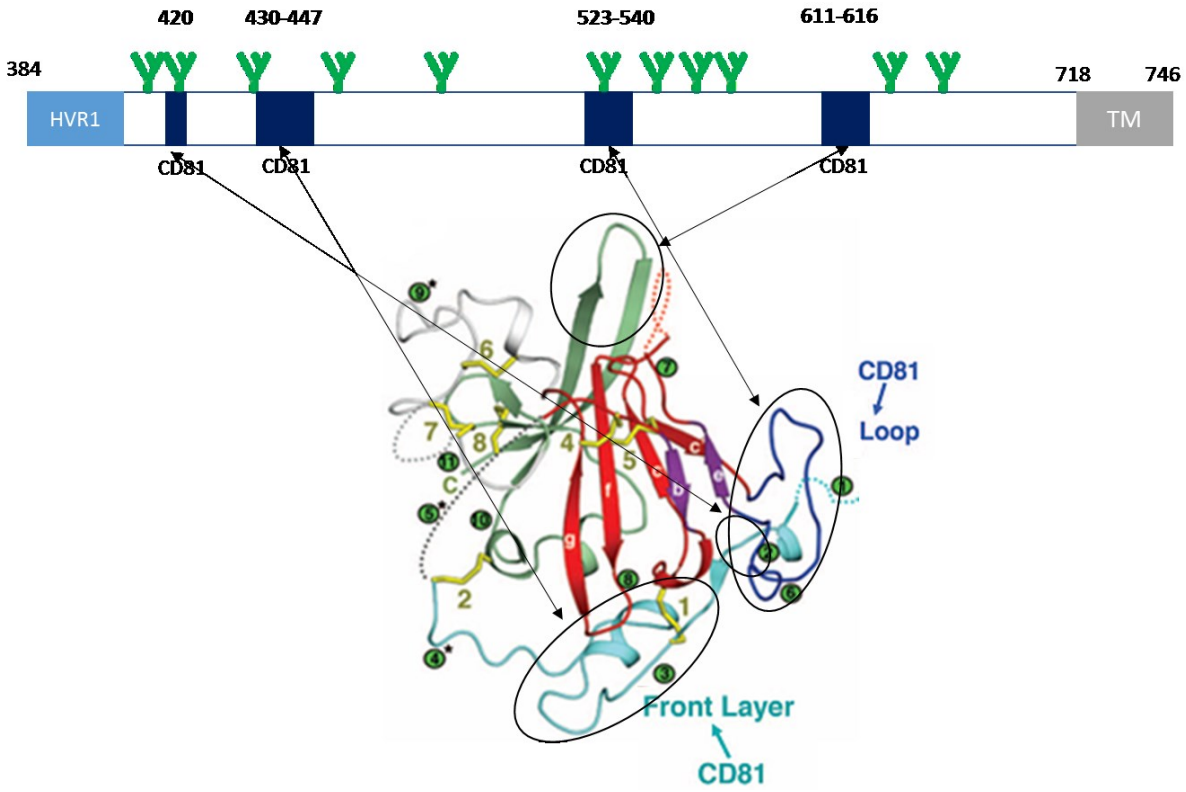


Figure 1.10 Location of E2 residues essential to CD81 binding.
The E2 protein sequence is displayed at the top of figure. The locations and amino acids (based on H77 numbering) of HVR1 and the transmembrane domain are indicated. The green symbols indicate the 11 N-glycosylation sites in E2. The four dark blue segments along with the amino acids indicate the four clusters of residues known to be essential for E2-CD81 interaction. These residues are as follows: W420, N428, G436, W437, G440, L441, F442, Y443, Y527, W529, G530, D535, Y613, and W616 (Owsianka et al. 2006). Below is the E2 crystal structure (PDB reference: 4MWF) and the location of each CD81 region is indicated. Figure adapted from (Kong et al. 2013).

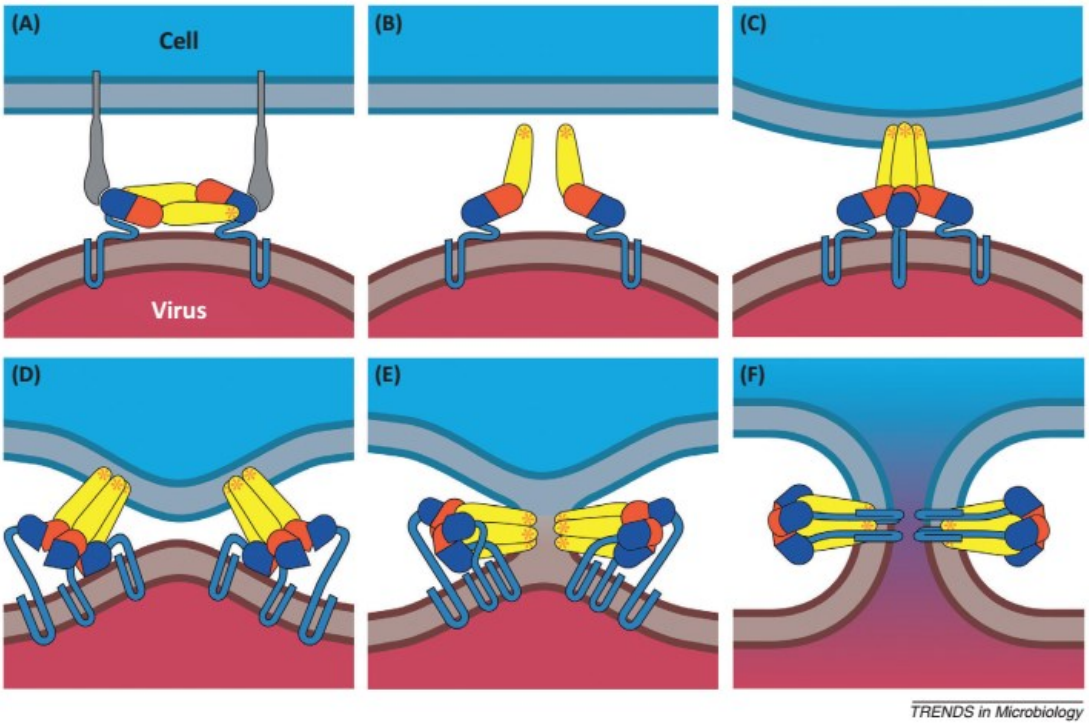


Figure 1.11 Membrane fusion mechanism of enveloped viruses using a class II fusion protein as an example.
(A) The viral envelope protein binds to a cell surface receptor that leads to endocytosis. (B) An environmental cue – for example endosomal pH (depicted here) or coreceptor binding (not shown) cause the ectodomain of the fusion protein to hinge away from the viral surface in conformational changes to expose a hydrophobic fusion peptide. (C) The fusion peptide inserts into the cell membrane, and this leads to a trimeric formation of the fusion protein. (D) The fusion protein folds back on itself which directs the fusion peptide towards the C-terminal transmembrane anchor. This refolding energy drives the bending of the apposed membranes. (E) There is creation of new contacts during refolding of the fusion protein leads to hemifusion. (F) This leads to the formation of a lipidic fusion pore, full lipid mixing, and release of viral genetic material into the cytosol. Figure taken from (Li and Modis 2014).

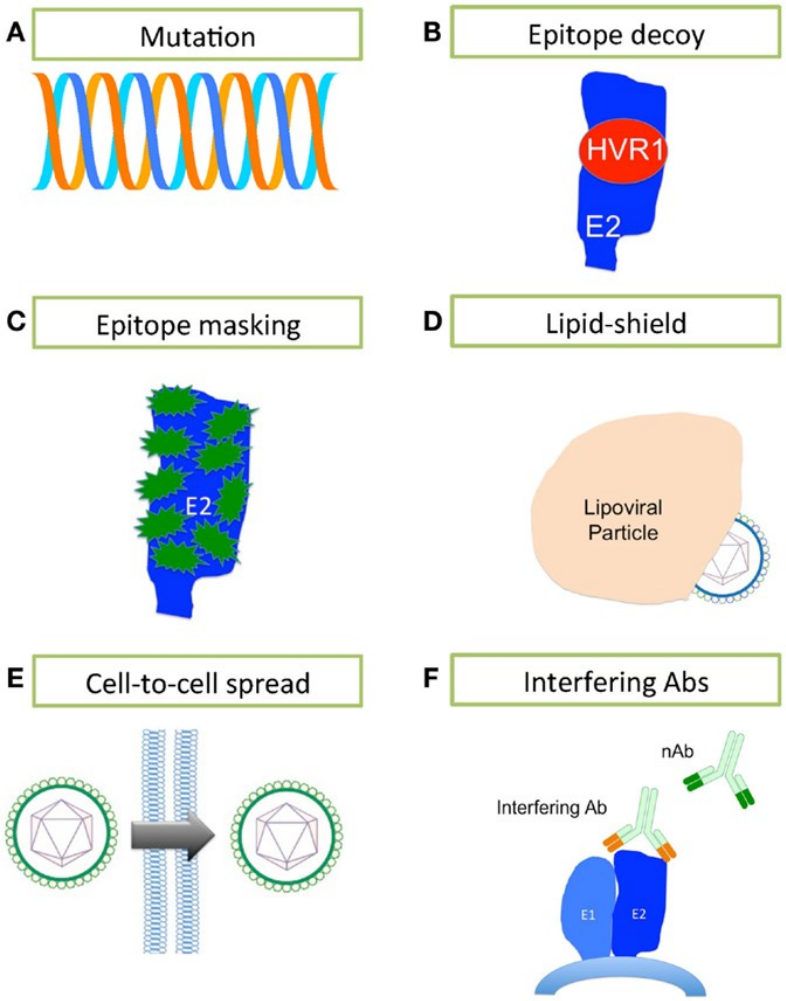


Figure 1.12 Escape mechanisms HCV utilizes to avoid the humoral immune response. HCV has several mechanisms that help it avoid being neutralized by antibodies. (A) It has an RNA-dependent RNA polymerase which has a very high rate of mutation as there is no proofreading function which leads to the generation of a quasispecies. (B) The N-terminus of E2 contains a hypervariable yet immunogenic region named HVR1 that may act as an immunological decoy to mask a conserved hydrophobic region that is necessary for entry. (C) Both E1 and E2 are glycosylated with N-linked glycans that can mask the proteins from nAbs. (D) The nature of the HCV particle as a lipoviral particle associated with lipoproteins means that the particle is protected from nAbs. (E) There is evidence HCV may be able to spread cell-to-cell instead of going through the extracellular space and this would protect it from nAbs. (F) Antibodies elicited against certain epitopes may either be non-neutralizing or may even interfere with the binding of neutralizing antibodies. Taken from (Cashman, Marsden, and Dustin 2014).

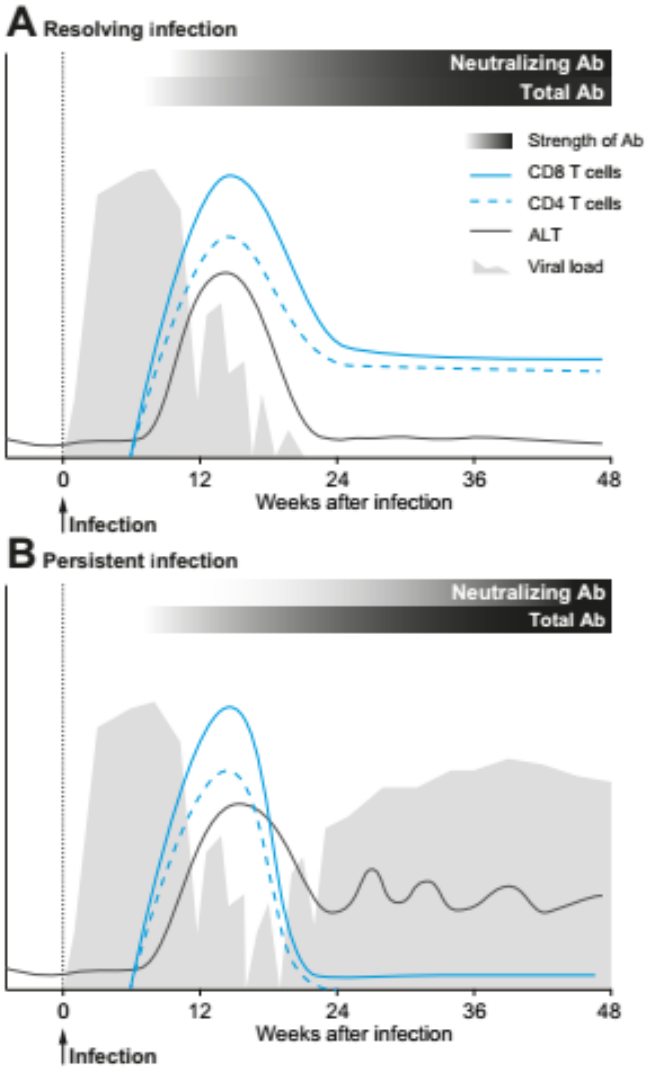


Figure 1.13 Dichotomous natural outcome of HCV infection.
Following exposure to HCV, patients have high levels of viral load within days. Adaptive immune responses develop after 6-10 weeks, and by this time viremia is at least partially controlled and elevated liver enzymes signaling liver damage appears. (A) Maintenance of CD4+ and CD8+ T-cell responses and early neutralizing antibodies is associated with spontaneous clearance within 6 months of infection. (B) Patients that lose CD4+ and CD8+ T cell responses and do not develop neutralizing antibodies until late are likely to become chronically infected. Figure taken from (Baumert et al. 2014).

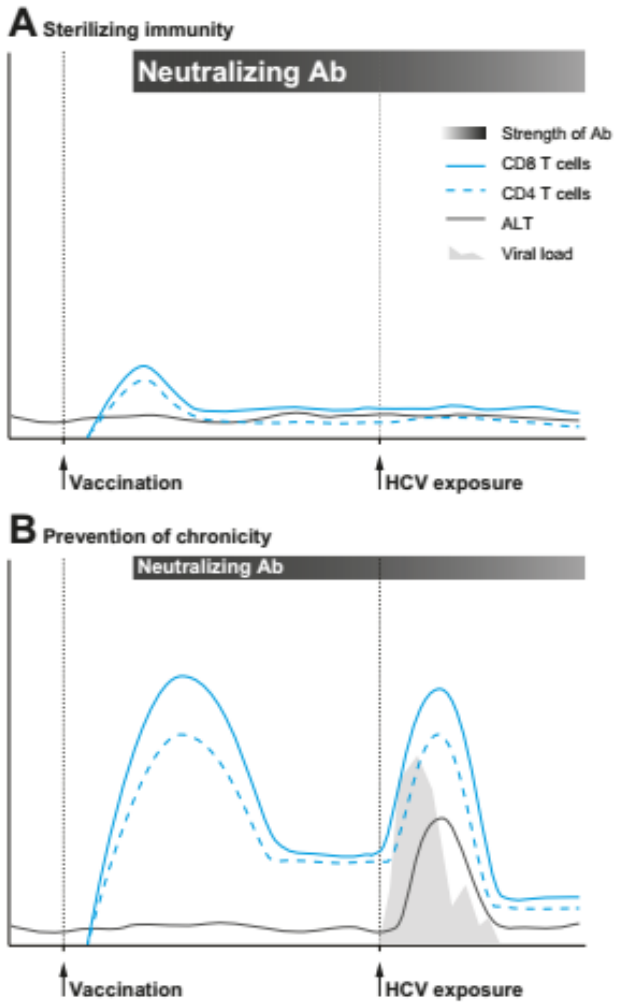


Figure 1.14 Two pathways for a successful prophylactic vaccine.
(A) An ideal HCV vaccine would induce sterilizing immunity and this response would eradicate HCV so quickly upon infection that there would be very low viremia upon infection. (B) However, a vaccine that can still induce adaptive immune responses strong enough to prevent chronic infection and provide the same immunity that leads to spontaneous clearance (depicted in Figure 1.12B) would be sufficient for an effective HCV prophylactic vaccine. Figure taken from (Baumert et al. 2014).

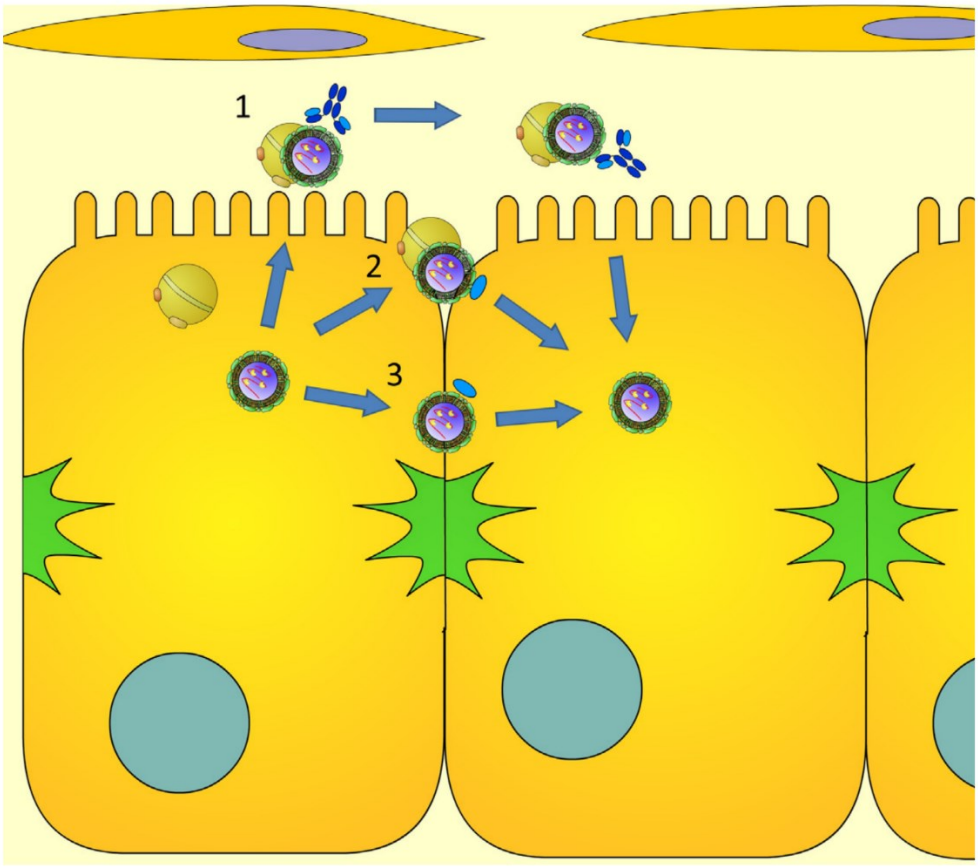


Figure 1.15 Neutralizing antibody activity on viral entry.
HCV lipoviral particles complexed with low-density lipoproteins circulate in the blood. Infection can occur either from extracellular virus entering a naïve hepatocyte or by direct cell-to-cell transmission between adjacent cells across the apical surface. Neutralization by antibodies can occur: (1) as particles are released from infected cells so that they can't infect naïve hepatocytes, (2) as cell-tethered particles are about to infect naïve adjacent cells, or (3) by inhibition of cell-to-cell transmission. It is currently thought that only nanobodies are capable of blocking the last two steps. Figure taken from (Ball, Tarr, and McKeating 2014).

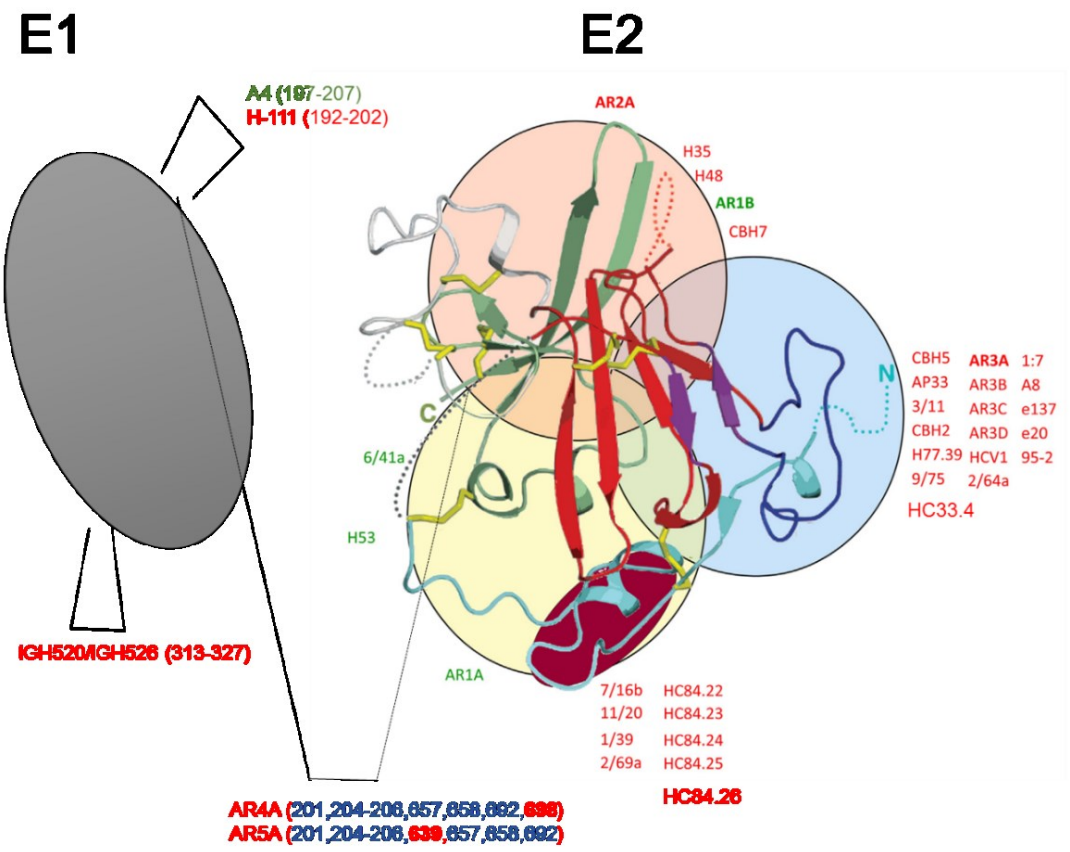


Figure 1.16 Antigenic organization of the E1E2 heterodimer.
Antibodies are indicated close to their epitope and green text indicates non-neutralizing and red text indicates neutralizing. The monoclonal antibodies were mapped primarily using alanine scanning mutagenesis, competition ELISAs, and peptide binding. (E1): There are three antibodies targeting E1 alone and the cluster of residues containing the critical binding residues are indicated. (E2): Antibodies targeting E2 alone are divided into different domains that are mapped on the crystal structure of E2 **(Kong et al. 2013)** (PDB reference: 4MWF). The blue circle includes the CD81 binding loop and the N-terminus of E2 which includes HVR1, regions that are important for SRB1 and CD81 interaction. The bulk of the highly cross-neutralizing anti-E2 antibodies that have been isolated target to this region. The pink circle includes regions a β-sheet containing less well conserved neutralization epitopes. Antibodies to this region tend to be moderately neutralizing but poorly cross-neutralizing. One region important to CD81 binding is located in this circle. The yellow circle contains a less organized region containing mostly epitopes of non-neutralizing epitopes. There is also the burgundy oval which contains a helical region between 428-442 that contains conserved cross-neutralization epitopes. (E1E2): AR4A and AR5A are the only two nAbs that require natively folded E1E2 heterodimer for binding and not E1 or E2 alone. The epitopes of AR4A and AR5A are not fully understood and although they do not compete for binding they share most of the critical binding residues (blue residues) – these residues are likely necessary for proper E1E2 folding. The unique residues that are likely contact residues are in red. Adapted from (Ball, Tarr, and McKeating 2014).

2 CHAPTER II: MATERIALS AND METHODS

2.1 Chemicals, reagents, antibodies

Epigallocatechin gallate (EGCG), Ezetimibe, and Bafilomycin A1 (BafA1) were purchased from Santa Cruz Biotechnology (Mississauga, ON, Canada). These compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Oakville, ON, Canada) and stored at -20°C.

Monoclonal antibodies (mAbs) mouse anti-SRB1 Clone 25 (BD Biosciences, Mississauga, ON, Canada); mouse anti-CD81 clone JS-81 (BD Biosciences); rat anti-CLDN1 clone MAB4618 (R&D Systems); mouse isotype control IgG1 clone MAB002 (R&D Systems, Minneapolis, MN, USA); and rat isotype control IgG1 clone MAB005 (R&D Systems) were purchased from suppliers. Anti-HCV mAbs were generously provided by collaborators. H77.16 (Sabo et al. 2011) and H77.39 (Sabo et al. 2011) were provided by Dr. Michael Diamond (Washington University School of Medicine in St. Louis, St. Louis, MO, USA), A4 (Dubuisson et al. 1994), H52 (Cocquerel et al. 1998), and H53 (Cocquerel et al. 1998) were provided by Dr. Jean Dubuisson (Institut Pasteur de Lille, Lille, France), 6F5 (unpublished) was obtained from Drs. Elizabeth Elrod and Arash Grakoui (Emory University, Atlanta, GA, USA), IGH526 (Meunier, Russell, Goossens, et al. 2008), AR3B (Law et al. 2008), AR4A (Giang et al. 2012), AR5A (Giang et al. 2012), and human anti-HIV antibody B6 were provided by Dr. Mansun Law (The Scripps Research Institute, San Diego, CA, USA), AP33 (Owsianka et al. 2005) was provided by Dr. Arvind Patel (University of Glasgow, Glasgow, Scotland), HC33.4 (Keck et al. 2013), HC33.1, CBH-7 (Keck et al. 2005, 2007), CBH-23 (Keck et al. 2012), HC-1 (Keck et al. 2011), and HC-11 (Keck et al. 2011) were obtained from Dr. Steven Foung (Stanford University, Stanford, CA, USA), 1:7 (Johansson et al. 2007) was provided by Dr. Mats Persson (Karolinska Institutet, Solna, Sweden), and anti-mouse NS5A (9E10) (Lindenbach et al. 2005) was provided by Drs. Tim Tellinghuisen and Charlie Rice (Rockefeller University, New York City, NY, USA).

2.2 Cells

Human hepatoma Huh-7.5 cells were obtained from Dr. Charles Rice. Buffalo Rat Liver cells (BRL3A; catalog number CRL-1442) and Chinese Hamster Ovary cells (CHO/dhFr-; catalog number CRL-9096) were obtained from the American Type Culture Collection (Manassas, VA, USA). Human Embryonic Kidney Cells (HEK293T; catalog number CRL-3216) were provided by D. Lorne Tyrrell (University of Alberta, Edmonton, AB, Canada).

All tissue cultured cells were grown at 37°C with 5% CO_2 unless specifically stated. Huh-7.5 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, Rockland, IL, USA) supplemented with 10% heat inactivated fetal bovine calf serum (FBS; Omega Scientific, Tarzana, CA, USA); 0.1mM nonessential amino acids (NEAA; Invitrogen, Burlington, ON, Canada); and 100 units/mL penicillin and 100µg/mL streptomycin (PenStrep; Invitrogen). HEK293T and BRL3A cells were cultured in DMEM (Gibco); 10% heat inactivated FBS (Gibco); 0.1mM NEAA (Invitrogen); and 100 units/mL penicillin and 100µg/mL streptomycin (Invitrogen). CHO cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Gibco); 0.1mM/0.016mM sodium hypoxanthine/thymidine (HT; Gibco); 0.002mM methotrexate (Sigma-Aldrich); 0.1mM NEAA (Gibco); and 100 units/mL penicillin and 100µg/mL streptomycin (Invitrogen).

2.3 Generation of recombinant envelope glycoproteins

Recombinant protein of full length E1E2 (rE1E2) derived from the genotype 1a HCV-1 (GenBank accession number: M62321.1) and H77 (GenBank accession number: AF009606.1) strains (aa192-746) was provided by Dr. Michael Logan in our group. Methods of purifying rE1E2 from cells have been previously described (Ralston et al. 1993; Frey et al. 2010; Logan et al. 2016). The E1E2 coding regions preceded by the signal peptide sequence for tissue plasminogen activator (tPA) was inserted into the SpeI/MluI site of the pTRIP lentiviral vector. The vector also contained an internal ribosome entry site (IRES)-*Aequorea coerulescens* green fluorescent protein (ACGFP reporter) (Schoggins et al. 2011). For Fc-tagged rE1E2 constructs, a duplication of aa384-385 (ET) was inserted at the N-terminus of E2, followed by the human IgG1 Fc-tag (227aa) and the PreScission protein/human rhinovirus protease 3C (HRV3C) sequence (Logan et al. 2017). The pTRIP vectors along with vectors containing lentiviral gag-pol

and VSVG were used to produce lentiviral particles in HEK293T cells according to a previous method (Schoggins et al. 2011). Packaged lentiviruses were used to transduce CHO cells. GFP-positive cells were sorted by flow cytometry using a BD FACSAria III cell sorter (BD Biosciences) and suspension adapted in ProCHO4 media (Lonza, Walkersville, MD, USA) with 6% FBS in 250mL shaker flasks (Corning, Corning, NY, USA). The cells were then expanded in 3L spinner flasks (Corning). Recombinant E1E2 lacking the Fc-tag was purified from CHO cell extracts using *Galanthus nivalis* antigen (GNA)-agarose (Vector Laboratories, Burlingame, CA, USA) and then the eluate was then purified again through a hydroxyapatite (HAP) column (Bio-Rad, Hercules, CA, USA). The HAP column flowthrough was then concentrated with a 50K centrifugal filter unit (EMD Millipore, Billerica, MA, USA). Final antigen purity was at least 90%. Recombinant E1E2 containing the Fc-tag was purified from CHO cell extracts using a Protein G Sepharose 4 Fast Flow column (GE Healthcare, Piscataway, NJ, USA) and the resin was digested with His6-GST-HRV3C protease (Thermo Fisher Scientific) overnight at 4°C. The digested resin was then run through a Glutathione-Sepharose 4B column (GE Healthcare) to remove the protease. The flowthrough containing rE1E2 with the Fc-tag cleaved was applied to a HAP column and then concentrated.

Soluble E2 (sE2) derived from the HCV-1, H77, and J6 (GenBank accession number: JN180452.1) strains were generously provided by Dr. Joseph Marcotrigiano (NIH, Bethesda, MD, USA) (Khan et al. 2014). In short, pJG vectors containing HCV-1, H77, or J6 E2 followed by a C-terminal PreScission protein/human rhinovirus protease 3C (HRV3C) sequence and a Protein-A tag along with vectors containing HIV gag-pol and VSVG were packaged into recombinant lentiviruses in HEK293T cells. Lentiviruses were used to transduce GnTI-HEK293T cells which were then expanded and seeded into an adherent cell bioreactor (ESCO Technologies Inc., Trevose, PA, USA). Supernatant containing sE2 was run through a human IgG FF column (GE Healthcare) and PreScission protease was used to digest the resin. The protein was purified with a heparin affinity column followed by size-exclusion chromatography over a Superdex200 column.

Recombinant E1E2 derived from the H77 strain lacking the E1N3 (N234Q), E2N1 (N417Q), and E2N6 (N532Q) glycosylation sites were attained by site directed mutagenesis converting the asparagine amino acid to a glutamine amino acid. These proteins were generated and purified as described above for rE1E2 lacking an Fc-tag. Recombinant H77 rE1E2 lacking

HVR1 (aa384-411 were deleted) were generated and purified as described above for rE1E2 lacking an Fc-tag. Soluble E2 from the H77 and J6 strains (aa384-411 were deleted) were obtained from Dr. Joseph Marcotrigiano. The T-cell epitopes were derived from the HCV NS3 region of a genotype 1 consensus sequence and were designed by Dr. Amir Landi (personal communication with Dr. Amir Landi). The T-cell antigens were inserted in the Fc-tagged rE1E2 constructs between the human IgG1 Fc-tag and E2. The T-cell modified rE1E2 antigens were generated and purified as described above for Fc-tagged rE1E2 and the Fc-tag is removed by PreScission Protease cleavage exposing the T-cell epitope at the N-terminus of E2. Biochemical analysis of the antigens was performed by adding sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Loading Buffer including beta-mercaptoethanol to 1μg of each antigen and they were heated at 95°C for 5 minutes. These samples were subjected to SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada) for western blotting. Denatured E1 and E2 were detected with murine mAbs A4 and H52 respectively at a dilution of 1:2000. Alexa Fluor 750 conjugated goat anti-mouse antibody (1:10000; Invitrogen) were added as secondary antibodies. Antibodies were diluted in PBS/0.1% Tween-20/0.5% BSA. Then the image of the Western Blot was scanned using the LI-COR Odyssey Scanner (LI-COR, Lincoln, NE, USA). The images were analyzed with LI-COR Image Studio Lite (LI-COR).

2.4 Generation of antisera/immunization schedule

All goat immunizations and blood draws were conducted by Rockland Immunochemicals (Pottstown, PA, USA). Goats were used because of the advantage of a large amount of antisera that can be collected. The similarity of B-cell makeup between goats and humans is not well understood but the V(D)J recombination mechanism across all tetrapods is quite similar (Das et al. 2011). Goats were immunized five times with 10μg of rE1E2 (rE1E2) derived from a genotype 1a isolate (HCV-1) (Novartis, East Hanover, NJ, USA), sE2 derived from a genotype 1a isolate (HCV-1), or sE2 derived from a genotype 2a isolate (J6) (GenBank accession number: AF177036.1) (Rockland Immunochemicals). For the first three immunizations the vaccine was formulated with the adjuvant Addavax™ (Invivogen, San Diego, CA, USA), a squalene-based oil-in-water emulsion similar to MF59 on days 32, 53, and 102. This was followed by one

immunization formulated with Complete Freund's Adjuvant (CFA) on day 165, and one immunization formulated with Incomplete Freund's Adjuvant (IFA) on day 172. The three antisera samples examined in this study were collected in blood draws performed prior to the immunizations on day 0 (**Pre**), after the 3 rounds of immunizations using the Addavax adjuvant on day 112 (**Post-Addavax**), and after the last two immunizations with CFA/IFA on day 182 (**Post-Freund's**). Goat immunizations were performed by Rockland Immunochemicals under approved Institutional Animal Care and Use Committee (IACUC) protocols. Two goats (G757 and G714) were immunized with rE1E2 derived from a genotype 1a isolate (HCV-1), two goats (G786 and G799) were immunized with sE2 derived from a genotype 1a isolate (HCV-1), and two goats (G773 and G766) were immunized with sE2 derived from a genotype 2a isolate (J6).

Human antisera against HCV glycoproteins were obtained from the Phase 1 clinical trial (DMID 01-012) where safety and immunogenicity of a vaccine constituting a rE1E2 immunogen derived from a genotype 1a isolate (HCV-1) formulated with the MF59C.1 adjuvant was tested (Frey et al. 2010). The study was performed at the National Institute of Allergy and Infectious Diseases' Vaccine Treatment Unit at St. Louis University (St. Louis, MO, USA) and approval was granted by the St. Louis University Institutional Review Board. In this trial, sixty volunteers were immunized with 3 different doses of the recombinant protein (4μg, 20μg, and 100μg) intramuscularly at day 0 and weeks 4, 24, and 48. Post-immunization antisera were collected on day 0 prior to immunization, and weeks 4, 6, 8, 24, 26, 28, 48, 50, 52, and 64. Two samples from the 100μg group were used for this study: prior to immunization (**Pre**) and 2 weeks post 3rd immunization (26 weeks) (**Post**).

Chronically infected patient sera were provided by Dr. D. Lorne Tyrrell (originally drawn by the Provincial Laboratory for Public Health). These samples are described in regard to sex, age at time of blood sample taken, and subgenotype in **Table 3.4**. In the experiments these sera were compared to normal human sera pooled from HCV negative subjects (also provided by Dr. D. Lorne Tyrrell).

Mouse antisera against HCV glycoproteins in the GNA-derived versus Tag-derived study were obtained from immunizing female CB6F1 mice (5 to 7 weeks old) (Charles River Laboratories, Montreal, QC, Canada) under Canadian Council on Animal Care (CCAC) guidelines. The experimental methods were reviewed and approved by the University of Alberta Health Sciences Animal Welfare Committee. Recombinant protein antigens (2μg) were mixed in

a 1:1 ratio of 75μg alum and 7.5μg monophosphoryl Lipid A (MPLA) (VacciGrade; Invivogen). These preparations were used to immunize mice intramuscularly (35μL final volume) on days 0, 14, 28, and 56. Pre-immunization sera were collected at day 0 and post-immunization antisera were collected at day 42.

Small animal antisera against HCV glycoproteins in the WT versus ΔHVR1 study were obtained from immunizing female CB6F1 mice (5 to 7 weeks old) (Charles River Laboratories, Montreal, QC, Canada) or female Hartley guinea pigs (Medimabs, Montreal, QC, Canada) under CCAC guidelines. The experimental methods were reviewed and approved by the University of Alberta Health Sciences Animal Welfare Committee. Recombinant protein antigens (2μg for mice; 7.5μg for guinea pigs) were mixed in a 1:1 ratio of 75μg alum and 7.5μg monophosphoryl Lipid A (MPLA Vaccigrade (Invivogen, San Diego, CA, USA). These preparations were used to immunize mice intramuscularly (35μL final volume) on days 0, 14, and 28. Guinea pigs were intramuscularly immunized (100μL final volume) on days 0, 14, 42, and 90. Pre-immunization sera were collected at day 0 and post-immunization antisera were collected at day 42 for mice and day 104 for guinea pigs.

All antisera (goats, humans, guinea pigs, and mice) were heat-inactivated at 56°C for 30 minutes prior to all assays in order to inactivate complement.

2.5 *In vitro* HCV preparation

Cell culture derived chimeric HCV (HCVcc) were produced using a previously described protocol (Lindenbach et al. 2005; Gottwein et al. 2009). DNA templates containing a T7 polymerase promoter and HCV RNA based on the JFH-1 strain expressing Core-NS2 regions from the various strains were provided by Dr. Jens Bukh (University of Copenhagen, Kobenhaven, Denmark) (Gottwein et al. 2009). The phylogenetic tree with these strains is shown in **Figure 2.1.** First, plasmid DNA was linearized by XbaI (New England Biolabs, Whitby, ON, Canada) for 3 hours at 37°C for run-off transcription. Then the linearized DNA was cleaned up with MinElute PCR purification columns (Qiagen, Toronto, ON, Canada). Linearized template DNA was diluted to 0.25μg/μL in RNase free H_2O. RNA transcription was completed with the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Madison, WI, USA)

and DNase (Qiagen) was used to digest template DNA. RNA purification was achieved through the RNeasy Mini Kit (Qiagen). RNA was then diluted to 0.5μg/μL and stored.

6 x 10^6 Huh7.5 cells were mixed with 5μg of in vitro transcribed HCV RNA in a 2mm gap electroporation cuvette. Five pulses of 860 V (99μs, 1.1s interval) were delivered using the ElectroSquare Porator ECM 830 electroporator (BTX, Holliston, MA, USA) followed by 10 minutes recovery at RT. Then, cells were diluted in 30mL DMEM/10% FBS/0.1mM NEAA and incubated at 37°C and 5% CO_2. Supernatant was collected as virus stocks at 3 and 5 days post-electroporation. Supernatant was filtered through a 0.22μm filter (EMD Millipore, Etobicoke, ON, Canada). In some instances, HCVcc were further concentrated using Amicon Ultra 0.5mL Centrifugal Filters 100kDa cutoff centrifugal filter (EMD Millipore).

Virus titer was calculated as 50% tissue culture infectious dose ($TCID_{50}$) as described (Lindenbach et al. 2005). 96-well plates were coated with 40μL of poly-L-lysine for 5 minutes at RT and then Huh-7.5 were seeded at 10^4 cells/well in Huh-7.5 media. 24 hours after, ten-fold serial dilutions of the viral stock were prepared in DMEM and used to infect cells for 12 hours before media were removed and replenished with fresh media. After 48 hours, cells were washed once with PBS and followed by fixing with cold methanol for at least 30 minutes at -20°C. Immunohistohemical staining of NS5A was then conducted. The cells were washed unless otherwise stated by twice with PBS and then once with PBS/0.1% Tween-20 (v/v). The cells were first blocked with PBS/0.5% Tween-20 (v/v)/5% skim milk powder (m/v) for 30 minutes at RT. Then cells were incubated with 4% H_2O_2 for 5 minutes followed by washing. Infection were detected by incubating mouse monoclonal anti-NS5A antibody (9E10) diluted at 1:10000 in PBS/0.1% Tween-20 overnight at 4°C. After another round of washing, HRP-conjugated secondary antibody goat anti-mouse diluted at 1:200 in PBS/0.1% Tween-20 was added to the cells for 30 minutes at RT. Then the cells were washed and the colour substrate from the Liquid DAB+ Substrate Chromogen System (Agilent Technologies, Santa Clara, CA, USA) was used to develop the stain. Once the infected cells were visualized, the number of wells containing infected cells at each dilution of virus stock was determined and $TCID_{50}$ was calculated based on method described by Reed and Muench (Reed and Muench 1938).

2.6 Neutralization assays

2.6.1 HCVcc neutralization assays

Neutralization was evaluated using HCV produced from tissue culture (HCVcc). Briefly, dilutions of antisera from immunized goats and humans or mAbs were pre-incubated with 300 $TCID_{50}$ of HCVcc for 1 hour at 37°C. They were then added to 96-well plates seeded with Huh-7.5 cell monolayers (10^4 cells/well) 24 hours prior. Media was removed 12 hours post-infection and replaced with fresh media. Either 48 or 72 hours post infection, the number of infected cells was quantified with one of three methods. In all three methods, percent neutralization was calculated by this formula: 100-((A-B)/(C-B)*100) where A is the level of infection quantitated in the presence of viral entry inhibitors or antisera; B is the level of infection quantitated in cells lacking virus; and C is the level of infection quantitated without addition of inhibitors or antisera.

Method 1: Immunohistochemistry detection. As described above (see HCV preparation), virus infected cells were detected using the anti-NS5A antibody 9E10 followed by HRP-conjugated secondary antibody. Foci of infected cells were detected and counted either manually or with the CTL ImmunoSpot S6 Analyzer (CTL, Cleveland, OH, USA).

Method 2: Flow cytometry detection of infected cells with anti-NS5A antibody (9E10). Cells were fixed with 2% paraformaldehyde (PFA; Sigma-Aldrich) 48 hours post-infection. Mouse anti-NS5A antibody (9E10) or mouse isotype control IgG1 (R&D Systems) were added in 1μg/ml as a primary antibody followed by incubation with an Alexa Fluor 647-conjugated goat anti-mouse secondary antibody at 1:400 (Molecular Probes). Cells were counted using the FACS LSRFortessa (BD Biosciences) for NS5A positive cells. Results were analyzed with FlowJo 10.4 (TreeStar).

Method 3: Luciferase detection of cells infected with HCVcc encoding nanoluciferase gene. HCVcc reporter viruses encoding a nanoluciferase gene in the NS5A protein were used for some of the assays (Eyre et al. 2017). Level of infection was indirectly measured by determination of luciferase activity. In order to quantitate infection, cells were assayed with the NanoLuc® Luciferase Kit (Promega) according to the manufacturer's protocol. Luminescence was measured by the Enspire™ 2300 Multilabel Plate Reader (Perkin-Elmer, Waltham, MA, USA).

2.6.2 HCVcc synchronized Time-of-addition Neutralization Assays

On day 1, $1x10^5$ cells/well of Huh7.5 cells were seeded on 96-well plates. Then on day 2, 1000 $TCID_{50}$ of HCVcc were incubated on the cell monolayer for 2 hours at 4°C, a temperature that allows for virus binding but not efficient entry (t=-2). Unbound virus was then washed off the cells with cold PBS followed by shifting the cells to 37°C in warm media (t=0) to trigger virus entry. Antibodies, vaccinated antisera, or compounds affecting HCV entry were added at different times (t=-2,0,+1,+2,+6 or up to 12 hour post temperature shift). Subsequently, media was removed and replenished with fresh media. For the t=-2 addition, antibodies, antisera, or tested compounds were present during the 2 hour period while the cells were at 4°C, and then antibodies or antisera was replenished at t=0. After 72 hours of infection, the level of viral infection was measured by either NS5A staining in a flow cytometry based assay or by measuring luciferase activity if the HCVcc strains encoded a luciferase reporter are used. A diagram of the protocol is shown in **Figure 2.2**.

2.6.3 HCVpp production and neutralization assays

HCVpp expressing a luciferase reporter were generated as described previously (Hsu et al. 2003). HCVpp pseudotyped with E1E2 from the H77 strain lacking HVR1 has been previously described and encodes two compensatory mutations (H261R, Q444R) (Prentoe et al. 2014). In neutralization assays, Huh-7.5 cells were plated on poly-L-lysine coated 96-well plates 1 day prior to infection. HCVpp were diluted and pre-incubated with diluted antisera for 1 h followed by addition to Huh 7.5 cells. At 6 hours post-infection, the mixture was replaced with fresh culture medium. Cells were processed at 48 hours post-infection using the Bright-Glo luciferase assay system (Promega). Luminescence was measured using an Enspire™ 2300 Multilabel Plate Reader (Perkin-Elmer). Neutralization activity normalized to pre-vaccination sera was calculated using the formula listed above in **Section 2.6.1**.

2.7 ELISAs

2.7.1 Binding to unpurified E1E2 glycoproteins

Microtiter plates (Corning, Corning, NY, USA) were coated with *Galanthus nivalis* lectin antigen (GNA) (20μg/ml; Sigma-Aldrich, St. Louis, MO, USA) and the plates were blocked with blocking buffer (phosphate buffered saline (PBS)/1% casein (m/v)/0.5% Tween-20 (v/v)). 100μg of lysates from CHO cells stably expressing H77 rE1E2 or non-transfected CHO cells as a control were incubated in each well. Antisera from goats and humans or mAbs were then added to test binding. For titration experiments, mAbs were added in 6-fold dilutions starting at 36μg/mL or at 2.5-fold dilutions starting at 24μg/mL. Alkaline phosphatase (ALP) conjugated goat anti-human, goat anti-mouse, or rabbit anti-goat secondary antibody (1:10000; Jackson ImmunoResearch, West Grove, PA, USA) was incubated in the wells. The signal of antibody binding was developed using para-Nitrophenylphosphate) (pNPP; Sigma-Aldrich). Absorbance was read at 405nm in an Enspire™ 2300 Multilabel Plate Reader (Perkin-Elmer).

2.7.2 Binding to purified E1E2 glycoproteins

Microtiter plates (Corning) were coated with 25ng of purified envelope glycoproteins (H77 rE1E2, H77 sE2, HCV-1 sE2, J6 sE2) in carbonate coating buffer (15mM Na_2CO_3/35mM $NaHCO_3$/pH 9.6) and the plates were blocked with blocking buffer. Antisera from goats and humans or mAbs were then added to test binding. For titration experiments, mAbs were added in 6-fold dilutions starting at 36μg/mL or at 2.5-fold dilutions starting at 24μg/mL. ALP conjugated goat anti-human, goat anti-mouse, or rabbit anti-goat secondary antibody (1:10000; Jackson ImmunoResearch) was incubated in the wells and binding of antibody were detected using pNPP (Sigma-Aldrich). Absorbance (405-495nm) was read in an Enspire™ 2300 Multilabel Plate Reader (Perkin-Elmer).

In other anti-E1E2 ELISAs using WT and ΔHVR1 rE1E2 antigens, microtiter plates (Corning) were coated with GNA (20μg/ml; Sigma-Aldrich) in PBS overnight at 4°C and then they were blocked for 1 hour in 4% bovine serum albumin (BSA; Sigma-Aldrich) in PBS containing 0.2% Tween-20 (PBS-T). Lysates containing WT or ΔHVR1 rE1E2 (2μg/ml) were added for 1 hour. Anti-E2 mAbs (H77.16, HC33.4, HC84.26, AR3B), anti-E1E2 (AR4A, AR5A), or a control mAb (B6) were added for 1 hour and detected by species-specific secondary antibody conjugated to horseradish peroxidase (HRP) (1:10000; Jackson ImmunoResearch) and KPL peroxidase substrate (SeraCare Life Sciences, Milford, MA, USA). Absorbance (450-

507nm) was read using an Enspire™ 2300 Multilabel Plate Reader (Perkin-Elmer, Waltham, MA, US).

2.7.3 Binding to purified E2 glycoproteins (Horseradish Peroxidase)

Microtiter plates (Corning) were coated with H77 sE2 (aa384-656) or ΔHVR1 sE2 (aa412-656) overnight at 4°C in PBS. Wells were blocked in 4% BSA in PBS for 1 hour. Sera from vaccinated mice were diluted in PBS-T and added to the plates for 1 hour. Signal was developed using an HRP-conjugated species-specific secondary antibody (1:10,000; Cedarlane Laboratories, Burlington, ON, Canada) and KPL peroxidase substrate (SeraCare Life Sciences). Absorbance (450-507nm) was read using an Enspire™ 2300 Multilabel Plate Reader (Perkin-Elmer).

2.7.4 Competition ELISAs

GNA-captured unpurified rE1E2 lysates or purified rE1E2 protein was used as the target antigen. Goat or human antisera in different dilutions were added for 1 hour prior to replacement with a specific detecting mAb for 1 hour. I used a concentration of detecting mAb resulting in 70% maximal binding which was pre-determined by testing binding of each mAb to rE1E2. Binding of this detecting mAb was measured with AP-conjugated anti-human or anti-mouse secondary antibodies (1:10000; Jackson ImmunoResearch). If the blocking antisera and the detecting mAb were derived from the same source (human or murine), the detecting mAb was biotinylated using the EZ-Link® Micro NHS-PEG4-Biotinylation Kit (Thermo Fisher Scientific) with the protocol described by the manufacturer. Binding was detected using AP-conjugated streptavidin (1:4000; Sigma-Aldrich). The signal of antibody binding was developed using pNPP (Sigma-Aldrich). Absorbance was read at 405 nm in an Enspire™ 2300 Multilabel Plate Reader (Perkin-Elmer). IC_{50} values were calculated with Prism 7 (GraphPad Software, Inc.).

2.8 Peptide binding assays

2.8.1 CelluSpot peptide binding assays

The CelluSpot peptide array synthesis technology (Intavis Bioanalytical Instruments; Koeln, Germany) involves the synthesis of arrays of peptides on individual disks consisting of a modified cellulose support on a planar surface such as a glass slide. Up to 768 peptides can be synthesized in parallel on two frames. This allows for a single preparation of antibodies or protein to be incubated on the full slide testing the binding to all these different peptides at once.

The peptides used for the CelluSpot assay were designed by Dr. Aviad Levin and synthesized onto glass slides (Levin et al. 2014). The CelluSpot assay slide was composed of 15aa polypeptides overlapped by 8aa along the complete consensus polyprotein sequence of HCV genotype 1. CelluSpot arrays were incubated in blocking buffer (Tris buffered saline (TBS)/0.05% Tween-20 (v/v)/10% skim milk (m/v)) overnight at 4°C on a nutator rocker at a gentle speed. Then the peptide arrays were washed three times with washing buffer (TBS/0.05% Tween-20 (v/v)) for 5 minutes at RT. The peptide arrays were then incubated for 4 hours with antisera from humans vaccinated with HCV-1 rE1E2 diluted in blocking buffer at a dilution of 1:50 at RT. Peptide arrays were then washed followed by incubation with HRP conjugated goat anti-human IgG secondary antibody at 1:1000 dilution (Jackson ImmunoResearch). Signal was detected through HRP based chemiluminescence with ECL detection reagent (GE Healthcare, Pittsburgh, PA, USA) exposed onto Fuji RX film (Fujifilm). Reactivity was quantified by MultiGauge V2.0 software (Fujifilm, Valhalla, NY, USA).

2.8.2 Peptide ELISAs

N-terminal biotinylated peptides (GLBiochem, Shanghai, China) were reconstituted in acetonitrile and water in a 1:1 proportion to 1mg/ml. Peptides were further diluted to 10µg/mL in blocking buffer (PBS/1% Casein/1% Triton X-100/0.5M NaCl/1mM EDTA) and added on NeutrAvidin coated microtiter plates (Thermo Fisher Scientific). Following washing to remove unbound peptides, antisera from either vaccinated goats and human mAbs were first diluted in blocking buffer and then added to the prepared plates. Binding of antisera or mAbs were detected by ALP-conjugated secondary antibodies diluted to 1:10000 in a 1:1 ratio of peptide blocking buffer and Odyssey® blocking buffer (LI-COR Biosciences). The peptides used in this study are detailed in **Table 3.3**. For peptide competition assays, serial dilutions of goat antisera were added to the peptides for 1 hour then replaced with mAb for 1 hour. The signal of antibody binding was

developed using pNPP (Sigma-Aldrich). Absorbance was read at 405-495nm in an Enspire™ 2300 Multilabel Plate Reader (Perkin-Elmer).

For other peptide assays, microtiter plates (Corning) were coated with an N-terminal biotinylated peptide comprising H77 residues 387-417aa (Biotin-CETHVTGGNAGRTTAGLVGLLTPGAKQNIQLINTN; GLBiochem, Shanghai, China). Wells were blocked in 4% BSA in PBS for 1 hour. Sera from vaccinated mice were diluted in PBS-T and added to the plates for 1 hour. Signal was developed using an HRP-conjugated species-specific secondary antibody (1:10,000; Cedarlane Laboratories) and KPL peroxidase substrate (SeraCare Life Sciences). Absorbance (450-507nm) was read using an Enspire™ 2300 Multilabel Plate Reader (Perkin-Elmer, Waltham, MA, US).

2.9 Inhibition of glycoproteins binding to CHO cells expressing HCV entry receptors

2.9.1 Generation and selection of CHO cells expressing HCV entry receptors

MLV based lentiviral vectors carrying HCV entry receptors were generously provided by Drs. Bertrand Boson and François-Loic Cosset (University of Lyon, Lyon, France). The vectors carrying HCV entry receptors expressed either SRB1 or CD81 along with Neomycin/Geneticin or Blasticidin resistance genes respectively. The MLV pseudotyped particle were obtained by co-transfecting MLV plasmids along with packaging plasmids pMLV gag-pol and pVSVG (encoding the glycoprotein of VSV). HEK293T cells ($3.5x10^6$) were seeded on a P100 dish (Corning) precoated with poly-L-lysine. After 24 hours of incubation, the 293T growth medium was changed to 3% FBS. One hour later, a mixture of plasmids (one vector containing the glycoprotein from VSV (4μg), one vector containing MLV gag-pol (8 μg), and one vector containing the HCV entry receptor (8μg)) were diluted in lipofectamine 2000 (Invitrogen) and Opti-MEM Reduced Serum Media (Gibco), and then added onto HEK293T cells. 24 hours after transfection, media were replaced with fresh media containing 3% FBS. The packaged retroviral particles in the supernatant were collected at 48 and 72 hours post transfection. Media was filtered through a 0.22μm filter (EMD Millipore) and then supplemented with polybrene

(4μg/mL; Sigma-Aldrich) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (20mM; Gibco). Individual aliquots were stored at -80°C until future usage.

For viral transduction, CHO cells ($4x10^5$ cells/well) were seeded on 6-well plates. On day 1 after seeding, the retroviral particles were first diluted at 1:4 with 3% FBS containing medium supplemented with polybrene (4μg/mL; Sigma-Aldrich) and HEPES (20mM; Gibco) then added onto CHO cells. The retroviral particles were spinoculated onto cells by spinning at 1200 rpm (335 x g) for 1 hour at 37°C. After 6 hours incubation at 37°C, the media was replaced with standard CHO medium. Transduced cells were then passaged every 3 days or when they reached confluency.

Transduced cells were selected with either Geneticin (Gibco) and Blasticidin (Invivogen). To determine the optimal amount of selection antibiotic, non-transduced CHO cells were seeded at 10^5 in 24-well plates and passaged in media containing various concentrations of Geneticin or Blasticidin. It was found that 400μg/mL of Geneticin and 10μg/mL of Blasticidin were barely sufficient for selecting against non-transduced CHO cells and therefore optimal for selection of transduced cells. Subsequently, CHO overexpressing SRB1 were selected with Geneticin and CHO overexpressing CD81 were selected with Blasticidin accordingly. Expression of each receptor was monitored by flow cytometry.

5 x 10^5 CHO cells overexpressing either CD81 or SRB1 were washed with PBS/2%BSA (Gemini Bio-Products, West Sacramento, CA, USA), stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (1:1000; Molecular Probes, Life Technologies Inc., Burlington, ON, Canada), washed again, and then were probed for expression of these cell receptors with anti-SRB1 and anti-CD81 (BD Biosciences), along with mIgG1 and rIgG1 (R&D Systems) as isotype controls, all at the concentration of 0.5μg/mL. After another wash, Alexa Fluor 647 conjugated secondary antibody goat anti-mouse (1.6μg/mL; Invitrogen) was added. After another round of washes, the cells were fixed in 2% PFA and then samples were processed using the FACS LSRFortessa (BD Biosciences). Results were analyzed with FlowJo 10.4 (TreeStar).

2.9.2 Inhibition of glycoprotein interaction with CHO cells overexpressing HCV entry receptors

To measure inhibition of glycoprotein/receptor interaction, H77 rE1E2 or J6 sE2 protein (2.5µg/ml) were pre-incubated with HCV mAbs or vaccinated goat antisera for 1 hour at 37°C prior to adding the mixture to the cells for 1 hour at 37°C. After staining for live/dead cells as described above, glycoprotein binding onto CHO receptor overexpressing cells were detected with 5µg/mL biotinylated anti-E2 mAbs (H53 or 6F5) followed by Alexa Fluor 647 conjugated Streptavidin (1µg/mL; Jackson ImmunoResearch). After staining, the cells were fixed in 2% PFA and were processed using the FACS LSRFortessa (BD Biosciences). Results were analyzed with FlowJo 10.4 (TreeStar).

2.11 Inhibition of the binding of HCV envelope glycoproteins to GST-tagged CD81 LEL

Vectors containing GST-tagged CD81 Large Extracellular Loop or GST alone were provided by Dr. Joseph Marcotrigiano (NIH). The pGEX-4T1 vectors were transformed into Rosetta-gami™ 2 Competent Cells (Novagen, Merck, Kirkland, QC, Canada). Glutathione-Sepharose 4B beads (GE Healthcare) were washed three times with immunoprecipitation (IP) buffer (20mM Tris/150mM NaCl/0.5% Triton-X100 (v/v)/pH 7.5). Then 3X excess in volume of lysates containing GST-CD81 LEL or GST were added to the washed beads for one hour at RT. After aliquoting lysates/beads mixtures for each sample, 2µg H77 rE1E2 or 4µg J6 sE2 pre-incubated with antibodies or antisera for 30 minutes was added. After a 1 hour incubation, the samples were washed and SDS-PAGE Loading Buffer including beta-mercaptoethanol was added to the samples and they were heated at 95°C for 5 minutes. These samples were subjected to SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad) for Western Blotting. CD81 LEL pulled down H77 E2 or J6 E2 were detected with murine mAbs H53 or 6F5 respectively at a dilution of 1:2000. The pulled down CD81 protein was detected with rabbit anti-GST antibody (1:1000; Sigma-Aldrich). Alexa Fluor 750 conjugated goat anti-mouse antibody (1:10000; Invitrogen) and Alexa Fluor 680 conjugated goat anti-rabbit (1:10000; Invitrogen) were added as secondary antibodies. Antibodies were diluted in PBS/0.1% Tween-20/0.5% BSA. Then the image of the Western Blot was scanned using the LI-COR Odyssey Scanner (LI-COR). The images were analyzed with LI-COR Image Studio Lite (LI-COR).

2.12 Statistics

Statistical methods are provided in the applicable figure legends. All statistical tests were performed with Prism 7 (GraphPad Software, Inc.). One-tailed paired t-test was utilized for experiments comparing neutralizing responses of human antisera before and after vaccination. For all experiments determining statistical significance between groups of vaccinated mice in **Chapter 5** (competition ELISAs, E2 ELISAs, and neutralizing responses), one way ANOVA was used. As Gaussian distribution could not be assumed, the Kruskal-Wallis ANOVA test and the Dunn's multiple comparisons post hoc test were used. In all figures, (*) designated $P<0.05$, (**) designated $P<0.01$, and (***) designated $P<0.001$. In several experiments, statistical significance was not determined or reported, as it was deemed that statistical testing would be inappropriate (Cumming, Fidler, and Vaux 2007). In the case of experiments using goat antisera, the number of goats was deemed too low for statistical testing. In other experiments, experiments were repeated with the same aliquots and statistical testing would have been limited to testing inter-assay variability.

Table 2.1 Antibodies

Reagent name	Source	Catalog number
B6, IGH526, AR3B, AR4A, AR5A	Dr. Mansun Law	N/A; (Meunier, Russell, Goossens, et al. 2008; Law et al. 2008; Giang et al. 2012)
CBH-7, CBH-23, HC-1, HC-11, HC33.1, HC33.4, HC84.26	Dr. Steven Foung	N/A; (Keck et al. 2005, 2007, 2008, 2011, 2012, 2013; Owsianka et al. 2008)
1:7	Dr. Mats Persson	N/A; (Johansson et al. 2007)
H77.16, H77.39	Dr. Michael Diamond	N/A; (Sabo et al. 2011)
A4, H52, H53	Dr. Jean Dubuisson	N/A; (Dubuisson et al. 1994)
6F5	Dr. Elizabeth Elrod, Dr. Arash Grakoui	N/A
AP33	Dr. Arvind Patel	N/A; (Owsianka et al. 2005; Tarr et al. 2006; Desombere et al. 2017)
9E10 (Anti-NS5A)	Dr. Tim Tellinghuisen, Dr. Charles Rice	N/A
Mouse Anti-SRB1 Antibody (CLA-1/Clone 25)	BD Biosciences (Mississauga, ON, Canada)	610882
Mouse Anti-CLDN1 Antibody	R&D Systems (Minneapolis, MN, USA)	MAB4618
mIgG1 isotype control	R&D Systems (Minneapolis, MN, USA)	MAB002
Mouse Anti-Human CD81 (JS81)	BD Biosciences (Mississauga, ON, Canada)	555675
Mouse Anti-Human CD81 (5A6)	BioLegend (San Diego, CA, USA)	349501
Rat IgG2a isotype control	R&D Systems (Minneapolis, MN, USA)	MAB006

Table 2.2 ELISA Reagents

Reagent name	Source	Catalog number
Human hepatoma Huh-7.5 cells	Dr. Charles Rice (Rockefeller University, New York City, NY, USA)	N/A
Buffalo Rat Liver BRL3A cells	American Type Culture Collection (Manassas, Virginia, USA)	CRL-1442
Chinese Hamster Ovary CHO/dhFr- cells	American Type Culture Collection (Manassas, Virginia, USA)	CRL-9096
Human Embryonic Kidney HEK293T Cells	American Type Culture Collection (Manassas, Virginia, USA)	CRL-3216

Table 2.3 Cell Culture Reagents

Reagent name	Supplier	Catalog number
Dulbecco's Minimal Eagle Medium (DMEM) (Glucose 4.5 g/L, Glutamine 4 mM)	Invitrogen (Burlington, ON, Canada)	11965
Dulbecco's Minimal Eagle Medium (DMEM) (Glucose 4.5 g/L, Glutamine 4 mM, Sodium Pyruvate 1 mM)	Invitrogen (Burlington, ON, Canada)	11995
DMEM (phenol red-free)	Invitrogen (Burlington, ON, Canada)	11054-001
Methotrexate hydrate	Sigma-Aldrich (Oakville, ON, Canada)	M8407
Iscove's Modified Dulbecco's Medium (IMDM)	GE Healthcare Bio-Sciences (Pittsburgh, PA, USA)	SH30228.FS
MEM Sodium Pyruvate (100x)	Invitrogen (Burlington, ON, Canada)	11360-070
HT Supplement	Gibco (Thermo Fisher Scientific, Rockland, IL, USA)	11067-030
Nonessential Amino Acids	Gibco (Thermo Fisher Scientific, Rockland, IL, USA)	11140-050
Fetal Bovine Serum (FBS)	Omega Scientific (Tarzana, CA, USA)	FB-11
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific (Rockland, IL, USA)	12483
Penicillin/Streptomycin (10000 U/mL)	Gibco (Thermo Fisher Scientific, Rockland, IL, USA)	15140122
Trypsin/EDTA (0.5%)	Invitrogen (Burlington, ON, Canada)	15400-054
Accutase	BD Biosciences (Mississauga, ON, Canada)	561527
Amicon Ultra Centrifugal Filters 15ml 100K	EMD Millipore (Etobicoke, ON, Canada)	UFC910024
Nano-Glo Luciferase System	Promega (Madison, WI, USA)	N1130
Bright-Glo Luciferase Assay System	Promega (Madison, WI, USA)	E2620

Reagent name	Supplier	Catalog number
Poly-L-Lysine	R&D Systems (Cultrex, Minneapolis, MN, USA)	3438-100-01
Collagen I Rat Tail	Corning (VWR, Radnor, PA, USA)	CACB354236
Trypsin-EDTA (0.05%), phenol red	Gibco (Thermo Fisher Scientific, Rockland, IL, USA)	25300-062
2mm gap cuvettes	BTX (Holliston, MA, USA)	45-0125
Polybrene	Sigma-Aldrich (Oakville, ON, Canada)	AL-118
Geneticin	Gibco (Thermo Fisher Scientific, Rockland, IL, USA)	10131-035
Lipofectamine 2000	Life Technologies (Burlington, ON, Canada)	11668-019
Hygromycin B	Thermo Fisher Scientific (Rockland, IL, USA)	10687010
Blasticidin	Invivogen (San Diego, CA, USA)	anti-bl-05

Table 2.4 Antibodies and immunostaining reagents

Reagent name	Supplier	Catalog number
Mouse IgG anti-HCV core	Enzo Life Sciences (Farmingdale, NY, USA)	ALX-804-277-C100
Goat Anti-Mouse IgG (H+L) Antibody Alexa Fluor 488	Thermo Fisher Scientific (Rockland, IL, USA)	A-11001
Normal goat serum	Sigma-Aldrich (Oakville, ON, Canada)	G9023
Bovine serum albumin (BSA)	Gemini Bio-Products (West Sacramento, CA, USA)	700-100P
Liquid DAB+ Substrate Chromogen System	Agilent Technologies (Santa Clara, CA, USA)	K3468
ECL Anti-Mouse IgG, Horseradish Peroxidase linked whole antibody (from sheep)	GE Healthcare Bio-Sciences (Pittsburgh, PA, USA)	LNA931V/AG

Table 2.5 Western Blot Reagents

Reagent name	Supplier	Catalog number
Rabbit Anti-Glutathione-S-Transferase	Sigma-Aldrich (Oakville, ON, Canada)	G7781
Goat Anti-Mouse IgG (H+L) Alexa Fluor 750	Invitrogen (Thermo Fisher Scientific, Rockland, IL, USA)	A-21037
Goat Anti-Rabbit IgG (H+L) Alexa Fluor 680	Invitrogen (Thermo Fisher Scientific, Rockland, IL, USA)	A-32734
Heparin Sepharose 6 Fast Flow	GE Healthcare Life Sciences (Mississauga, ON, Canada)	17099825
Glutathione Sepharose 4 Fast Flow	GE Healthcare Life Sciences (Mississauga, ON, Canada)	17513202

Table 2.6 ELISA Reagents

Reagent name	Source	Catalog number
Lectin from Galanthus nivalis (snowdrop) lyophilized powder	Sigma-Aldrich (Oakville, ON, Canada)	L8275
Unconjugated Galanthus Nivalis Lectin (GNL)	Vector Laboratories (Burlingame, CA, USA)	L-1240
para-Nitrophenylphosphate	EMD Millipore (Etobicoke, ON, Canada)	ES009
para-Nitrophenylphosphate	Thermo Fisher Scientific (Rockland, IL, USA)	37621
Zeba Spin Desalting Columns 2mL	Thermo Fisher Scientific (Rockland, IL, USA)	89889
Recombinant Protein A	Pierce (Thermo Fisher Scientific, Rockland, IL, USA)	21184
Donkey Anti-Guinea Pig IgG (H+L) Alkaline Phosphatase	Jackson ImmunoResearch (West Grove, PA, USA)	706-055-148
Goat Anti-Mouse IgG (Fcγ) Alkaline Phosphatase	Jackson ImmunoResearch (West Grove, PA, USA)	115-055-071
Goat Anti-Human IgG (H+L) Alkaline Phosphatase	Jackson ImmunoResearch (West Grove, PA, USA)	109-055-088
Goat Anti-Rabbit IgG (H+L) Alkaline Phosphatase	Jackson ImmunoResearch (West Grove, PA, USA)	111-055-144
NeutrAvidin Protein Alkaline Phosphatase	Thermo Fisher Scientific (Rockland, IL, USA)	31002
Recombinant Human SRB1	Origene (Rockville, MD, USA)	TP310264
Recombinant Human CD-81	Origene (Rockville, MD, USA)	TP317508
EZ-Link NHS-PEG4 Biotinylation Kit	Thermo Fisher Scientific (Rockland, IL, USA)	21455
NeutrAvidin Coated High Sensitivity Plates, Clear, 8-Well Strips	Thermo Fisher Scientific (Rockland, IL, USA)	15530

Table 2.7 Chemicals and drugs

Reagent name	Source	Catalog number
Ezetimibe	Santa Cruz Biotechnology (Mississauga, ON, Canada)	sc-205690
EGCG	Santa Cruz Biotechnology (Mississauga, ON, Canada)	sc-200802A
Bafilomycin A1	Santa Cruz Biotechnology (Mississauga, ON, Canada)	sc-201550
Concanamycin A	Sigma-Aldrich (Oakville, ON, Canada)	C9705
Heparan sulfate	Sigma-Aldrich (Oakville, ON, Canada)	H3149
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (Oakville, ON, Canada)	D2650
Heparan Sulfate	Sigma-Aldrich (Oakville, ON, Canada)	H3149
Octadecyl rhodamine B chloride (R18)	Molecular Probes (Life Technologies Inc., Burlington, ON, Canada)	O-246
EZMix N-Z-Amine A	Sigma-Aldrich (Oakville, ON, Canada)	C4644
Citric acid anhydrous	Sigma-Aldrich (Oakville, ON, Canada)	251275
Normal goat serum	Sigma-Aldrich (Oakville, ON, Canada)	G9023

Table 2.8 Flow Cytometry

Reagent name	Source	Catalog number
Permeabilization Wash Buffer (10x)	BioLegend (San Diego, CA, USA)	421002
LIVE/DEAD Fixable Violet Cell Stain	Life Technologies (Burlington, ON, Canada)	L34955
Streptavidin-PE	eBioscience (Thermo Fisher Scientific, Rockland, IL, USA)	12-4317-87
$F(ab')_2$-Goat Anti-Mouse IgG (H+L) Antibody Alexa Fluor 647	Life Technologies (Burlington, ON, Canada)	a21237
Goat anti-mouse IgG Alexa Fluor 647	Thermo Fisher Scientific (Rockland, IL, USA)	A-21235
Streptavidin Alexa Fluor 647	Jackson ImmunoResearch (West Grove, PA, USA)	016-600-084

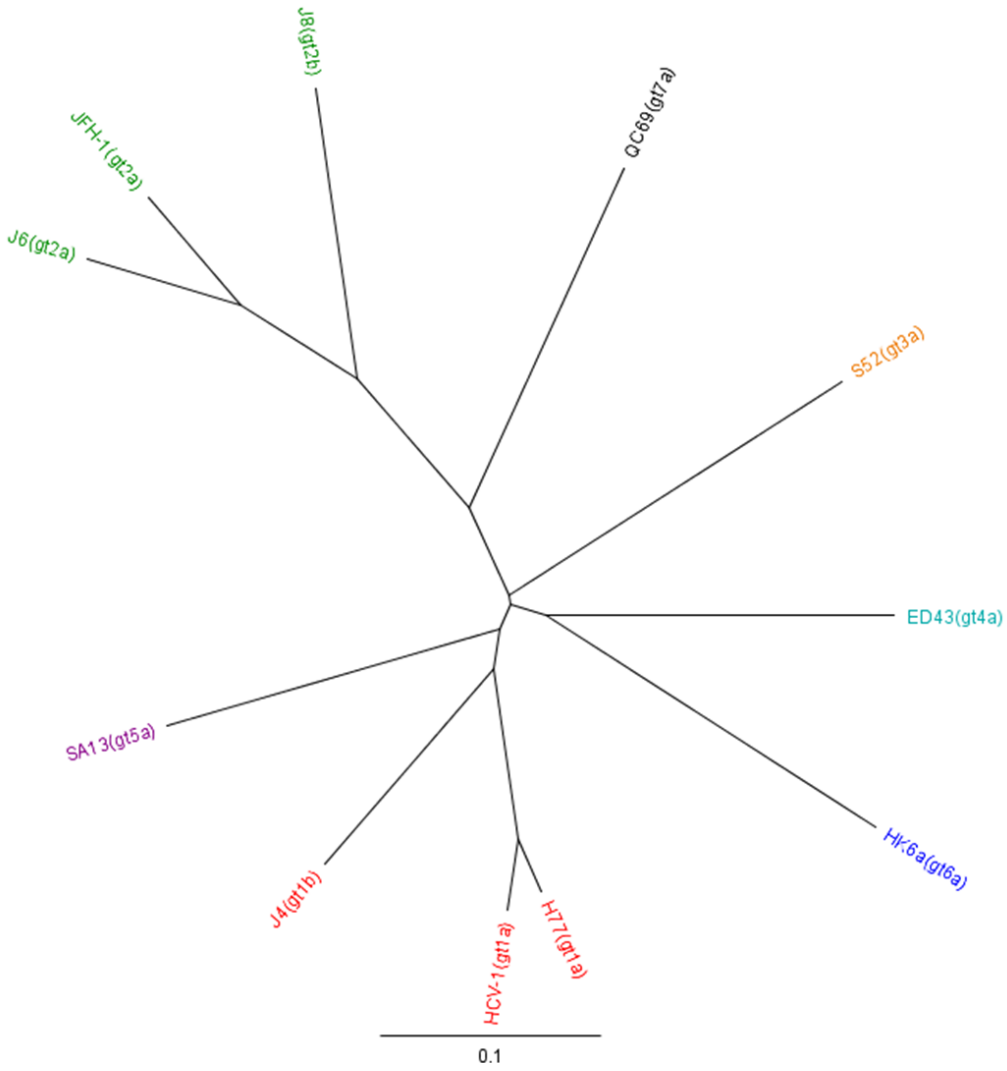


Figure 2.1 A Neighbor-Joining (NJ) phylogeny based on the E1E2 protein sequences of the 11 HCV strains relevant to this thesis.
Strains are coloured by genotype and the subgenotypes of the strains are indicated.

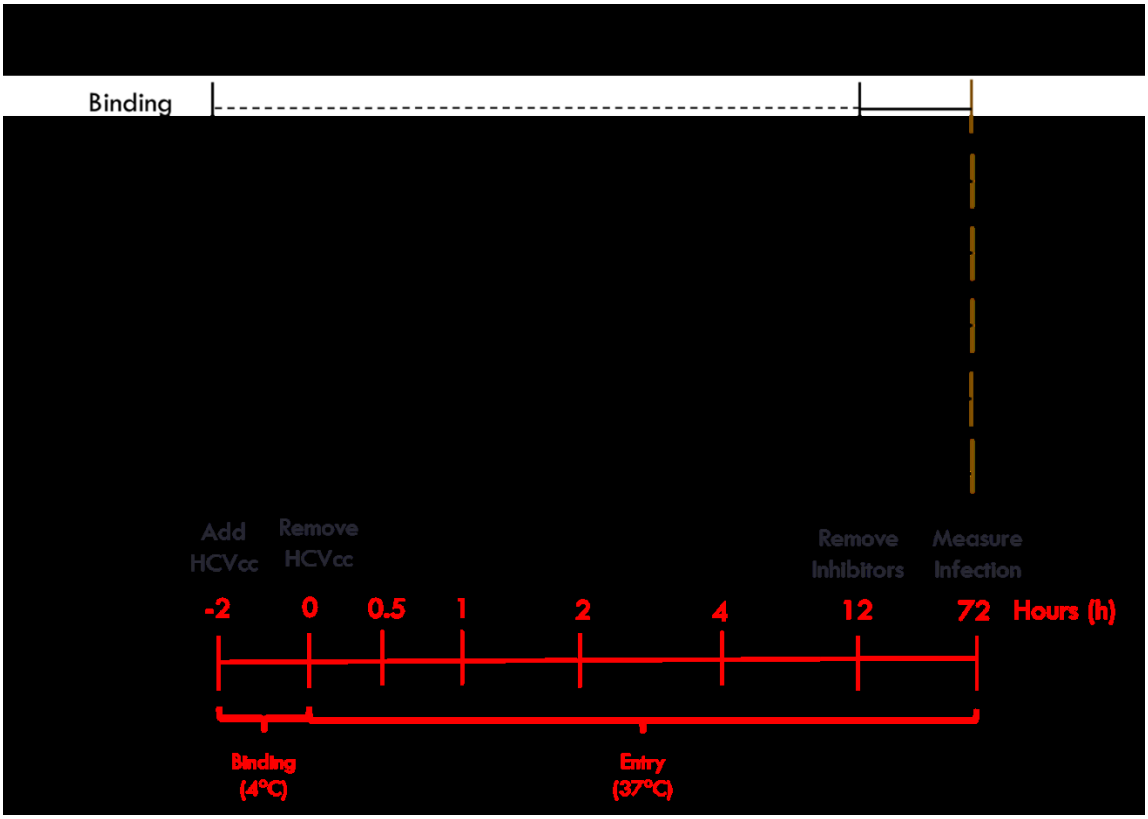


Figure 2.2 Schematic of time-of-addition synchronized neutralization assays. Neutralization assay were performed with HCVcc. Inhibitors or antisera were added at the indicated intervals relative to t = 0, the timepoint where unbound virus was washed off and the cultures were shifted from 4°C to 37°C. An anti-CD81 antibody that blocks E2-CD81 interaction (JS81) and a murine IgG1 isotype control were used at 1μg/ml, and endosomal acidification inhibitor bafilomycin A1 (BafA1) was used at 10nM. Prior to T=0, cells are kept at 4°C. This allows binding of HCVcc on cell surface but not efficient entry into cells. Dotted lines indicate the time frame where inhibitor or antisera is present. At T=12, cells were washed and replaced with fresh media. The level of infection was determined 3 days post-infection.

3 CHAPTER III: AN HCV ENVELOPE GLYCOPROTEIN VACCINE DERIVED FROM A SINGLE GENOTYPE 1A STRAIN GENERATES CROSS-NEUTRALIZING ANTIBODIES THAT ARE DIRECTED TOWARDS MULTIPLE NEUTRALIZING EPITOPES IN THE E1E2 HETERODIMER

Data in this chapter were published in the following paper:

Law, J.L., Chen, C., **Wong, J.A.,** Hockman, D., Santer, D.M., Frey, S.E., Belshe, R.B., Wakita, T., Bukh, J., Jones, C.T., Rice, C.M., Abrignani, S., Tyrrell, D.L., and Houghton, M. **2013.** A hepatitis C virus (HCV) vaccine comprising envelope glycoproteins gpE1/gpE2 derived from a single isolate elicits broad cross-genotype neutralizing antibodies in humans. ***PLoS ONE*** *8(3):e59776*.

I was responsible for the optimizations of protocols for neutralization assays. J.L. Law and M. Houghton were responsible for concept formation. J.L. Law was responsible for manuscript composition. M. Houghton was responsible for manuscript edits. J.L. Law and C. Chen were responsible for the bulk of the experimental data.

Wong, J.A., Bhat, R., Hockman, D., Logan, M., Chen, C., Levin, A., Frey, S.E., Belshe, R.B., Tyrrell, D.L., Law, J.L.M., and Houghton, M. **2014.** Recombinant Hepatitis C Virus Envelope Glycoprotein Vaccine Elicits Antibodies Targeting Multiple Epitopes on the Envelope Glycoproteins Associated with Broad Cross-Neutralization. ***J. Virol.*** *88(24): 14278-14288*.

I was responsible for the majority of the experimental data and manuscript composition. M. Houghton, J.L. Law, and I were responsible for concept formation. M. Houghton and J.L. Law assisted with manuscript edits. Generation of rE1E2 was performed by M. Logan. Virus generation and neutralization assays were performed by J.L. Law, D. Hockman, J. Johnson, A. Dahiya, C. Chen, and me.

3.1 Introduction

As indicated above, one of the major overarching goals of the Houghton Lab is to generate an effective prophylactic HCV vaccine designed to elicit broad cross-neutralizing antibodies and cross-reactive CD4+ and CD8+ T cell responses against all the world's diverse clades. Such immune responses have been shown to correlate with spontaneous recovery from acute HCV infection and a vaccine constituting CHO-derived recombinant E1E2 heterodimer along with nonstructural antigens is intended to recapitulate these responses. My thesis work has focused on the characterization of cross-neutralizing antibodies elicited by the E1E2 heterodimer. Much early research in the field concluded that anti-E1E2 neutralizing antibody responses would largely be strain-specific and that there would be little cross-genotype neutralization (Farci et al. 1994, 1996; Shimizu et al. 1996; Zibert, Schreier, and Roggendorf 1995). However, a prototypical rE1E2 vaccine derived from the gt1a strain HCV-1 has been shown to be effective at protecting chimpanzees from chronic infection following both homologous and heterologous genotype 1a challenge and surprisingly, these chimpanzee antisera were shown to cross-neutralize representative HCVpp and HCVcc from HCV genotypes 1 through 6, albeit to different extents (Meunier et al. 2011; Houghton 2011). This cross-neutralization was supported by data that guinea pigs vaccinated with the same vaccine immunogen and adjuvant were also capable of cross-neutralization of HCVpp expressing E1E2 from different gt1a, gt1b, and gt2a strains (Stamataki et al. 2007). These promising data led to a Phase I clinical trial for safety and immunogenicity performed in healthy, HCV-negative human volunteers. These volunteers exhibited strong anti-E1E2 ELISA titers, CD4+ T cell responses, and capacity for inhibiting the interaction between sE2 and CD81 (Frey et al. 2010).

As described previously, many monoclonal antibodies (mAbs) have been isolated recently that have potent neutralizing and even broadly neutralizing activity. Some of these antibodies have been well-characterized to determine where the antibody binds on the E2 surface and its mechanism of neutralization. Most of these antibodies cluster around the CD81 binding site (CD81bs) (includes E2 Epitope I and II) which is a well-defined surface on the E2 protein and inhibit the E2-CD81 interaction (Owsianka et al. 2005; Potter et al. 2012; Keck et al. 2013; Sabo et al. 2011; Keck et al. 2012; Law et al. 2008). A few antibodies inhibit the E2-SRB1 interaction and these are directed toward the C-terminal end of HVR1 and E2 Epitope I (Hsu et

al. 2003; Sabo et al. 2011; Scarselli et al. 2002). The other antibodies directed against E1 (IGH520, IGH526) and the natively folded E1E2 heterodimer (AR4A, AR5A) can be potently broad cross-neutralizing but are not well characterized (Meunier, Russell, Goossens, et al. 2008; Giang et al. 2012).

While many of these antibodies are directed against a conformational epitope, several are directed against linear peptide epitopes, the important ones being E1 Epitope I, E2 HVR1, E2 Epitope I, and E2 Epitope II. These peptide epitopes are very valuable as they allow for mAb-peptide complexes to be crystallized to understand the nature of neutralization (Krey et al. 2013; Kong et al. 2016, 2015; Potter et al. 2012; Li et al. 2015). These data are valuable as they can inform on rational vaccine design (He et al. 2015). Indeed, antisera from mice hyperimmunized with these peptides have shown anti-HVR1 and anti-E2 Epitope I antisera were neutralizing against HCVpp, while anti-E2 Epitope II antisera was not (Torresi et al. 2007).

The goals of the work covered by this chapter was to determine if a recombinant E1E2 vaccine derived from a single strain (gt1a HCV-1) would exhibit broad cross-neutralizing activity in human volunteers and use peptide mapping and competition ELISAs with well described cross-neutralizing mAbs to interrogate the antigenic regions of E1E2 that were recognized by antibodies present in neutralizing antisera.

Based on the earlier data from vaccinated chimpanzees, I hypothesized that the antibodies found in the vaccinated subjects would be cross-neutralizing *in vitro* and furthermore, that these antibodies would be binding to at least one conserved region important for critical functions such as E2- CD81 interaction.

3.2 Results

3.2.1 Humans immunized with E1E2 produce cross-neutralizing antibodies

Based on the guinea pig and chimpanzee *in vitro* neutralizing data, it was expected that there would be a broader response than a purely isolate-specific neutralizing response in the human volunteers (Stamataki et al. 2007; Meunier et al. 2011). In the first analysis of the phase I clinical trial, the maximal anti-E1E2 ELISA titers were seen at two weeks post third immunization (Frey et al. 2010). Therefore, this antisera time-point from 13 volunteers from the 100µg gt1a HCV-1 rE1E2 dose group were tested for neutralization against the heterologous

gt1a strain H77/JFH-1 HCVcc at a dilution of 1:50 after heat inactivation for 30 minutes at 56°C to remove complement. After incubation, this mixture was used to infect the Huh-7.5 cells and the resulting infection was quantified 2 days post-infection by immunohistochemistry or flow cytometry using an anti-NS5A antibody to detect the number of infected cells. Variable background in the pre-immunization sera samples is typical and likely due to the non-specific effect of components in human sera on virus entry (Bartosch et al. 2005; Meunier et al. 2005). Comparing pre-vaccination to post-vaccination sera neutralizing activity revealed that vaccination induced statistically significant higher neutralizing activity (**Figure 3.1A**). As can be seen in **Figure 3.1B**, after normalization to the pre-immunization sera, 5/13 of the volunteers exhibited moderate neutralization (>25%), and 3/13 of the volunteers exhibited strong neutralization of HCVcc infectivity (>70%).

Three antisera samples, 1, 5, and 7, were chosen to examine for cross-neutralizing activity as these had the strongest neutralizing titers against H77/JFH-1 HCVcc. Chimeric HCVcc encoding the Core-NS2 proteins derived from nine strains from all seven major global genotypes were obtained from a collaborator (Gottwein et al. 2009). Testing these antisera samples against the nine strains of HCVcc and normalizing neutralization to the pre-vaccination samples showed that antisera from both volunteers 5 and 7 were broadly cross-neutralizing (**Figure 3.1C**). However, neutralization was relatively low for gt2a, gt2b, gt3a, and gt7a. Although antisera from volunteer 1 showed generally weaker levels of cross-neutralization, all three samples showed strong neutralization against gt6a.

3.2.2 Goats immunized with E1E2 produce cross-neutralizing antibodies

Two goats (G757 and G714) were immunized with gt1a HCV-1 rE1E2, the same antigen previously utilized in a phase I clinical trial (Frey et al. 2010). The first three vaccinations were formulated with the adjuvant Addavax followed by two sequential immunizations using Complete Freund's Adjuvant and Incomplete Freund's Adjuvant (CFA/IFA). The vaccine was immunogenic: Post-Addavax and Post-Freund's antisera exhibited binding to E1E2 derived from the gt1a H77 sequence, as measured by ELISA (**Figure 3.2A**). Goat antisera also neutralized infection of heterologous gt1a strain H77/JFH-1 chimeric HCVcc. Of the two goats, antisera from G757 exhibited higher neutralizing activity than antisera from G714 (**Figure 3.2B**). The

immunogenicity was improved after boosting with the CFA/IFA formulation: there was an increase in E1E2 binding and neutralization potency after the boosts with CFA/IFA for both goats. Goat 757 was studied for neutralizing activity against HCVcc of genotype 1 to 6 strains (representing most of the clinically relevant genotypes around the world). The antisera were found to have broadly cross-neutralizing activity and notably, the neutralizing profile was comparable with human vaccinee antisera, with high levels of neutralization against gt1a, gt1b, gt4a, gt5a, and gt6a and lower levels of neutralization against gt2a, gt2b, and gt3a (**Figure 3.2C**, **Figure 3.1C**). In the subsequent characterizations of the immune response, I focused on antisera from G757 instead of G714 due to its greater neutralizing activity.

3.2.3 Cross-neutralizing antisera from vaccinated goats compete with the binding of different cross-neutralizing monoclonal antibodies

Competition ELISAs were used to determine if vaccine-elicited antibodies targeted one or more epitopes of previously described broadly cross-neutralizing mAbs. **Table 3.1** indicates the cross-neutralizing antibodies examined in this chapter and **Figure 1.16** provides a model describing the epitopes on E1E2. Five of the antibodies (AP33, HC33.4, HC84.26, 1:7, and AR3B) are capable of binding soluble E2. These mAbs target the disparate regions of E2 that form the CD81 receptor binding site (CD81bs) to block the interaction between E2 and CD81 (Law et al. 2008; Owsianka et al. 2005; Potter et al. 2012; Tarr et al. 2006; Johansson et al. 2007; Keck et al. 2012, 2013; Sabo et al. 2011). Two other antibodies (AR4A and AR5A) recognize unique epitopes outside the CD81bs and bind to the native E1E2 heterodimer and do not bind denatured E1E2 or either E1 or E2 alone (Giang et al. 2012). A4 and IGH526 recognize epitopes in E1. A4 is a non-neutralizing mAb and IGH526 is capable of cross-neutralizing various genotypes of HCV (Dubuisson et al. 1994; Helle et al. 2010; Meunier, Russell, Goossens, et al. 2008).

To determine the concentration of mAb to use in the competition studies, I titrated one of the mAbs, HC84.26, in 3-fold dilutions down from 12μg/mL on GNA-captured recombinant E1E2 derived from genotype 1a strain H77 (H77 rE1E2) in ELISAs (**Figure 3.3A**). I also tested the competition of a fixed amount of G757 antisera (1:100 dilution) in competition ELISAs against the mAbs in 3-fold dilutions down from 12μg/mL (**Figure 3.3B**). I found that a sub-

saturating amount (approximately 70% maximal binding) was associated with the highest level of competition with Post-Freund's. Therefore, for all remaining mAbs, I titrated them at the same concentrations on GNA-captured H77 E1E2 and then selected the concentration of each mAb resulting in closest to 70% maximal binding for competition studies. Both G757 Post-Addavax and Post-Freund's antisera were capable of partially inhibiting the binding of all mAbs to H77 E1E2 while G757 Pre demonstrated minimal nonspecific inhibition (**Figure 3.4A-C**). The dilution of antisera capable of inhibiting the binding of the mAbs by 50% (IC_{50}) was calculated (**Table 3.2**). Both Post-Addavax and Post-Freund's antisera were able to inhibit the binding of AP33, HC33.4, HC84.26, 1:7, AR3B, and AR5A by more than 50% at the lowest dilution of antisera tested (1:5). While substantial competition was observed with IGH526, A4, and AR4A, it was noticeably less than for the other mAbs; either Post-Addavax (IGH526 and A4) or both post-immunization bleeds (AR4A) were incapable of competing with the binding of these mAbs by at least 50% at a dilution of 1:5 (**Table 3.2**). Our results showed that goat antisera competed with the binding of mAbs targeting a variety of epitopes in E1, E2, and the E1E2 heterodimer. While the anti-E1 and anti-E2 mAbs varied in their dependence on native folding for binding, both the anti-E1E2 mAbs were conformation sensitive (**Table 3.1**, **Figure 1.16**). Interestingly, although E1 is usually considered much less immunogenic than E2, we found that the goat antisera competed with the binding of two anti-E1 antibodies (**Figure 3.4C**).

Antisera from the other rE1E2-vaccinated goat (G714) showed a very similar pattern of competition with the binding of mAbs in the panel but competition was less efficacious compared to G757 antisera since the vaccine was generally less reactive in G714 (**Figure 3.5, Figure 3.2A-C**).

3.2.4 Competition of mAb binding by goat antisera was specific

A major concern for the competition data seen above was that the antibodies present in the goat antisera were not preventing the binding of mAbs to their epitopes by recognizing the same epitopes but instead were merely occluding the epitope through steric hindrance. I attempted to address this issue with the mAbs HC33.4 and HC84.26 which bind to synthetic peptides derived from the E2 Epitope I and E2 Epitope II, respectively (Keck et al. 2012, 2013). Consistent with the literature, HC33.4 and HC84.26 bound linear synthetic peptides P409

(derived from E2 Epitope I) and P433 (derived from E2 Epitope II), respectively (**Table 3.3**, **Figure 3.6A**). I found G757 Post-Freund's antisera also directly bound these peptides as shown in **Figure 3.6B**. While immunization elicited antibodies in G757 capable of binding both peptides, the reactivity was higher for P433. Consistently, I observed better competition with G757 antisera against HC84.26 than HC33.4 (**Figure 3.4A**, **Table 3.2**). To explore the specificity of the competition with binding of mAbs to E1E2, we studied the ability of goat antisera to compete with the binding of HC33.4 and HC84.26 to their respective peptides. We found G757 antisera could compete with the binding of these mAbs to their respective peptides but did not affect nonspecific binding to an irrelevant peptide (**Figure 3.6C**). This indicates G757 antisera competes with the binding of HC33.4 and HC84.26 by directly blocking interaction with their target epitopes.

3.2.5 Cross-neutralizing antisera from vaccinated humans compete with the binding of cross-neutralizing monoclonal antibodies

I extended these findings to the rE1E2-vaccinated human volunteers and investigated if antibodies targeting the epitopes of various cross-neutralizing mAbs were elicited. Competition assays were conducted with antisera from five of the volunteers vaccinated in the clinical phase I trial in the 100µg dose group. The Pre and Post antisera samples were from volunteers 2, 5, 7, 10, 14. Post samples from two of these volunteers (5 and 7) were found to have broad cross-neutralizing activity against chimeric HCVcc expressing the structural proteins from strains from all 7 HCV genotypes (**Figure 3.1C**; (Law et al. 2013)). Due to the paucity of vaccinees' antisera, we tested competition with just five mAbs targeting epitopes within either E1 alone, E2 alone or the E1E2 heterodimer (IGH526, AP33, AR3B, AR4A, AR5A) (**Figure 3.7A**,**B**). Since all the mAbs except for AP33 were human derived, these mAbs were biotinylated to enable detection in competition ELISAs. Pre-vaccination antisera from all the volunteers exhibited no effect on binding of all tested mAbs compared to no serum control (**Figure 3.7A**). However, antibodies recognizing epitopes of these mAbs were detected after vaccination. There was a similar pattern of competition exhibited by all the volunteers with competition being superior against the anti-E2 mAbs and anti-E1E2 mAb AR5A and weaker against anti-E1 mAb IGH526 and anti-E1E2 mAb AR4A. Post-vaccination antisera from volunteer 5 blocked binding of the mAbs to an

extent comparable with antisera from G757 taken after the initial three immunizations with Addavax-adjuvanted E1E2 (**Figure 3.7B**). This is consistent since both species received a similar immunogen and adjuvant formulation and immunization regimen. We also observed a dose-dependent effect as competition was enhanced at 1:10 compared to 1:30.

3.2.6 Sera from chronically infected patients compete with the binding of cross-neutralizing monoclonal antibodies

I showed that both goat and humans vaccinated with HCV-1 rE1E2 produced antibodies that competed with the binding of different cross-neutralizing mAbs and furthermore, both species exhibited a similar pattern with similar levels of competition between the anti-E2 mAbs, all of which bound to different regions of the CD81 binding surface, along with weaker competition against mAbs binding rarer epitopes (anti-E1 and anti-E1E2 mAbs).

I used sera from chronically infected HCV patients to determine if the pattern of competition of the mAb panel would be similar to the vaccinated humans and goats. I obtained seven chronically infected patient sera samples and the patient information is listed in **Table 3.4**. I first tested the ability of the sera, which was from patients infected with diverse HCV genotypes, for the ability to bind GNA-captured H77 rE1E2 lysates (**Figure 3.8A**). I found that all the sera samples bound the target protein significantly – in fact, the samples with the highest responses were nearly saturating at the highest dilution of 1:1600 – while the control sera (Pooled Negative Sera; PNS) displayed much lower background binding to H77 rE1E2. I also assayed for the ability of these HCV patient sera samples to neutralize H77 HCVpp infectivity and all samples could potently neutralize infectivity at a dilution of 1:50, while control sera showed low non-specific neutralization (**Figure 3.8B**,**C**). Based on both assays, I chose four sera samples to study further: two high responders (CS174 and CS178), one of the weakest responders (CS185), and the control PNS. I tested the ability of these chronically infected patient sera samples to compete with the binding of cross-neutralizing mAbs IGH526, AP33, AR3B, AR4A, and AR5A – the same mAbs that I tested against vaccinated goats and humans (**Figure 3.9A**,**B**). At dilutions of 1:25 and 1:100, PNS showed very low nonspecific competition, while CS174 and CS178 showed high levels of competition against all the mAbs. Comparisons with the vaccinated goats (**Figure 3.4A-C**, **Figure 3.5**) show an interesting finding – it appears that

the CS174 and CS178 competes with all the mAbs relatively equally. However, the goat antisera competition data indicates AR4A and IGH526 represented rarer epitopes more difficult to elicit antibodies towards. In keeping with the evidence competition is tied to anti-E1E2 ELISA data, CS185 competition was lower than for the other CS samples (**Figure 3.8A**, **Figure 3.9A**,**B**). Interestingly, despite the lower competition and anti-E1E2 ELISA titers, CS185 still possessed potent neutralizing activity against H77 HCVpp (**Figure 3.8B**,**C**).

3.2.7 Human antisera recognize linear peptides from E1E2 sequence

I sought to assay the human vaccinee antisera for reactivity to synthetic linear peptides derived from the E1E2 sequence. However, the extreme paucity of vaccinee antisera remained a major limitation. There were too little antisera to conduct standard ELISAs with peptides coated on a monolayer surface. Therefore, I used a technology named CelluSpot™ (Intavis Bioanalytical Instruments, Koeln, Germany) to determine the linear epitopes recognized by the vaccine-induced sera. This technology allows for peptides to be synthesized on a small spot on a 3D-modified cellulose membrane that results in 1000-fold more copies of the peptide as compared with conventional monolayer deposition. Furthermore, up to 384 peptides in duplicate can be contained on a single planar surface (glass slide). The signal is developed akin to a western blot. The combination of the high copy number of the peptide (allowing for high sensitivity) and the large number of peptides that can be assayed at once allowed for the conservation of the vaccinee antisera, but this method does not provide strong quantitative data.

Developing CelluSpot™ arrays produced a similar result to a Western Blot with each spot representing the reactivity of the antibodies in the antisera sample to each peptide (**Figure 3.10A**). The reactivity was quantified by MultiGauge V2.0 software (FujiFilm, Valhalla, NY, USA). There was much nonspecific binding by the pre-vaccination sera to the peptides on the array; the specific binding was determined by analyzing the change between pre-vaccination and post-vaccination antisera. This change was scored on a scale as (---), representing a negative change with post-vaccination antisera being less reactive to the peptide than pre-vaccination sera, and (+++), representing a positive change and that post-vaccination antisera was more reactive to the peptide than pre-vaccination sera (**Figure 3.10B**). For convenient analysis of the results, I built the results into a heat map seen in **Figure 3.11**, which shows the results from four

neutralizing human vaccinee samples and one non-neutralizing sample. The breadth and strength of reactivity partly corresponded with the level of measured neutralizing activity.

While the breadth of positive reactivity was generally inconsistent with neutralizing activity to the heterologous gt1a H77/JFH-1 virus seen in **Figure 3.1B**, all the vaccinees that showed significant neutralizing activity (>25%) (vaccinees 1, 2, 3, 5, 6, and 7) were reactive to one or more linear epitopes associated with neutralization (E1 Epitope 1 (aa313-327), the C-terminal half of HVR1 (aa396-407), E2 Epitope I (412-423), and E2 Epitope II (434-446). On the other hand, the poorly neutralizing vaccinees (vaccinees 9, 10, 11) either did not show any reactivity to any of the peptides or did not show reactivity to the same neutralizing epitopes as the neutralizing vaccinees (**Figure 3.11**, **Figure 3.1B**). Vaccinee 9 recognized an HVR1 peptide – however this was the N-terminal half of HVR1 that is not associated with neutralization. Surprisingly, although cross-neutralizing vaccinee 7 showed the highest breadth of recognized epitopes across all tested vaccinees, including the 4 neutralizing linear epitopes mentioned above and one region containing residues highly critical for E2-CD81 interaction (aa523-535), vaccinee 5, which was also cross-neutralizing, exhibited substantial negative change and only showed recognition of a single peptide (E2 Epitope II (aa434-446)) (**Figure 3.11**, **Figure 3.1C**). Many of these very positive changes seen with the neutralizing vaccinees' antisera were seen in regions in E1 and E2 not associated with any characterized neutralizing mAb and they included a number of viral fusion peptide candidates in E1 and E2 as well as regions suspected to be important for proper E1E2 heterodimerization and folding to support proper conformation of the AR4A and AR5A epitopes (N-terminus of E1 and Stem region of E2) (Lavillette et al. 2007; Giang et al. 2012; Albecka et al. 2011; Kachko et al. 2011).

3.2.8 Cross-neutralizing goat antisera recognize peptides from E1E2 sequence

The results from the CelluSpot™ arrays were then used to design a series of biotinylated synthetic peptides to further probe the peptide recognition profiles of goats vaccinated with HCV-1 rE1E2 in a system providing strong quantitative data. These peptides were used for a conventional peptide ELISA using biotinylated peptides coated to NeutrAvidin-treated plates. The peptides were derived from the gt1a HCV-1 strain and were modeled after the hotspots from the CelluSpot™ arrays. These peptides are listed in **Table 3.3** and included the E1 Epitope I, E2

Epitope I, and E2 Epitope II neutralizing epitopes, as well as novel peptides that were found to react with neutralizing human vaccinee antisera – this included two peptides from the stem region and a few peptides from the predicted viral fusion regions. One of these predicted fusion regions aa496-515 was shown to be associated with neutralization from chimpanzees vaccinated with H77 rE1E2 (Kachko et al. 2011). Since the vaccinated human volunteer antisera was in short supply and the biotinylated peptide ELISA assay required so much antisera, G757 antisera was used instead. These biotinylated peptides were bound to NeutrAvidin plates and then G757 antisera was added at a 1:50 dilution and signal was developed with AP-conjugated secondary antibodies and para-Nitrophenylphosphate (pNPP) substrate. The results were normalized to the binding of pre-vaccination sera (**Figure 3.12A**). This assay was also repeated using purified IgG from G757 sera to reduce background binding (**Figure 3.12B**). As can be seen from the data, while G757 Post-Addavax antisera recognized only P433 (containing E2 Epitope II) weakly, G757 Post-Freund's showed high reactivity for P409 (containing E2 Epitope I), P433 (containing E2 Epitope II), and for the two peptides derived from the stem region (P661 and P681).

3.3 Discussion

Previously, it was shown that a genotype 1a rE1E2 (strain HCV-1) vaccine formulated with various oil-in-water adjuvants was able to prevent heterologous and homologous chronic infection upon genotype 1a challenge with strong statistical significance (Houghton 2011). It was also found that the antisera from vaccinated chimpanzees and guinea pigs had broad cross-genotype neutralizing activity *in vitro* (Meunier et al. 2011; Stamataki et al. 2007). This led to the HCV-1 rE1E2 vaccine formulated in the MF59C.1 oil-in-water adjuvant being used to vaccinate healthy human volunteers. This vaccine was found to be safe and immunogenic in assays testing the CD4+ T-cell and antibody responses of the volunteers and vaccinated human antisera was shown to have neutralizing activity against gt1a HCV-1 HCVpp and gt1a H77/JFH-1 HCVcc *in vitro* (Frey et al. 2010; Ray et al. 2010). We found the human volunteers that received the highest dose (100μg) showed a statistically significant increase in neutralization after vaccination and three responders showed strong neutralization (**Figure 3.1A**,**B**). One of the moderate responders showed relatively weak cross-neutralization while the two strong responders showed broad cross-neutralization to most of the strains (**Figure 3.1C**). While there

was variation and the low number of subjects (n=3) does not allow for clear conclusions to be formed, neutralization was generally moderate to high against genotypes 1a, 1b, 2a, 4a, 5a, and 6a and weaker against genotypes 2b, 3a, and 7a. This suggested that while there must be antibodies in the antisera recognizing highly conserved epitopes on E1E2, there must also be variable neutralizing epitopes that are being targeted – epitopes that are conserved between some genotypes and not others. This also indicated that concerns over a strain-specific neutralizing antibody response were incorrect according to the three subjects studied and that a vaccine based on the envelope glycoproteins derived from a single strain could elicit broad cross-neutralization. This also suggests the possibility that a small cocktail of rE1E2 antigens derived from just 2 or 3 strains could provide optimal cross-neutralization of all the world's highly heterogeneous strains.

Given the paucity of the human vaccinees samples, the same vaccine antigen was used to immunize two goats (G757 and G714) as a surrogate model formulated with Addavax™ (an oil-in-water adjuvant similar to MF59C.1) for the first three immunizations, followed by boosts with the Complete and Incomplete Freund's Adjuvant. Therefore, the Post-Addavax goat antisera sample was comparable to the human vaccinee antisera in vaccine formulation while the Post-Freund's immunizations elicited a stronger response. Post-Addavax and Post-Freund's antisera from both goats were able to bind H77 rE1E2 strongly relative to Pre, which showed a very low background (**Figure 3.2A**). Post-Freund's bound E1E2 much stronger than Post-Addavax. This difference was echoed in the H77/JFH-1 HCVcc assay, where Post-Freund's showed stronger neutralization than Post-Addavax in both goats (**Figure 3.2B**). However, G757 possessed stronger neutralizing activity than G714, which was surprising as both goats showed comparable E1E2 binding in ELISAs – indicating G714 possessed an antibody response consisting of a higher level of non-neutralizing antibodies and/or antibodies that interfered with neutralization (**Figure 3.2A,B**). The existence of antibodies within anti-HCV antisera that can interfere with neutralization has been reported before (Keck et al. 2016; Kachko et al. 2015; Zhang et al. 2009, 2007).

It is interesting that the G757 Post-Freund's antisera showed a somewhat similar pattern to the human volunteer antisera for cross-neutralization: strong against most of the genotypes but relatively low against gt2a, gt2b, and gt3a. Overall, for the goat antisera this difference is far more striking. The gt2a, gt2b, and gt3a strains show very low neutralizing activity – lower than that of the human vaccinees' antisera – yet the others show strong and uniform neutralization

(**Figure 3.2C**). Notably, G757 Post-Freund's antisera was very ineffective at neutralizing gt2a HCVcc but 2/3 of the human vaccinees tested could neutralize the virus moderately well (~50% neutralization). The enhanced response against some of the genotypes by the goat antisera relative to the human vaccinee antisera could be attributed to the extended adjuvant schedule that included stronger and harsher adjuvants, but the lower activity seen against gt2a, gt2b, and gt3a is not as easy to explain. It is possible either the CFA/IFA adjuvants modify the E1E2 protein in a way that could elicit that kind of genotype-specific response – indeed CFA/IFA is somewhat denaturing and is known to have a structural effect on the immunogen it is carried with – or it could be indicative of the different humoral immune responses between goats and humans (Barinova et al. 2017). If it is in part due to the former option, studying this interaction further could help determine the neutralizing epitopes that are altered by the CFA/IFA adjuvanted boosts.

Of note, there are two adaptive mutations in the E1E2 proteins in the gt6a HK6a/JFH-1 strain (F350S in E1 and N417T in E2). N417 is an N-glycosylation site within the E2 Epitope I (aa412-423), an epitope associated with cross-neutralizing antibodies. It has been shown that N417S/T causes a glycan shift to N415 which was shown to make the virus insensitive to neutralization by mAbs directed against E2 Epitope I (Pantua et al. 2013). Further experimentation should be performed to determine if this glycan shift could be the reason for the near complete neutralization of HK6a/JFH-1 by G757 and all three human volunteers (**Figure 3.1C**, **Figure 3.2C**).

As expected, G757 Post-Freund's showed higher competition activity against the mAbs than Post-Addavax – this was consistent with the higher rE1E2 ELISA titers and neutralizing activity (**Figure 3.2A-C**, **Figure 3.4A-C**). Despite significant differences in neutralization, both goats showed a similar pattern in competition ELISAs across different mAbs which indicated the two goats reacted similarly against the antigen (**Figure 3.2B**, **Figure 3.5**). Antisera from the human volunteers was also shown to have similar competition ELISA profiles compared to antisera from the goats – this indicated the goat immune response to the vaccine was similar to the human immune response (**Figure 3.5**, **Figure 3.7A**,**B**).

Competition was strongest against anti-E2 mAbs blocking E2-CD81 interaction for Post-Freund's from both goats (**Figure 3.4A-C**, **Figure 3.5**). This was consistent with the previous finding that the human vaccinees could block this interaction (Frey et al. 2010). There was strong

competition seen against AR5A by Post-Freund's from both goats, but competition against the other antibodies targeting E1 and E1E2 were weaker compared to the other mAbs except for AR5A. This was disappointing as anti-E1E2 mAb AR4A was found to be potently and broadly cross-neutralizing and AR4A is currently being considered for post-transplant treatment (Giang et al. 2012; Carlsen et al. 2014). However, both AR4A and AR5A are considered to be rare mAbs as they were only discovered through exhaustive panning techniques using anti-E2 mAbs to mask already identified epitopes (Giang et al. 2012). Although recognition of AR5A requires native E1E2, binding to E1E2 was shown to be effectively competed by CBH-7, an anti-E2 mAb that inhibits the E2-CD81 interaction (Giang et al. 2012; Owsianka et al. 2008). As such, it is possible that the antibodies present in vaccine antisera compete with the binding of AR5A in a similar fashion to CBH-7 and prevent E2-CD81 interaction. As the AR5A epitope is still poorly understood, it is very difficult to determine the specificity of the competition. Competition with IGH526 by goat and human vaccine antisera was also reduced in comparison with the other mAbs (**Figure 3.4A-C**, **Figure 3.5**, **Figure 3.7A**,**B**). E1 has been regarded as a poorly immunogenic protein and more attention has been paid to E2 as a neutralizing antibody target. With only one neutralizing epitope exclusively in E1 characterized so far (aa313-327), there is likely much more to learn about neutralizing epitopes on E1 and their role in protection (Meunier, Russell, Goossens, et al. 2008). Recombinant E1 protein derived from a genotype 1b strain elicited neutralizing antibodies in two chimpanzees and protected them from heterologous genotype 1b viral challenge (Verstrepen et al. 2011). Along with evidence that rE1E2 showed higher immunogenicity than sE2 in vaccinated chimpanzees and the lower variability of E1, there is potential in improving E1 as a vaccine antigen in future vaccines.

When neutralizing human vaccinees' antisera were tested to determine competition against mAbs, it was found that there was moderate competition against all the mAbs tested and while the pattern of competition was mostly similar to the Post-Freund's from the goats, the difference in competition against different mAbs was not as dramatic (**Figure 3.7A,B**). These data show the immune responses from both goats and humans were quite similar, but Post-Freund's goat antisera competed strongly and specifically with AP33, AR3B, and AR5A. As this is not seen with Post-Addavax, it's feasible that the CFA/IFA adjuvant regimen may have directed a high response to these epitopes (**Figure 3.7B**).

A critical issue was whether the antisera were competing with the binding of the mAbs specifically by containing antibodies that targeted the same epitope as the mAbs or if it was a matter of less specific or non-specific steric inhibition. This was not an easy question to answer as most of the epitopes of the mAbs were highly discontinuous. However, three of the mAbs do have an epitope that can be approximately represented by a peptide: HC33.4 binds aa412-423 and HC84.26 binds aa434-446. It was found that G757 Post-Freund's could bind these peptides directly and could also specifically compete with the binding of the mAbs to their respective peptide (**Figure 3.6B**,**C**). This indicated that at least in the cases of these two mAbs, there was specific binding to the epitope. Creating scaffolds presenting specific mimics of discontinuous epitopes is a technique currently being used and in the future it is likely specificity of competition can be verified for these anti-HCV mAbs with highly discontinuous epitopes as their epitopes are better understood (Mulder et al. 2013).

The data showed in **Figure 3.4A-C**, **Figure 3.5**, and **Figure 3.7A**,**B** demonstrated the HCV-1 rE1E2 vaccine elicits cross-neutralizing antibodies that can compete with a wide variety of mAbs targeting distinct epitopes associated with E2-CD81 interaction and other unique neutralizing epitopes formed by E1 and E1E2. As the elicited immune response appears to target distinct neutralizing epitopes, this indicates this vaccine would likely still be effective against HCV escape mutants to just one or two neutralizing epitopes, which adds great promise to this vaccine. In addition, there could be other neutralizing epitopes that have not been identified yet that were targeted by vaccine-elicited antibodies.

Sera from chronically infected patients were tested in these assays to determine if the rarer epitopes were exposed differently on the E1E2 on the surface of the native virion than on rE1E2. Sera samples were shown to have high binding to H77 E1E2 and high neutralizing activity against H77/JFH-1 HCVcc (**Figure 3.8A**,**B**). The chronically infected strong responders were capable of strong competition while the chronically infected moderate responder showed very weak competition (**Figure 3.9A**,**B**). The observed competition across the mAbs was relatively consistent – this was seen in the human vaccinee antisera as well, albeit with less overall competition activity (**Figure 3.4A-C**, **Figure 3.5**, **Figure 3.7A**,**B**, **Figure 3.9A**,**B**). Although small sample sizes limit a conclusion to be made, it does appear that the Post-Freund's goat antisera were especially good at competing with the binding of AP33, AR3B, and AR5A. It

will be important to find a safer adjuvant that can similarly enhance antibodies targeting critical neutralizing epitopes in humans.

Peptide mapping was performed on human vaccine antisera with CelluSpot™ arrays. This presented highly variable data – however, all the vaccine antisera samples that showed clear neutralizing responses against H77/JFH-1 HCVcc (>25% neutralization) bound to at least one peptide or more that were associated with neutralization (these peptides are indicated in **Figure 3.11** outlined in blue boxes and include aa313-327 (E1 Epitope 1), aa385-407 (HVR-1), aa409-423 (E2 Epitope I), aa433-447 (E2 Epitope II), and 521-535 (E2 CD81 binding loop)) while the non-neutralizing antisera did not bind to peptides associated with neutralization. Indeed, although non-neutralizing volunteer 9 bound to an HVR1 peptide, it did not bind the section associated with neutralization (396-410) ((**Figure 3.11**); (Sabo et al. 2011; Flint, Thomas, et al. 1999)). The notable outlier was volunteer 3 which was less than 25% neutralizing against H77/JFH-1 but still bound E2 Epitope I. The lack of a tight correlation is likely in part due to the fact the peptide sequences were from a genotype 1 consensus sequence and not the homologous gt1a HCV-1 strain that was used to vaccinate the human volunteers along with high background nonspecific binding. As well, neutralizing activity was very likely partly due to antibodies not binding linear peptide epitopes. However, these assays did provide interesting data and led to the design of biotinylated peptides for a quantitative assay with peptides derived from the gt1a HCV-1 sequence that sharply reduced background nonspecific binding.

When tested with G757 antisera, this assay revealed a specificity for four peptides, E2 Epitope I, E2 Epitope II, and two novel peptides in the E2 stem region (**Figure 3.12A**,**B**). As mentioned before, E2 Epitope I and E2 Epitope II have been reported a number of times to be associated with cross-neutralization (Tarr et al. 2006; Keck et al. 2012; Owsianka et al. 2005; Torresi et al. 2007). A deletion of aa651-681 encompassing the novel peptide region P661 (aa661-677) was derived from shows this region is critical for HCVpp infectivity, but no other roles for this region have been identified (Albecka et al. 2011). It has been shown that residues within the conserved hydrophobic membrane-proximal heptad repeat region of E2 (aa675-699), which encompasses all the residues found within P681 (aa681-699), was shown to be required for proper heterodimerization and viral entry (Drummer and Poumbourios 2004). Consistently, an insertion at aa692, which falls within the P681 (aa681-699) region, was shown to interfere with E1E2 heterodimerization (Rychłowska et al. 2011). A synthetic peptide derived from

aa671-705, which encompasses 7 of the residues within P661 (aa661-677) and all the residues within P681 (aa681-699) from the gt1a H77 strain was found to broadly and potently inhibit fusion of HCVcc (Chi et al. 2016). Overall, these data indicate that the region the two novel peptides were derived from are associated with E1E2 heterodimerization and fusion. Considering these are critical functions of the virus, this is a highly conserved region, and our vaccine could elicit strong responses to these peptides, this warrants further study.

In conclusion, despite very high viral heterogeneity, an HCV vaccine consisting of recombinant envelope glycoproteins derived from a single gt1a strain can elicit a very broad cross-neutralizing antibody response. This provides hope for an effective prophylactic vaccine that can combat the huge global diversity of the virus and assuages fears that the neutralizing response against a single strain would be mostly strain-specific. These data indicate it is likely that highly conserved neutralizing epitopes are present despite the high genetic variation. Consistently, I have shown the antibodies elicited by the vaccine can compete with the binding of cross-neutralizing mAbs targeting various linear and discontinuous epitopes within E1, E2, and the E1E2 heterodimer. This will make it harder for the virus to evade such a broadly cross-neutralizing antibody response in vaccines. Lastly, the importance of adjuvant was also clear from my studies indicating the importance of selecting a powerful but safe adjuvant in the future clinical development of this vaccine candidate.

Monoclonal Antibody	Species	Protein Targeted	Conformation Dependent?	Binds Peptide?	Critical Binding Residues	Interaction Targeted	Neutralization Potency	Reference
AP33	Mouse	E2	No	Yes 412-423	413,415,418,420	CD81	1a,1b,2a,4a,5a,6a,7a (HCVcc) 1a,1b,2a,3a,4a,5a,6a (HCVpp)	(Owsianka et al. 2005; Tarr et al. 2006; Desombere et al. 2017)
HC33.4	Human	E2	No	Yes 412-423	408,413,420	CD81	1a,2a,3a,4a,5a,6a (HCVcc)	(Keck et al. 2013)
HC84.26	Human	E2	Yes	Yes 434-446	429,441,442,616	CD81	1a,2a,3a,4a,5a,6a (HCVcc)	(Keck et al. 2012)
1:7	Human	E2	Yes	N/A	415,417,484,491, 523,525,526,527, 529,530,533,535, 540	CD81	2a (HCVcc) 1a,1b,2a,2b,3,4,5,6 (HCVpp)	(Johansson et al. 2007)
AR3B	Human	E2	Yes	N/A	412,416,418,423, 424,523,525,530, 535,540	CD81	2a (HCVcc) 1a,1b,2a,2b,4,5 (HCVpp)	(Law et al. 2008)
A4	Mouse	E1	No	Yes 197-207	197-207	N/A	No	(Dubuisson et al. 1994)
IGH526	Human	E1	N/A	Yes 313-327	313-327	N/A	1a,1b,2a,4a,5a,6a (HCVcc) 1a,2a,4a,5a,6a (HCVpp)	(Meunier, Russell, Goossens, et al. 2008)
AR4A	Human	E1E2	Yes		487,692	N/A	1a,1b,2a,3a,4a,5a,6a (HCVcc) 1a,1b,2a,2b,2i,2x,3a,4,5,6 (HCVpp)	(Giang et al. 2012)
AR5A	Human	E1E2	Yes		639	N/A	1a,2a,4a,5a,6a (HCVcc) 1a,1b,4,5,6 (HCVpp)	(Giang et al. 2012)

Table 3.1 List of monoclonal antibodies used in the competition ELISAs.
Neutralization potency is determined by an IC_{50} cutoff of less than 100μg/mL. Critical binding residues are determined by 25% or less binding to antigen after mutagenesis of residue to alanine. All amino acid numbering is based on the H77 isolate strain (GenBank accession number: AF009606) as specified by international consensus (Kuiken and Simmonds 2009).

	G757 Antisera (IC_{50})	
Monoclonal Antibody	Post-Addavax	Post-Freund's
AP33	1:7.4	1:145
HC33.4	1:12.4	1:252
HC84.26	1:30	<1:400
1:7	1:18.0	1:296
AR3B	1:22.7	<1:400
AR4A	>1:5	>1:5
AR5A	1:17.2	1:244
IGH526	>1:5	1:8.75
A4	>1:5	1:44.3

Table 3.2 Dilution of G757 goat antisera capable of inhibiting binding of mAbs by 50% (IC50).
The IC50 values were calculated using a range of dilutions from 1:5 to 1:400 and curve fit method in Prism 7 (GraphPad Software, Inc.). ">1:5" indicates the immunized goat antisera sample was incapable of inhibiting binding of a mAb by at least 50% with the lowest dilution (1:5).

Name	Source	Peptide Sequence	AA	Notes
P258	HCV-1 (gt1a)	LRRHIDLLVGSATLC	258-272	
P272	HCV-1 (gt1a)	CSALYVGDLCGSVFL	272-286	Putative FP
P313	HCV-1 (gt1a)	ITGHRMAWDMMMNWS	313-327	E1 Epitope I
P409	HCV-1 (gt1a)	QNVQLINTNGSWHLN	409-423	E2 Epitope I
P433	HCV-1 (gt1a)	LNTGWLAGLFYHHKF	433-447	E2 Epitope II
P503	HCV-1 (gt1a)	CGPVYCFTPSPVVVG	503-517	FP?
P553	HCV-1 (gt1a)	TWMNSTGFTKVCGAPPC	553-569	FP?
P581	HCV-1 (gt1a)	CPTDCFRKHPDATYSR	581-596	
P597	HCV-1 (gt1a)	CGSGPWITPRCLVDYPY	597-613	FP?
P625	HCV-1 (gt1a)	TIFKIRMYVGGVEHC	625-638	
P644	HCV-1 (gt1a)	CNWTRGERCDLEDRDR	644-659	
P661	HCV-1 (gt1a)	ELSPLLLTTTQWQVLPC	661-677	Stem
P681	HCV-1 (gt1a)	TLPALSTGLIHLHQNIVC	681-699	Stem
C100c	HCV gt1a Consensus	ILRRHVGPGEGAVQWMNRLIAFASRGNHVSPTHY VPESDA		NS4A
GE-13	Rice protein (*Oryza sativa japonica)* – irrelevant peptide	GYIRGLFPNVLRE	N/A	

Table 3.3 List of peptides used in the biotinylated peptide assay and used to immunize animals.

The peptides used in biotinylated peptide assays are listed. The strain the sequence was derived from, the sequence, the amino acid numbering, and notes are indicated. Some peptides were used to immunize small animals and a terminal cysteine was required for conjugation to key limpet hemocyanin, a T-cell antigen. In some cases, a cysteine had to be added although it was not in the original HCV sequence (indicated in red). The notes indicate the peptides that contain regions associated with neutralization (HVR1, E1 Epitope I, E2 Epitope I, E2 Epitope II), peptides that are found in the E2 stem region, and peptides that contain at least part of a putative fusion peptide (FP). All amino acid numbering is based on the H77 isolate strain (GenBank accession number: AF009606) as specified by international consensus (Kuiken and Simmonds 2009).

Patient Sera Sample	Genotype	Sex	Age at sample	Est. Length of Infection	Additional Notes
CS15	gt1a	Female	62	<20 years	Cirrhosis
CS27	gt2b	Female	50	<20 years	Normal Liver
CS174	gt1a	Female	64	N/A	N/A
CS178	gt1a	Male	62	N/A	N/A
CS181	gt1a	Male	54	N/A	N/A
CS185	gt1a	Male	52	N/A	N/A
CS189	gt1a	Male	60	N/A	N/A
CS195	gt1a	Male	62	N/A	N/A

Table 3.4 Details on chronically infected patient sera samples.
These patient sera samples were drawn by the Provincial Laboratory for Public Health (samples were provided by the laboratory of Dr. D. Lorne Tyrrell).

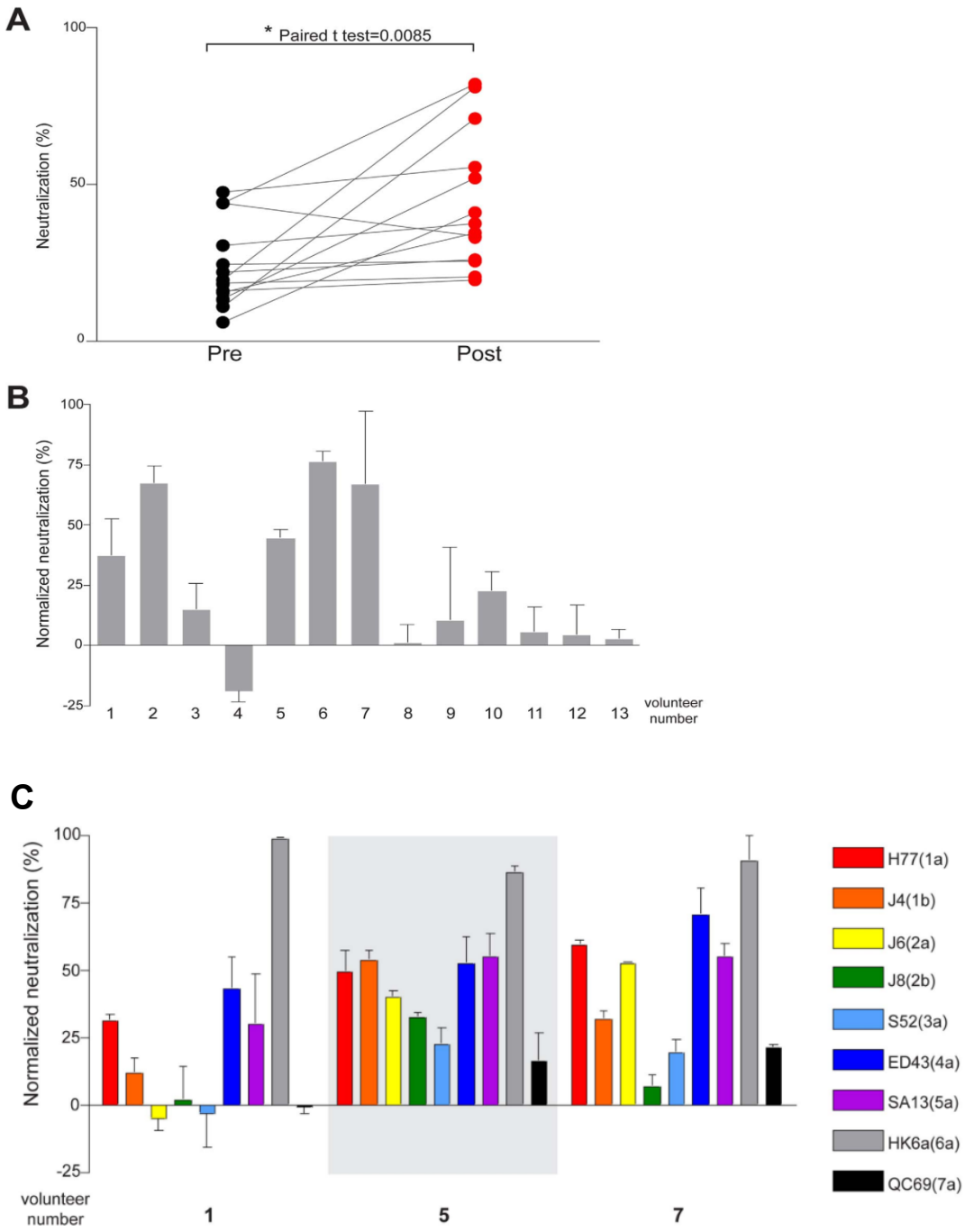

A
* Paired t test=0.0085
Neutralization (%)
100
50
0
Pre
Post
B
Normalized neutralization (%)
100
75
50
25
0
-25
1 2 3 4 5 6 7 8 9 10 11 12 13
volunteer number
C
Normalized neutralization (%)
100
75
50
25
0
-25
volunteer number
1
5
7
H77(1a)
J4(1b)
J6(2a)
J8(2b)
S52(3a)
ED43(4a)
SA13(5a)
HK6a(6a)
QC69(7a)

Figure 3.1 Antisera from humans vaccinated with gt1a strain HCV-1 rE1E2 neutralizes HCVcc infectivity in cell culture.
(A and B) Pre (prior to vaccination) or Post antisera (2 weeks post-3rd immunization) at a dilution of 1:50 was incubated with chimeric H77/JFH-1 HCVcc and then the mixture was used to infect Huh-7.5 cells – infectivity was detected 2 days later by NS5A immunostaining. Neutralization activity was normalized to a negative control lacking antisera (0%) and to a no virus added control (100%). (A) The samples were plotted comparing the neutralizing activity between the Pre and Post samples and the grey line connects the samples from the same volunteer. The paired t-test score indicates a significant change between Pre and Post. (B) Neutralization activities from each volunteer are shown. The Post neutralizing activity of each volunteer was normalized to the Pre neutralizing activity of the same volunteer. (C) Antisera from the volunteers 1, 5, and 7 were further tested for neutralization activity against the chimeric HCVcc strains gt1a (H77), gt1b (J4), gt2a (J6), gt2b (J8), gt3a (S52), gt4a (ED43), gt5a (SA13), gt6a (HK6a), and gt7a (QC69). This was performed with both Pre and Post antisera from each patient at a dilution of 1:50. Post neutralizing activity was normalized with Pre. These experiments were performed in two independent experiments in triplicate. The means from two independent experiments are shown and the error bars represent standard deviation. Statistical analysis (one-tailed paired t-test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. This figure was taken from (Law et al. 2013).

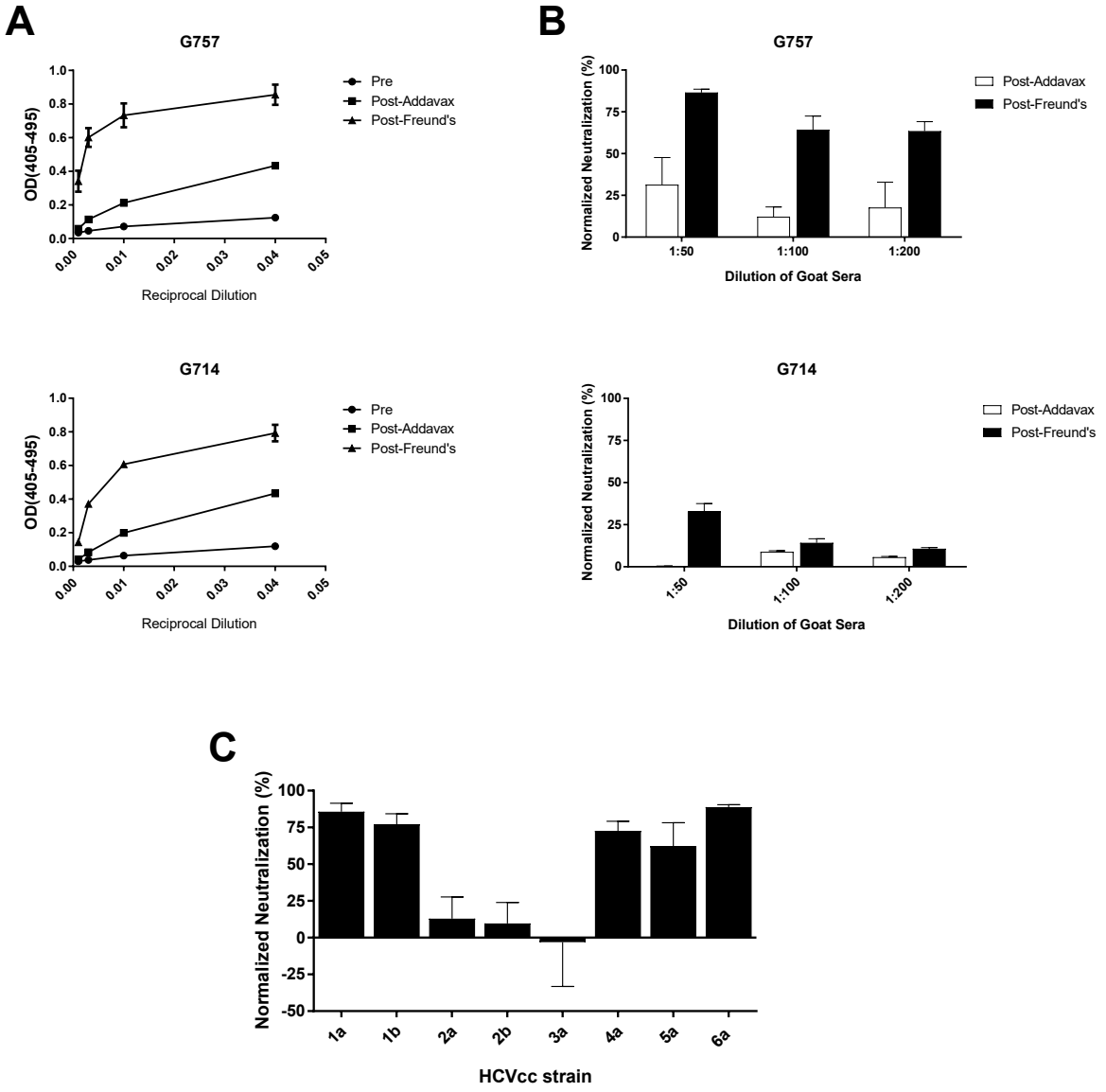
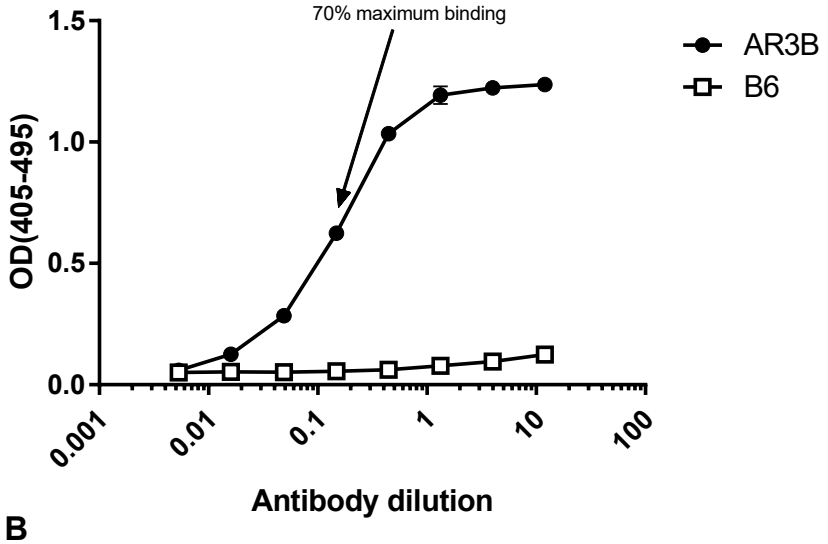

A
G757
OD(405-495)
Reciprocal Dilution
Pre
Post-Addavax
Post-Freund's
G714
B
G757
Normalized Neutralization (%)
Dilution of Goat Sera
1:50
1:100
1:200
G714
C
Normalized Neutralization (%)
HCVcc strain
1a
1b
2a
2b
3a
4a
5a
6a

Figure 3.2 Antisera from two goats (G757 and G714) immunized with recombinant E1E2 (strain HCV-1) were tested for E1E2 binding (A) and activity to prevent HCV infection (B and C).

Three antisera samples were tested from each goat: Pre, Post-Addavax, and Post-Freund's. (A) ELISA microtiter plates were first coated with *Galanthus nivalis* antigen (GNA), then with lysate from CHO cells stably expressing recombinant E1E2 (strain H77c). Serially diluted goat antisera (1:25, 1:100, 1:400, 1:1600) were added to ELISA microtiter wells containing E1E2. Antibody binding was detected with an alkaline phosphatase (AP) conjugated anti-goat secondary antibody. This experiment was performed in two independent experiments in triplicate. The mean from one representative experiment is shown and the error bars represent standard deviation. (B) Neutralization of cell-culture derived HCV (HCVcc) expressing the chimeric H77/JFH-1 by immunized goat antisera at dilutions of 1:50, 1:100, and 1:200. HCVcc were pre-incubated with diluted goat antisera and used to infect Huh-7.5 cells. Cells were fixed 48 hours post-infection followed by staining with an anti-NS5A mAb. The infected cells were detected by flow cytometry. Results are shown as percent neutralizing activity by post-vaccination antisera normalized to activity by pre-vaccination antisera. This experiment was performed in three independent experiments in singlicate for G757 and two independent experiments in singlicate for G714. The means between the experiments and standard deviation are shown. (C) Neutralization activities by Post-Freud's antisera from G757 (1:50) against chimeric HCVcc expressing the Core-NS2 regions of the H77c (1a), J4 (1b), J6 (2a), J8 (2b), S52 (3a), ED43 (4a), SA13 (5a), and HK6a (6a) strains are shown. This experiment was performed in three independent experiments in singlicate for G757. The means between the experiments and standard deviation are shown. This figure was taken from (Wong et al. 2014).

A


70% maximum binding
AR3B
B6
OD(405-495)
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
100
Antibody dilution

B

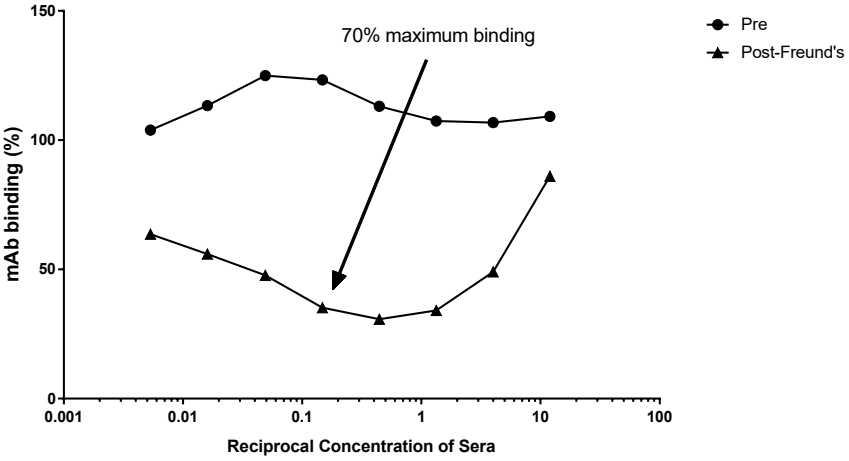
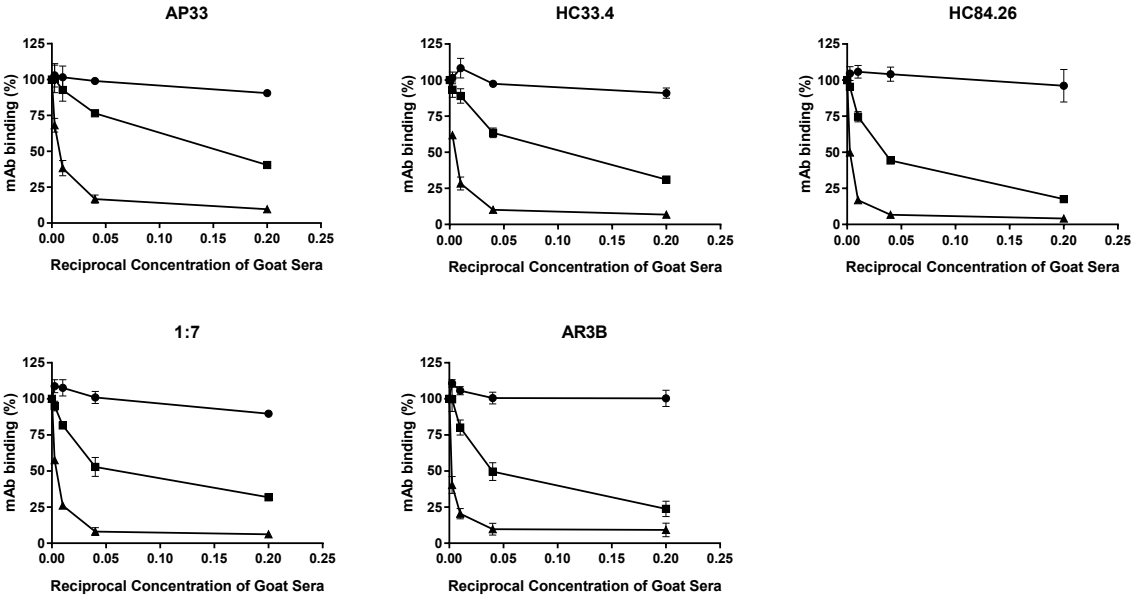
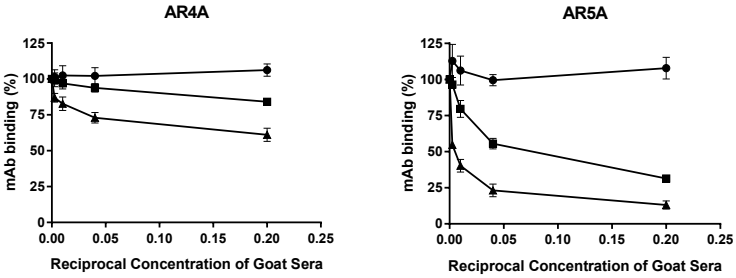

70% maximum binding
Pre
Post-Freund's
mAb binding (%)
150
100
50
0
0.001
0.01
0.1
1
10
100
Reciprocal Concentration of Sera

Figure 3.3 Selecting mAb concentration for competition studies.
(A) One of the mAbs (AR3B and a human isotype control B6) on the antibody panel was titrated at 3-fold dilutions starting at 12μg/mL on GNA-captured recombinant E1E2 derived from the gt1a H77 strain. AP-conjugated anti-human IgG secondary and pNPP were used to develop the signal and the absorbance titration curve is shown. The experiment was performed once in triplicate and the error bars represent the standard error. (B) G757 Pre-bleed and G757 Post-Freund's antisera at a dilution of 1:100 was incubated on GNA-captured H77 E1E2 for 1 hour before adding the same concentrations used in **Figure 3.3A**. AP-conjugated anti-human IgG secondary and pNPP were used to develop the signal. The percentage of mAbs binding in the presence of goat antisera normalized to binding in the absence of any antisera was calculated. The experiment was performed once in triplicate and the error bars represent the standard error.

A


AP33
HC33.4
HC84.26
1:7
AR3B
mAb binding (%)
Reciprocal Concentration of Goat Sera

B


AR4A
AR5A
mAb binding (%)
Reciprocal Concentration of Goat Sera

C

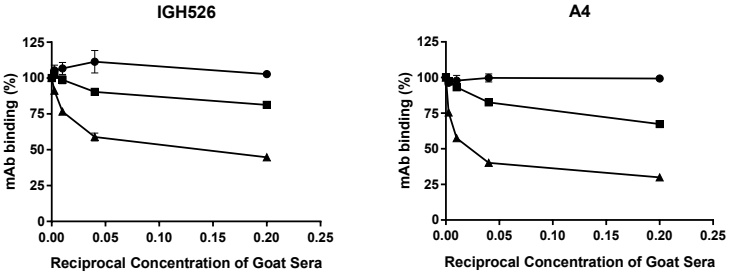

IGH526
A4
mAb binding (%)
Reciprocal Concentration of Goat Sera

Figure 3.4 Competition studies with G757 goat antisera (● Pre, ■ Post-Addavax, ▲ Post-Freund's) and a panel of monoclonal antibodies (mAbs).
Microtiter wells containing GNA-captured E1E2 of H77c were first incubated with diluted antisera samples from G757 (1:5, 1:25, 1:100, 1:400) for an hour, followed by incubation of the indicated mAb. Monoclonal antibody was added at a concentration resulting in 70% maximal binding to E1E2, as determined in prior titration experiments. Binding of the mAbs was detected with species-specific AP-conjugated secondary antibodies. The percentage of mAbs binding in the presence of goat antisera normalized to binding in the absence of any antisera was calculated. The experiment was performed twice in triplicate. The means between the experiments and standard deviation are shown. The eight mAbs used in competition ELISAs are grouped based on their reactivity: (A) antibodies that bind E2; (B) antibodies that require E1E2 for binding; and (C) antibodies that target E1. This figure was taken from (Wong et al. 2014).

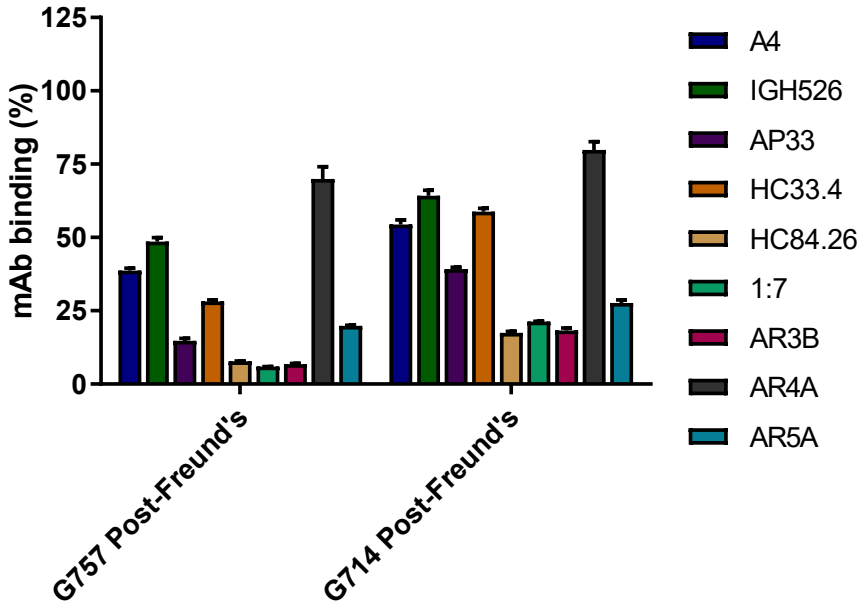


Figure 3.5 G757 and G714 Post-Freund's display similar patterns of competition. Microtiter wells containing GNA-captured E1E2 of H77c were first incubated with diluted antisera samples from G757 and G714 (1:100) for an hour, followed by incubation of the indicated mAb. Monoclonal antibody was added at a concentration resulting in 70% maximal binding to E1E2, as determined in prior titration experiments. Binding of the mAbs was detected with species-specific AP-conjugated secondary antibodies. The percentage of mAbs binding in the presence of goat post-vaccination (Post-Freund's) antisera normalized to binding in the presence of pre-vaccination (Pre) antisera was calculated. Data is reported as the mean values of at least 2 experiments performed in triplicate. Error bars represent the standard deviation of all replicates.

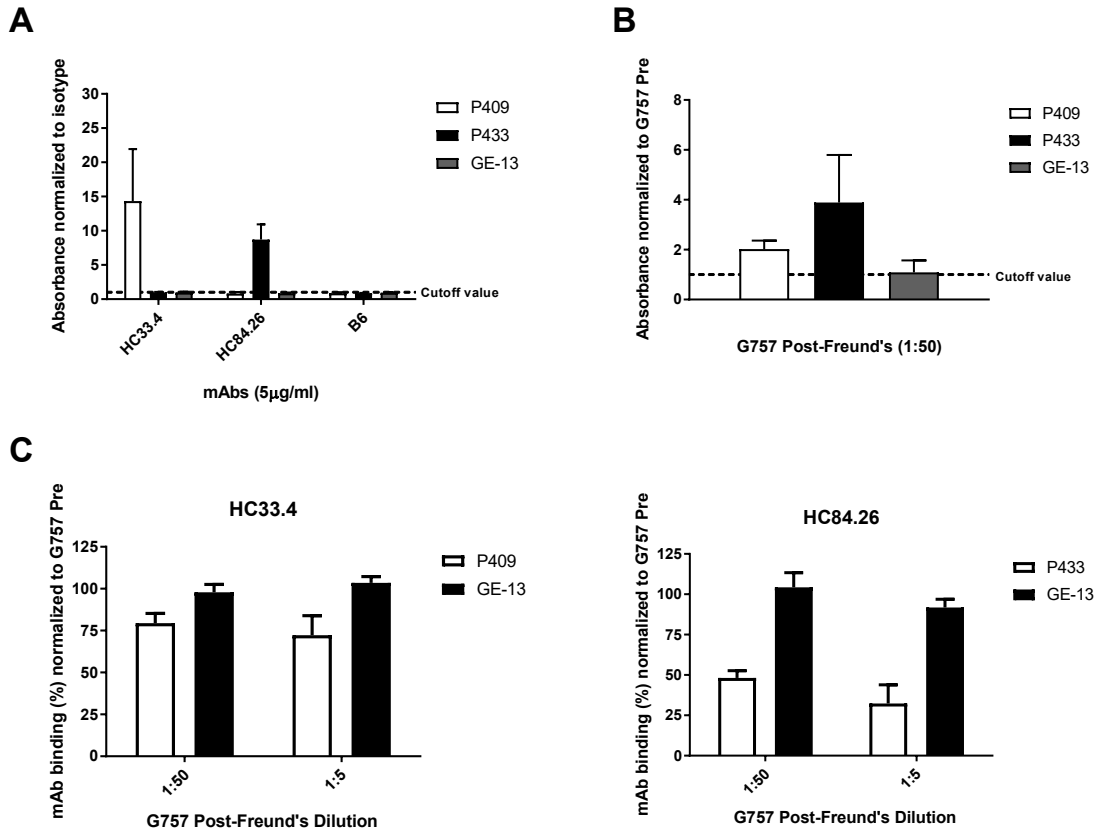


Figure 3.6 Binding of immunized goat antisera and mAbs to linear-synthetic biotinylated peptides by ELISA.

(A) Human mAbs (HC33.4, HC84.26, and human IgG1 isotype control B6) in 5µg/ml or (B) immunized goat antisera from G757 diluted 1:50 were incubated with biotinylated peptides pre-captured in NeutrAvidin-coated microtiter plate wells. Two peptides from E2 (aa 409-423 and aa 433-447) and an irrelevant biotinylated peptide of similar length were used (see Materials and Methods for sequences). Binding was detected with species-specific AP-conjugated secondary antibodies. Results are presented as fold binding signal minus background ($OD_{405-495}$) to peptide over cutoff value. Cutoff value was determined by the mean binding to the same peptide + 3SD by either (A) G757 Pre or (B) human IgG1 isotype control mAb B6. (C) The ability of goat sera to compete with mAb binding to peptide was determined. Diluted goat antisera were added to wells containing peptide for an hour, followed by incubation of the mAb in 5µg/ml. The mAb and the target peptide are indicated. Binding of the mAbs was detected and the percentage of mAb binding in the presence of G757 antisera was normalized to binding in the presence of G757 Pre. Error bars are shown as standard deviation of all replicates from at least two independent experiments done in triplicate. This figure was taken from (Wong et al. 2014).

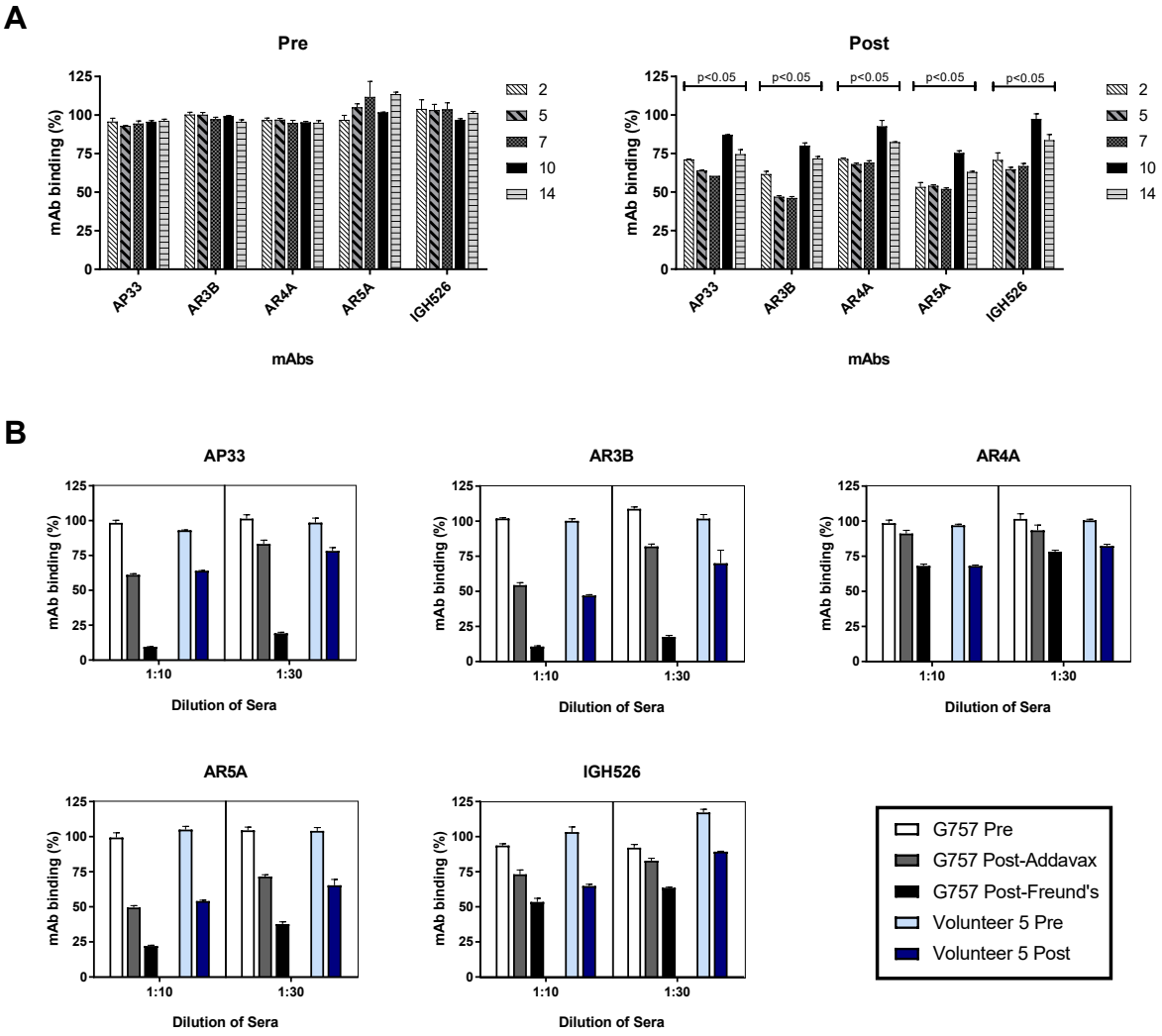

A
Pre
Post
mAb binding (%)
125
100
75
50
25
0
2
5
7
10
14
p<0.05
AP33
AR3B
AR4A
AR5A
IGH526
mAbs
B
AP33
AR3B
AR4A
AR5A
IGH526
1:10
1:30
Dilution of Sera
G757 Pre
G757 Post-Addavax
G757 Post-Freund's
Volunteer 5 Pre
Volunteer 5 Post

Figure 3.7 Competition studies with human volunteers' antisera and a panel of neutralizing monoclonal antibodies (mAbs).
Antisera samples collected prior to immunization (Pre) and 2 weeks post 3rd immunization (Post) from five volunteers (2, 5, 7, 10, 14) were tested. Microtiter wells containing GNA-captured E1E2 of H77c were first incubated with diluted volunteers' antisera or G757 for an hour, followed by incubation of the indicated mAb. Each mAb (AP33, AR3B, AR4A, AR5A, IGH526) was added at a concentration resulting in 70% maximal binding. AR3B, AR4A, AR5A, and IGH526 were biotinylated to enable specific detection. Binding was detected with AP-conjugated NeutrAvidin or AP-conjugated anti-mouse secondary antibody. Results are shown as the percentage binding of mAbs in the presence of human or goat antisera normalized to binding in the absence of antisera. (A) Competition assay results from antisera samples from five volunteers at a dilution of 1:10. Statistical analysis (one-way ANOVA, Kruskal-Wallis, and Dunn's multiple comparisons test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$. (B) Results comparing immunized goat antisera and matched antisera samples from one of the volunteers (5) at dilutions of 1:10 and 1:30. Due to the paucity of human vaccinee antisera, experiments were only performed once in duplicate with a 1:10 dilution and twice in duplicate with a 1:30 dilution. Error is shown as standard deviation between replicates. This figure was taken from (Wong et al. 2014).

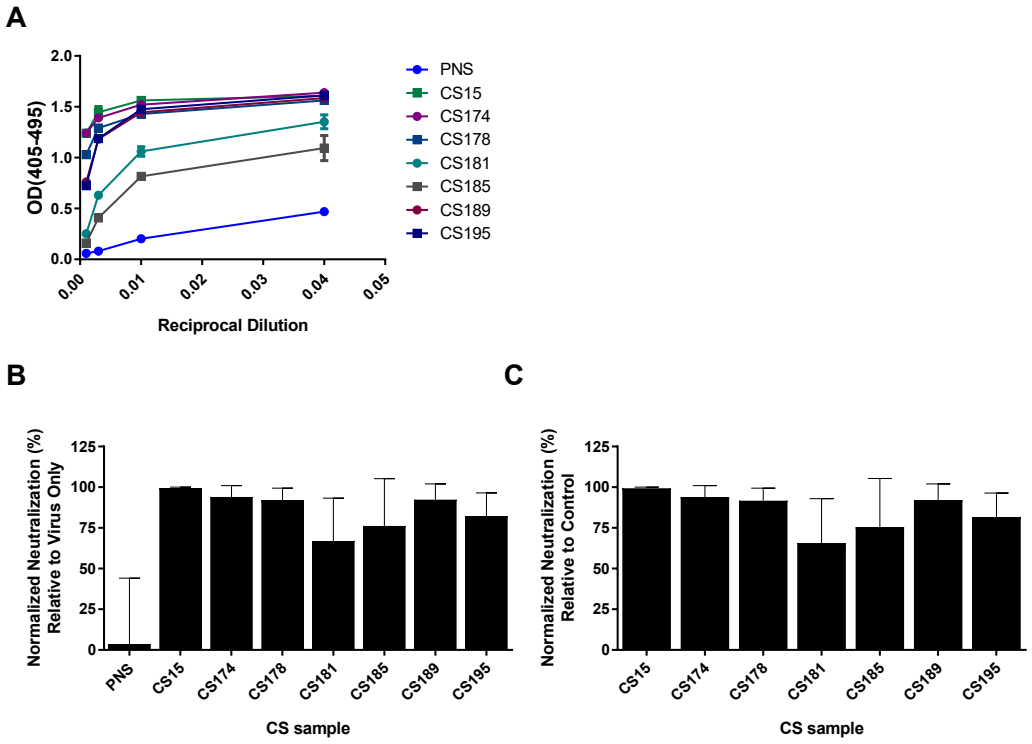


Figure 3.8 Sera from chronically infected patients bind rE1E2 and neutralize HCVpp infectivity.

(A) Sera from chronically infected patients or a pooled negative sera sample at various dilutions (1:25, 1:100, 1:400, 1:1600) were added to a microtiter well with GNA-captured H77 E1E2 lysates. Signal was developed with an alkaline-phosphatase conjugated goat anti-human secondary antibody and pNPP. (B and C) H77 HCVpp was mixed with sera from chronically infection patients or a pooled negative sera sample at a dilution of 1:100. The mixture was used to infect Huh-7.5 cells and after 2 days infection was determined by detecting luciferase activity with a plate reader. The signal is displayed (B) normalized to virus only (0%) and cells only (100%) or (C) normalized to PNS (0%) and cells only (100%). Error bars represent the standard deviation and results represent two independent experiments performed in triplicate.

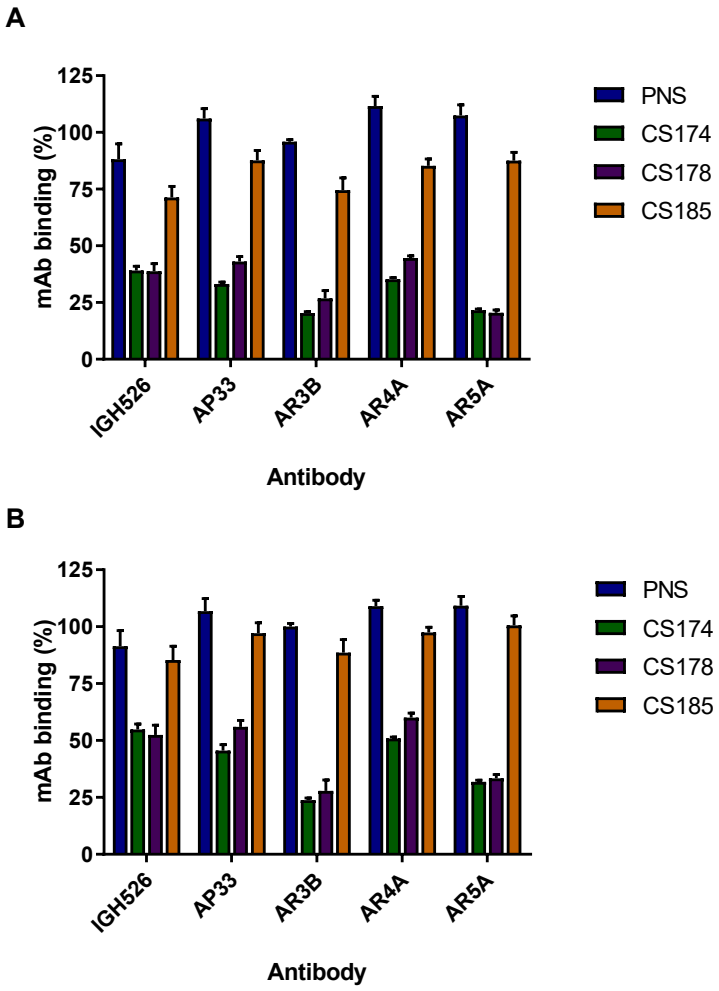


Figure 3.9 Sera from chronically infected patients can inhibit the binding of cross-neutralizing monoclonal antibodies.
Sera from chronically infected patients or a pooled negative sera sample at a dilution of (A) 1:25 or (B) 1:100 were added to GNA-captured E1E2 lysates in a microtiter well. After incubation with the indicated mAb (human antibodies IGH526, AR3B, AR4A, and AR5A required biotinylation as the patient sera was human), the signal was developed with appropriate alkaline-phosphatase conjugated secondary antibodies and pNPP. Signal is displayed relative to wells without any sera. Error bars represent the standard deviation and results represent two independent experiments performed in triplicate.

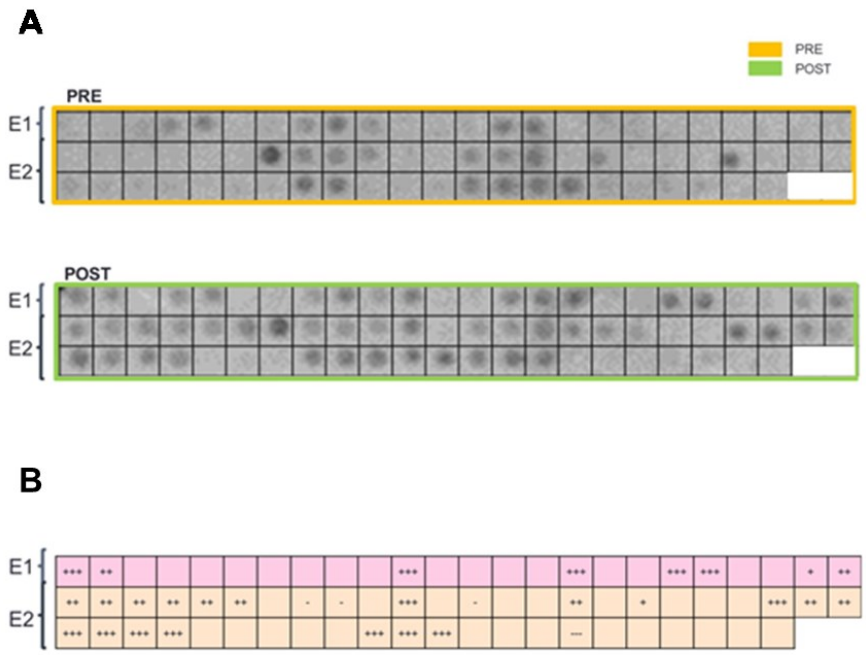


Figure 3.10 Example of the results of the CelluSpot™ assays.
(A) CelluSpot™ assays were performed with Pre and Post antisera from each human volunteer tested. The regions containing the E1 and E2 peptides are indicated. After development, a signal developed from each spot of peptides if there was reactivity. Densitometry was measured by MultiGauge software (Fujifilm). (B) The scoring was based on the intensity of the signal with Post antisera normalized to the signal with Pre antisera. Positive signs indicate peptides where incubation with Post antisera resulted in a stronger signal than with Pre antisera and negative signs indicate peptides where incubation with Pre antisera resulted in a stronger signal than with Post antisera. Peptides where binding signals were equivalent were scored blank.


Vaccinee
1
2
3
5
6
7
9
10
11
Notes
Neut (>25%)
+
+
-
+
+
+
-
-
-
193-207
201-215
209-223
217-231
225-239
233-247
241-255
249-263
257-271
265-279
273-287
281-295
289-303
297-311
305-319
313-327
321-335
329-343
337-351
345-359
353-367
361-375
369-383
377-391
385-399
393-407
401-415
409-423
417-431
425-439
433-447
441-455
449-463
457-471
465-479
473-487
481-495
489-503
497-511
505-519
513-527
521-535
529-543
537-551
545-559
553-567
561-575
569-583
577-591
585-599
593-607
601-615
609-623
617-631
625-639
633-647
641-655
649-663
657-671
665-679
673-687
681-695
689-703
697-711
705-719
713-727
721-735
729-743
737-751
745-759
FP? (272-288)
E1 Epitope I (313-327)
HVR1 (384-411)
HVR1 (384-411)
E2 Epitope I (412-423)
FP? (418-432)
E2 Epitope II (434-446)
FP? (496-515)
CD81 binding loop (523-540)
FP? (549-569)
FP? (549-569)
FP? (597-621)
FP? (597-621)
CD81bs back layer (613-616)
Stem (651-715)
Stem (651-715)
Stem (651-715)
Stem (651-715)
Stem (651-715)
Stem (651-715)
Stem (651-715)
TM (716-746)
TM (716-746)
TM (716-746)
TM (716-746)
TM (716-746)
TM (716-746)
--- -- - 0 + ++ +++

Figure 3.11 Results from peptide mapping sera from selected volunteers with CelluSpot™ assays.

Ten of the volunteers tested for neutralization against H77/JFH-1 HCVcc were tested with CelluSpot peptide binding arrays. The neutralizing activity against H77/JFH-1 HCVcc (greater or less than 25% neutralization) is indicated. Results are summarized in a heat map format (legend is shown below the figure). Positive hits were coloured red (higher intensity with post-immunization antisera than with pre-immunization antisera) and negative hits were coloured green (higher intensity with pre-immunization antisera than with post-immunization antisera) and no difference between pre- and post-immunization antisera in white. The left column lists the 70 peptides in order from top to bottom, with the E1 derived peptides in pink and the E2 peptides in orange. The locations of areas of E1E2 of note including neutralizing epitopes (HVR1, E1 Epitope I, E2 Epitope I, E2 Epitope II, CD81 binding loop) are outlined in blue. E2 stem region, and putative fusion peptides (FP?) are indicated.

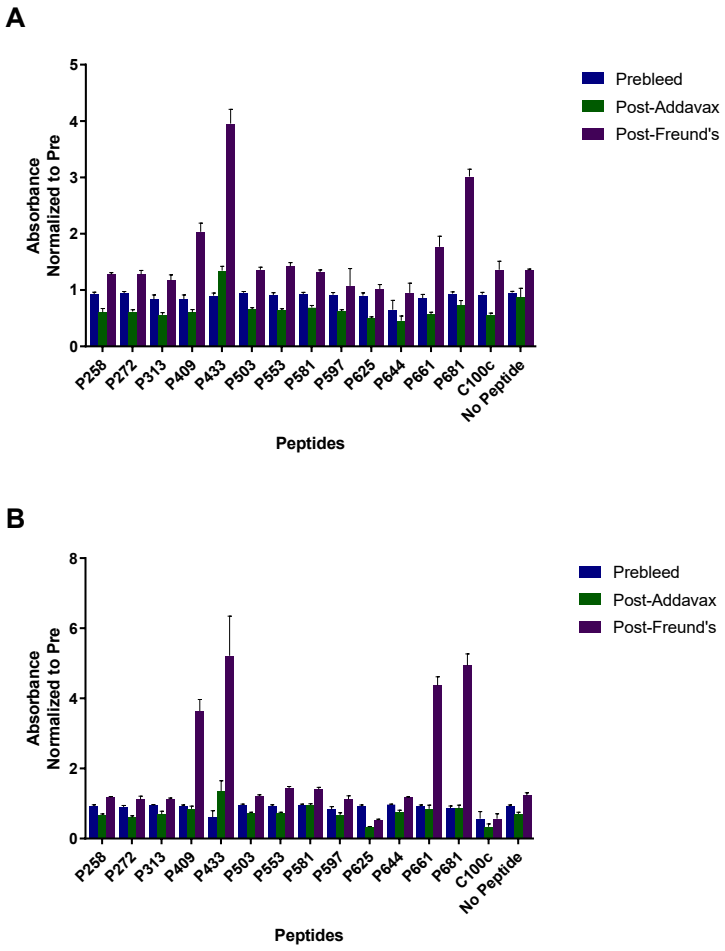


Figure 3.12 Vaccinated G757 antisera recognizes E1E2 peptides specifically.
(A) Immunized goat antisera from G757 or (B) purified IgG from G757 diluted 1:50 were incubated with biotinylated peptides pre-captured in NeutrAvidin-coated microtiter plate wells. Binding was detected with species-specific AP-conjugated secondary antibodies. Results are presented as fold binding signal minus background ($OD_{405-495}$) to peptide over cutoff value. Cutoff value was determined by the mean binding to the same peptide + 3SD by G757 Pre. Irrelevant peptide from the HCV NS4A region (C100c) and no peptide controls were included. Error bars are shown as standard deviation of all replicates and results are from two independent experiments done in triplicate.

4 CHAPTER IV: VACCINATION WITH HCV ENVELOPE GLYCOPROTEINS INDUCES ANTIBODIES THAT PREVENT THE INTERACTION OF E1E2 WITH CRITICAL CELL ENTRY RECEPTORS

Data in this chapter are being prepared for publication:

Wong, J.A., Hockman, D., Logan, M., Chen, C., Frey, S.E., Belshe, R.B., Law, J.L., and Houghton, M. **2017.** Vaccination with HCV envelope glycoproteins induces antibodies that prevent the interaction of E1E2 with critical cell entry receptors. In preparation.

I was responsible for the majority of the experimental data and manuscript composition. M. Houghton, J.L. Law, and I were responsible for concept formation. M. Houghton and J.L. Law assisted with manuscript edits. D. Hockman was responsible for some of the neutralization assay data. Generation of rE1E2 was performed by Michael Logan. Soluble E2 was provided by J. Marcotrigiano (NIH).

4.1 Introduction

Understanding both the epitope of a neutralizing antibody and its mechanism of action in neutralizing viral infectivity is very important to rational vaccine design. Understanding the step that is being blocked by an antibody with a known epitope can also inform on the structure-function relationship of that area of the protein.

The entry of HCV is an especially complicated process relative to that of other viruses. As discussed in **Chapter 1**, the viral entry process can be separated into early binding events (involving HSPG, Syndecan molecules, and SRB1), post-binding events (involving SRB1, CD81, CLDN1, and OCLN1), prefusion events (possibly involving NPC1L1 and TfR1), fusion with the endosomal membrane, and genome uncoating. The vast majority of well-characterized neutralizing monoclonal antibodies derived from chronically infected patients are directed against the E2 protein, and most of these anti-E2 antibodies have been found to either block E2-

SRB1 interaction by binding to residues in the C-terminal half of HVR1 (Sabo et al. 2011; Flint, Thomas, et al. 1999) or block E2-CD81 interaction by binding to the various disparate residues that form the CD81 binding surface (Law et al. 2008; Johansson et al. 2007; Keck et al. 2012, 2013; Sabo et al. 2011; Owsianka et al. 2005; Potter et al. 2012). Interestingly, one anti-E2 mAb binding to E2 Epitope I, which contains a critical binding residue for CD81 interaction, prevents the E2-SRB1 and E2-CD81 interactions (Sabo et al. 2011). There are other anti-E2 mAbs with neutralizing activity that do not block SRB1 or CD81 interaction with E2 and their mechanisms are currently unknown (Owsianka et al. 2008; Keck et al. 2012). There are other neutralizing mAbs that are either specific to the E1 protein alone or require the properly folded E1E2 heterodimer for binding and these do not appear to act at the SRB1 or CD81 step (Giang et al. 2012; Meunier, Russell, Goossens, et al. 2008; Keck, Sung, et al. 2004). Immunization with the sE1 protein alone can confer protective responses in humans and chimpanzees – however, due to low subject numbers (n=2), this could not be confirmed to be statistically significant in the HCV challenge chimpanzee model (Verstrepen et al. 2011; Leroux-Roels et al. 2005; Nevens et al. 2003). Interestingly, in the same HCV challenge chimpanzee study an sE2 vaccine was not seen to be protective in the chimpanzee model (Verstrepen et al. 2011). Together, these data suggest that antibodies targeting both E2 as well as E1 are important in viral neutralization.

There are two highly cross-neutralizing mAbs currently known to require natively folded E1E2 heterodimer for binding (Giang et al. 2012). This demonstrates that there are important neutralizing epitopes present beyond E2-specific epitopes. Along with evidence that a rE1E2 vaccine was more immunogenic than an sE2 vaccine in chimpanzees and a rE1E2 vaccine is still currently the only HCV vaccine shown to elicit statistically significant protection in the HCV challenge chimpanzee model, these data indicate there could be an advantage to a recombinant E1E2 vaccine over a soluble E2 vaccine (Houghton 2011).

Interrogating the neutralizing mechanisms of polyclonal antisera has been understudied compared to monoclonal antibodies. However, one study analyzing the neutralizing activity of polyclonal antisera from chronically infected patients found that sera did not appear to inhibit the early binding step and the activities occurred at a post-binding step with kinetics similar to an anti-CD81 mAb and that these sera blocked fusion in an *in vitro* fusion assay (Haberstroh et al. 2008). Antisera elicited in human volunteers vaccinated with the HCV-1 E1E2 vaccine were shown to directly block E2-CD81 interaction in an ELISA assay (Frey et al. 2010).

Following from the epitope mapping in **Chapter 3**, I further interrogated the entry steps that are blocked by antisera from vaccinated animals. I tested antisera from goats vaccinated with three different antigens (gt1a HCV-1 strain rE1E2, gt1a HCV-1 strain sE2, and gt2a J6 strain sE2) in assays to determine at what point of the entry process vaccine-elicited neutralizing antibodies block viral entry. Based on the data covered above, I hypothesized that goat antisera would primarily inhibit the CD81 binding step but would also show evidence of targeting other parts of the virus entry process.

4.2 Results

4.2.1 Goats immunized with different envelope glycoprotein antigens elicit different neutralization profiles

Three sets of goats were used in this study: G757 and G714 were immunized with HCV-1 (gt1a) full-length rE1E2 heterodimer, G786 and G799 were immunized with HCV-1 (gt1a) sE2, and G773 and G766 were immunized with J6 (gt2a) sE2 (**Table 4.1**). G757 was previously tested for neutralization against 8 strains of HCVcc to determine the breadth of the response (**Figure 3.2C**; (Wong et al. 2014)). This goat was shown to neutralize genotypes 1a, 1b, 4a, 5a, 6a very well but not genotypes 2a, 2b, and 3a at a 1:50 dilution. I used neutralization assays with these 6 goats at a 1:50 dilution against HCVcc derived from three different strains: H77(gt1a)/JFH-1, J6(gt2a)/JFH-1, and JFH-1 (gt2a). As explained in **Chapter 1**, these chimeric HCVcc strains were generated by substituting the Core-NS2 regions of other HCV strains in the backbone of the JFH-1 strain (the first strain to be discovered to have highly efficient growth in cell culture) (Wakita et al. 2005). The strains used in this study were kindly provided by Jens Bukh (University of Copenhagen) (Gottwein et al. 2009). I tested two different bleeds: pre-vaccination bleed taken on day 0 and the first post-vaccination bleed following the last of five immunizations (three with Addavax adjuvant (an oil-in-water adjuvant similar to MF59), one with Complete Freund's Adjuvant (CFA), and one with Incomplete Freund's Adjuvant (IFA) taken on day 182.

G757/714 and G786/G799 efficiently neutralized H77(1a)/JFH-1 and minimally neutralized J6(2a)/JFH-1, while G773/766 efficiently neutralized J6(2a)/JFH-1 and minimally neutralized H77(1a)/JFH-1 (**Figure 4.1**). However, although JFH-1 HCVcc is a genotype 2a

strain, G757/714 displayed efficient neutralization against JFH-1 HCVcc similar to the neutralization against H77/JFH-1. G773/766 showed minimal neutralization against JFH-1 HCVcc similar to the neutralization against H77/JFH-1. The similarity of neutralization profiles between H77/JFH-1 and JFH-1 and the dissimilarity of neutralization profiles between JFH-1 and J6/JFH-1 was unexpected.

4.2.2. Synchronized HCV cell entry experiments suggest that envelope glycoproteins elicit antibodies that block early in the cell entry process

HCV viral entry is a complex process involving multiple interactions between the envelope glycoproteins and cell surface proteins to guide binding, post-binding, migration to tight junctions, internalization, and fusion events. It is possible that antibodies binding the envelope glycoproteins could be blocking any of these steps. Therefore, synchronized time-of-addition neutralization assays were performed in order to investigate which step(s) antisera were inhibiting. These assays involve synchronized infections and adding antibodies/inhibitors at different time-points to examine the kinetics of neutralization. The earlier the time-points at which the antibodies/inhibitors show a reduction in neutralization, the earlier in the viral entry process the antibodies/inhibitors are actively inhibiting entry. In this assay (outlined in **Figure 2.2**), HCVcc is incubated at 4°C on Huh-7.5 cells for 2 hours – this allows for the virus to bind to the cells but not efficiently enter and allows for synchronization of entry (Koutsoudakis et al. 2006). The unbound virus is then washed off with cold PBS and cultures are shifted to 37°C to allow for entry. Antibodies or chemical inhibitors of entry are added at various times (with the addition of virus or with the 37°C shift, or at 1, 2, or 4 hours after the temperature shift) and are present until 12 hours post temperature shift, at which point media containing antibodies or inhibitors are removed and wells are replenished with fresh media followed by further incubation and assay for infection levels.

I compared the kinetics of neutralization by goat antisera and two controls: an anti-CD81 antibody that blocks the interaction between E2 and CD81, and Balifomycin A1 (BafA1), an inhibitor which prevents endosomal acidification which is triggered after clathrin-mediated endocytosis and directly before fusion. G757 Pre and Post-Freund's antisera were tested against JFH-1 HCVcc and G773 Pre and Post-Freund's antisera were tested against J6/JFH-1 HCVcc.

Figure 4.2 shows that while anti-CD81 and BafA1 act at a post-binding step, as neutralizing activities at t=-2 and t=0 were very similar, most of the neutralizing activity by anti-CD81 against JFH-1 HCVcc was prevented by t=+1 whereas BafA1 required longer to exert its full inhibition against JFH-1 HCVcc (t≥+2). This was consistent with what has been seen in the literature (Zeisel et al. 2007; Sourisseau, Michta, et al. 2013). Antisera from both animals exerted most of their inhibition of infectivity at a post-binding stage and lost activity by t=+1, more closely resembling the observed kinetics using anti-CD81 rather than BafA1. This suggests that both vaccines elicit antibodies that primarily act to prevent binding to CD81 rather than prevent the later, post-binding fusion step. A minor but consistent result showed G773 acted at a step earlier than the activity of the anti-CD81 mAb while the activity of G757 neutralization tightly resembled the activity of the anti-CD81 mAb. However, it should be emphasized that these data only allow preliminary conclusions because of the lack of additional animal samples for testing and the high inter-assay variation. Nonetheless, a tentative conclusion was that both goats' antisera appeared to act at or prior to the CD81 engagement step.

4.2.3 Goats immunized with E1E2 from a genotype 1a strain blocks the interaction of E2 with SRB1

SRB1 is essential for viral entry and has three major functions in that regard (explained further in **Chapter 1**). Two of these functions, "attachment" and "access", do not require direct engagement with the envelope glycoproteins, but the "enhancement" function requires both the presence of HDL and direct interaction between E2 and SRB1. The authors showed that this enhancement function is not essential for entry but greatly increases entry efficiency and also makes the virus less vulnerable to neutralizing antibodies as a result (Thi et al. 2012). Antibodies binding the N-terminus region of E2 (including HVR1 and the 412-423 E2 Epitope 1 region) can block this interaction to reduce entry (Sabo et al. 2011). CHO cells overexpressing human SRB1 were used to determine if vaccine-elicited antibodies could block the binding of H77 rE1E2 to SRB1. These CHO-SRB1 cells generated by transduction with Murine Leukemia Virus particles carrying the human SRB1 gene were confirmed to express SRB1 using an anti-SRB1 mAb (**Figure 4.3A**). Notably, this expression did not exhibit the expected single peak of SRB1 expression as has previously been reported (Sabo et al. 2011), but instead exhibited two peaks,

both of which possessed a higher signal than non-transduced cells. Transduction of BRL3A Buffalo rat liver cells with MLV particles carrying the human SRB1 gene has shown to generate stably transfected cells possessing a strong expression of SRB1 (personal communication with Dr. Florian Douam;(Douam et al. 2014). After transducing BRL3A cells with MLV particles carrying the human SRB1 gene, I also found that these cells expressed the SRB1 receptor in two peaks above non-transduced background in similar proportions to the CHO-SRB1 cells (**Figure 4.4**). CHO-SRB1 cells were ultimately chosen for consistency with the CHO-CD81 experiments.

H77 rE1E2 binding to CHO-SRB1 cells was detectable using an anti-E2 antibody. Background binding of H77 rE1E2 to non-transduced CHO cells was also monitored (**Figure 4.3B**). Consistent with the double peak of SRB1 expressing cells, the anti-E2 antibody signal was also composed of two peaks. However, only the second peak showed higher signals than the control non-transduced cells. These peaks coincided with the two peaks of CHO cells expressing SRB1 in **Figure 4.3A**. This indicated that although the lower expressing CHO-SRB1 cells still expressed SRB1 higher than non-transduced cells, the expression was not sufficient to result in higher binding by rE1E2. I attempted to detect the binding of J6 sE2 to CHO-SRB1 using a panel of anti-E2 antibodies to detect J6 sE2, but overall differences in binding between CHO-SRB1 and non-transduced cells were too minimal and inconsistent between experiments for reliable data (**Figure 4.5**). Vaccine-elicited antibodies or control mAbs were then incubated with rE1E2 for 1 hour at 37°C prior to adding the mixture to the CHO-SRB1 cells. Bound E2 was detected with anti-E2 antibodies to determine if the antisera/mAbs could block the interaction. For positive control mAbs, I used H77.16 which binds to residues within HVR1 and H77.39 which binds residues within E2 Epitope I. Both mAbs have been shown to block the E2-SRB1 interaction (Sabo et al. 2011). For a negative control mAb, I used A4, which is non-neutralizing and binds to the N-terminus of E1 (Dubuisson et al. 1994). Consistent with the literature, H77.16 and H77.39 reduced binding by >70% in my assays while A4 only minimally affected binding (**Figure 4.3C**). I then tested the vaccinated antisera from the goats immunized with either HCV-1 rE1E2 or HCV-1 sE2 at dilutions of 1:100 and 1:400. I found antisera from G757 and G714 inhibited the interaction drastically at 1:100 with near 100% reduction in binding (**Figure 4.3D**). Antisera from G786 and G799 were slightly less effective, with around 40% and 20% reduction in binding, respectively. When these goat antisera were tested at 1:400, only G757 remained clearly effective relative to the control mAb A4. An important concern was that antisera and

mAbs could be binding to the E2 protein and competing with the binding of the detecting mAb without interfering with the E2-SRB1 binding interface. To control for this, I performed competition ELISAs to determine if the antisera and mAbs were preventing the binding of the detecting mAb to the target protein (H77 rE1E2). I found that there was no appreciable background competition between the mAbs and antisera tested in **Figure 4.3** with the detecting mAb H53 for H77 rE1E2 (**Figure 4.6A**,**B**).

4.2.4 Goats immunized with E1E2 from a genotype 1a strain blocks the interaction of E2 with CD81

CD81 is a required entry receptor for HCV and is the most well understood of the receptors. This is partially due to the fact that the majority of potent cross-genotype neutralizing antibodies neutralize entry by directly preventing the interaction between E2 and CD81 (Keck et al. 2012, 2013, 2009; Keck, Beeck, et al. 2004; Law et al. 2008; Johansson et al. 2007). I investigated if vaccine antisera from the three different groups of goats could inhibit the interaction between E2 and CD81 using two independent assays. Similar to the E2-SRB1 assay, I used CHO cells overexpressing human CD81 and added either H77 rE1E2 or J6 sE2 pretreated with vaccine antisera or mAbs. CHO-CD81 cells generated by transduction with MLV particles carrying the human CD81 gene were confirmed to express CD81 using an anti-CD81 antibody in flow cytometry (**Figure 4.7A**). Unlike with CHO-SRB1 cells, detection of CD81 on CHO-CD81 cells resulted in a single peak of CD81 expression, as reported previously (Sabo et al. 2011). I used anti-E2 antibodies to detect H77 rE1E2 and J6 sE2 binding to CHO-CD81 cells. I washed the cells to remove unbound glycoprotein and detected the presence of E2 with antibodies specific for H77 or J6 E2 – H53 and 6F5 respectively. Consistent with the single peak of CD81 expression on CHO-CD81 cells, detection of H77 rE1E2 and J6 sE2 binding to CHO-CD81 cells presented as a single peak separated from binding to non-transduced CHO cells (**Figure 4.7B**,**C**). I tested mAbs H77.39 and AR3B as positive controls and mAb AR4A as a negative control for inhibition between envelope glycoproteins and CHO-CD81. H77.39 binds residues located within the E2 Epitope I and blocks both the E2-SRB1 and E2-CD81 interactions (Sabo et al. 2011). AR3B binds to the CD81 binding loop and prevents the E2-CD81 interaction (Law et al. 2008). AR4A binds to a poorly understood epitope requiring the natively folded E1E2

heterodimer and is potently cross-neutralizing without affecting the E2-CD81 interaction (Giang et al. 2012). Assays with these mAbs confirmed that H77.39 and AR3B potently block the interaction between E2 and CD81 for both H77 rE1E2 and J6 sE2 (>75% inhibition) and AR4A showed poor inhibition of this interaction with either antigen (<25% inhibition) (**Figure 4.7D**,**E**). Vaccine antisera from the goats immunized with HCV-1 rE1E2 or HCV-1 sE2 were tested in this assay at a dilution of 1:100 or 1:400 against H77 rE1E2 binding to CHO-CD81. After the signal with post-immunization antisera was normalized to the signal with pre-immunization antisera, I found that all four goats were able to prevent H77 rE1E2 interaction with CD81, with G786 possessing the lowest inhibition (~75% inhibition) at 1:100 dilution (**Figure 4.7F**). At a 1:400 dilution, G714, G786, and G799 showed minimal inhibition while G757 still showed effectiveness (~75%). Vaccine antisera from the goats immunized with J6 sE2 were tested in this assay at a dilution of 1:100 or 1:400 against J6 sE2 binding to CHO-CD81. Surprisingly, I found antisera from the J6 sE2 immunized goats were weak at preventing the J6 E2 interaction with CD81 at both dilutions of 1:100 and 1:400 (<25% inhibition) (**Figure 4.7G**). As with the CHO-SRB1 assay, I controlled for irrelevant competition using a competition ELISA to see if the antisera or mAbs were preventing the binding of the detecting mAbs and found there was no appreciable background competition (**Figure 4.6A-D**).

I sought to test this with a different assay to confirm these results. I used a biochemical pulldown assay allowing H77 rE1E2 and J6 sE2 pretreated with mAbs or vaccine antisera to bind to recombinant CD81 Large Extracellular Loop (CD81 LEL) expressed with a GST tag and captured onto Glutathione-Sepharose beads. After reducing SDS-PAGE, Western Blotting to detect E2 from H77 rE1E2 or J6 sE2 with anti-E2 mAbs specific for each protein was conducted (**Figure 4.8A**,**B**). As detection was performed after reducing SDS-PAGE, irrelevant competition was not a complicating factor. Similar to that found in the CHO-CD81 assay, AR3B was very effective at preventing the interaction between both H77 rE1E2 and J6 sE2 with GST-CD81 LEL and AR4A was poorly effective against both interactions – although there was still some minor inhibition (<25% inhibition) (**Figure 4.8C**,**D**). I used mAb HC84.26 as a positive control – this cross-neutralizing antibody binds to the region known as E2 Epitope II to prevent E2-CD81 interaction (Keck et al. 2012). I found this antibody effectively prevented the E2-CD81 interaction as expected. I tested the HCV-1 rE1E2 vaccine antisera for inhibition of the interaction between H77 rE1E2 and CD81 and confirmed that the antisera could prevent H77

rE1E2-CD81 interaction effectively (**Figure 4.8E**). As well, I found that these antisera could not prevent J6 sE2-CD81 interaction which is consistent with the data that these antisera could not neutralize the entry of HCVcc encoding the J6 structural proteins (**Figure 4.8F**, **Figure 3.2C**, **Figure 4.1**). Agreeing with the previous data, I discovered that J6 E2 vaccine antisera could not block the interaction between either J6 sE2 or H77 rE1E2 with CD81 LEL (**Figure 4.8G**,**H**).

4.3 Discussion

Previously, antisera from human volunteers immunized with genotype 1a rE1E2 (strain HCV-1) were found to have cross-genotype neutralizing activity (Law et al. 2013). Using antisera from vaccinated humans and goats, I characterized the cross-neutralizing epitope repertoire within these antisera and found the antibodies targeted numerous epitopes associated with broad cross-neutralization (**Chapter 3;** (Wong et al. 2014)). In this study, I investigated vaccine antisera to understand the specific mechanisms of neutralization to further our knowledge of immune responses to HCV recombinant envelope glycoprotein vaccines and potentially to improve vaccine design.

I studied vaccine antisera from three sets of goats: immunized with HCV-1 (gt1a) rE1E2, HCV-1 (gt1a) sE2, and J6 (gt2a) sE2. This allowed me to compare the differences in immune responses between heterologous strains and between sE2 alone and heterodimeric rE1E2 I found that goats immunized with HCV-1 rE1E2 or HCV-1 sE2 were able to neutralize H77 (gt1a) and JFH-1 (2a) strains efficiently but neutralized J6 (gt2a) very poorly and the inverse was true for J6 sE2 vaccinated goats (**Figure 4.1**). It was a surprising finding that a vaccine immunogen derived from a gt2a strain (J6) would not be able to elicit antibodies able to neutralize a heterologous but genetically similar gt2a strain (JFH-1) (87.7% identity at primary amino acid sequence within the E1E2 region) but a vaccine immunogen derived from a gt1a strain (HCV-1) could generate antibodies capable of neutralizing the JFH-1 strain despite the higher genetic difference (66.8% identity). Current work by another graduate student in the lab is underway using chimeric viruses expressing different regions from the J6 and JFH-1 strains to determine the mechanism of this finding. This is a very worthy field of study as it could determine the minimal epitopes that resulted in the high level of neutralization of JFH-1. More work on unexpected neutralization patterns such as this one could lead to an optimized vaccine prioritizing the elicitation of the

minimal epitopes for global cross-neutralization, which could create an optimal vaccine while easing the burden of manufacturing and distribution.

The clear majority of isolated neutralizing mAbs have been shown to block the E2-CD81 interaction and therefore, the greatest amount of attention so far has been paid to this part of the entry step. Nevertheless, I hypothesized that polyclonal vaccine antisera would affect more than one step of the entry cycle. Therefore, I performed time-of-addition neutralization assays to establish a time frame for the neutralization to allow me to determine which specific assays I would have to do to interrogate the separate steps of entry affected by the vaccine antisera.

This assay has been used to demonstrate that attachment to heparan sulfate proteoglycans occurs very early (by using soluble heparin inhibitor) (Haberstroh et al. 2008), followed by post-binding events with SRB1 (SRB1 has both early attachment and post-binding roles in HCV entry) and CD81 (by using anti-SRB1 and anti-CD81 antibodies) (Zeisel et al. 2007). An anti-CLDN1 antibody has also been shown to inhibit cell entry activity at a step after HCV binding to SRB1 and CD81 (Evans et al. 2007), while an anti-OCLN antibody was shown to inhibit entry after the action of an anti-CLDN1 antibody (Sourisseau, Michta, et al. 2013). While the process of events involving TfR1 and NPC1L1 are not well understood, it has been shown that inhibitors of these receptors act after anti-CD81 (Sainz Jr et al. 2012; Martin and Uprichard 2013).

G757 post-immunization antisera exhibited neutralization kinetics similar to the anti-CD81 mAb (**Figure 4.2A**). Antisera from a goat immunized with J6 sE2 also possessed kinetics similar to the anti-CD81 mAb. Interestingly, G773 antisera appeared to act just earlier than the anti-CD81 antibody – however, due to the high inter-assay variability and the fact only one goat was tested, it was not possible to make this conclusion firmly (**Figure 4.2B**). However, my data suggested that both goats possessed neutralization kinetics similar to an anti-CD81 antibody and so I investigated the steps around CD81 engagement in more detail. In a paper studying the neutralization kinetics of sera from several patients chronically infected with HCV, it was found that some of the sera samples had neutralization kinetics very similar to anti-CD81 while other sera samples had neutralization kinetics that seemed to act sooner than anti-CD81 (Haberstroh et al. 2008). Interestingly, this appears to mirror my findings with HCV-1 rE1E2 antisera and J6 sE2 antisera.

Because an anti-SRB1 polyclonal antibody (pAb) preparation that is capable of blocking viral entry was shown to possess similar kinetics as the JS81 anti-CD81 antibody in the time-of-

addition neutralizing assays (Zeisel et al. 2007), I focused on the interactions of E2 with SRB1 and CD81. I first investigated the SRB1 binding step utilizing flow cytometry to detect the interaction between sE2 and SRB1 expressed on CHO cells in the presence or absence of goat antisera and found that the HCV-1 rE1E2 and HCV-1 sE2 antisera were able to block the E2-SRB1 binding step potently (**Figure 4.3D**). This is consistent with data showing antisera from mice vaccinated with H77 rE1E2 were reactive to a peptide derived from the HVR1 sequence – HVR1 is critical for the interaction between E2 and SRB1 that leads to an HDL-dependent enhancement of infection (Thi et al. 2012). I showed that this interaction can be inhibited by vaccinated goat antisera. I wanted to test J6 sE2 vaccinated goat antisera for the ability to prevent the binding of J6 sE2 to SRB1 but unfortunately, recognition of J6 sE2 bound to CHO-SRB1 was poor and inconsistent (**Figure 4.5**). In the future, possible solutions to this issue include the generation of J6 rE1E2 (in case the full heterodimer binds SRB1 better than sE2), the generation of cell lines with superior expression of SRB1, and using ELISAs with recombinant SRB1 to detect the E2-SRB1 interaction.

I then tested if goat antisera could block the interaction between E2 and CD81. I found that HCV-1 rE1E2 and HCV-1 sE2 goat antisera were able to block the CD81 binding step effectively in two independent assays – one using flow cytometry to detect the interaction between H77 rE1E2 and CD81 expressed at the surface of CHO cells and one using protein complex immunoprecipitation (Co-IP) and immunoblotting (IB) to detect the interaction between H77 sE2 and CD81 LEL (**Figure 4.7F**, **Figure 4.8E**). Importantly, the antisera from HCV-1 rE1E2 vaccinated goats were not able to inhibit the interaction between J6 sE2 and CD81 (**Figure 4.8F**). This is consistent with the poor neutralization by antisera from these goats against J6/JFH-1 HCVcc (**Figure 3.2C**, **Figure 4.1**). As well, vaccine antisera from goats and humans immunized with rE1E2 and sE2 from the HCV-1 strain were shown to compete with the binding of a panel of neutralizing antibodies that block the E2-CD81 interaction (**Figure 3.4**, **Figure 3.5**). These results indicated that both the E1E2 heterodimer and the soluble E2 from the same strain elicited a similar neutralizing antibody response: the neutralizing trends against H77/JFH-1 and J6/JFH-1 HCVcc strains were comparable and the antibody response from both could block both E2-SRB1 and E2-CD81 interaction. This is very significant as this showed the vaccine could elicit a broad response targeting at least two different important entry steps.

Given the fact that all the antisera samples showed potent inhibition of the E2-CD81 interaction and G773 antisera possessed kinetics fairly similar to the anti-CD81 mAb, I expected the same for J6 sE2 goat antisera. Surprisingly, I found that in both assays antisera from both goats vaccinated with J6 sE2 could not block binding between J6 sE2 and CD81 and the antisera also could not block binding between H77 rE1E2 and CD81 (**Figure 4.7G**, **Figure 4.8G**,**H**). The data suggest that there could be a different neutralization mechanism for J6 sE2 antisera compared to HCV-1 rE1E2 and sE2 antisera, likely at a step prior to the CD81 binding step according to the neutralization kinetics experiments. However, more vaccinated animals would have to be analyzed to confirm this but preliminarily, these possible mechanistic differences seem to be more based on genotypic differences than construct differences (E1E2 heterodimer vs soluble E2).

There are assays to evaluate if compounds, mAbs, and antisera block inhibition of early attachment to cells (Colpitts and Schang 2014). It has been seen that HCVpp but not sE2 is capable of interacting with CLDN1 expressed on the surface of BRL3A cells, enabling inhibition of binding experiments (Douam et al. 2014). Future research should interrogate the early attachment and post-binding steps to gain a more complete view of how G757 and G773 antisera are preventing entry. The specifics of OCLN, NPC1L1, TfR1, fusion, and genome uncoating in terms of interaction with E1E2 are poorly understood at this moment and it is currently unknown if there are any mAbs or neutralizing epitopes that specifically interfere with these steps. There is evidence that a synthetic peptide and HCV chronically infected patient antisera can block fusion of HCV, but the specificity of this inhibition and the sequence of the fusion peptide are currently unknown (Chi et al. 2016; Haberstroh et al. 2008). Once these interactions are better understood, this late entry step also deserves to be interrogated with vaccinees' antisera.

My study has added to the knowledge surrounding our HCV vaccine and has also some potential practical applications – the E2-SRB1 and E2-CD81 inhibition assays have value for testing antisera from our future clinical trials. Multi-genotype cocktail vaccines combining antigens from different strains that elicit weak cross-genotype neutralizing antibody responses have already been developed (e.g. Polio virus and human papillomavirus vaccines) and there is good reason to believe this concept can be applied to HCV. As we already showed, a recombinant E1E2 heterodimer vaccine from a single genotype 1a vaccine was capable of eliciting a cross-neutralizing response in healthy human volunteers and this response was more

effective against some strains than others suggesting a cocktail vaccine may be optimal for a global HCV vaccine (Law et al. 2013). Furthermore, if certain strains from different genotypes can elicit a neutralizing response with differing dominant neutralization mechanisms, this might improve vaccine potency and prevent the appearance of escape mutants to vaccination. Furthermore, more powerful adjuvants are also being investigated in order to further increase immune responses. The addition of rE1E2 to a vaccine component eliciting conserved HCV CD4+ and CD8+ T cell responses could elicit broadly protective responses from both the humoral and cell-mediated arms of the immune system and offer enhanced protection. Therefore, there is much promise for improving this vaccine candidate, which is the only candidate that has existing statistically significant pre-clinical efficacy (Houghton 2011). The more we can understand about possible mechanisms and directed epitopes associated with neutralization, the better we can improve the vaccine to create a truly globally effective vaccine and alleviate the high disease burden affecting hundreds of millions of people around the globe.

Goat ID	Vaccine Antigen
G757	HCV-1 (gt1a) rE1E2
G714	HCV-1 (gt1a) rE1E2
G786	HCV-1 (gt1a) sE2
G799	HCV-1 (gt1a) sE2
G773	J6 (gt2a) sE2
G766	J6 (gt2a) sE2

Table 4.1 List of goats vaccinated with HCV envelope glycoproteins.
The goat identification number is displayed along with the vaccine construct and strain the construct was derived from.

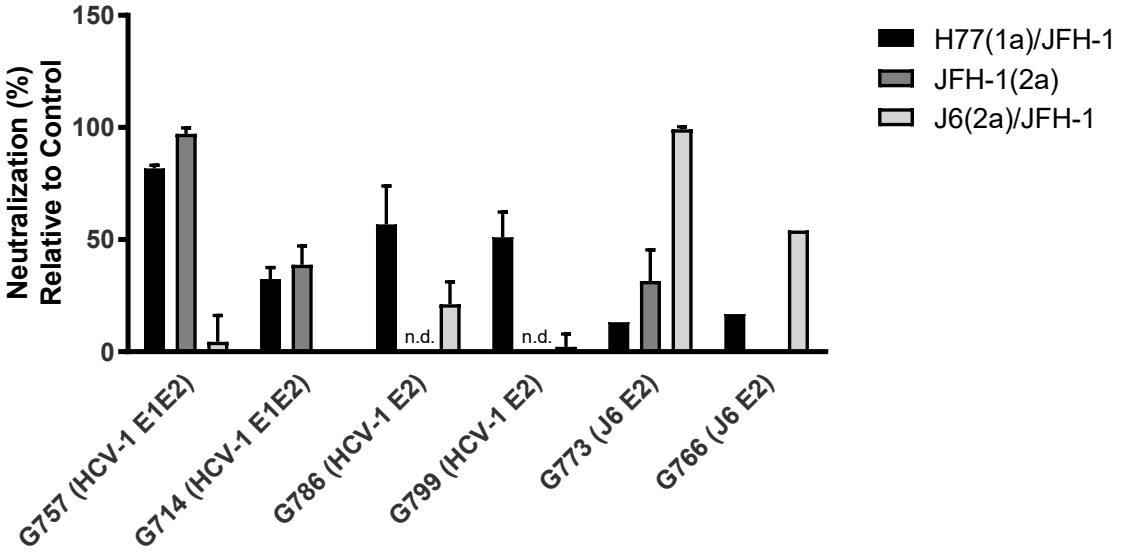
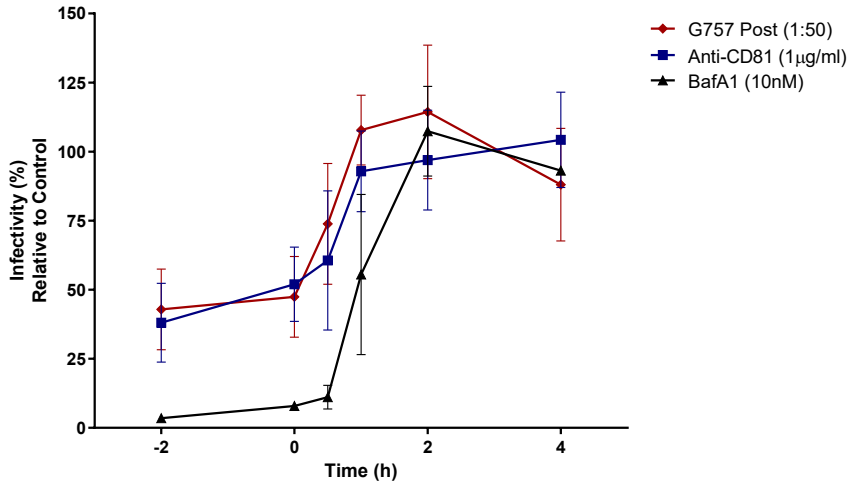


Figure 4.1 Neutralization against various HCVcc strains by vaccinated goat antisera. Neutralization of cell-culture derived HCV (HCVcc) expressing the Core-NS2 regions of the H77c (1a), JFH-1 (2a), or J6 (2a) strains by immunized goat antisera. HCVcc were pre-incubated with goat antisera heat-inactivated at 56°C for 1 hour diluted 1:50 for 1 hour at 37°C and then the mixture was used to infect Huh-7.5 cells. After 48 hours post-infection, infected cells were detected by counting focus forming units after immunohistochemistry staining (counting for NS5A+ foci) or flow cytometry (counting for NS5A+ cells). Results are shown as percent neutralizing activity by post-vaccination antisera normalized to activity by pre-vaccination antisera. This experiment was performed in three independent experiments in singlicate. The results are displayed as the means from three independent experiments and the error bars represent standard deviation.

A


150
125
100
75
50
25
0
Infectivity (%)
Relative to Control
-2
0
2
4
Time (h)
G757 Post (1:50)
Anti-CD81 (1μg/ml)
BafA1 (10nM)

B

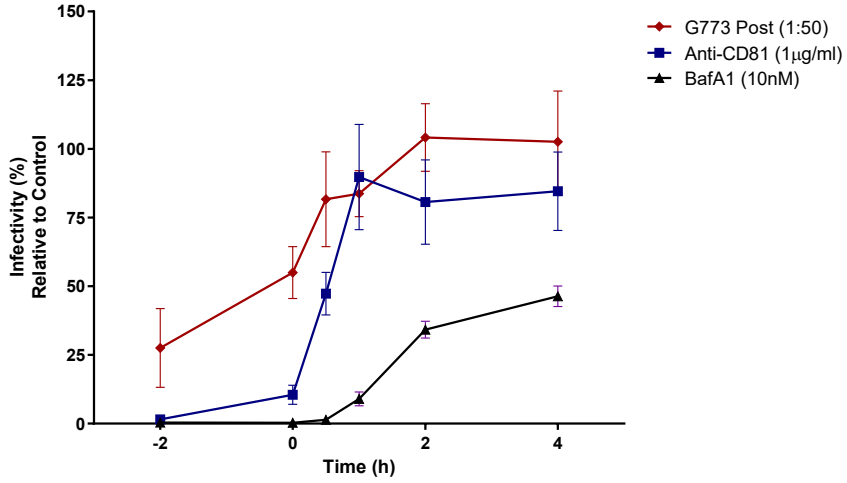

150
125
100
75
50
25
0
Infectivity (%)
Relative to Control
-2
0
2
4
Time (h)
G773 Post (1:50)
Anti-CD81 (1μg/ml)
BafA1 (10nM)

Figure 4.2 Vaccine antisera possess neutralizing activity at the early steps of the viral entry cycle.
Time-of-addition synchronized neutralization assays (schematic is shown in **Figure 2.2**) were performed with cell-culture derived HCV (HCVcc) expressing the Core-NS2 regions of (A) genotype 2a JFH-1 strain or (B) genotype 2a J6 strain. Inhibitors or antisera were added at the indicated intervals relative to t = 0, the timepoint where unbound virus was washed, and the cultures were shifted from 4°C to 37°C. An anti-CD81 antibody that blocks E2-CD81 interaction (JS81) and a murine IgG1 isotype control were used at 1μg/ml, and endosomal acidification inhibitor bafilomycin A1 (BafA1) was used at 10nM. (A) Goat 757 Pre and Post-Freund's antisera were used at 1:50 dilution against JFH-1 HCVcc and (B) Goat 773 Pre and Post-Freund's antisera were used at 1:50 dilution against J6/JFH-1 HCVcc. After 12 hours post-infection, media containing inhibitors were removed and replaced with fresh media without inhibitors. Infectivity was measured by (A) flow cytometry or (B) luciferase activity 72 hours post-infection. Results are shown as percent neutralizing activity by post-vaccination antisera normalized to activity by the relevant control: Pre/Post-Freund's, mIgG1/Anti-CD81, and DMSO/BafA1. This experiment was performed in three independent experiments in singlicate. The results are displayed as the means from three independent experiments and the error bars represent standard deviations.

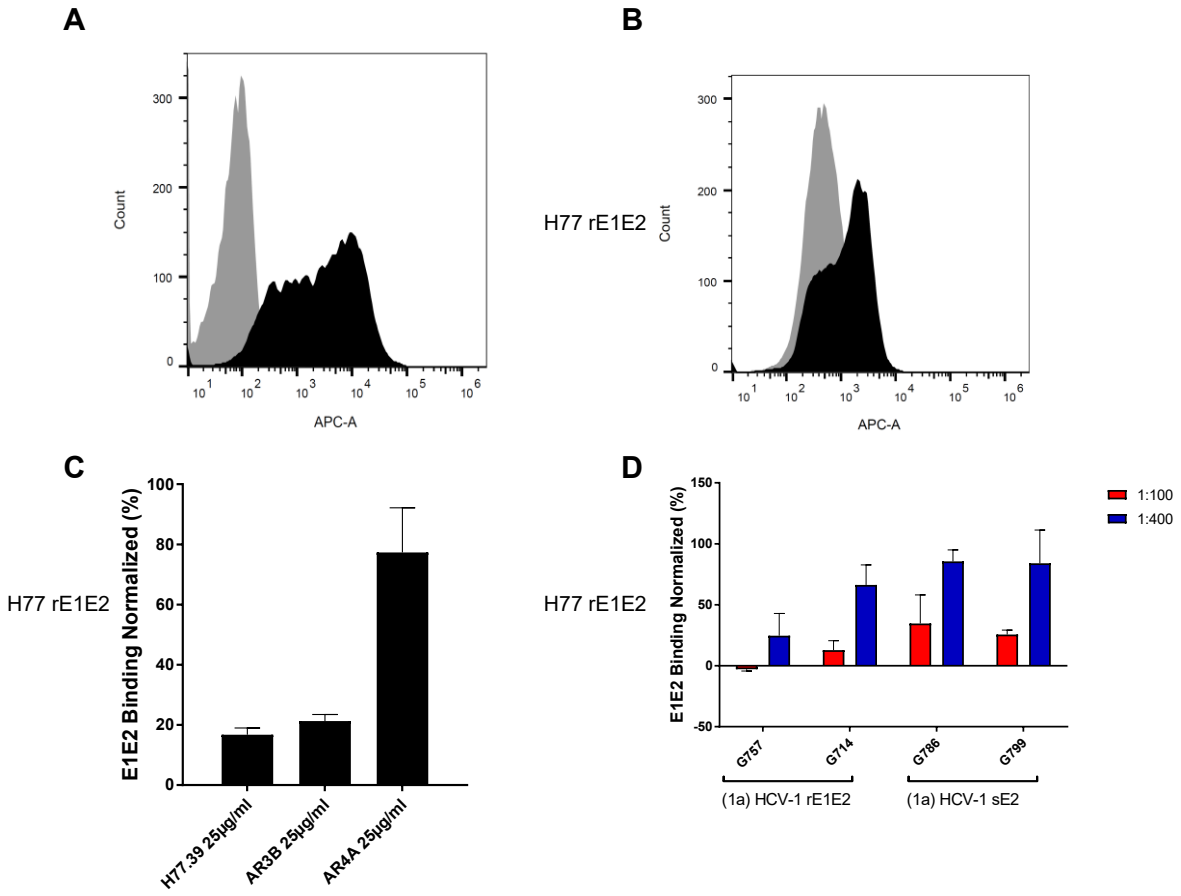

A
B
H77 rE1E2
Count
APC-A
C
D
H77 rE1E2
E1E2 Binding Normalized (%)
H77.39 25µg/ml
AR3B 25µg/ml
AR4A 25µg/ml
1:100
1:400
G757
G714
G786
G799
(1a) HCV-1 rE1E2
(1a) HCV-1 sE2

Figure 4.3 Vaccinated goat antisera block the interaction between the envelope glycoproteins and CHO-SRB1.
(A) Stable expression of HCV entry receptor SRB1 on CHO cells transduced with MLV particles encoding the gene for this receptor. Either non-transduced (gray histograms) or transduced (black histograms) cells were incubated with murine anti-SRB1 (0.5µg/mL) prior to incubation with a goat anti-mouse secondary linked to Alexa Fluor 647 (1.6µg/mL) and fixed and analyzed by flow cytometry. (B) CHO cells expressing SRB1 were incubated with H77 rE1E2 (0.5µg) and binding was detected with anti-E2 antibody H53 (5µg/mL) (modified with a biotin linker). After incubation with a Streptavidin secondary linked to Alexa Fluor 647 (1µg/mL), cells were fixed and analyzed by flow cytometry. Detection by the anti-E2 antibodies is shown for these cells in the absence (gray histograms) and presence (black histograms) of H77 rE1E2. Histograms are displaying units of mean fluorescence intensity (MFI) and are representative of at least three independent experiments performed in singlicate. (C and D) H77 rE1E2 (0.5µg) was mixed with (C) monoclonal antibodies or (D) goats immunized with HCV-1 rE1E2 or HCV-1 sE2 prior to binding to CHO-SRB1 cells. After detection with biotinylated H53 (5µg/mL) for H77 rE1E2, results were processed by flow cytometry. The result is displayed as the geometric mean determined with mAbs or goat antisera normalized to the geometric mean determined with the relevant control (mIgG1/B6 control antibodies or goat pre-immunization antisera). Histograms are displaying units of mean fluorescence intensity (MFI) and are representative of at least three independent experiments performed in singlicate. Results from inhibition of binding experiments are displayed as the means from three independent experiments performed in singlicate and the error bars represent standard deviation.

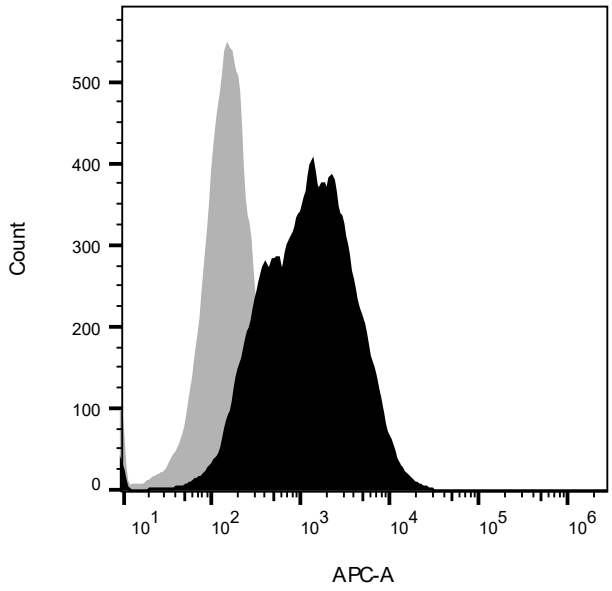


Figure 4.4 Expression of SRB1 on the surface of BRL3A Buffalo rat liver cells.
Stable expression of HCV entry receptor SRB1 on BRL3A cells transduced with MLV particles encoding the gene for this receptor. Either non-transduced (gray histograms) or transduced (black histograms) cells were incubated with murine anti-SRB1 (0.5μg/ml) prior to incubation with a goat anti-mouse secondary linked to Alexa Fluor 647 (1.6μg/mL) and fixed and analyzed by flow cytometry. Histograms are displaying units of mean fluorescence intensity (MFI) and are representative of at least three independent experiments performed in singlicate.

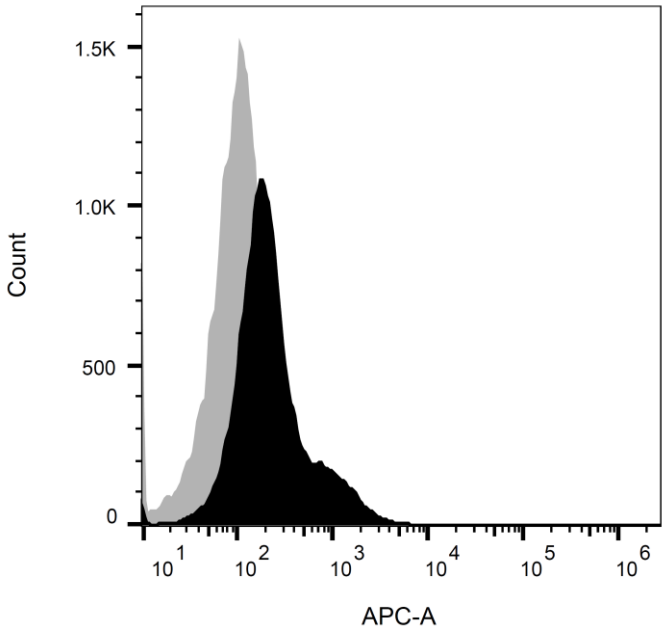


Figure 4.5 Detection of the binding of J6 sE2 on the surface of CHO-SRB1 cells.
CHO cells expressing SRB1 were incubated with J6 sE2 (2µg) and binding was detected with anti-E2 antibody 6F5 (5µg/mL). After incubation with a goat anti-mouse secondary linked to Alexa Fluor 647 (1.6µg/mL), cells were fixed and analyzed by flow cytometry. Detection by the anti-E2 antibodies is shown for these cells in the absence (gray histograms) and presence (black histograms) of J6 sE2. Histograms are displaying units of mean fluorescence intensity (MFI) and are representative of at least three independent experiments performed in singlicate.

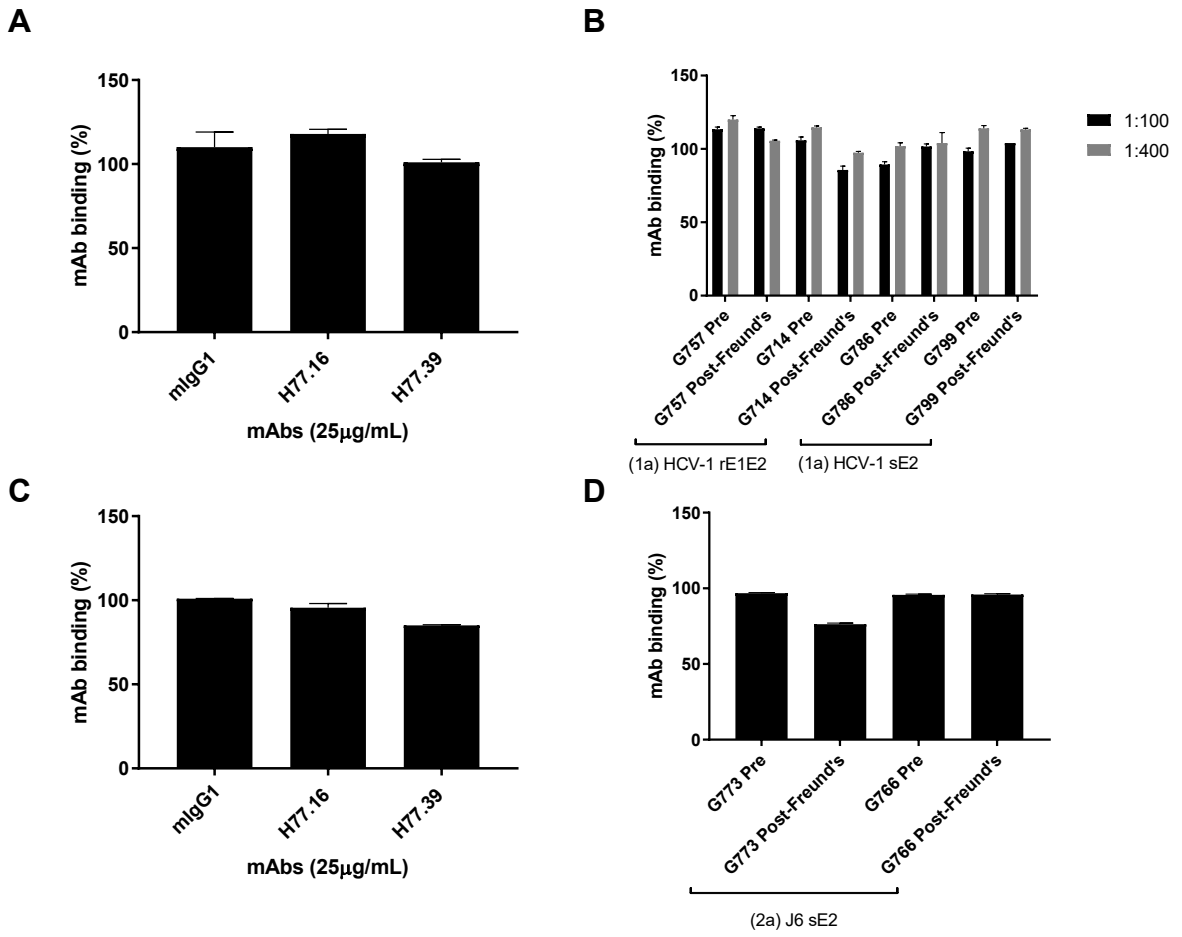
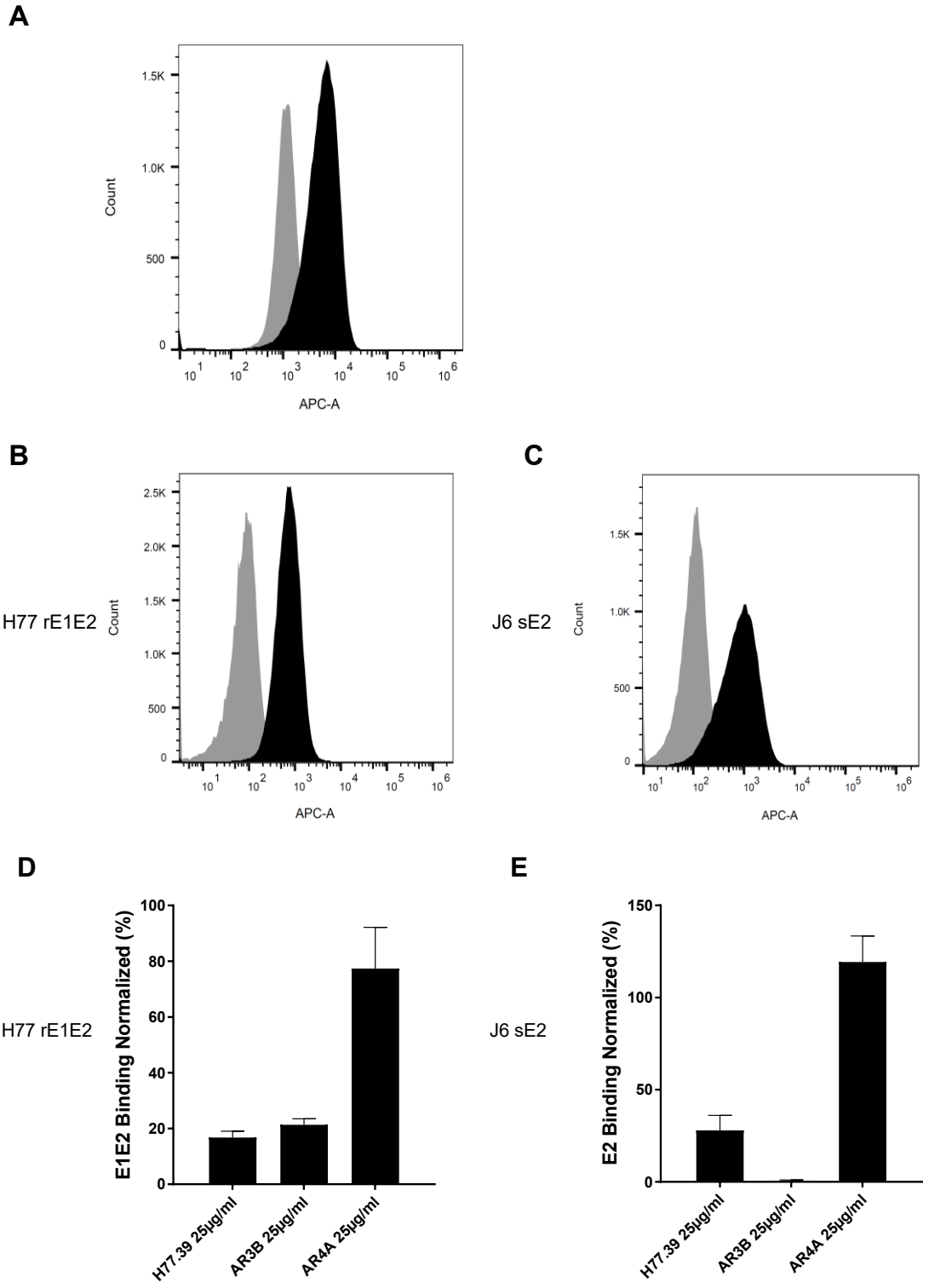


Figure 4.6 Goat antisera and mAbs do not interfere significantly with the detection of bound E2 by the detecting mAbs H53 (H77 E2) and 6F5 (J6 E2).
0.1µg of (A and B) H77 rE1E2 or (C and D) J6 sE2 was coated to the bottom of a microtiter well. (A and C) mAbs (25µg/mL) or (B and D) goat antisera (dilutions of 1:100 and 1:400) were incubated with the antigen for an hour prior to removal and then detecting mAbs (A and B) biotinylated H53 or (C and D) biotinylated 6F5 (5µg/mL) were incubated for an hour. Then the binding of the detecting antibody (biotinylated H53 or 6F5) was detected with a Streptavidin secondary linked to alkaline phosphatase (1:4000). The percentage of the detecting antibody binding in the presence of goat antisera/mAbs normalized to binding in the absence of any antisera was calculated. This experiment was performed in three independent experiments in singlicate. The results are displayed as the means from three independent experiments and the error bars represent standard deviation.


A
1.5K
1.0K
500
0
Count
APC-A
B
H77 rE1E2
2.5K
2.0K
1.5K
1.0K
500
0
Count
APC-A
C
J6 sE2
1.5K
1.0K
500
0
Count
APC-A
D
H77 rE1E2
E1E2 Binding Normalized (%)
100
80
60
40
20
0
H77.39 25µg/ml
AR3B 25µg/ml
AR4A 25µg/ml
E
J6 sE2
E2 Binding Normalized (%)
150
100
50
0
H77.39 25µg/ml
AR3B 25µg/ml
AR4A 25µg/ml

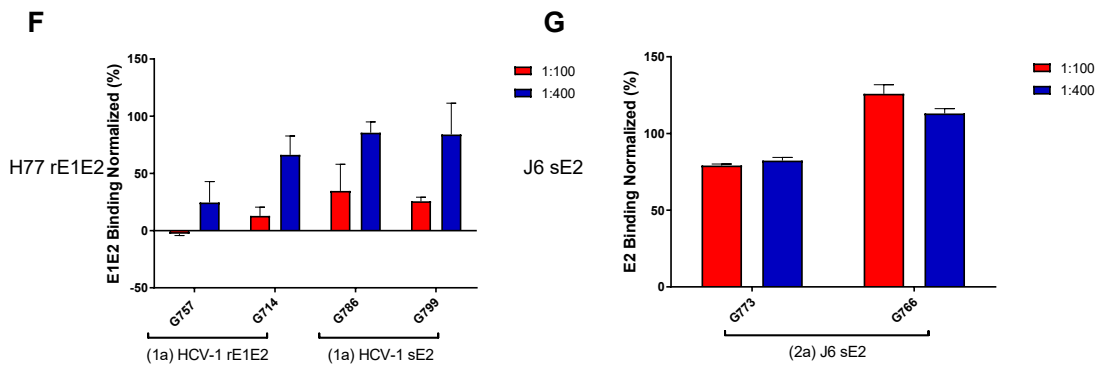
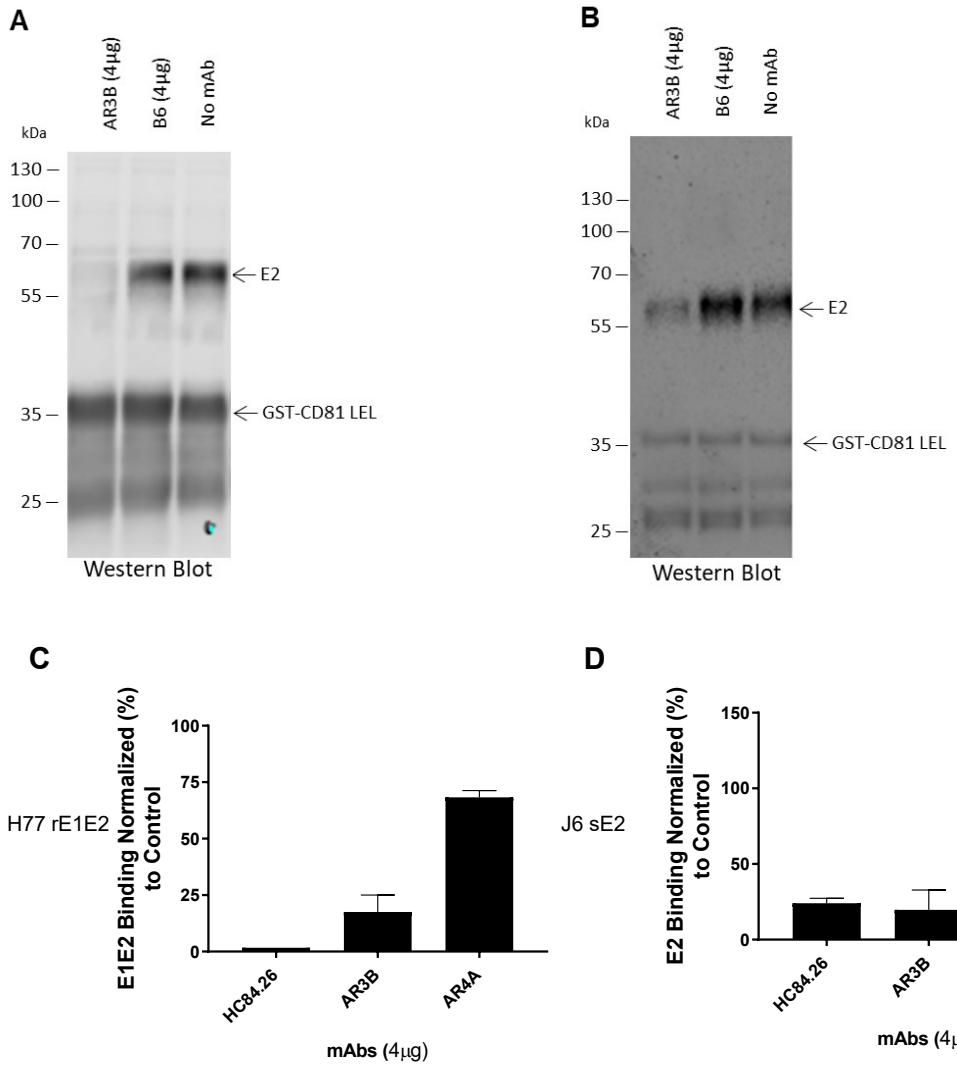


Figure 4.7 Vaccinated goat antisera block the interaction between the envelope glycoproteins and CHO-CD81.

(A) Stable expression of HCV entry receptor CD81 on CHO cells transduced with MLV particles encoding the gene for this receptor. Either non-transduced (gray histograms) or transduced (black histograms) cells were incubated with murine anti-CD81 (0.5µg/mL) prior to incubation with a goat anti-mouse secondary linked to Alexa Fluor 647 (1.6µg/mL) and fixed and analyzed by flow cytometry. (B and C) CHO cells expressing CD81 were incubated with either (B) H77 rE1E2 (0.5µg) or (C) J6 sE2 (0.5µg) and binding was detected with anti-E2 antibodies (B) H53 (5µg/mL; modified with a biotin linker) or (C) 6F5 (5µg/mL; modified with a biotin linker). After incubation with a Streptavidin secondary linked to Alexa Fluor 647, cells were fixed and analyzed by flow cytometry. Detection by the anti-E2 antibodies is shown for these cells in the absence (gray histograms) and presence (black histograms) of either H77 rE1E2 or J6 sE2. Histograms are displaying units of mean fluorescence intensity (MFI) and are representative of at least three independent experiments. (D and F) H77 rE1E2 or (E and G) J6 sE2 (0.5µg) was mixed with (D and E) monoclonal antibodies or (F and G) goats immunized with HCV-1 rE1E2, HCV-1 sE2, or J6 sE2 prior to binding to CHO-CD81 cells. After detection with (D and F) biotinylated H53 (5µg/mL) or (E and G) biotinylated 6F5 (5µg/mL), results were processed by flow cytometry. The result is displayed as the geometric mean determined with mAbs or goat antisera normalized to the geometric mean determined with the relevant control (mIgG1/B6 control antibodies or goat pre-immunization antisera). Histograms are displaying units of mean fluorescence intensity (MFI) and are representative of at least three independent experiments performed in singlicate. Results from inhibition of binding experiments are displayed as the means from three independent experiments performed in singlicate and the error bars represent standard deviation.


A
AR3B (4µg)
B6 (4µg)
No mAb
kDa
130
100
70
55
35
25
E2
GST-CD81 LEL
Western Blot
B
AR3B (4µg)
B6 (4µg)
No mAb
kDa
130
100
70
55
35
25
E2
GST-CD81 LEL
Western Blot
C
H77 rE1E2
E1E2 Binding Normalized (%) to Control
100
75
50
25
0
HC84.26
AR3B
AR4A
mAbs (4µg)
D
J6 sE2
E2 Binding Normalized (%) to Control
150
100
50
0
HC84.26
AR3B
AR4A
mAbs (4µg)

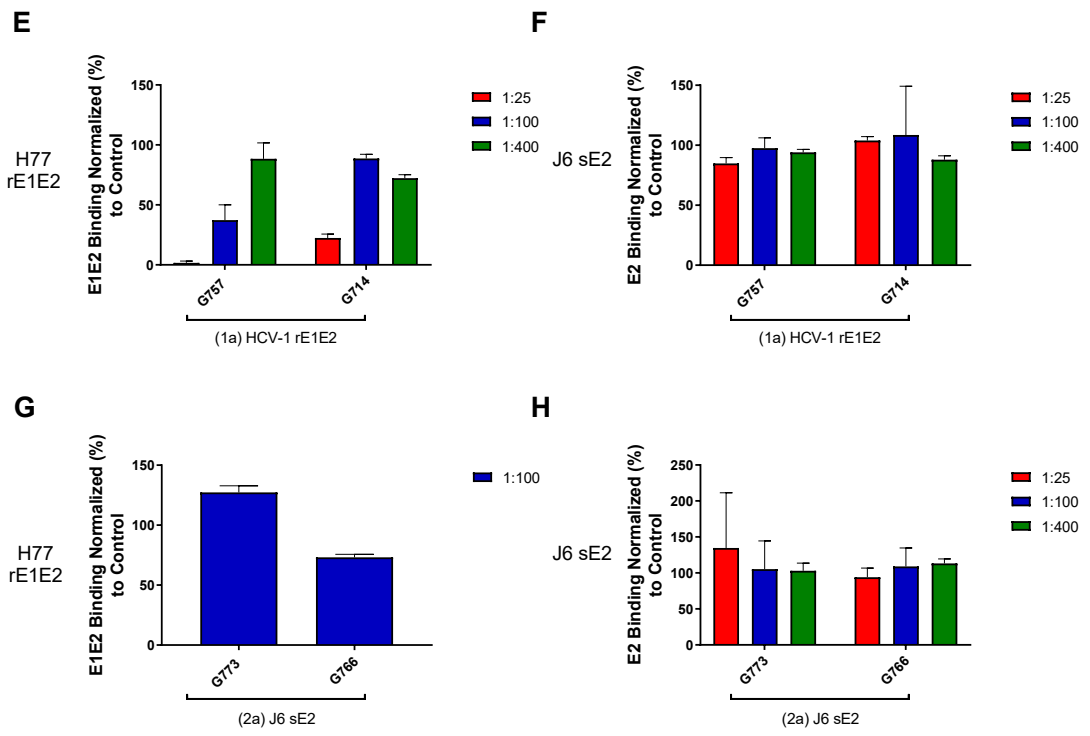


Figure 4.8 Vaccinated goat antisera block the interaction between the envelope glycoproteins and recombinant CD81 Large Extracellular Loop.
(A and B) Representative Western Blots are shown where (A) H77 rE1E2 (2µg) or (B) J6 sE2 (4µg) was pre-incubated in the presence of mAb AR3B (4µg) that blocks the E2-CD81 interaction, in the presence of isotype control B6 (4µg), or in the absence of mAbs. The mixture was then added to CD81 LEL bound to Glutathione-Sepharose beads. After SDS-PAGE, Western Blotting with mAbs H53 or 6F5 (1:2000) specific for H77 E2 or J6 E2, respectively, was performed. (C and E and G) H77 rE1E2 (2µg) or (D and F and H) J6 sE2 (4µg) was mixed with (C and D) monoclonal antibodies, (E and F) goats immunized with HCV-1 rE1E2, or (G and H) goats immunized with J6 sE2 prior to binding to CD81 LEL bound to Glutathione-Sepharose beads. After SDS-PAGE, Western Blotting with mAbs H53 or 6F5 (1:2000) specific for H77 E2 or J6 E2, respectively, was performed. The result is displayed as the densitometric signal determined with mAbs or goat antisera normalized to the densitometric signal determined with the relevant control (mIgG1/B6 control antibodies or goat pre-immunization antisera). This experiment was performed in three independent experiments in singlicate. The results are displayed as the means from three independent experiments and the error bars represent standard deviation.

5 CHAPTER V: HCV VACCINE ENGINEERING: EVALUATING NEW HCV ENVELOPE GLYCOPROTEIN VACCINE CANDIDATES USING IMMUNOLOGICAL ASSAYS

Data in this chapter were published in the following two papers:

Logan, M., Law, J.L., **Wong, J.A.**, Hockman, D., Landi, A., Chen, C., Crawford, K., Kundu, J., Baldwin, L., Johnson, J., Dahiya, A., LaChance, G., Marcotrigiano, J., Law, M., Foung, S., Tyrrell, D.L., and Houghton, M. **2016.** Native Folding of a Recombinant gpE1/gpE2 Heterodimer Vaccine Antigen from a Precursor Protein Fused with Fc IgG. ***J. Virol.*** *91(1): e01552-16*.

I was responsible for the E1E2 ELISAs, mAb binding ELISAs, and competition ELISAs. M. Logan and M. Houghton were responsible for concept formation. M. Logan was responsible for manuscript composition. Virus generation, neutralization assays, and animal work were performed by J.L. Law, D. Hockman, J. Johnson, and C. Chen. Generation of rE1E2 antigens, biochemical assays, and E2 ELISAs were performed by M. Logan.

Law, J.L., Logan, M., **Wong, J.A.**, Kundu, J., Hockman, D., Landi, A., Chen, C., Crawford, K., Wininger, M., Johnson, J., Prince, C.M., Dudek, E., Mehta, N., Tyrrell, D.L., and Houghton, M. **2018**. Role of the E2 Hypervariable Region (HVR1) in the Immunogenicity of a Recombinant HCV Vaccine. ***J. Virol.*** *92(11): e02141-17.*

I was responsible for the E1E2 ELISAs, mAb binding ELISAs, and competition ELISAs. J.L. Law and M. Houghton were responsible for concept formation. J.L. Law was responsible for manuscript composition. Virus generation, neutralization assays, and animal work were performed by J.L. Law, D. Hockman, J. Johnson, and C. Chen. Generation of rE1E2 antigens, biochemical assays, and E2 ELISAs were performed by M. Logan.

5.1 Introduction

The prototypical rE1E2 vaccine derived from the HCV-1 strain has shown great promise as a vaccine antigen as shown in **Chapter 3** and reviewed in **Chapter 1**. However, modifications to the vaccine antigen from rational vaccine design could result in improved vaccine efficacy, whether through increased immunogenicity; greater exposure of conserved neutralizing epitopes; removal of immunological decoys or elements that elicit non-neutralizing or neutralization-interfering antibodies; or stabilization of the glycoprotein structure. In addition, boosting cell-mediated responses tends to be a high priority for subunit vaccines protecting against intracellular pathogens (Benmira and Schmid 2010). Basic research into the structure and immunogenicity of the HCV glycoproteins has provided a great deal of knowledge, which can be used to engineer novel antigens that may provide improved vaccine efficacy.

Novel antigens were generated in attempts to provide the laboratory with an improved vaccine antigen for an upcoming Phase I clinical trial to enhance efficacy over the HCV-1 E1E2 vaccine antigen. Four approaches were attempted. Our laboratory generated H77 rE1E2 antigen generated through an Fc-tag precursor to optimize purification costs and time relative to the GNA method. We also generated recombinant envelope glycoproteins lacking the HVR1 region to determine if HVR1 acted as an immunological decoy or inhibitor during elicitation of immune responses and if removing HVR1 would therefore boost responses against important neutralizing epitopes. We also generated mutants lacking N-glycosylation sites to determine if the glycans act as an immunological decoy or shield during elicitation of immune responses. Finally, in an attempt to boost CD4+ and CD8+ T-cell responses, short and immunogenic T-cell antigens were taken from the HCV NS3 region and fused to rE1E2.

These novel antigens were tested in binding ELISAs with well described cross-neutralizing mAbs targeting a variety of epitopes to determine how these modifications affected recognition of neutralizing epitopes within E1 and E2. After immunization of mice, antisera were tested in ELISAs using rE1E2 or sE2 as the target antigen for immunoreactivity and in HCVpp and HCVcc neutralizing assays. Competition ELISAs with the panel of cross-neutralizing mAbs was also used for epitope mapping of the mouse antisera. These assays were used to test if these modifications would enhance neutralizing antibody responses.

I tested if the Fc-tag derived antigen and GNA-derived antigen possessed differences in presenting various cross-neutralizing epitopes and hypothesized that there would not be any differences between the two antigens. As other publications have shown that removal of HVR1 and certain N-glycosylation sites does not compromise E1E2 structure and function, I hypothesized these modifications would enhance neutralizing activity through uncovering of critical neutralizing epitopes without sacrificing antigen integrity. The T-cell epitope modified rE1E2 antigens were generated to boost cellular immune responses and enhance neutralizing antibody responses against E1E2 via cross-help. Since these sequences were substantial in length, ranging from 29-228aa, I hypothesized there would be some steric hindrance to mAbs binding epitopes on the heterodimer (particularly in the case of longer fusion partners sequences) or that there may be some structural disruption of E1E2.

5.2 Results

5.2.1 Panel of monoclonal antibodies used in these studies

Table 5.1 indicates the cross-neutralizing mAbs examined in this chapter and **Figure 1.16** provides a model describing the epitopes on E1E2. Twelve of the antibodies (H77.16, H77.39, AP33, HC33.1, HC33.4, HC84.26, 1:7, AR3B, HC-1, HC-11, CBH-7, and CBH-23) are capable of binding soluble E2. H77.16 and H77.39 can inhibit the interaction between E2 and SRB1. The mAbs H77.39, AP33, HC33.1, HC33.4, HC84.26, 1:7, AR3B, HC-1, and HC-11 target the disparate regions of E2 that form the CD81 receptor binding site (CD81bs) to block the interaction between E2 and CD81 (Law et al. 2008; Sabo et al. 2011; Keck et al. 2013, 2012; Johansson et al. 2007; Owsianka et al. 2005; Potter et al. 2012; Tarr et al. 2006; Keck et al. 2008, 2011). CBH-7 and CBH-23 are neutralizing mAbs that bind to E2 but their epitopes are not well understood (Owsianka et al. 2008; Keck et al. 2005, 2007, 2008, 2011). Two other antibodies (AR4A, AR5A) recognize unique epitopes outside the CD81bs and bind to the native E1E2 heterodimer and do not bind denatured E1E2 or either E1 or E2 alone (Giang et al. 2012). A4 and IGH526 recognize epitopes in E1 alone. A4 is a non-neutralizing mAb and IGH526 is capable of cross-neutralizing various genotypes of HCV (Dubuisson et al. 1994; Meunier, Russell, Goossens, et al. 2008; Helle et al. 2010).

5.2.2 Both Wild-Type and Fc-Tag derived E1E2 antigens are recognized similarly by the antibody panel

The previous process using GNA-lectin affinity chromatography to purify rE1E2 from lysates from CHO cells is labour intensive and difficult to scale-up for manufacturing purposes. Since rE1E2 remains tethered in the lumen of the rough ER and therefore needs to be isolated by lysing the cell in detergent, these N-linked glycoproteins are still largely associated with immature branched high-mannose chains while proteins that go through the Golgi complex, such as sE2, undergo alterations that result in the addition of complex oligosaccharide chains (Alberts et al. 2002). The purification process for the prototypical rE1E2 vaccine uses *Galanthus nivalis* antigen (GNA) agarose affinity chromatography for this purification step. GNA has high affinity for the branched high-mannose chains abundant on rE1E2 which brings the disadvantages of lack of specificity (branched high-mannose chains is a common motif) and a lack of target protein affinity. Supplies of GNA lectin agarose for large-scale manufacturing are also limiting. Therefore, our laboratory aimed to develop a method of purification that would improve on these disadvantages (Logan et al. 2017). Our laboratory inserted an Fc domain from human IgG1 at the N-terminus of E2 (shown in a schematic in **Figure A.1A**). It was shown by Dr. Michael Logan that both WT H77 rE1E2 antigen and Fc-tagged H77 rE1E2 antigen had affinity for GNA, but only Fc-tagged antigen could be captured by Protein G Sepharose (**Figure A.1B**). Once captured by Protein G Sepharose, the Fc-tag was cleaved by digestion with PreScission Protease. It was shown that the Fc-tag and purification process using Protein G Sepharose affinity chromatography to generate Fc-derived (Fc-d) antigen did not affect heterodimerization, protein size, or formation of noncovalently-linked complexes relative to GNA-derived (WT) antigen purified by GNA affinity chromatography (**Figure A.1B-D**).

Despite no differences between the two antigens seen in the biochemical assays, it was still unknown if the Fc-d rE1E2 had differences in folding leading to altered accessibility of neutralizing epitopes. Therefore, I coated ELISA plates with Fc-d rE1E2 and WT rE1E2 and I interrogated the presentation of various neutralizing epitopes with the panel of cross-neutralizing mAbs. I found that all the mAbs bound to both antigens very similarly (**Figure 5.1A-C**). This indicates that the Fc tag did not interfere with E1E2 heterodimer formation, folding, and

presentation of neutralizing epitopes across E1, E2, and E1E2, while offering a much improved purification step for our E1E2 vaccine component.

5.2.3 Both WT and Fc Tag-derived antigens elicit antibodies that compete similarly with the mAb panel

Antisera from three groups of mice (vaccinated with sham control, H77 WT rE1E2, and H77 Fc-d rE1E2 by Darren Hockman in the laboratory) were tested in E2 ELISAs using sE2 derived from the gt1a HCV-1 strain by Dr. Michael Logan in the laboratory. Homologous target antigen (H77 sE2) would have been ideal, but this was not available at the time of experiments (however, the H77 and HCV-1 strains are both of genotype 1a and share 94% identity at the primary amino acid level within the E1E2 region). At all dilutions tested, WT rE1E2 and Fc-d rE1E2 mice antisera were able to bind sE2 very similarly (with no statistical difference between the two groups but both statistically higher than sham control) (**Figure A.2**). Antisera was tested by Dr. John Law in the laboratory for neutralization of infectivity of the homologous gt1a H77 HCVpp and a heterologous gt5a SA13 HCVpp, normalizing post-vaccination antisera neutralizing activity to pre-vaccination sera. Both the WT and Fc mice antisera potently neutralized H77 HCVpp but there was no statistical difference between the two antigen groups (**Figure A.3A**). While Fc-d vaccine antisera could neutralize SA13 HCVpp with statistical significance relative to sham, WT vaccine antisera could not – however, there was no significant difference between WT and Fc-d groups (**Figure A.3B**). These data indicate that Fc-derived antigen is at least as effective in generating anti-E2 ELISA titers and neutralizing antibodies as WT rE1E2.

To determine if the similar neutralization is also consistent with a similar epitope targeting profile, I conducted competition ELISAs with a panel of cross-neutralizing mAbs. While sham sera did not block binding of any of the mAbs, both WT and Fc-derived rE1E2 antisera showed marginal to moderate competition with the panel of mAbs, with the weakest competition against HC33.4 and AR4A (**Figure 5.2**). Interestingly, while WT rE1E2 antisera was only statistically significant in competing with HC33.4 as compared with sham sera, Fc-d rE1E2 antisera showed significant competition against all 5 mAbs although there was no significant difference between WT and Fc-derived antisera competition. These competition

ELISA results suggest the elicitation of antibodies targeting conserved neutralized epitopes by GNA-derived and Fc-d antigen are similar, although only Fc-d E1E2 antisera were consistently significant compared with sham sera at the tested dilution.

5.2.4 N-glycosylation E1E2 mutants affect the recognition by cross-neutralizing monoclonal antibodies

There is evidence that the extensive glycosylation of the HCV envelope plays important roles in E1E2 folding, viral particle secretion, and entry, as well as shielding important neutralization epitopes (Helle et al. 2010, 2007; Chen et al. 2014; Helle, Duverlie, and Dubuisson 2011; Goffard et al. 2005; Falkowska et al. 2007). There are 4 N-glycosylation sites in E1 and 11 N-glycosylation sites in E2 – these are depicted in **Figure 5.3**. As shown in the literature, each glycosylation site has been investigated to determine each site's major impact on HCVpp and HCVcc infectivity, core release, and sensitivity to neutralization. Therefore, we hypothesized that removing certain glycosylation sites that are located around known neutralizing epitopes but not critical for entry or core release would expose neutralizing epitopes and allow better accessibility to these important regions by B-cells. It was believed that this would result in a stronger and more specific immune response against critical regions without impairing the folding of the E1E2 heterodimer.

Our laboratory selected three N-glycosylation sites: the third glycosylation site in E1 (E1N3; N234), the first glycosylation site in E2 (E2N1; N417), and the sixth glycosylation site in E2 (E2N6; N532). We selected these residues as all three did not lead to critically impaired core release and infectivity, and mutations at E2N1 and E2N6 have been shown to lead to a higher sensitivity to neutralization (Helle, Duverlie, and Dubuisson 2011). These residues were converted to glutamine residues by site-directed mutagenesis by Dr. Michael Logan. This removed the consensus sequence necessary for the attachment of the glycan to the protein, which requires a sequence of Asn-X-Ser/Thr where X is any amino acid besides proline, while keeping the amino acid itself similar to asparagine (Kaplan, Welply, and Lennarz 1987). E2N1 is located within the neutralizing linear epitope 412-423, which contains a critical binding residue for interaction with CD81 (W420) and antibodies that bind to this region have been shown to inhibit E2-SRB1 and E2-CD81 interaction (Owsianka et al. 2006; Keck et al. 2013; Sabo et al. 2011;

Tarr et al. 2006). The E2N6 N-glycosylation site is located close to the CD81 binding loop (including critical CD81 binding residues 527, 529, 530, and 535) and antibodies that bind to these residues potently neutralize entry and prevent E2-CD81 interaction (Kong et al. 2013; Johansson et al. 2007; Law et al. 2008). As E2N1 and E2N6 are located within neutralizing epitopes and mutations at these sites are associated with increased sensitivity to neutralization, Dr. Michael Logan in our laboratory generated a double mutant of E2N1 and E2N6, named E22N, in the possibility that removing both glycosylation sites associated with neutralizing epitopes would further enhance accessibility to B-cells. Our laboratory also generated a construct with a mutation of the third N-glycosylation site in E1 (E1N3) – this site is not associated with a neutralizing epitope or with increased sensitivity to neutralization (Helle, Duverlie, and Dubuisson 2011). Only WT rE1E2, E2N1 rE1E2, and E2N6 rE1E2 were purified (with GNA affinity chromatography) and analyzed with biochemical assays. While it could be seen that the N417Q and N532Q mutations resulted in a minimally smaller protein size, these mutations did not compromise heterodimerization (**Figure A.4A,B**). We were not able to confirm this for E1N3 rE1E2 or E22N rE1E2.

To analyze how the N-glycosylation mutations affect recognition of neutralizing epitopes, I used mAb binding ELISAs with three anti-E2 mAbs: AR3B, 1:7, and AP33. These three mAbs were chosen as all three bind epitopes containing the E2N1 and/or E2N6 N-glycosylation sites and are therefore likely to show how the N-glycosylation site mutations affect exposure of local neutralizing epitopes. There is no isolated mAb that has been shown to bind near the E1N3 N-glycosylation site. As mentioned above, AP33 binds to the aa412-423 E2 Epitope I neutralizing epitope which contains the E2N1 N-glycosylation site at N417 (Owsianka et al. 2005; Potter et al. 2012). The mAbs 1:7 and AR3B have been shown to have critical dependence on the CD81 binding loop residues local to E2N6 and moderate dependence on residues in the E2 Epitope I region (Johansson et al. 2007; Law et al. 2008). An antibody with critical dependence on the CD81 binding loop but no dependence on E2 Epitope I would have been ideal, but our laboratory did not possess any antibodies with these properties at the time of this study. The E1N3 mutant did not affect the binding of these mAbs and nonspecific binding of lysates from non-transduced by the mAbs was very low (**Figure 5.4**). AP33 showed lower binding to both E2N1 and E22N mutants, with E2N1 appearing to possess lower binding than E22N, and similar binding to E2N6. 1:7 binding was unaffected by E2N1 but was able to bind

E2N6 better than WT. 1:7 also appeared to be able to bind E22N antigen better than E2N6 antigen. AR3B binding was unaffected by E2N1 and E2N6 alone but showed higher binding to E22N. Overall, these data showed that the E22N double mutant is the best way to enhance binding of antibodies that require the CD81 binding residues within the CD81 binding loop for binding – however, the presence of the E2N1 mutant lowers the binding of an antibody that binds to the E2 Epitope I region, AP33.

5.2.5 E1E2 N-glycosylation site mutants do not elicit antibodies that compete better than WT with cross-neutralizing mAbs

Mice were vaccinated in four groups: sham control, WT, E2N1, and E2N6 by Darren Hockman in the laboratory. The post-vaccination bleeds of these mice were tested for immunogenicity against HCV-1 sE2 in ELISAs. At a dilution of 1:1000, all three test groups of mice were significantly able to bind sE2 better than sham control mice (**Figure A.5**). Although E2N1 and E2N6 mouse antisera bound lower than WT, there was no significant difference between the test groups of mice.

I then interrogated the resulting antisera in competition ELISAs using H77 sE2 as target antigen to determine if the removal of glycans led to augmentation of antibodies to neutralizing epitopes. AP33 and HC33.4 were used to test if there was enhanced elicitation of antibodies directed against E2 Epitope I and 1:7 and AR3B were used to test if there was enhanced elicitation of antibodies directed against the CD81 binding loop (Keck et al. 2013; Owsianka et al. 2005; Johansson et al. 2007; Law et al. 2008). I found competition was modest and significant for mice immunized with WT or E2N6 antigen compared to control for AP33 (no difference between the two), while E2N1 did not show statistical significance from control (**Figure 5.5**). There was no significant competition for any of the mouse groups against HC33.4 relative to control. WT and E2N6 mouse antisera were able to significantly compete modestly (no difference between the two) with the binding of 1:7 relative to control, while E2N1 was not able to significantly compete with 1:7 relative to control. All three groups of mice were able to compete with AR3B moderately and significantly relative to control (but with no difference between them).

These data indicated that WT and E2N6 antigens had similar antibody generation in respect to the tested mAbs, with E2N1 antigen likely being an inferior antigen, although there was no statistical significance between competition activities of WT, E2N1, and E2N6.

5.2.6 Fusion of various T-cell peptides to an E1E2 vaccine antigen affects the recognition by cross-neutralizing monoclonal antibodies

A HCV vaccine candidate currently in clinical testing consists of an adenoviral vector and a Modified Vaccinia Ankara vector encoding the NS3-NS5B proteins from a gt1b strain in an attempt to generate protective CD4+ and CD8+ T-cell responses (Swadling et al. 2014; Barnes et al. 2012). The use of T-cell epitopes in vaccines chosen through rational vaccine design has exciting potential and has resulted in an approved therapeutic vaccine against HPV, and such vaccines against HIV, influenza, and some bacterial pathogens are currently in development and/or testing (Kenter et al. 2009; Pleguezuelos et al. 2012; Rosendahl Huber et al. 2014; Holanda et al. 2017). The prototypical HCV-1 rE1E2 vaccine was able to generate strong CD4+ T-cell responses (Frey et al. 2010) but our laboratory sought to enhance the CD4+ & CD8+ T-cell responses while keeping the strong B-cell responses generated by the rE1E2 heterodimer. Therefore, Dr. Amir Landi in our laboratory selected sequences from the HCV NS3 region predicted to contain strong CD4+ T helper & CD8+ T cell epitopes (personal communication with Dr. Amir Landi; **Table 5.2**). These sequences were inserted by Dr. Michael Logan into the Fc-tagged H77 rE1E2 constructs with the T cell epitopes fused between the Fc-tag and E2 to produce E1E2 heterodimers containing various T-cell epitopes fused to the N-terminus of E2 (**Figure A.6A**). These sequences varied in length and in biochemical properties and so it was important to determine if the presentation and availability of important epitopes associated with neutralization were affected by the insertion of the different T-cell epitopes – whether by improper folding, improper heterodimerization, or occlusion of these neutralizing epitopes. Early analysis of these constructs performed by Dr. Michael Logan showed no misfolded aggregates and normal heterodimerization (**Figure A.6B-C**). I hypothesized that these T-cell epitopes when fused to E2 within the E1E2 heterodimer could alter its conformation and immunoreactivity. To test this hypothesis, I interrogated these rE1E2 fusion heterodimers using immunological assays.

I coated microtiter plate wells with the purified rE1E2 antigens of the different constructs and conducted mAb binding ELISAs with a panel of anti-E2 and anti-E1E2 cross-neutralizing mAbs. Surprisingly and as seen in **Figure 5.6**, all the T-cell epitope fusions reduced the binding of all the mAbs. The length of the T-cell epitope correlated roughly with the reduction in binding but not as expected since TP138 and TP138L reduced binding minimally, TP100 and TP228 reduced binding moderately, while the two shortest tags TP29 and TP52 reduced binding severely. These trends were consistent across the numerous mAbs tested – an exception being TP228 and HC33.4. TP138L contained an extra Glycine-Serine linker (GGGSGGGS) between the T-cell epitope and the start of the E2 protein to determine if a short flexible linker could prevent occlusion of the epitopes – this data shows the linker did not improve availability of the epitopes. According to the data generated, it appears that TP138 is the tag most likely to result in an E1E2 heterodimer exhibiting an immunoreactivity profile approaching that of wild-type unfused E1E2.

Mice were vaccinated in five groups: sham control, WT, TP29, TP52, and TP100 by Darren Hockman in the laboratory. Post-vaccination antisera were tested for immunogenicity against HCV-1 sE2. This revealed that only WT and TP100 groups of mice recognized antigen significantly better than sham, while TP29 and TP52 groups of mice could not (**Figure A.7**). All the TP antigens appeared to elicit antisera with weaker ELISA titers relative to WT. However, the only significant difference was between WT and TP52.

5.2.6 Deletion of the hypervariable 1 region (HVR1) at the N-terminus of E2 within the E1E2 heterodimer reduces binding by cross-neutralizing mAbs targeting the HVR1 region but does not affect binding by other cross-neutralizing mAbs and elicits antibodies with lower neutralizing activity in mice than WT

As detailed in **Chapter 1**, there is evidence suggesting HVR1 plays an important role in humoral immune evasion as an immunological decoy or by affecting neutralizing epitopes elsewhere in E1E2. While antibodies to HVR1 can be neutralizing, this seems to be strain-specific (Farci et al. 1996; Shimizu et al. 1996; Zibert, Schreier, and Roggendorf 1995; Kato et al. 1994). Deleting HVR1 enhances interaction between E2 and CD81 and a mAb targeting HVR1 (H77.16) was capable of interfering with the binding of broadly cross-neutralizing

antibodies targeting E2 Epitope I (aa412-423) (Keck et al. 2016; Sabo et al. 2011; Scarselli et al. 2002; Roccasecca et al. 2003). Furthermore, deleting the HVR1 region from a panel of HCVcc strains from different HCV genotypes led to their enhanced neutralization by a panel of cross-neutralizing mAbs targeting diverse epitopes. Importantly, while inter-genotypic neutralization against the WT virus strains by the mAbs was highly variable (24-fold to 130-fold differences in 50% inhibitory concentration values (IC_{50})), deleting HVR1 resulted in more uniform inter-genotypic neutralization for most of the mAbs (<6-fold differences in IC_{50}), – this indicated HVR1 is a critical determinant of genotype-specific neutralization (Prentoe et al. 2016; Bankwitz et al. 2010).

The WT and ΔHVR1 (lacking the first 27aa at the N-terminus of E2 (aa384-411) H77 rE1E2 constructs were created using GNA affinity chromatography by Dr. Michael Logan in the laboratory (Law et al. 2018). His biochemical analysis of these constructs showed no misfolded aggregates and normal heterodimerization (**Figure A.8**). I then interrogated the purified protein of these two constructs with a panel of diverse cross-neutralizing mAbs. As expected, mAb H77.16, which requires three residues from the C-terminal end of HVR1 for binding, was found to bind H77 E1E2 WT but not H77 E1E2 ΔHVR1 (**Figure 5.7A-C**; (Sabo et al. 2011)). Surprisingly, the mAb HC33.4, which targets E2 Epitope I (aa412-423), produced the same result as H77.16 (Keck et al. 2013). This could be explained by the fact that HC33.4 requires a residue within the HVR1 region for binding (aa408) (Keck et al. 2013). However, when I performed the ELISA using HC33.1 and H77.39, two mAbs that also bind residues within the E2 Epitope I region (aa412-423) but do not require any residues within the HVR1 according to alanine scanning mutagenesis data, this same result was seen (Sabo et al. 2011; Keck et al. 2013). This suggests HVR1 could possibly be necessary for the proper presentation of E2 Epitope I, at least in the context of the E1E2 heterodimer. For the one mAb primarily binding E2 Epitope II (aa434-446), HC84.26, and the four mAbs primarily binding the CD81 binding loop in E2 (aa523-540), 1:7, AR3B, HC-1, and HC-11, I saw that these mAbs bound rE1E2 ΔHVR1 very similar to or slightly weaker than they bound rE1E2 WT. These data indicate that the CD81 binding sites found on E2, including residues found in E2 Epitope I, E2 Epitope II, and the CD81 binding loop were not more accessible after deleting HVR1 from rE1E2. For the CBH-7 and CBH-23 mAbs that bind E2 and require no residues found in HVR1, E2 Epitope I, E2 Epitope II, and the CD81 binding loop, there was a moderate reduction in the binding of these mAbs to

ΔHVR1 antigen relative to WT protein. A moderate reduction was also seen for the two mAbs (AR4A and AR5A) binding an epitope requiring the E1E2 heterodimer, and the mAb (A4) binding an E1 epitope. These data suggest that in contrast to the data obtained with infectious virus, deletion of HVR1 does not increase accessibility of the rE1E2 protein for a variety of neutralizing mAbs and can drastically reduce the accessibility of some mAbs for their epitopes (Prentoe et al. 2016; Bankwitz et al. 2010).

5.2.7 Deletion of the hypervariable 1 region (HVR1) at the N-terminus of E2 within the E1E2 heterodimer does not elicit antibodies that compete better than WT with cross-neutralizing mAbs

The H77 rE1E2 WT and H77 rE1E2 ΔHVR1 antigens were used to vaccinate mice by Darren Hockman in our laboratory. Final bleeds from the three groups of mice (sham, H77 E1E2 WT, and H77 E1E2 ΔHVR1) were tested for ELISA titers by Dr. Michael Logan against H77 sE2 WT and H77 sE2 ΔHVR1. There were strong antibody responses with very similar titers between WT and ΔHVR1 groups with both target antigens used (**Figure A.9A**). While it seemed the responses from the group of mice immunized with ΔHVR1 were slightly higher relative to the group of mice immunized with WT to both target antigens, this difference was not statistically significant. Mice vaccinated with rE1E2 WT vaccinated mice showed strong responses to a synthetic peptide containing HVR1 and the first 6 residues downstream of HVR1 (aa384-417), while the rE1E2 ΔHVR1 vaccinated mice were not responsive to this peptide even at the lowest dilution tested (1:100) (**Figure A.9B**). This confirmed the presence of an HVR1-specific immune response.

Pre-immunization and post-immunization antisera from the WT and ΔHVR1 vaccinated groups were then tested in neutralizing assays against HCVpp virions pseudotyped with E1E2 from the H77 strain. Pre-vaccination sera from both groups did not show any neutralizing activity. Interestingly, post-immunization antisera from the WT group showed strong neutralizing activity but post-immunization antisera from the ΔHVR1 group did not show significant neutralization over pre-immunization sera (**Figure A.10**).

An HCVpp virus pseudotyped with E1E2 from the H77 strain lacking the HVR1 region was also generated for further experiments – this virus contained compensatory mutations in E1

and E2 (H261R, Q444R). As expected, H77.16 (targeting the HVR1 region) was effective against WT but not against ΔHVR1 viruses (**Figure A.11A**). The anti-CD81 antibody was similarly effective against both viruses, although it was slightly more effective against WT virus (not significant). Despite previous studies implicating HVR1 as an inhibitor of other neutralizing epitopes by showing that removal of HVR1 led to enhanced neutralization by a diverse panel of mAbs (Prentoe et al. 2016; Bankwitz et al. 2010), AR3B (targeting the CD81 binding loop region) was unaffected by the deletion of HVR1 and was effective against both viruses. Antisera from the WT and ΔHVR1 mouse groups were tested against WT and ΔHVR1 HCVpp. The WT vaccinated mouse group potently neutralized both viruses (**Figure A.11B**). Notably, the ΔHVR1 vaccinated mouse group potently neutralized the ΔHVR1 virus but not the WT virus. This indicated that both antigens elicited antibodies targeting outside of HVR1 with similar neutralizing activity and suggested the difference of effectiveness between WT and ΔHVR1 vaccinated mice antisera against WT HCVpp was due to anti-HVR1 antibodies.

The two antigens were also used to vaccinate guinea pigs by Darren Hockman, which have been shown to exhibit stronger immune responses upon vaccination relative to mice (Stamataki et al. 2007). When antisera were tested for neutralization against H77 HCVpp, it was found that both antigens were capable of eliciting potently neutralizing antisera at the dilution tested – however WT antisera had statistically higher neutralization than ΔHVR1 antisera (**Figure A.12A**). The antisera were also tested against HCVpp encoding the envelope glycoproteins of a gt3a strain (S52), a gt4a strain (ED43), and a gt5a strain (SA13) to determine cross-neutralizing potency. Both antigens again showed equivalent capability of eliciting neutralizing antibodies against the heterologous viruses (**Figure A.12B**). These data indicated there is a difference in the immune response elicited in mice and guinea pigs as ΔHVR1 antisera was only marginally less neutralizing than WT antisera (but still statistically significantly lower) in guinea pigs. These data also showed that removing the HVR1 region did not appear to enhance cross-neutralizing antibodies.

To determine the mechanism of the reduced neutralization of antisera from ΔHVR1 vaccinated mice compared to WT antigen, I performed competition ELISAs with a panel of cross-neutralizing antibodies to determine the differences in how the antisera from the two groups of mice targeted these neutralizing epitopes. I used mAbs H77.16 and HC33.4 – both require at least one residue in HVR1 – as well as mAbs HC84.26, AR3B, AR4A, and AR5A. I

found there was a very similar pattern in the competition profiles between the two groups of mice (**Figure 5.8**). Competition against antibodies HC84.26, AR3B, and AR5A showed moderate competition and competition against H77.16, HC33.1, HC33.4, and AR4A was minimal or nonexistent at the tested dilution. For HC33.1 and AR3B, there was statistically significant competition in ΔHVR1 vaccinated mice but not in WT vaccinated mice relative to Sham – however there was no significant difference between ΔHVR1 and WT vaccinated mice. Competition against H77.16 and HC33.4 showed no significant competition at the concentration of antisera tested for either group. These data indicate that both antigens elicited similar levels of antibodies recognizing E2 and E1E2 epitopes outside of HVR1 and that neither antigen generated high enough levels of anti-HVR1 antibodies to compete with the two mAbs dependent on HVR1 residues.

5.3 Discussion

5.3.1 Fc-derived rE1E2 heterodimer as a vaccine antigen

The previous approach to recombinant E1E2 purification utilized GNA affinity chromatography, which has the disadvantages of low affinity and low specificity. Our laboratory developed a method with higher protein affinity, specificity, and scalability using a human IgG1 Fc-tag placed at the N-terminus of E2. Despite the large size of the tag, E1E2 heterodimerization appeared unaffected and Fc-d antigen appeared indistinguishable from GNA-derived antigen (**Figure A.1B-D**). To ensure there were no effects on folding or epitope presentation, I conducted binding ELISAs with a panel of cross-neutralizing mAbs and found very similar binding profiles (**Figure 5.1A-C**). My data matched the work of my colleagues who showed in vaccinated mice that both forms of E1E2 elicited similar ELISA titers and neutralization profiles (**Figure A.2**, **Figure A.3A**,**B**). This was also consistent with the mAb competition ELISAs that I performed which showed similarity between the two antigen groups (**Figure 5.2**). As mentioned in **Chapter 3**, there are concerns that competition with mAbs by a polyclonal sera sample may partially represent allosteric inhibition and steric hindrance rather than direct binding to the epitope. This was addressed in **Chapter 3** and shown that in the cases of two mAbs that bound a linear epitope, there was indeed direct competition by goat antisera. It is likely that most of the competition seen in **Chapter 3** and **Chapter 5** are at least partially due to direct binding. While

the Fc-d group showed significant competition with a few of the mAbs and neutralization of gt5a HCVpp relative to control while the WT group did not, there was no significant difference between Fc-d and WT groups, so I concluded that Fc-d antigen has a more efficient method of production and purification without negatively affecting immunoreactivity or immunogenicity.

5.3.2 N-glycosylation site mutants as HCV vaccine antigens

The heavy glycosylation of E1E2 has been thought to be important in immune evasion/shielding. Indeed, some glycans are located close to neutralizing epitopes and removing certain glycans increases neutralization (Helle, Duverlie, and Dubuisson 2011). Our laboratory generated H77 E1E2 antigens with asparagine to glutamine mutations at E1N3, E2N1, E2N6, and a double mutation at E2N1 and E2N6 (E22N). As expected, the E1N3 mutation, which has not been shown to have an effect on neutralization and is not located near any neutralizing epitopes, did not affect binding by any of the neutralizing mAbs in my mAb binding ELISAs (**Figure 5.4**). AP33 bound the E2N1 and E22N mutants poorer than WT which was unexpected as I predicted the removal of the N-glycosylation site would open E2 Epitope I for enhanced AP33 binding. It is possible that even though the N417Q mutation was chosen to conserve the amino acid properties as best as possible, this change could have negatively affected AP33 binding. Consistent with this, AP33 showed reduced neutralization against N417D and N417G mutated HCVcc (these mutations also remove the N417 glycan) (Pantua et al. 2013). Interestingly, another mAb that binds E2 Epitope I, 3/11, was shown to have increased neutralization against HCVpp with an E2N1 mutation (Helle et al. 2007). The two mAbs AP33 and 3/11 bind to two very different conformations of the E2 Epitope I region and it's possible this is the reason why they react differently to alterations at N417 even though both do not require the 417 residue for binding (Meola et al. 2015). In the future, it would be of interest to test mAb binding with 3/11.

I found that the double E22N mutant achieved the best enhancement of binding by AR3B and 1:7 as compared with E2N1 or E2N6, possibly indicating there was an additive or synergistic effect. While AR3B and 1:7 have not been tested against N-glycosylation site mutants in the literature, mAb CBH-5, which has been shown to primarily require residues within the CD81 binding loop along with moderate dependence on residues within the E2 Epitope I region (very

similar to AR3B and 1:7), showed increased neutralizing activity to HCVpp containing N-glycosylation site mutants at both E2N1 and E2N6 (Helle et al. 2007; Owsianka et al. 2008). These data indicate that for antibodies that require the binding of residues within the CD81 binding loop and E2 Epitope I, mutations in the E2N1 and E2N6 N-glycosylation sites – and especially a double E2N1/E2N6 mutation – can enhance binding and subsequent neutralizing activity. Interestingly, mAb H48 also showed heightened neutralization against both E2N1 and E2N6 HCVpp mutants and has critical dependence on CD81 binding loop residues but no dependence on residues in the E2 Epitope I region, indicating that the removal of the N417 glycan could open the larger part of the CD81 binding site (Owsianka et al. 2006; Helle et al. 2007).

Although the E2N1, E2N6, and E22N mutations appear to benefit binding and neutralization by antibodies binding to the CD81 binding loop in both the literature and in my work, this effect was only less than a 2-fold increase ((**Figure 5.4**); (Helle et al. 2007)). Similarly, the neutralizing activity of mAb 3/11 increased less than 2-fold after E2N1 mutation (Helle et al. 2007). Considering the E2N1 mutation may reduce binding and neutralizing activity of potent neutralizing antibodies binding the E2 Epitope I such as AP33 and HCV1, N-glycosylation mutants may not offer a superior vaccine antigen (Pantua et al. 2013). Consistent with this, E2N1 and E2N6 vaccinated mice did not bind sE2 better than WT and competition ELISAs with vaccinated mouse antisera showed no statistical benefit of removing either one of the N-glycosylation sites in E2 (**Figure A.5**, **Figure 5.5**). From these data, it appears that removal of the N-glycosylation sites does not lead to higher accessibility by antibodies and B-cells, or if it does, it impacts the neutralizing epitopes only minimally.

5.3.3 T-cell epitopes to boost CD4+ and CD8+ T-cell responses within an E1E2 vaccine antigen

In an attempt to enhance the CD4+ T-cell, CD8+ T-cell, and humoral immune responses to the rE1E2 vaccine, our laboratory fused T-cell epitopes of varying lengths from the HCV NS3 region to the N-terminus of E2 within the context of the E1E2 heterodimer. Since I expected that these sequences could interfere with the binding of neutralizing mAbs and subsequent neutralizing activity, I used mAb binding ELISAs to interrogate the various constructs. I found

all the T-cell epitopes interfered with binding of all the antibodies and that TP138 was the fusion that interfered with binding the least (**Figure 5.6**). It is possible that the longer the fusion domain, the more structured it is resulting in less disruption of the downstream E2. T-cell fusion domains could affect the structure of E1E2, create steric hindrance of neutralizing epitopes, and/or cause allosteric inhibition of the epitopes.

The specific residues within the T-cell epitopes could also affect the presentation of E1E2 epitopes. Only TP29, TP52, and TP100 antigens were used to immunize mice. Only WT and TP100 vaccinated mice antisera were able to bind target antigen significantly better than sham (**Figure A.7**). There seemed to be a correlation between mAb binding ELISAs and sE2 ELISAs – TP29 and TP52 vaccinated mice antisera showed the lowest binding followed by TP100. However, the only significant difference between the test groups of mice was between WT and TP52. This further strengthened the validity of using mAb binding ELISAs when evaluating rE1E2 vaccine antigens. Neutralization assays and T-cell assays will be necessary to determine if the tags offer an overall improved vaccine.

5.3.4 HVR1-deleted E1E2 heterodimer as a vaccine antigen

As mentioned in **Chapter 1**, HVR1 appears to play a role in immune escape; HVR1 undergoes changes under selection from HVR1 targeting neutralizing antibodies (Farci et al. 2000; von Hahn et al. 2007; Weiner et al. 1992; Liu et al. 2010). In addition, viruses lacking HVR1 were more sensitive to neutralization by mAbs targeting a variety of epitopes outside of HVR1 (Prentoe et al. 2016; Bankwitz et al. 2010). These data suggested the possibility that a vaccine lacking ΔHVR1 would be more effective.

Results from probing H77 E1E2 ΔHVR1 with mAbs showed a near complete ablation of binding for four antibodies dependent on residues found in HVR1 and/or the proximal E2 Epitope 1 region for binding (**Figure 5.7A-C**). These findings indicated that HVR1 may be necessary for the proper presentation of the important aa412-423 epitope. While deleting HVR1 did affect the binding of most of the mAbs binding outside of HVR1 and E2 Epitope I, in none of these cases was binding to ΔHVR1 antigen enhanced over WT but instead moderately reduced. This was unexpected as the removal of HVR1 enhanced the neutralization of HCVcc, often dramatically, by most of the mAbs that I used in my studies (Bankwitz et al. 2010; Prentoe et al.

2016). It was shown that HCV RNA from HVR1-deleted HCVcc was captured in higher amounts relative to WT HCVcc following immunoprecipitation of HCVcc with mAbs, indicating the binding affinities of mAbs differed between HCVcc and H77 rE1E2 (Prentoe et al. 2016). Therefore, the binding differences are most likely due to either structural differences between a recombinant E1E2 heterodimer protein and the E1E2 incorporated into the virion and/or to other components complexed with HCVcc such as lipoproteins that may be mediated at least in part by HVR1 (Freedman et al. 2017; Falson et al. 2015; Jiang et al. 2012). Apparently, HVR1 acts as an important immunological decoy when present in these higher order structures complexed with lipoproteins while not interfering in the recombinant heterodimer E1E2.

Work by another research group has shown that when recombinant H77 sE2 was used as the target antigen, HC33.4 binding was reduced drastically when HVR1 was removed, while binding of HC33.1, HC-1, and HC84.26 was modestly enhanced when HVR1 was removed, and binding of HC-11 and CBH-7 was identical between WT and ΔHVR1 (Keck et al. 2016). The difference between HC33.1 and HC33.4 was thought to be due to HC33.4 requiring a residue within HVR1 for binding but HC33.1 does not. This is partially consistent with my findings – I saw no change for HC-11 and CBH-7 but a reduction of HC33.4 was seen. However, there was no enhancement seen with any of the mAbs and HC33.1 binding was as poor for ΔHVR1 antigen as HC33.4. The many differences between the antigen generation and purification, and ELISA protocols, such as the use of H77 rE1E2 as opposed to H77 sE2 and the use of purified protein as opposed to unpurified cell lysates and GNA coated plates, could be sufficient to explain the differences between the Z.-Y. Keck et al., 2016 findings and my findings with H77 rE1E2. These data could suggest that presentation of the aa412-423 epitope could be dependent on HVR1 in the context of the E1E2 heterodimer but not the soluble E2 protein and that the HVR1 could act as an immunological decoy by blocking other neutralizing epitopes in the context of sE2 and HCVcc but not rE1E2.

The lack of increased mAbs binding implied that the rE1E2 ΔHVR1 antigen would not elicit a higher level of neutralizing antibodies, as it seemed that neutralizing regions distant to HVR1 were not rendered any more accessible and neutralizing antibodies targeting regions close to HVR1 were reduced. Indeed, neutralization against H77 HCVpp was significantly reduced in ΔHVR1 vaccine antisera, while neutralization against three heterologous strains of HCVpp was unchanged (**Figure A.10**, **Figure A.12**). Combined with the ELISA data showing similar E2

ELISA titers but undetectable levels of anti-HVR1 antibodies in ΔHVR1 vaccine antisera, this suggests that the HVR1 directed antibodies that were found in WT antisera but not ΔHVR1 antisera enhanced the neutralization of the homologous virus while neutralization of variant strains was dependent on other neutralizing epitopes within E1E2 (**Figure A.9B**). This was further supported by data showing that removing HVR1 from HCVpp led to neutralization by ΔHVR1 vaccinated mice antisera to the same level as WT vaccinated mice antisera (**Figure A.11B**). The similarity in E2 ELISA titers indicated that the majority of reactive antibodies were directed outside of HVR1 and this was consistent was the similar competition ELISA results (**Figure 5.8**). This indicates that as predicted by the mAb binding ELISAs, the two vaccines generated similar antibodies with the exception of HVR1 directed antibodies. This difference between the results of neutralization of WT and ΔHVR1 HCVpp from mice and guinea pigs could possibly be explained by previous evidence that guinea pigs tend to show stronger neutralizing responses than mice in HCV vaccine immunizations and sufficiently stronger titers to epitopes outside of HVR1 could have been responsible for the neutralization (**Figure A.10**, **Figure A.12A**). This possibility could be evaluated by assaying guinea pig antisera for E2 and HVR1 peptide ELISA titers.

Overall, these data show that removing HVR1 from H77 rE1E2 has deleterious effects on homologous neutralization without improving the role of cross-neutralizing epitopes elsewhere in E1E2.

5.3.5. Conclusions

Although there is evidence that removing HVR1 and some N-glycosylation sites leads to enhanced neutralization of HCV virions, my interrogations of the resulting antigens and their antigenicities showed that these changes did not lead to an improved rE1E2 vaccine antigen. Similarly, attempts at broadening T-cell responses by fusing HCV T cell epitopes to rE1E2 resulted in reduced binding of neutralizing mAbs and no improvement in the immunoreactivity or antigenicity of rE1E2. However, using these methods, I was able to confirm that producing E1E2 via a Fc fusion did not alter the presentation and immunogenicity of numerous neutralizing epitopes located throughout E1E2. This Fc-d rE1E2 expression and purification process greatly facilitates large scale production and delivery of our vaccine.

Monoclonal Antibody	Species	Protein Targeted	Conformation Dependent?	Binds Peptide?	Critical Binding Residues	Interaction Targeted	Neutralization Potency	Reference
H77.16	Mouse	E2	N/A	N/A	406,408,410	SRB1	1a (HCVcc)	(Sabo et al. 2011)
H77.39	Mouse	E2	N/A	N/A	415,417	SRB1/CD81	1a,2a,4a,5a (HCVcc)	(Sabo et al. 2011)
AP33	Mouse	E2	No	Yes 412-423	413,415,418,420	CD81	1a,1b,2a,4a,5a,6a,7a (HCVcc) 1a,1b,2a,3a,4a,5a,6a (HCVpp)	(Owsianka et al. 2005; Tarr et al. 2006; Desombere et al. 2017)
HC33.1	Human	E2	No	Yes 412-423	413,418,420	CD81	1a,2a,3a,4a,5a,6a (HCVcc)	(Keck et al. 2013)
HC33.4	Human	E2	No	Yes 412-423	408,413,420	CD81	1a,2a,3a,4a,5a,6a (HCVcc)	(Keck et al. 2013)
HC84.26	Human	E2	Yes	Yes 434-446	429,441,442,616	CD81	1a,2a,3a,4a,5a,6a (HCVcc)	(Keck et al. 2012)
1:7	Human	E2	Yes	N/A	415,417,484,491,523, 525,526,527,529,530, 533,535,540	CD81	2a (HCVcc) 1a,1b,2a,2b,3,4,5,6 (HCVpp)	(Johansson et al. 2007)
AR3B	Human	E2	Yes	N/A	412,416,418,423,424, 523,525,530,535,540	CD81	2a (HCVcc) 1a,1b,2a,2b,4,5 (HCVpp)	(Law et al. 2008)
HC-1	Human	E2	Yes	N/A	529,530,535	CD81	1a,2a (HCVcc) 1a,2a,5,6 (HCVpp)	(Keck et al. 2008, 2011)
HC-11	Human	E2	Yes	N/A	425,428,436,437,438, 442,443,530,535	CD81	1a,2a (HCVcc) 1a,2a,5 (HCVpp)	(Keck et al. 2008, 2011)
CBH-7	Human	E2	N/A	N/A	540	N/A	1a,1b (HCVpp) 2a (HCVcc)	(Owsianka et al. 2008; Keck et al. 2005, 2007, 2008, 2011, 2012)
CBH-23	Human	E2	N/A	N/A	N/A	N/A	N/A	(Keck et al. 2012)
A4	Mouse	E1	No	Yes 197-207	197-207	N/A	No	(Dubuisson et al. 1994)
IGH526	Human	E1	N/A	Yes 313-327	313-327	N/A	1a,1b,2a,4a,5a,6a (HCVcc) 1a,2a,4a,5a,6a (HCVpp)	(Meunier, Russell, Goossens, et al. 2008)
AR4A	Human	E1E2	Yes		487,692	N/A	1a,1b,2a,3a,4a,5a,6a (HCVcc) 1a,1b,2a,2b,2i,2x,3a,4,5,6 (HCVpp)	(Giang et al. 2012)
AR5A	Human	E1E2	Yes		639	N/A	1a,2a,4a,5a,6a (HCVcc) 1a,1b,4,5,6 (HCVpp)	(Giang et al. 2012)

Table 5.1 List of monoclonal antibodies used in the binding and competition ELISAs. Neutralization potency is determined by an IC_{50} cutoff of less than 100μg/mL. Critical binding residues are determined by 25% or less binding to antigen after mutagenesis of residue to alanine. All amino acid numbering is based on the H77 isolate strain (GenBank accession number: AF009606.1) as specified by international consensus (Kuiken and Simmonds 2009).

Name	Source	Peptide Sequence	AA	Notes
TP29	NS3	AIPLEVIKGGRHLIFCHSKKKCDELAAKL	1379-1407	
TP52	NS3	AIPLEVIKGGRHLIFCHSKKKCDELAAKLVALGINAVAYYRG LDVSVIPTSG	1379-1430	
TP100	NS3	VALSTTGEIPFYGKAIPLEVIKGGRHLIFCHSKKKCDELAAKL VALGINAVAYYRGLDVS VIPTSGDVVVVATDALMTGFTGDFDSVIDCNTCVTQTVDF	1365-1464	
TP138	NS3	VALSTTGEIPFYGKAIPLEVIKGGRHLIFCHSKKKCDELAAKL VALGINAVAYYRGLDVSVIPTSGDVVVVA TDALMTGFTGDFDSVIDCNTCVTQTVDFSLDPTFTIETTTLPQ DAVSRTQRRGRTGRGKPGIYRFV	1365-1502	
TP138L	NS3	VALSTTGEIPFYGKAIPLEVIKGGRHLIFCHSKKKCDELAAKL VALGINAVAYYRGLDVSVIPTSGDVVVVA TDALMTGFTGDFDSVIDCNTCVTQTVDFSLDPTFTIETTTLPQ DAVSRTQRRGRTGRGKPGIYRFVGGGSGGGS	1365-1502	Linker inserted between TP and start of E2
TP228	NS3	LHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAY MSKAHGIDPNIRTGVRTVTTGAPITYSTYGKFLADGGCSGGA YDIIICDECHSTDATTILGIGTVLDQAETAGVRLVVLATATPP GSVTVPHPNIEEVALGTTGEIPFYGKAIPLEVIKGGRHLIFCHS KKKCDELAAKLVGLGLNAVAYYRGLDVSVIPTSGDVVVVA TDALMTGFTGDFDSVIDCN	1228-1445	

Table 5.2 List of T-cell epitopes inserted in H77 rE1E2 vaccine proteins.
These sequences were designed by Dr. Amir Landi and were derived from the NS3 protein of a gt1a consensus sequence. TP138L contained a linker sequence (GGGSGGGS) in between the TP138 sequence and the E2 protein. All amino acid numbering is based on the H77 isolate strain (GenBank accession number: AF009606) as specified by international consensus (Kuiken and Simmonds 2009).

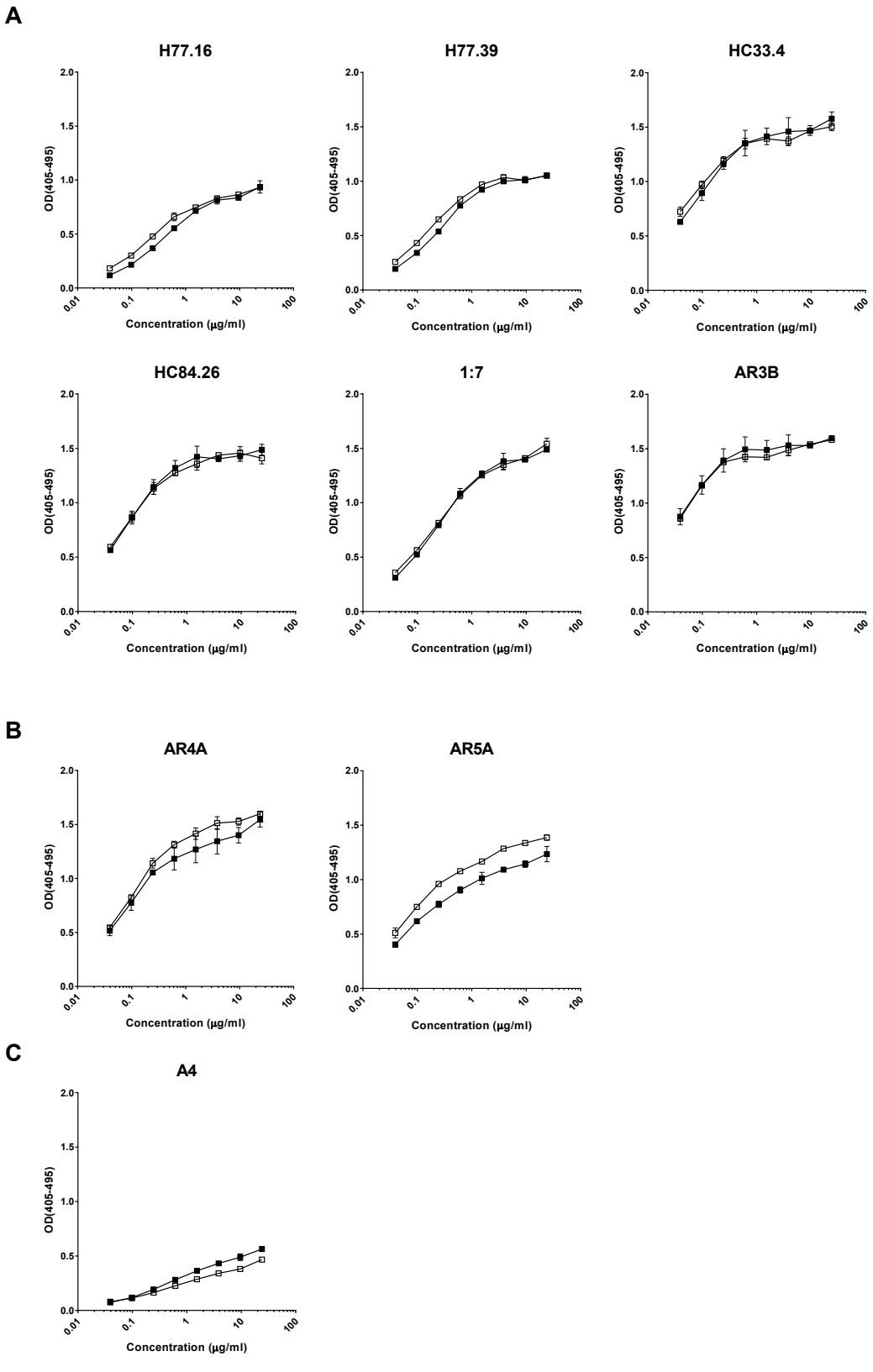

A
H77.16
H77.39
HC33.4
HC84.26
1:7
AR3B
B
AR4A
AR5A
C
A4
OD(405-495)
Concentration (µg/ml)
0.01
0.1
1
10
100
0.0
0.5
1.0
1.5
2.0

Figure 5.1 Effect of expression and purification techniques of H77 rE1E2 on the binding of well characterized cross-neutralizing monoclonal antibodies in ELISA experiments. H77 rE1E2 GNA-derived (WT) H77 rE1E2 Fc-tag derived (Fc-d). ELISA wells were coated with H77 rE1E2 GNA-derived or H77 rE1E2 Fc-tag derived. Unbound protein was washed off, and 2.5-fold dilutions from 24μg/mL of monoclonal antibodies were incubated on the bound protein. Signal was developed by species-specific secondary antibody conjugated to alkaline phosphatase and pNPP. Absorbance was recorded at 405nm (subtracting absorbance at 495nm to remove background). This was performed in two independent experiments in duplicate. The results are displayed as the means from one representative experiment and the error bars represent standard deviation. The nine mAbs used in binding ELISAs are grouped based on their reactivity: (A) antibodies that bind E2; (B) antibodies that require E1E2 for binding; and (C) antibodies that target E1.

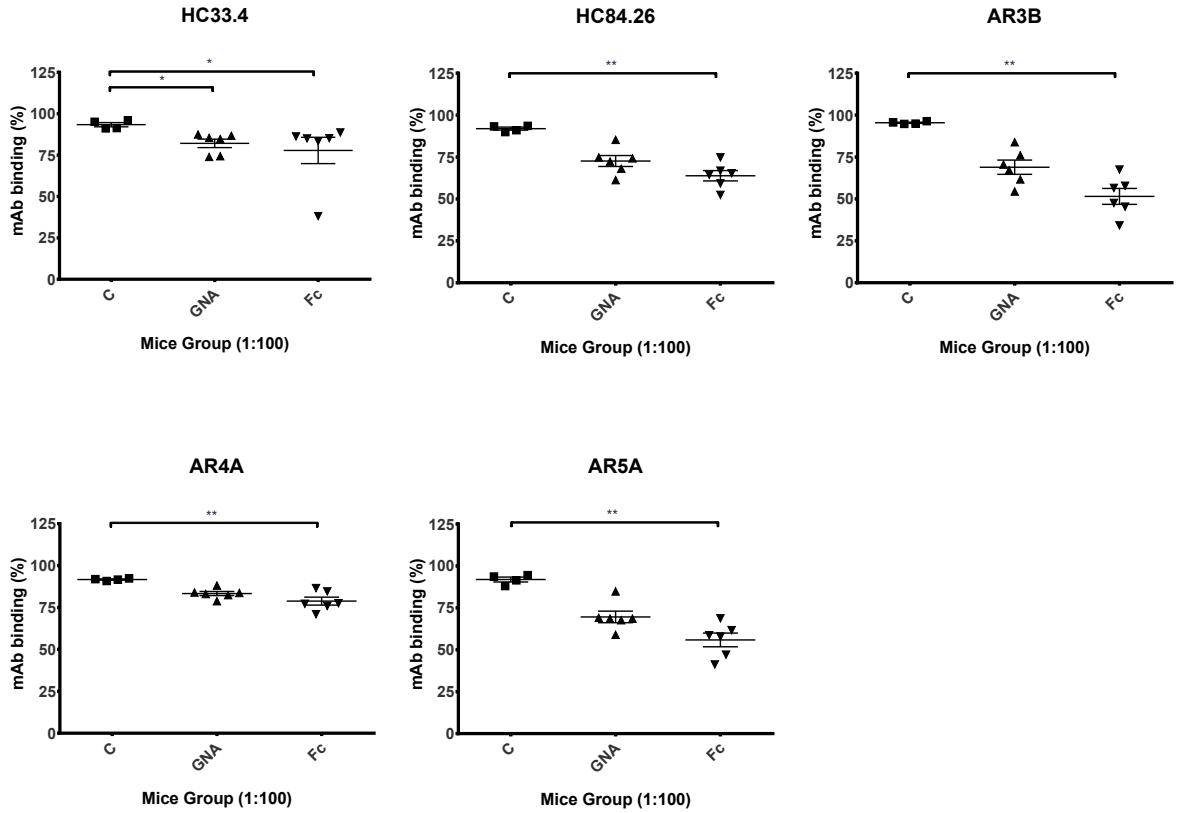


Figure 5.2 Competition studies with antisera from mice vaccinated with GNA-derived H77 rE1E2 and Fc-derived H77 rE1E2 and a panel of cross-neutralizing mAbs binding H77 rE1E2 GNA-derived antigen.

Microtiter wells containing purified H77 rE1E2 GNA-derived were first incubated with diluted antisera samples (1:100) from post-immunization antisera (final bleed) from three groups of mice (sham control (C), H77 rE1E2 GNA-derived (GNA), H77 rE1E2 Fc-derived (Fc)) prior to incubation with the indicated mAb. Monoclonal antibody was added at a concentration resulting in 70% maximal binding to rE1E2, as determined in prior titration experiments. Binding of the mAbs was detected with species-specific AP-conjugated secondary antibodies and pNPP. The percentage of mAbs binding in the presence of mouse antisera normalized to binding in the absence of any antisera was calculated. This experiment was performed in two independent experiments in duplicate. The results are displayed as the means from two independent experiments and the error bars represent standard error. Statistical analysis (one-way ANOVA, Kruskal-Wallis, and Dunn's multiple comparisons test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$.

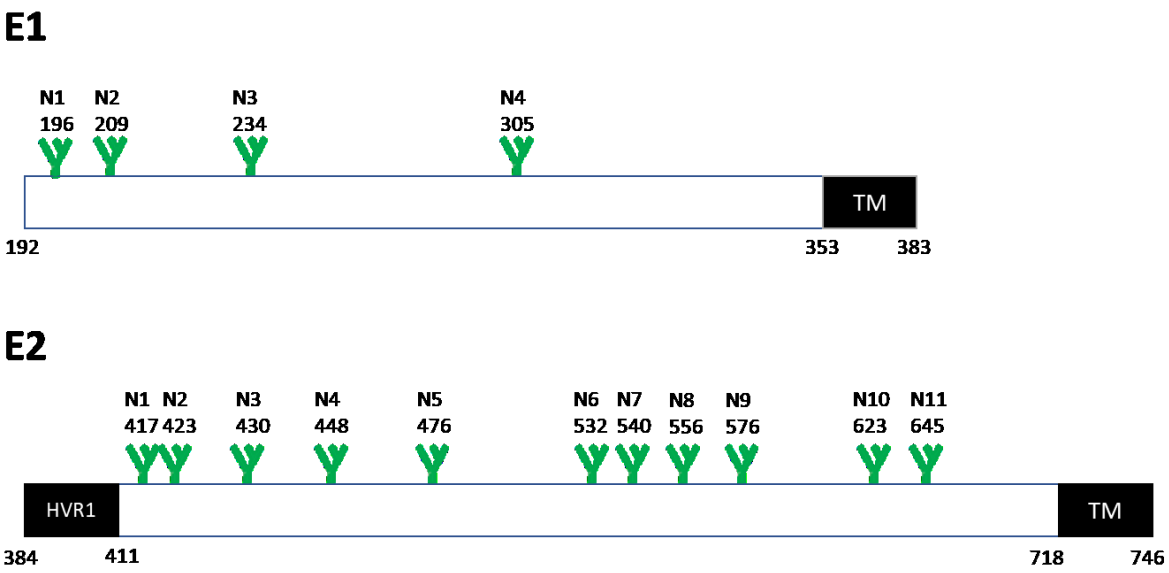


Figure 5.3 Pictorial representation of the N-glycosylation sites in E1 and E2.
The transmembrane domain (TMD) at the C-terminal ends of the proteins are shown along with amino acid numbers. The hypervariable region 1 (HVR1) of E2 is indicated with amino acid numbers. The 4 N-glycosylation sites in E1 and 11 N-glycosylation sites in E2 are indicated with green symbols along with amino acid numbers. Amino acid numbering is based on the polyprotein of reference gt1a isolate H77 (GenBank accession number: AF009606.1).

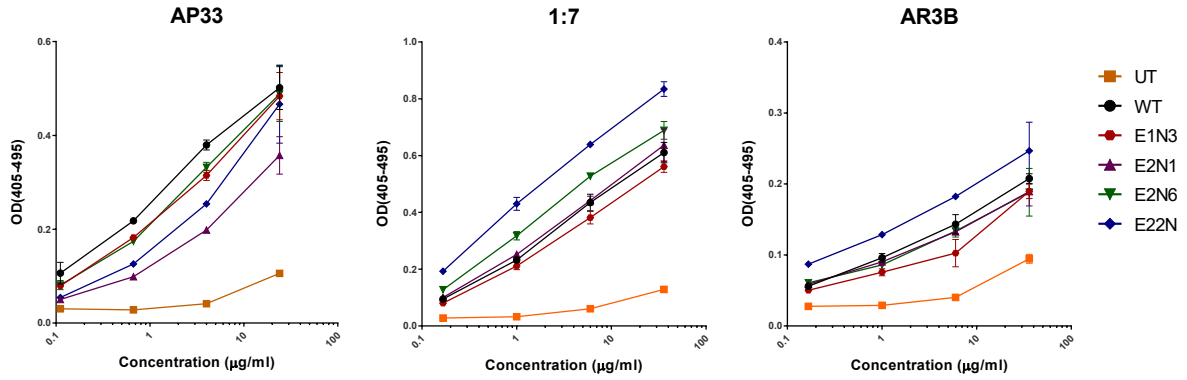


Figure 5.4 Effect of N-site mutations of H77 rE1E2 on the binding of well characterized cross-neutralizing monoclonal antibodies in ELISA experiments.
ELISA wells coated with *Galanthus nivalis* lectin were used to capture rE1E2 from lysates of CHO cells expressing the different constructs (Non-transduced (UT), H77 rE1E2 GNA (WT), H77 rE1E2 E2N1 (E2N1), H77 rE1E2 E2N6 (E2N6), H77 rE1E2 E2N1/E2N6 (E22N). Unbound protein was washed off, and 6-fold dilutions from 24µg/mL (AP33) or 36µg/mL (1:7 and AR3B) of monoclonal antibodies were incubated on the bound protein. An appropriate secondary antibody conjugated to alkaline phosphatase and the substrate pNPP were used to detect binding of the antibodies to the different forms of rE1E2. This experiment was performed in three independent experiments in triplicate. The results are displayed as the means from one representative experiment and the error bars represent standard deviation.

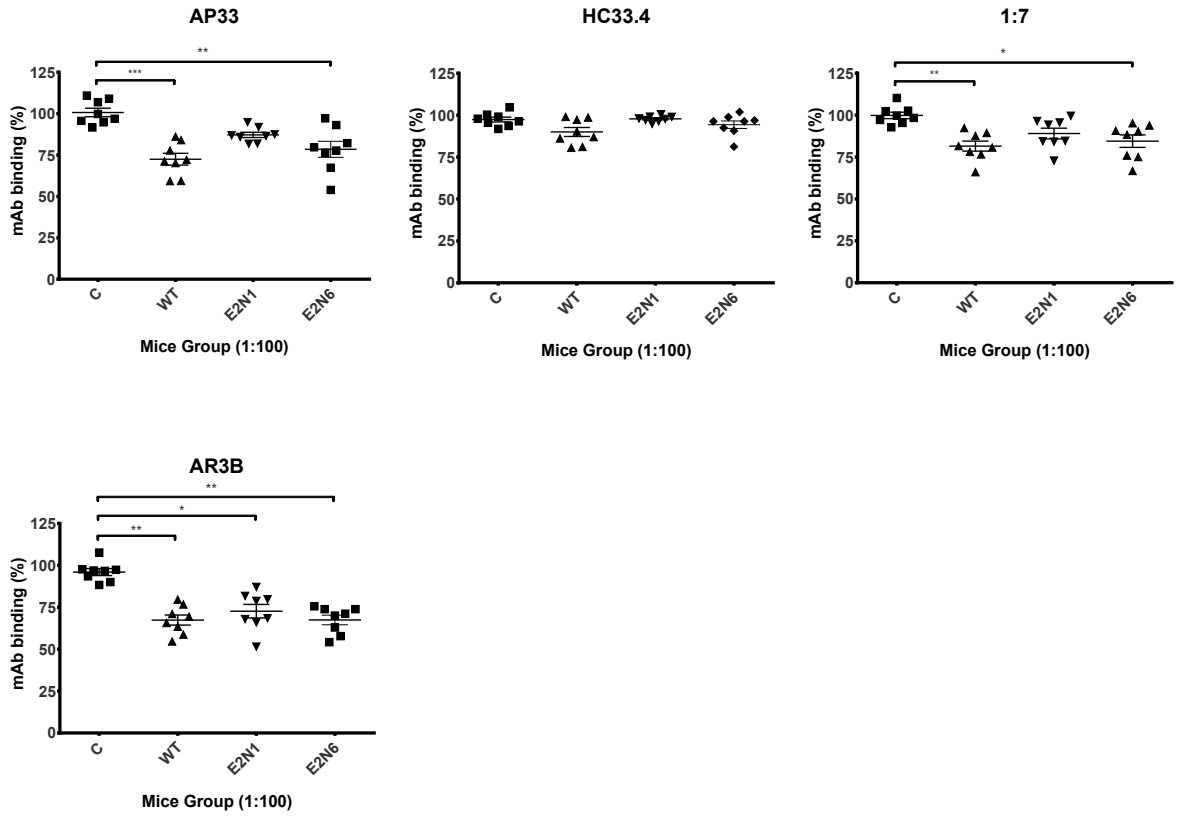


Figure 5.5 Competition studies with antisera from mice vaccinated with H77 rE1E2 containing N-glycosylation site mutations and a panel of cross-neutralizing mAbs to H77 E2 antigen.

Microtiter wells containing purified H77 sE2 were first incubated with diluted antisera samples (1:100) from post-immunization antisera (final bleed) from four groups of mice (sham control (C), H77 rE1E2 WT (WT), H77 rE1E2 E2N1 (E2N1), and H77 rE1E2 E2N6 (N6)) prior to incubation with the indicated mAb. Monoclonal antibody was added at a concentration resulting in 70% maximal binding to rE1E2, as determined in prior titration experiments. Binding of the mAbs was detected with species-specific AP-conjugated secondary antibodies and pNPP. The percentage of mAbs binding in the presence of mouse antisera normalized to binding in the absence of any antisera was calculated. This experiment was performed in two independent experiments in triplicate. The results are displayed as the means from two independent experiments and the error bars represent standard error. Statistical analysis (one-way ANOVA, Kruskal-Wallis, and Dunn's multiple comparisons test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$.

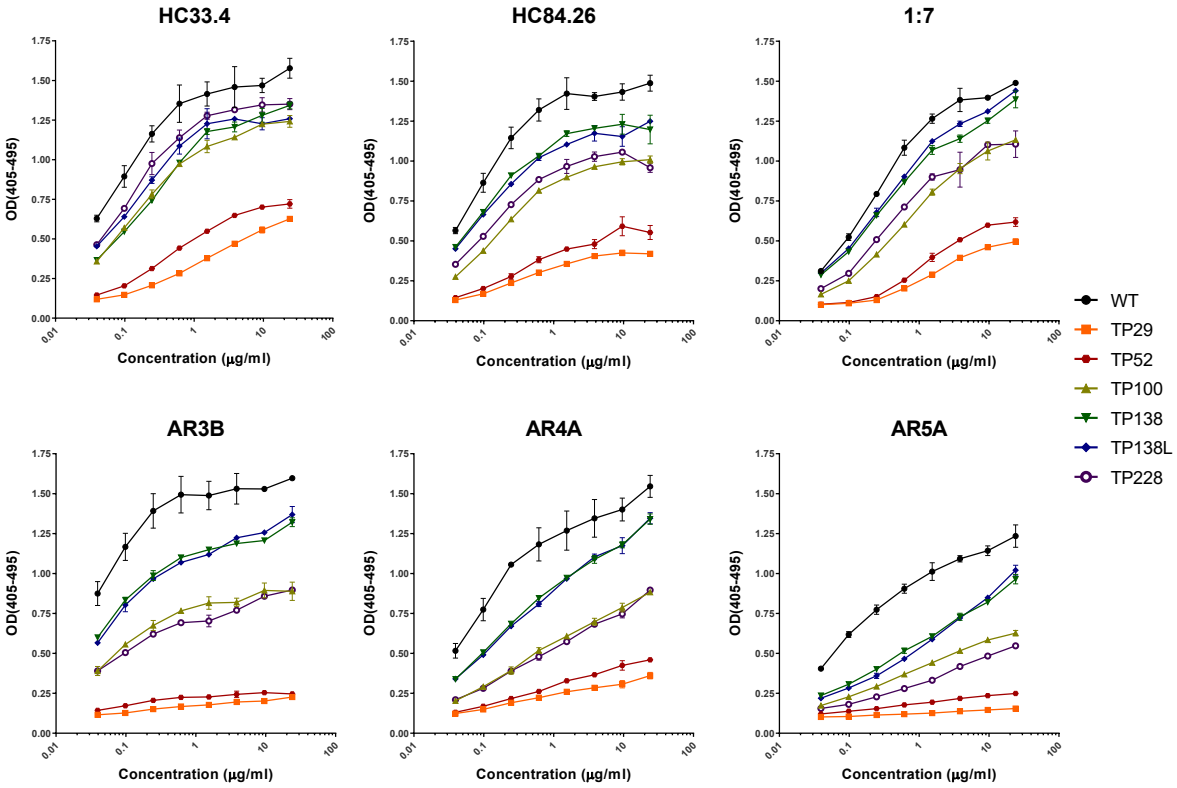
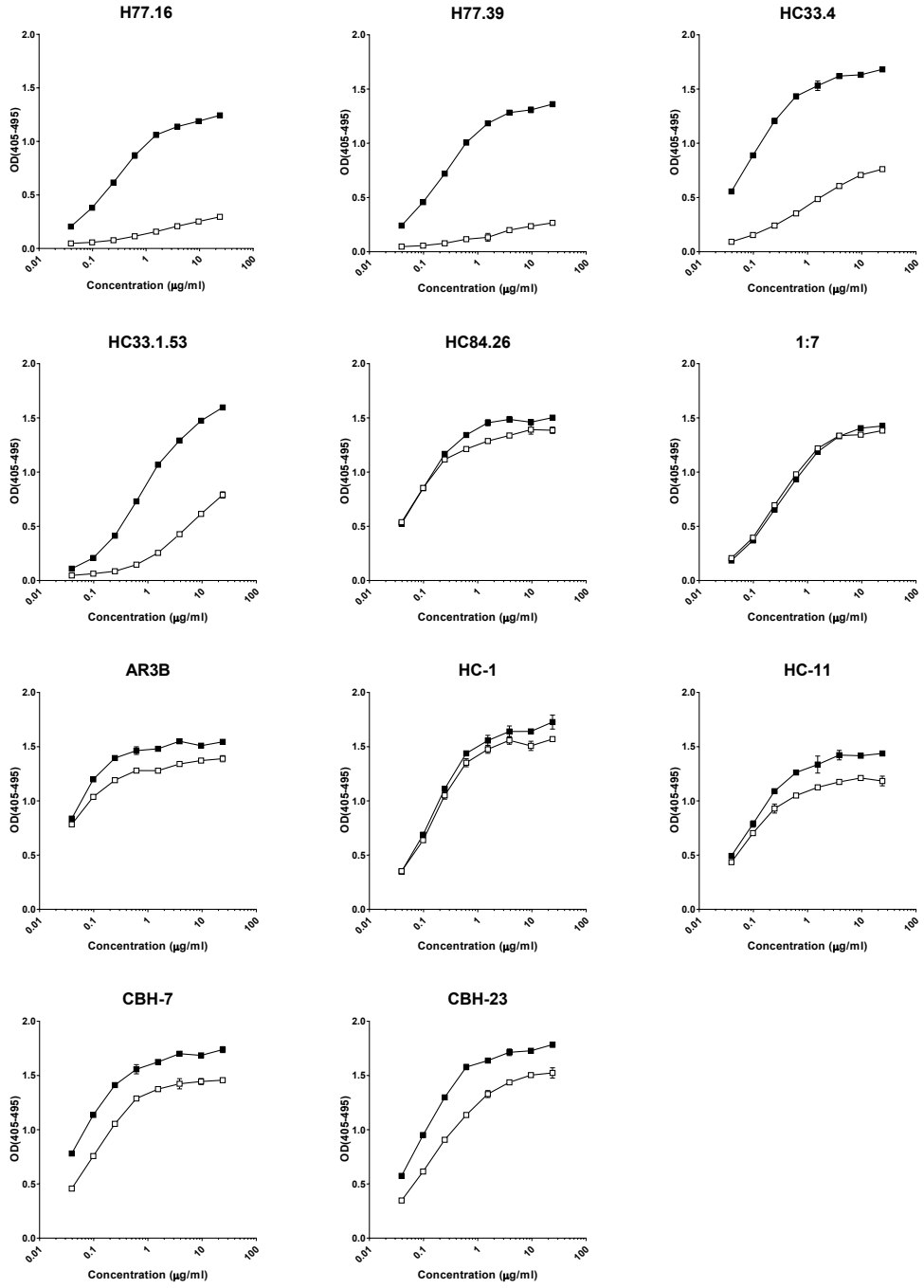


Figure 5.6 Effect of fusing T-cell epitopes on H77 rE1E2 on the binding of well characterized cross-neutralizing monoclonal antibodies in ELISA experiments.
ELISA wells were coated with H77 rE1E2 WT or fused to T-cell epitopes. Unbound protein was washed off, and 2.5-fold dilutions from 24µg/mL of monoclonal antibodies were incubated on the bound protein. Signal was developed by species-specific secondary antibody conjugated to alkaline phosphatase and pNPP. Absorbance was recorded at 405nm (subtracting absorbance at 495nm to remove background). This experiment was performed in two independent experiments in duplicate. The results are displayed as the means from one representative experiment and the error bars represent standard deviation.

A


H77.16
H77.39
HC33.4
HC33.1.53
HC84.26
1:7
AR3B
HC-1
HC-11
CBH-7
CBH-23
OD(405-495)
Concentration (µg/ml)

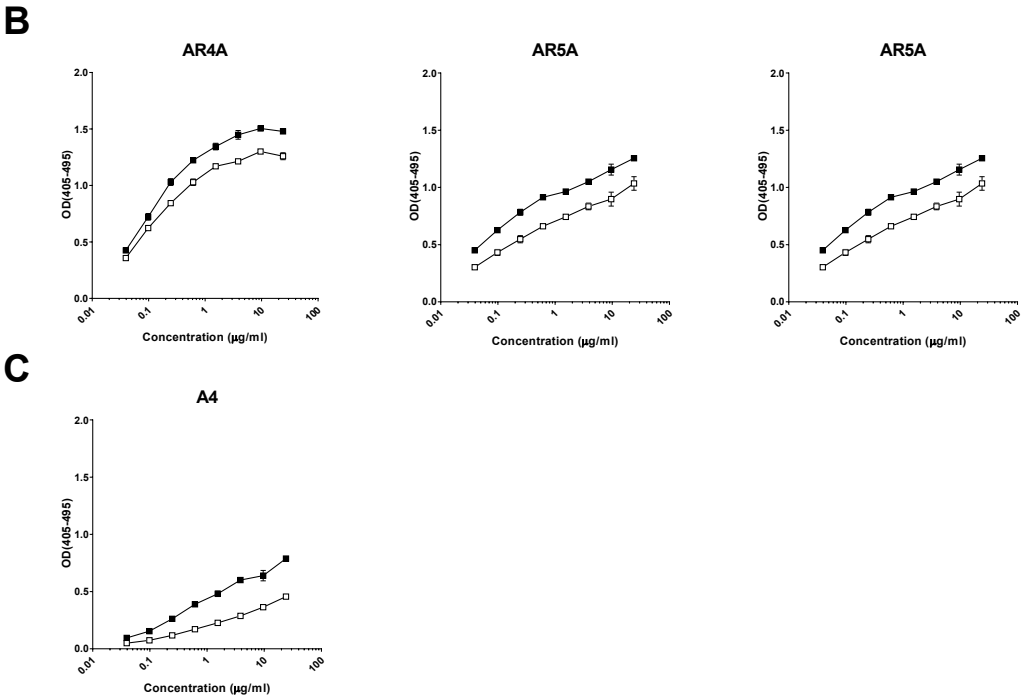


Figure 5.7 Effect of deleting HVR1 of H77 rE1E2 on the binding of well characterized cross-neutralizing monoclonal antibodies in ELISA experiments.
■ H77 rE1E2 WT (WT) □ H77 rE1E2 ΔHVR1 (ΔHVR1). ELISA wells were coated with H77 rE1E2 WT or H77 rE1E2 ΔHVR1. Unbound protein was washed off, and 2.5-fold dilutions from 24µg/mL of monoclonal antibodies were incubated on the bound protein. Signal was developed by species-specific secondary antibody conjugated to alkaline phosphatase and pNPP. Absorbance was recorded at 405nm (subtracting absorbance at 495nm to remove background). This experiment was performed in two independent experiments in duplicate. The results are displayed as the means from one representative experiment and the error bars represent standard deviation. The nine mAbs used in binding ELISAs are grouped based on their reactivity: (A) antibodies that bind E2; (B) antibodies that require E1E2 for binding; and (C) antibodies that target E1.

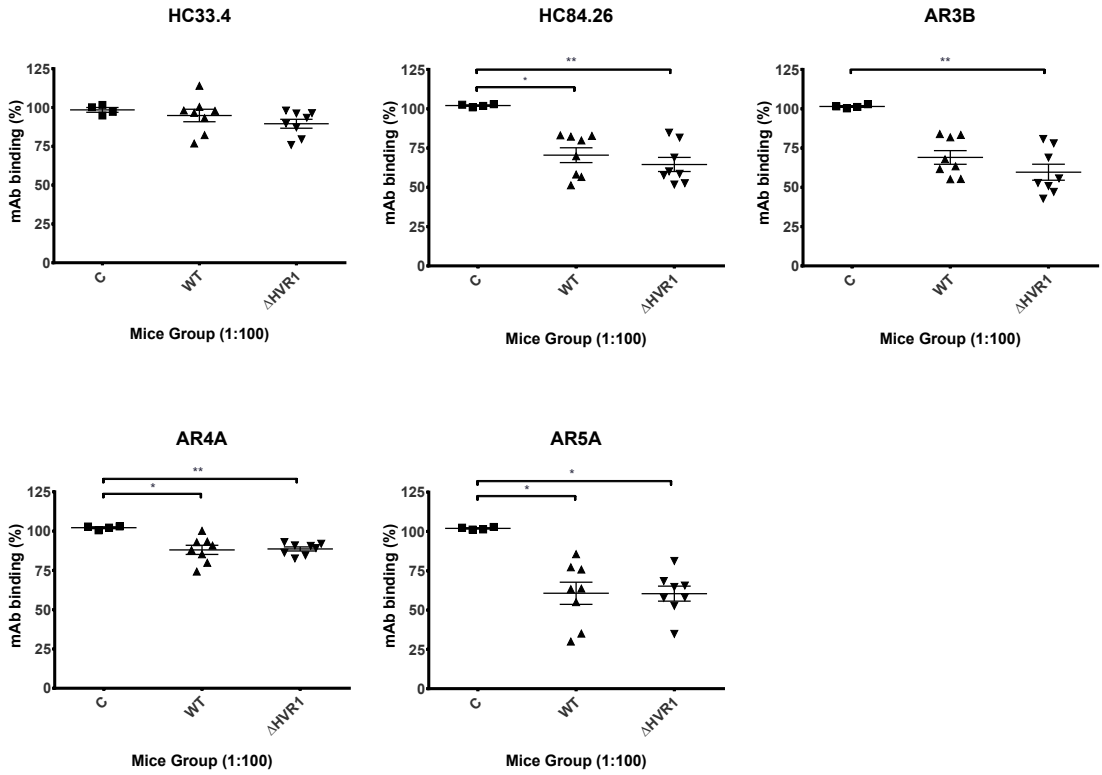


Figure 5.8 Competition studies with antisera from mice vaccinated with H77 rE1E2 WT and H77 rE1E2 ΔHVR1 and a panel of cross-neutralizing mAbs to H77 rE1E2 antigen. Microtiter wells containing purified H77 rE1E2 were first incubated with diluted antisera samples (1:100) from post-immunization antisera (final bleed) from three groups of mice (sham control (C), H77 rE1E2 WT (WT), H77 rE1E2 ΔHVR1 (ΔHVR1)) prior to incubation with the indicated mAb. Monoclonal antibody was added at a concentration resulting in 70% maximal binding to rE1E2, as determined in prior titration experiments. Binding of the mAbs was detected with species-specific AP-conjugated secondary antibodies and pNPP. For the mouse mAb H77.16, the antibody was conjugated to biotin and the binding of Biotin-H77.16 was detected with AP-conjugated streptavidin. The percentage of mAbs binding in the presence of mouse antisera normalized to binding in the absence of any antisera was calculated. This experiment was performed in two independent experiments in duplicate. The means from two independent experiments are shown and the error bars represent standard error. Statistical analysis (Kruskal-Wallis, and Dunn's multiple comparisons test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$.

6 CHAPTER VI: DISCUSSION

6.1 HCV neutralizing antibody response after vaccinating with different HCV envelope glycoprotein antigens

Since the discovery of HCV in 1989, an effective prophylactic vaccine for the virus has been a major goal. The development of a vaccine has been severely hindered by several factors, one being the vast diversity of the virus, stemming largely from the virus being an RNA virus utilizing an RNA-dependent RNA polymerase to copy its genome which results in highly frequent errors with an absence of proofreading (Ribeiro et al. 2012). Other reasons include the difficulty of growing HCV in cell culture, developing an assay for neutralizing antibodies, and the endangered and expensive chimpanzee being the only immunocompetent animal model to test vaccine efficacy. However, after extensive *in vitro* experimentation, a prototypical HCV-1 rE1E2 heterodimer vaccine was tested in preclinical models including both small animals (mice and guinea pigs) and chimpanzees (Stamataki et al. 2007; Houghton 2011). This vaccine candidate is still currently the only HCV vaccine to show statistically significant efficacy in the HCV challenge chimpanzee model (Houghton 2011). Data showing that antisera from small animals and chimpanzees possessed cross-neutralizing activity *in vitro* provided extra support for the vaccine (Meunier et al. 2011; Stamataki et al. 2007). The prototypical gt1a strain HCV-1 rE1E2 heterodimer was used in the first clinical trial of an HCV vaccine in humans (Frey et al. 2010). Although the Phase I clinical trial only studied safety and immunogenicity, the totality of the data including neutralizing assays, CD4+ T-cell responses, and protection after challenge in chimpanzees argues that the vaccine has strong promise (Frey et al. 2010; Ray et al. 2010; Meunier et al. 2011; Houghton 2011).

We investigated the vaccinated human antisera to determine if they contained cross-neutralizing potential as seen in the vaccinated chimpanzee antisera. We found that the majority of human volunteers had neutralizing activity against H77/JFH-1 HCVcc and overall post-vaccination antisera was more neutralizing than pre-vaccination sera (**Figure 3.1A**,**B**). Out of the three vaccinated volunteers tested for cross-neutralization, two of them showed overall moderate cross-neutralization against the 9 strains from all 7 genotypes (**Figure 3.1C**). I tested the goats

vaccinated with the prototypical vaccine and I found one of the two goats showed cross-neutralization with a similar profile to the human vaccinated volunteers (**Figure 3.2C**). Overall, these data confirmed the broad cross-neutralizing activity seen in the vaccinated chimpanzees, mice, and guinea pigs, and emphasized the potential of this vaccine to offer broad protection despite being derived from only a single strain. This was shown despite early research that predicted neutralizing antibody responses to the envelope glycoproteins would mostly be strain-specific (Farci et al. 1994, 1996; Shimizu et al. 1996; Zibert, Schreier, and Roggendorf 1995). While a multivalent vaccine comprising rE1E2 derived from more than one strain (as in the case of HPV) may be a way to create an optimal global vaccine, these data suggest it will require antigens from just a few strains.

Along with the goats vaccinated with HCV-1 rE1E2, goats vaccinated with HCV-1 sE2 and J6 sE2 were tested for neutralization against three different strains and surprisingly, it was found that HCV-1 rE1E2 vaccinated goats could not neutralize gt2a J6 HCVcc but it could neutralize gt2a JFH-1 HCVcc and gt1a H77/JFH-1 whereas J6 sE2 vaccinated goats could not neutralize JFH-1 HCVcc or H77/JFH-1 but could neutralize J6/JFH-1 HCVcc (**Figure 4.1**). Our laboratory is currently using chimeric viruses expressing different regions of the envelope glycoproteins from the J6 and JFH-1 strains in the HCVcc model to determine the mechanism of this unexpected finding. More work on unexpected neutralization patterns such as this one could lead to an optimized global vaccine.

6.2 Identification of multiple cross-neutralizing antibody epitopes within the 1a E1E2 vaccine

I next investigated the epitopes targeted by the vaccinated goats' antisera and found the antisera blocked the binding of all the cross-neutralizing mAbs tested (**Figure 3.4A-C**, **Figure 3.5**). This adds strength to this vaccine as it suggests it elicits antibodies that bind a variety of cross-neutralizing epitopes, leaving less chance for viral escape from neutralization. A very important caveat in interpreting the competition ELISA data is that these cannot distinguish between direct competition to an epitope (which is the desired reason), steric hindrance through binding nearby sites, and binding distant sites that could still possibly interfere with mAb

binding (allosteric inhibition). However, I was able to conclude direct competition against the HC33.4 and HC84.26 cross-neutralizing mAbs by performing peptide competition ELISAs with the goat antisera using linear peptides representing the mAbs' epitopes (**Figure 3.6C**). However, this is not currently possible for antibodies with highly discontinuous epitopes. AR5A was effectively competed by the vaccinated goat antisera. The anti-E2 mAb CBH-7 also effectively competes with AR5A, which is interesting as alanine scanning mutagenesis does not indicate any overlap of critical binding residues (Owsianka et al. 2008; Giang et al. 2012). It remains to be determined if these mAbs share a similar epitope or if this is a case of steric hindrance or allosteric inhibition. At the time of the study, I did not have access to CBH-7 so it is unclear if the goat antisera can compete with just AR5A or both mAbs. Research is ongoing elsewhere attempting to express discontinuous protein epitopes on scaffolds such as a triazacyclophane scaffold (Werkhoven et al. 2015). Combined with full structural knowledge of epitopes, competition ELISAs with scaffolds accurately representing both continuous and discontinuous protein epitopes will allow for highly specific epitope mapping with competition ELISAs.

There was stronger competition against anti-E2 mAbs in general, most of which bind to CD81 binding regions (**Figure 3.4A-C**, **Figure 3.5**, **Figure 3.7A**,**B**). There was weaker competition against the anti-E1 and anti-E1E2 mAbs. This is consistent with my data presented in **Chapter 4** that showed that the H77 rE1E2 goat antisera blocked E2-SRB1 and E2-CD81 binding and the synchronized time-of-addition data indicated the neutralizing response was acting primarily on these early post-binding steps in the cell entry process.

Peptide mapping utilizing CelluSpots aided in identifying interesting peptide epitopes to test in a quantitative peptide ELISA using biotinylated peptides. The results of the peptide ELISAs showed four reactive peptides – one was very similar to E2 Epitope I, one was very similar to E2 Epitope II, and two were novel (**Figure 3.12A**,**B**). E2 Epitope I and II have been shown to be associated with cross-neutralizing mAbs, with anti-E2 Epitope I mAbs blocking both the E2-SRB1 and E2-CD81 interactions and anti-E2 Epitope II mAbs blocking the E2-CD81 interaction (Sabo et al. 2011; Owsianka et al. 2005; Keck et al. 2012). Interestingly, E2 Epitope II is also associated with non-neutralizing antibodies as well as antibodies that can interfere with neutralization (Zhang et al. 2009; Duan et al. 2012).

The two novel peptides could be investigated further with peptide depletion experiments to determine if reactive antibodies found in G757 antisera contribute to neutralization. The P681

peptide (aa681-699) is of particular interest since it contains the majority of the heptad repeat sequence (aa675-699) found close to the transmembrane domain. This region is important for the heterodimerization of E1E2 and possible virus membrane fusion during entry and mAbs targeting the HIV-1 MPER were able to prevent entry (Rychłowska et al. 2011; Chi et al. 2016; Drummer and Poumbourios 2004; Song et al. 2009). Additionally, a synthetic peptide derived from aa671-705 that contains all of the residues within P681 was able to potently inhibit fusion of HCVcc expressing the structural proteins of a diverse range of genotypes (Chi et al. 2016). This is a highly conserved region (78.9% identity across 11 strains from every genotype) necessary for critical functions during entry that appears to be similarly immunogenic as E2 Epitope I and E2 Epitope II, at least in the goat tested (**Figure 3.12A**,**B**). The P661 peptide has not been studied as closely nor is it as conserved as P681 (56.3% identity across 11 strains from every genotype) – nevertheless, a deletion of aa651-677 critically impaired HCVpp infectivity and this peptide was also reactive to G757 antisera (Albecka et al. 2011). These peptides could represent components of important neutralizing epitopes.

In summary, my work indicates that the E1E2 vaccine contains multiple cross-neutralizing antibody epitopes making it difficult for the virus to escape from a polyclonal antibody response.

6.3 Characterizing the mechanism of neutralizing antisera.

Results from **Chapter 4** showed that there is not likely much neutralizing activity of G757 and G773 taking place later than the CD81 binding step based on the kinetics of neutralization (**Figure 4.2A**,**B**). According to the literature, the steps requiring CLDN1, OCLN, TfR1, and NPC1L1 all take place after CD81 binding – however, the only other control used in this assay was BafA1, an inhibitor of endosomal acidification which prompts viral fusion with the endosomal membrane (Meertens, Bertaux, and Dragic 2006; Sharma et al. 2011; Evans et al. 2007; Sourisseau, Michta, et al. 2013; Jia, Betters, and Yu 2011; Martin and Uprichard 2013).

G757 kinetics appeared to follow the anti-CD81 mAb kinetics closely, although there was high inter-assay variability. An anti-SRB1 pAb preparation possessed similar kinetics to an anti-CD81 mAb, which would seem to be consistent with the ability of G757 to prevent both E2-SRB1 and E2-CD81 interactions (Zahid et al. 2013). However, it appears that the anti-SRB1 pAb

preparation blocks the E2-independent lipid transfer function while anti-E2 mAbs that inhibit E2-SRB1 interaction block the E2-dependent role of SRB1 that leads to infection enhancement in the presence of HDL (Thi et al. 2012). It will be important in the future to test the kinetics of mAbs inhibition of infection that can block the E2-SRB1 interaction to determine if the kinetics are similar to the anti-SRB1 pAb preparation. As it's been shown anti-E2 mAbs that block E2-SRB1 interaction block at a post-binding step, it's likely the anti-E2 mAbs and the anti-SRB1 pAb preparation are kinetically similar (Sabo et al. 2011). Interestingly, G757 antisera was capable of blocking the E2-CD81 interaction when the antigen used was H77 rE1E2 but not when it was J6 sE2, consistent with the high neutralization against H77/JFH-1 virus and low neutralization against J6/JFH-1 virus (**Figure 4.7F**, **Figure 4.8E**,**F**, **Figure 3.2C**, **Figure 4.1**). Because of issues with detecting binding of J6 sE2 to CHO-SRB1 cells, it was not possible to determine if inhibition of the E2-SRB1 interaction was consistent with genotype-specific neutralization as in the case of inhibition of E2-CD81 interaction studies (**Figure 4.5**). Nonetheless, the data shown in **Chapter 4** indicates the potential of using inhibition of E2-CD81 binding assays as a high-throughput method to predict cross-neutralization.

The data shown in **Chapter 4** suggests the G773 antisera likely interacts prior to the CD81 engagement step. I did not gain useful data with CHO-SRB1 binding assays using J6 sE2 but solving the issue of the partially weak expression of SRB1 on the surface of both CHO and BRL3A cells is the first step to establishing this assay. Assays evaluating the ability of antisera to inhibit early attachment of the envelope glycoproteins to HSPG and Syndecan molecules and an inhibition of E2-SRB1 assay would be most likely to succeed in determining the mechanism of neutralization by J6 sE2 vaccinated antisera.

6.4 Application of ELISAs and competition ELISAs to rational vaccine design

Multiple modifications made to the H77 rE1E2 vaccine immunogen were probed in **Chapter 5** to determine how the modifications affected the immunogen. Inserting a human IgG Fc-tag into rE1E2 to facilitate purification of rE1E2 did not affect the immunogen (**Figure A.1-A.3**, **Figure 5.1-5.2**). The validation of this method greatly facilitates large-scale manufacturing

of the vaccine which previously relied on GNA-lectin affinity chromatography which has the problems of supply and scale-up unlike the use of Protein A/G affinity chromatography widely used in the commercial production of therapeutic antibodies.

Generating N-glycosylation site mutants showed that while it may enhance binding of certain mAbs for their epitopes by removing the large occluding glycan from the region, this can have negative effects on immunogenicity (**Figure 5.4**, **Figure A.5**). This has multiple possible reasons, the most obvious being compromising the immunogenicity of other neutralizing epitopes since AP33 binding was negatively affected by removal of the N-glycans. Even if removing a glycan makes a neutralizing epitope more accessible to a given set of mAbs, it does not necessarily mean that this epitope would be more antigenic during immunization and may indeed be less antigenic if the N-glycan was important for overall structural integrity. While we do not have published neutralizing data on the mice vaccinated with the E2N1 and E2N6 mutants, based on the immunogenicity and competition studies I do not expect these to be superior than WT, and likely worse than WT (**Figure 5.5**). E2N1 and E2N6 mutations were chosen because they are within neutralizing epitopes, removal of these sites was associated with higher sensitivity to neutralizing mAbs, and there was minimal effect of the mutation on infectious HCVcc virus including replication, E1E2 expression, and infectious viral production (Helle et al. 2007, 2010). The only other mutant tested with mAbs that showed a trend of high sensitivity to neutralizing mAbs when removed was E2N11, but this mutation was associated with a severe defect in particle assembly. E1N2, E1N3, E1N4, E2N2, and E2N4 do not affect replication, E1E2 expression, or infectious virus production but it is currently unknown if these mutations increase sensitivity to neutralizing mAbs. If they do show increased sensitivity, these may serve as potential targets for further research on N-glycosylation site mutants. At this moment, however, it appears that removing N-glycans are unlikely to be effective in enhancing a global vaccine.

All the known T-cell epitopes chosen from sequences found in NS3 that were predicted to elicit strong CD4+ and CD8+ T-cell responses negatively impacted recognition by mAbs binding various anti-E2 and anti-E1E2 epitopes (**Figure 5.6**). As it is unknown how stable these NS3 sequences are in the rE1E2 heterodimer and how they fold, it is difficult to understand the precise reasons behind differences in mAb recognition. However, it appears the shortest sequences have the most drastic consequence on mAb binding – it's likely these short sequences

are less stably structured and therefore are more likely to interfere with E2 folding. Most interestingly, even when the sequence severely affects mAb recognition it does not noticeably interfere with the interactions between E1 and E2 that mediate heterodimerization. The reduction of binding by mAbs correlated with a reduction in immunogenicity when testing antisera from mice immunized with three of the T-cell peptide fusion H77 rE1E2 proteins (**Figure A.7**). The immediate next step is to determine the CD4+ and CD8+ T-cell responses to determine if these sequences do indeed stimulate strong cell-mediated immunity. Neutralization assays are also critical because if CD4+ T-cell responses were boosted, this could potentially cross-help the neutralizing antibody response to the attached E1E2 moiety.

The E2 HVR1 region was deleted to determine if removal of this putative immune decoy would enhance immunogenicity to shielded epitopes. The mAb binding ELISAs cast serious doubt on this approach, as none of the mAbs showed enhanced binding to ΔHVR1 antigen – several mAbs showed modestly reduced binding, with mAbs binding the HVR1 and E2 Epitope I regions showing severely reduced binding (**Figure 5.7A-C**). Consistent with this unpromising data, the rE1E2 antigen lacking HVR1 was found not to be a superior vaccine antigen as compared with WT (**Figure A.9-A.12**). Overall, monitoring the binding of cross-neutralizing mAbs and analyzing the immune response in competition ELISAs has been useful in interrogating many rational vaccine designs, although so far, none have been shown to be superior to WT rE1E2. However, purifying rE1E2 via a precursor containing a Fc-E2 fusion was shown to retain the immunoreactivity and immunogenicity of the previously used prototype E1E2 while greatly facilitating its large scale production required for vaccinating the human population.

6.5 Future Directions

As mentioned in **Chapter 2**, goats allowed for large sera samples that enabled establishing multiple different assays. However, this came at the cost of very small sample sizes that disallowed the use of statistics. In the future it would be best to use a small animal model such as guinea pigs or mice in order to allow for statistically relevant data. Guinea pigs may be most ideal, as they would provide a larger amount of antisera, although care should be taken as it has been seen that guinea pig antisera tend to have higher neutralizing activities against HCV

relative to mice and this may result in overconfidence in preclinical assessments of vaccine antigens (Stamataki et al. 2007).

Human CLDN1 expressed on the surface of BRL3A cells has been used to show that HCVpp but not sE2 was able to bind to CLDN1, indicating that there are CLDN1 binding sites on the envelope glycoproteins and sE2 is not sufficient for binding CLDN1 (Douam et al. 2014). In the future it will be interesting to replicate these experiments with sE2, rE1E2, and HCVpp. Particularly, comparing the results with rE1E2 and HCVpp and HCVcc will inform whether rE1E2 contains the sufficient binding sites for CLDN1 or if the CLDN1 binding site is only formed by the higher order structure of E1E2 present on the surface of virions, which has been predicted to be a trimer of the E1E2 heterodimer (Falson et al. 2015; Freedman et al. 2017).

As mentioned above, testing G773 for the ability to prevent the entry steps prior to CD81 would be valuable. However, as the vaccine program continues, it would be beneficial to develop assays for each step of the viral entry cycle, so vaccine immunogens could be interrogated for each step of the cell entry process. This would include early attachment assays, binding assays using cells overexpressing SRB1, CD81, and CLDN1, and fusion assays. There are likely other assays that can capture other parts of viral entry, but it is not currently known if E1E2 directly interacts with entry co-receptors and co-factors OCLN, TfR1, and NPC1L1 or if they are indirectly necessary for entry. By studying the dominant mechanisms of neutralization elicited by different vaccine immunogens, it is possible a future multivalent vaccine may be able to elicit a very broad response involving multiple epitopes, active against most strains with multiple mechanisms of neutralization of infectivity.

While it has been shown that recombinant envelope glycoproteins from a single strain is capable at eliciting a broad immune response in humans, generating a multivalent vaccine containing the envelope glycoproteins from more than one strain as has been done for other vaccines such as the HPV vaccine may aid in broadening and optimizing the response (Szarewski 2010; Law et al. 2013). A trivalent vaccine comprising sE2 from strains from genotypes 1a, 1b, and 3a was shown to elicit antibodies with enhanced cross-neutralizing activity in rhesus macaques and mice relative to a monovalent vaccine (Wang et al. 2017). As HCV is an extremely diverse virus and that most countries have multiple and diverse genotypes afflicting its citizens, even a regional vaccine will likely benefit greatly from a multivalent vaccine.

While N-site glycosylation mutations of rE1E2 and deleting the HVR1 region from rE1E2 are unlikely to be successful strategies to developing a more effective global HCV vaccine, the inclusion of other HCV-specific T-cell epitopes are still likely to enhance the potency of a rE1E2 vaccine and future experimentation is important to find a T-cell peptide fused to E1E2 that boosts CD4+ and CD8+ T-cell responses without negatively affecting immunogenicity of rE1E2. Future work will also greatly benefit from deeper structural understanding of the structure of the rE1E2 vaccine antigen.

Our laboratory is also investigating other possible adjuvants to combine with rE1E2 in order to enhance neutralizing antibody responses and cellular responses. While Complete Freund's Adjuvant has long been deemed unsafe for human use due to high reactogenicity and toxicity, there are other adjuvants that may provide heightened immune responses without sacrificing safety standards (Petrovsky 2015). The laboratory evaluated several adjuvants with differing mechanisms and benefits including alum (generates strong humoral immunity) combined with MPLA (TLR4 agonist that skews towards a Th1 response) and MF59 (oil-in-water adjuvant that promotes a CD4+ T-cell response and generates antibodies), as well as novel adjuvants such as cyclic dinucleotide adenosine monophosphate (c-di-AMP) (activates stimulator of interferon genes (STING)), and archaeosomes, liposome vesicles comprised of ether lipids derived from *Archaea* which offer a stable antigen delivery system and generate strong cell-mediated and humoral immunity through induction of CD4+ and CD8+ T cells (Haq, Jia, and Krishnan 2016; Morefield et al. 2005; Agger et al., n.d.; Galli et al. 2009; Burdette et al. 2011; Woodward, Iavarone, and Portnoy 2010). It was found that while these adjuvants elicited antisera with similar neutralizing activity, the novel adjuvants c-di-AMP and archaeosomes were superior in eliciting a cellular immune response in the form of vaccine-specific poly-functional CD4+ T-cells (Landi et al. 2017).

6.6 Conclusions

Overall, I have shown that animals and humans vaccinated with the prototypical vaccine HCV-1 rE1E2 elicits neutralizing antibodies with cross-neutralizing activity. This cross-neutralizing activity was shown to be associated with competition against a panel of mAbs targeting diverse epitopes possessing strong cross-neutralizing activity. Most of these mAbs

neutralized the virus by inhibiting the E2-CD81 interaction. Vaccinated goat antisera were shown to neutralize viral entry primarily through targeting the E2-SRB1 and E2-CD81 interactions. The laboratory generated variants of H77 rE1E2 possessing modifications to improve the efficacy or efficiency of generation of the vaccine. Fc-derived E1E2 was tested in multiple preclinical experiments and while greatly improving the efficiency of purification, this did not negatively affect immunogenicity or neutralizing activity. However, many rational attempts to improve the efficacy of the rE1E2 vaccine was shown to affect immunoreactivity, immunogenicity and neutralizing activity meaning that future clinical testing will utilize WT rE1E2.

GRATITUDES

We thank Dr. Michael Diamond (Washington University) for providing H77.16 and H77.39; Drs. Elizabeth Elrod and Arash Grakoui (Emory University) for providing 6F5, Dr. Steven K. H. Foung (Stanford University) for providing CBH-7, CBH-23, HC-1, HC-11, HC33.1, HC33.4 and HC84.26; Dr. Mansun Law (Scripps Research Institute) for providing B6, AR3B, AR4A, AR5A, and IGH526; Dr. Arvind Patel (University of Glasgow) for providing AP33; Dr. Mats Persson (Karolinska Institutet) for providing 1:7, Dr. Jean Dubuisson (Université de Lille) for providing H52, H53, and A4; and Drs. Charlie Rice (Rockefeller University) and Tim Tellinghuisen (Scripps Florida) for providing 9E10. We thank Dr. Charlie Rice for providing Human hepatoma Huh-7.5 cells. We thank Dr. D. Lorne Tyrrell for providing Human Embryonic Kidney 293T cells, chronically infected patient sera, HCV negative sera, and JFH-1 cell-culture derived (HCVcc) virus stocks. We thank Dr. Jens Bukh for providing plasmids encoding chimeric JFH-1 HCVcc viruses containing the Core-NS2 regions of H77(gt1a), J4(gt1b), J6(gt2a), J8(gt2b), S52(gt3a), ED43(gt4a), SA13(gt5a), HK6A(gt6a), and QC69(gt7a). We thank Drs. Bertrand Boson and Dr. François-Loic Cosset (Université de Lyon) for providing MLV based lentiviral vectors carrying HCV entry receptors. We thank Dr. Aviad Levin for providing the design for the CelluSpot assays. We thank Dr. Joseph Marcotrigiano for providing vectors containing GST-tagged CD81 Large Extracellular Loop or GST alone and soluble E2 protein derived from strains HCV-1(gt1a) and J6(gt2a).

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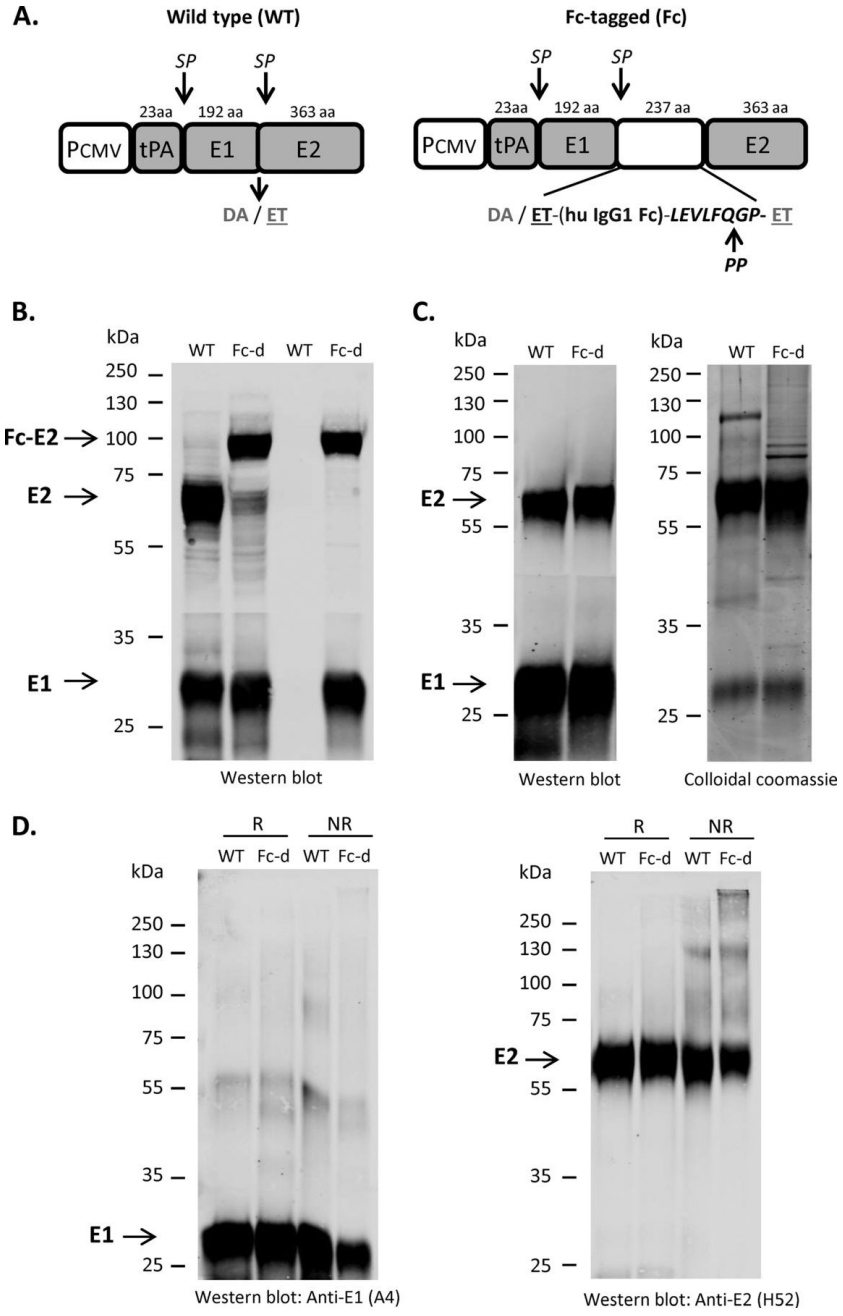
A. APPENDIX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SP
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tPA
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E2
DA / ET
DA / ET-(hu IgG1 Fc)-LEVLFQGP- ET
PP
B.
kDa
WT
Fc-d
Fc-E2
E2
E1
Western blot
C.
Western blot
Colloidal coomassie
D.
R
NR
Western blot: Anti-E1 (A4)
Western blot: Anti-E2 (H52)

Figure A.1 Purification of rE1E2 of GNA-derived (WT) and Fc-tag derived (Fc-d) forms. (A) Schematic representation of WT and Fc-d constructs and polypeptide processing. The polypeptide of H77 E1E2 (GenBank accession number: AF009606.1) was expressed under the control of the CMV promoter (P_{CMV}) and preceded by the signal sequence from tissue plasminogen activator (tPA). For Fc-d rE1E2, a duplication of the E2 N-terminal amino acids (384 and 385) (ET) was inserted, followed by the human IgG1 Fc tag (hu IgG1 Fc) and a PreScission protease (PP) recognition sequence (LEVLFQGP). Amino acid lengths of the polypeptide regions are shown at the top (aa) as well as cleavage sites by signal peptidase (SP). (B) WT or Fc-d rE1E2 from CHO cell extracts was captured with GNA and Protein G Sepharose (indicated above) and proteins were separated by SDS-PAGE and Western Blotted with anti-E1 (A4) and anti-E2 (H52) monoclonal antibodies (mAbs) **(Dubuisson et al. 1994)**. (C) Purified Fc-d rE1E2 antigen was treated with PP to remove the Fc-tag and PP recognition sequence. Purified rE1E2 antigens were separated by SDS-PAGE. (Left) Western blot with anti-E1 (A4) and anti-E2 (H52) mAbs (1µg/lane); (right) Coomassie brilliant blue G250 (2µg/lane). (D) WT and Fc-d rE1E2 antigens (1µg/lane) were denatured at 95°C for 5 min in Laemmli buffer with (R) or without (NR) 1% β-mercaptoethanol. Samples were separated by SDS-PAGE and blotted with anti-E1 (A4) and anti-E2 (H52) mAbs. Figure was generated by Dr. Michael Logan (Logan et al. 2017).

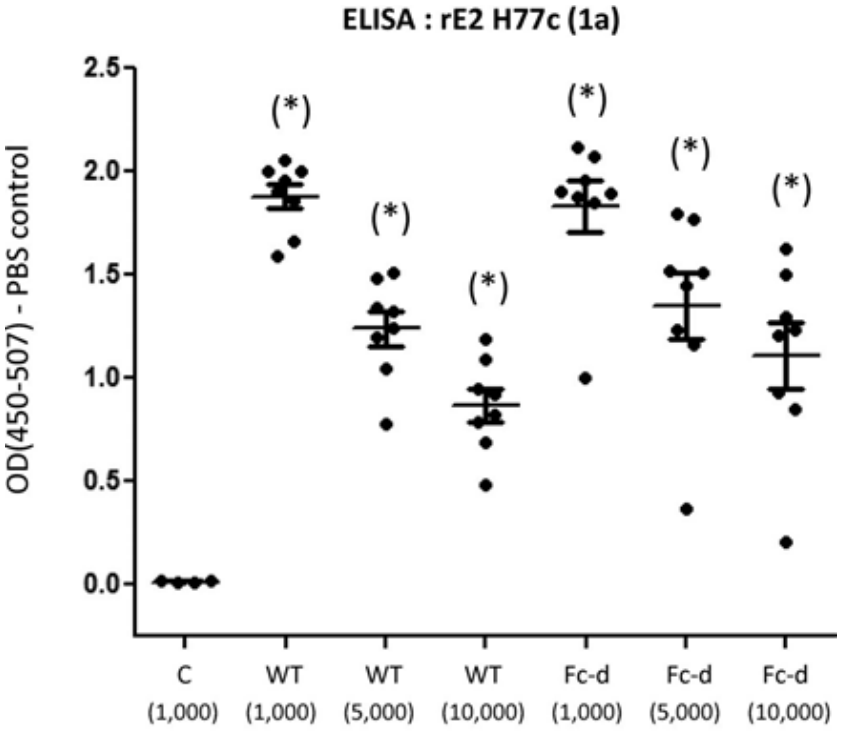

Figure A.2 Soluble E2 ELISA titers of antisera from mice immunized with H77 rE1E2 GNA-derived or H77 rE1E2 Fc-derived.
Purified recombinant sE2 (gt1a HCV-1 strain) was immobilized on GNA-coated microtiter wells. Heat inactivated mouse antisera from the post-vaccination bleed were tested at the indicated concentrations. The three groups were sham control (C), H77 rE1E2 GNA (GNA), and H77 rE1E2 Fc-derived (Fc). Signal was developed with anti-mouse secondary antibody conjugated to HRP and KPL peroxidase substrate. Absorbance was recorded at 450nm (subtracting absorbance at 507nm and PBS only control wells to remove background). This experiment was performed in three independent experiments in triplicate. The results are displayed as the means from three independent experiments and the error bars represent standard error. Statistical analysis (one-way ANOVA, Tukey's *post hoc* test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$. This figure was generated by Dr. Michael Logan (Logan et al. 2017).

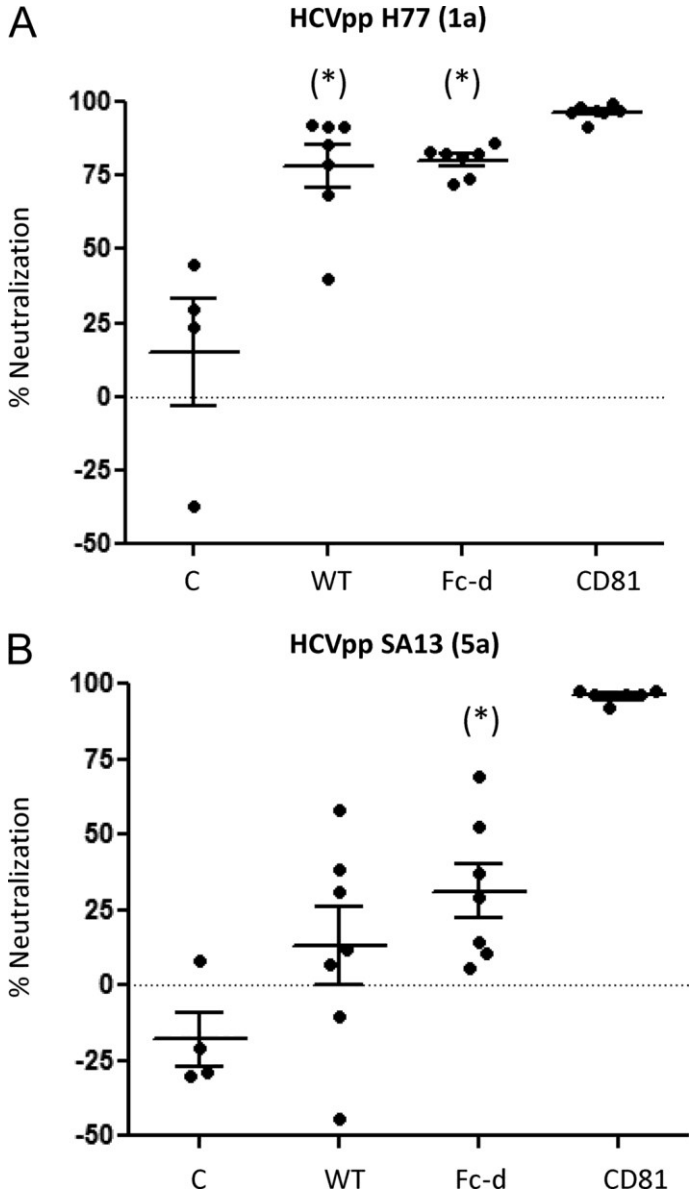
A
HCVpp H77 (1a)
(*)
(*)
100
75
50
25
0
-25
-50
% Neutralization
C
WT
Fc-d
CD81
B
HCVpp SA13 (5a)
(*)
100
75
50
25
0
-25
-50
% Neutralization
C
WT
Fc-d
CD81

Figure A.3 Neutralizing activity against H77 HCVpp by antisera from mice immunized with GNA-derived H77 rE1E2 and Fc-derived H77 rE1E2.
Pre-immunization and post-immunized antisera (1:50) were tested for neutralizing activity against HCVpp pseudotyped with E1E2 from (A) the H77 (gt1a) strain or the SA13 (gt5a) strain. Sham control (C), H77 rE1E2 GNA-derived (GNA), and H77 rE1E2 Fc-derived (Fc-d) groups of mice were tested, along with anti-CD81 (1μg/mL) positive control. HCVpp entry was quantitated by luciferase activity in cell extracts as described in Chapter 2. Neutralization activity of post-vaccination antisera was normalized to the luciferase levels in the presence of pre-vaccination antisera. This experiment was performed in three independent experiments in triplicate. The results are displayed as the means from three independent experiments and the error bars represent standard error. Statistical analysis (one-way ANOVA, Tukey's *post hoc* test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$. The positive control was an anti-CD81 mAb and was used at 1μg/mL. This figure was generated by Dr. John Lok Man Law (Logan et al. 2017).

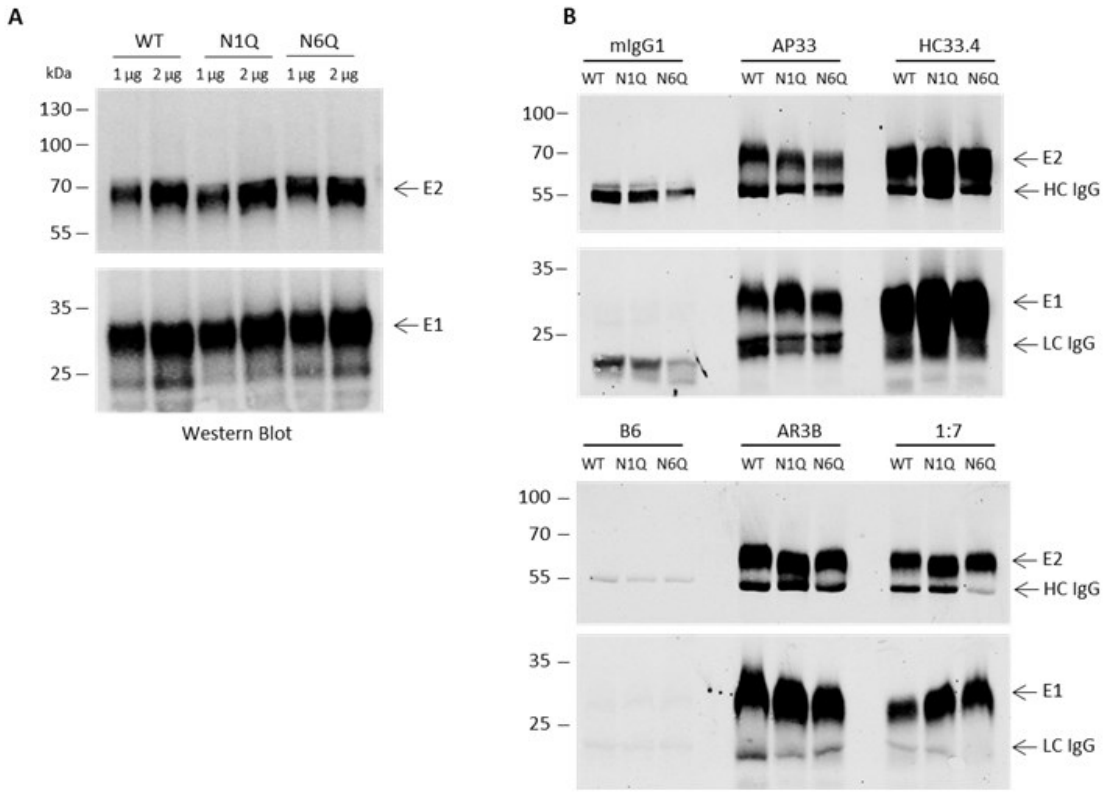

Figure A.4 Biochemical analyses of H77 rE1E2 N-glycosylation site mutants.
(A) WT rE1E2, E2N1Q rE1E2, and E2N6Q rE1E2 proteins were purified using GNA affinity chromatography and these proteins were separated by SDS-PAGE (either 1µg or 2µg was loaded) and Western Blotted with anti-E1 (A4) and anti-E2 (H52) mAbs (Dubuisson et al. 1994). (B) 1µg of purified protein was immunoprecipitated by anti-E2 mAbs AP33 (murine mAb), HC33.4 (human mAb), AR3B (human mAb), and 1:7 (human mAb) or control mAbs mIgG1 (murine IgG1 control) and B6 (human IgG1 control) (Owsianka et al. 2005; Keck et al. 2013; Law et al. 2008; Johansson et al. 2007). The proteins were then immunoblotted with anti-E1 (A4) and anti-E2 (H52) mAbs. This figure was generated by Dr. Michael Logan.

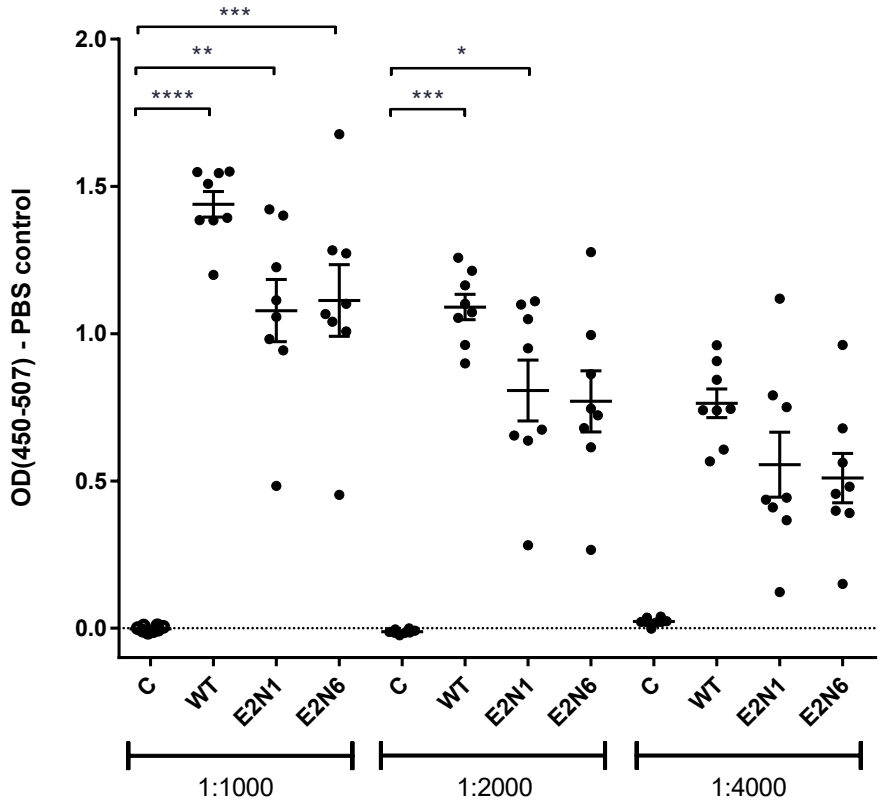

Figure A.5 Soluble E2 ELISA titers of antisera from mice immunized with H77 rE1E2 containing N-glycosylation mutations.
Purified recombinant sE2 (gt1a HCV-1 strain) was immobilized on GNA-coated microtiter wells. Heat inactivated mouse antisera from the post-vaccination bleed were tested at the indicated concentrations. The four groups were sham control (C), H77 rE1E2 GNA (WT), H77 rE1E2 E2N1 (E2N1), and H77 rE1E2 E2N6 (E2N6). Signal was developed with anti-mouse secondary antibody conjugated to HRP and KPL peroxidase substrate. Absorbance was recorded at 450nm (subtracting absorbance at 507nm and PBS only control wells to remove background). This experiment was performed in three independent experiments in triplicate. The means from three independent experiments are shown and the error bars represent standard error between mice. Statistical analysis (one-way ANOVA, Kruskal-Wallis, and Dunn's multiple comparisons test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$. This figure was generated by Dr. Michael Logan.

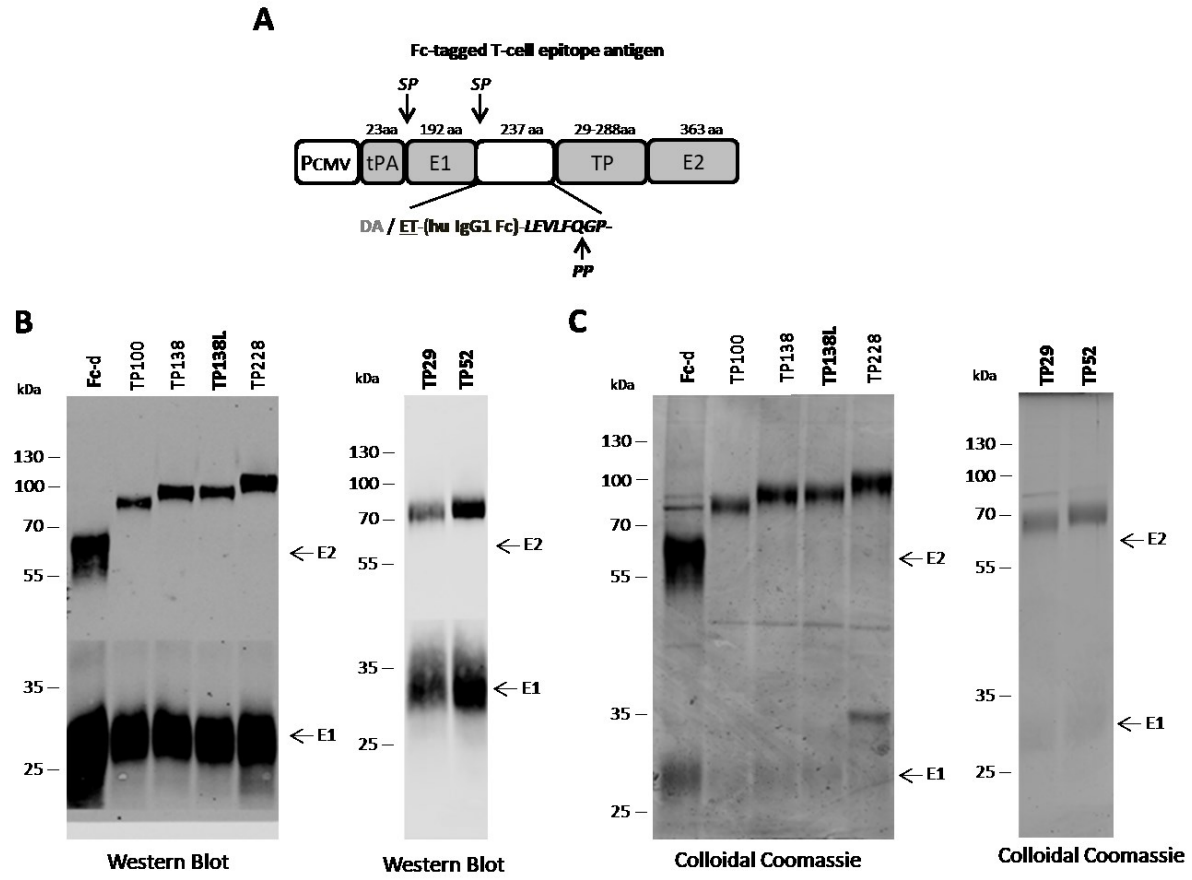

Figure A.6 Biochemical analyses of H77 rE1E2 immunogens containing T-cell epitopes. (A) Schematic representation of H77 rE1E2 proteins containing T-cell epitopes. (B) Purified Fc-tag derived H77 rE1E2 antigens containing T-cell epitopes (1µg) were separated by SDS-PAGE and Western Blotted with anti-E1 (A4) and anti-E2 (H52) monoclonal antibodies (mAbs) **(Dubuisson et al. 1994)**. (C) Purified Fc-tag derived H77 rE1E2 antigens containing T-cell epitopes (2µg) were separated by SDS-PAGE and bands were resolved with Coomassie brilliant blue G250. This figure was generated by Dr. Michael Logan.

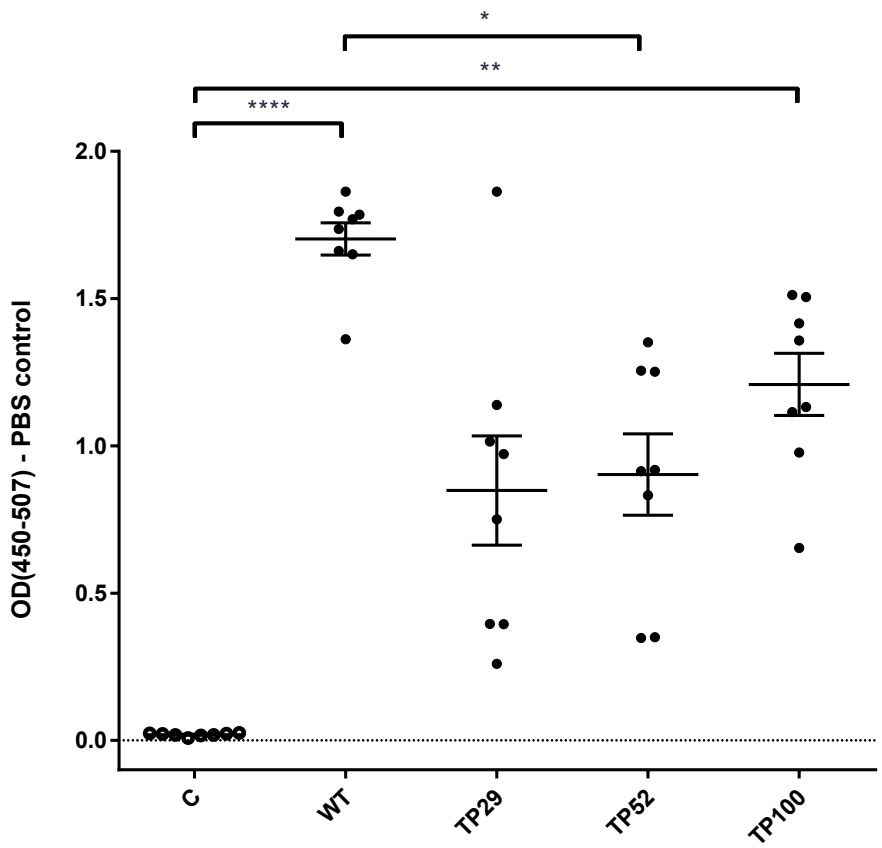

Figure A.7 Soluble E2 ELISA titers of antisera from mice immunized with H77 rE1E2 containing T-cell epitopes.
Purified recombinant sE2 (gt1a HCV-1 strain) was immobilized on GNA-coated microtiter wells. Heat inactivated mouse antisera from the post-vaccination bleed were tested at the indicated concentrations. The four groups were sham control (C), H77 rE1E2 GNA (WT), H77 rE1E2 TP29 (TP29), H77 rE1E2 TP52 (TP52), and H77 rE1E2 TP100 (TP100). Signal was developed with anti-mouse secondary antibody conjugated to HRP and KPL peroxidase substrate. Absorbance was recorded at 450nm (subtracting absorbance at 507nm and PBS only control wells to remove background). The experiment was performed in three independent experiments in triplicate. The means from three independent experiments are shown and the error bars represent standard error between mice. Statistical analysis (one-way ANOVA, Kruskal-Wallis, and Dunn's multiple comparisons test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$. This figure was generated by Dr. Michael Logan.

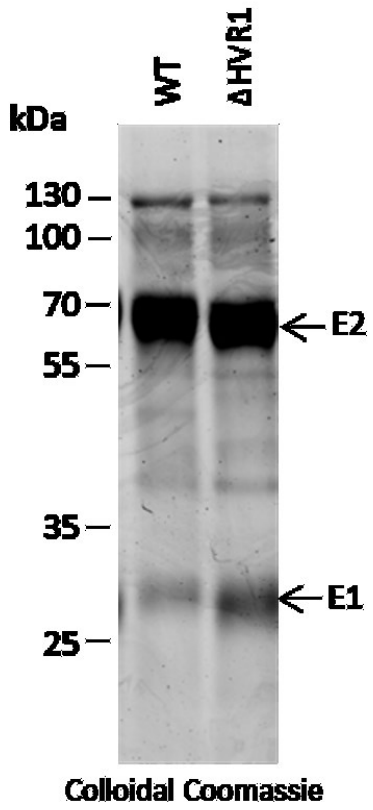

Figure A.8 Biochemical analyses of H77 rE1E2 immunogens with or without the HVR1 region.
Purified GNA-derived H77 rE1E2 proteins with and without HVR1 (2.5μg) were separated by SDS-PAGE and bands were resolved with Coomassie brilliant blue G250. This figure was generated by Dr. Michael Logan.

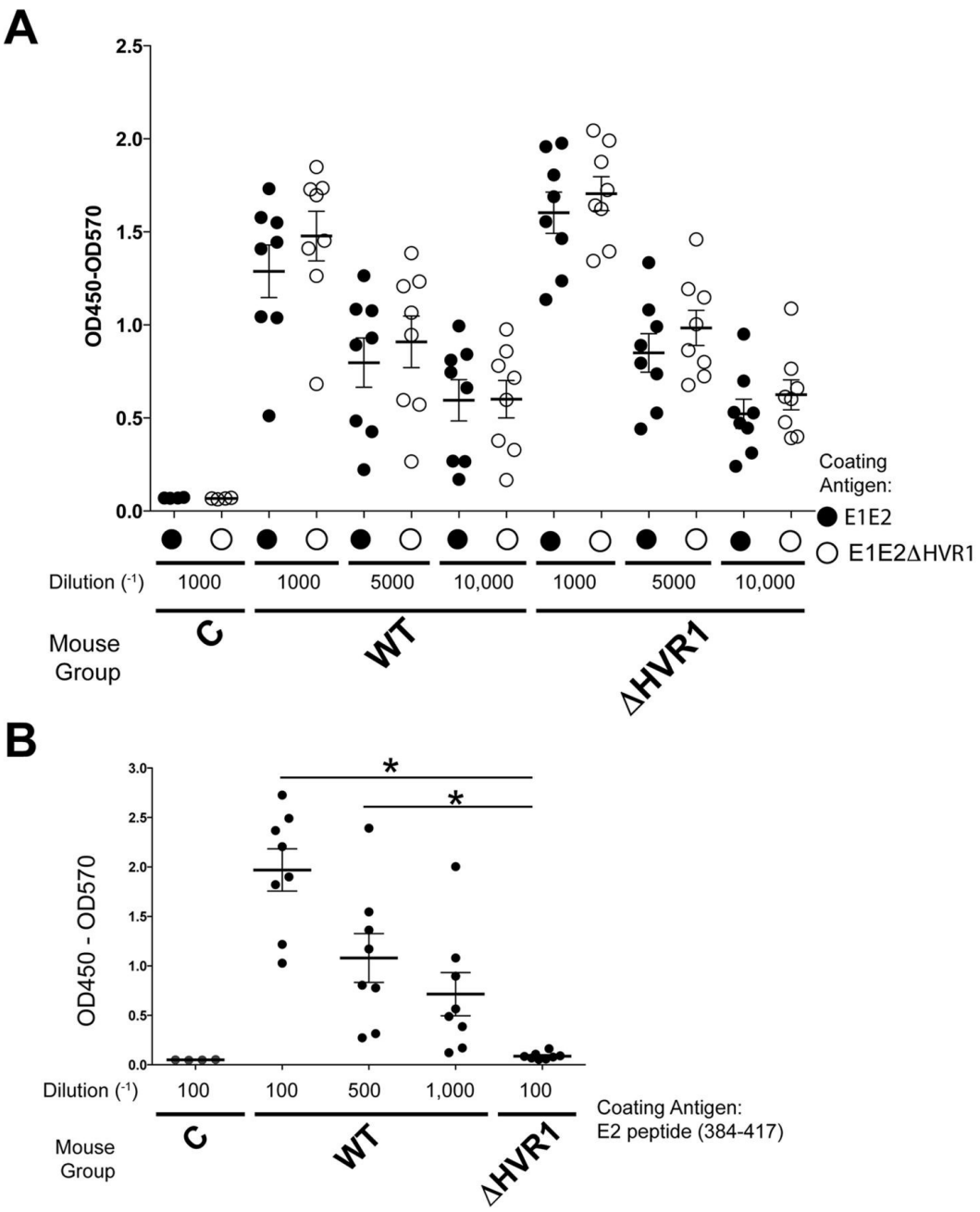
A
OD450-OD570
2.5
2.0
1.5
1.0
0.5
0.0
Coating Antigen:
E1E2
E1E2ΔHVR1
Dilution (-1)
1000
1000
5000
10,000
1000
5000
10,000
Mouse Group
C
WT
ΔHVR1
B
OD450 - OD570
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Dilution (-1)
100
100
500
1,000
100
Coating Antigen:
E2 peptide (384-417)
Mouse Group
C
WT
ΔHVR1

Figure A.9 (A) Anti-E2 and (B) anti-HVR1 ELISA titers of antisera from mice immunized with H77 rE1E2 WT or H77 rE1E2 ΔHVR1.
(A) Purified recombinant sE2 (gt1a H77 strain) containing or lacking the HVR1 region (aa384-411) was immobilized on GNA-coated microtiter wells. Heat inactivated mouse antisera from the final bleed were tested at the indicated concentrations. The three groups were sham control (C), H77 rE1E2 WT (WT), and H77 rE1E2 ΔHVR1 (ΔHVR1). Signal was developed with anti-mouse secondary antibody conjugated to HRP and KPL peroxidase substrate. Absorbance was recorded at 450nm (subtracting absorbance at 507nm and PBS only control wells to remove background). The results from at least two independent experiments in duplicate are shown and the error bars represent standard deviation. (B) The aa384-417 peptide was coated to the bottom of microtiter wells. The heat-inactivated final bleeds of the immunized mice antisera were tested at the indicated concentrations. Signal was developed with anti-mouse secondary antibody conjugated to HRP and KPL peroxidase substrate. Absorbance was recorded at 450nm (subtracting absorbance at 507nm and PBS only control wells to remove background). This experiment was performed in three independent experiments in triplicate. The means from three independent experiments are shown and the error bars represent standard error. Statistical analysis (one-way ANOVA, Tukey's *post hoc* test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$. This figure was generated by Dr. Michael Logan (Law et al. 2018).

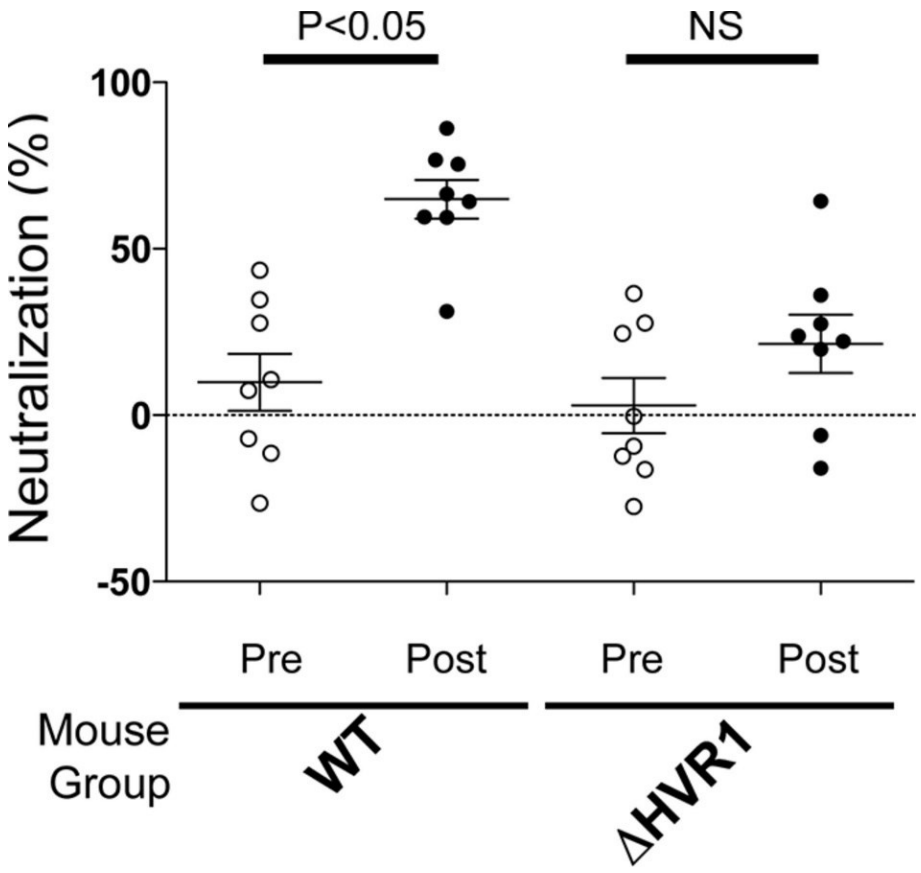

Figure A.10 Neutralizing activity against H77 HCVpp by antisera from mice immunized with H77 rE1E2 WT and H77 rE1E2 ΔHVR1.
Pre-immunization and post-immunized (final bleed) antisera (1:100) were tested for neutralizing activity against HCVpp pseudotyped with E1E2 from the H77 (gt1a) strain. HCVpp entry was quantitated by luciferase activity in cell extracts as described in **Chapter 2**. Neutralization activity was normalized to the luciferase levels of absence of sera (0% neutralization) and absence of virus (100%). This experiment was performed in three independent experiments in triplicate. The means from three independent experiments are shown and the error bars represent standard error. Statistical analysis (one-tailed paired t-test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. Statistical significance is indicated (NS means not significant). This figure was generated by Dr. John Lok Man Law (Law et al. 2018).

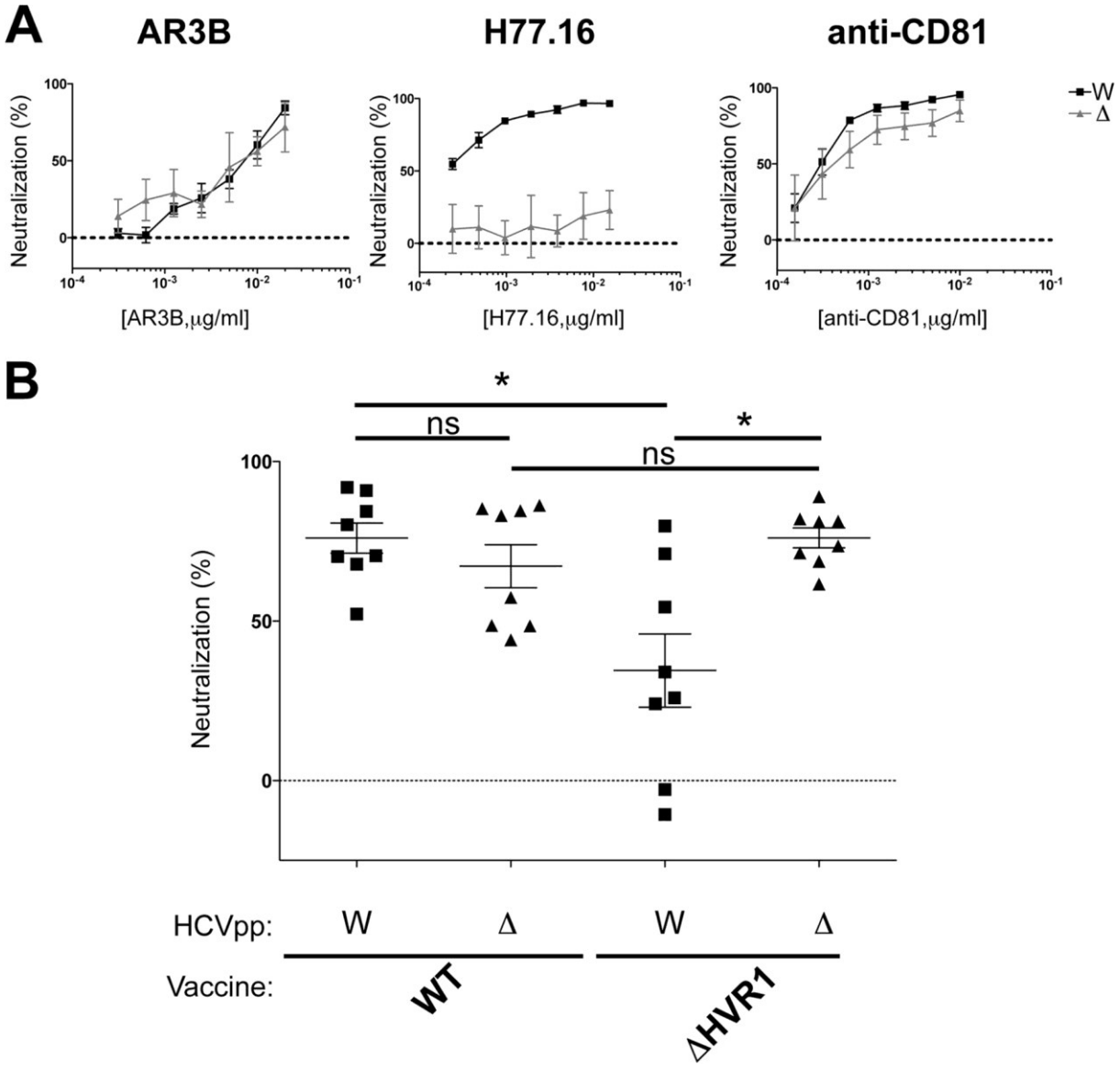
A
AR3B
H77.16
anti-CD81
Neutralization (%)
[AR3B,μg/ml]
[H77.16,μg/ml]
[anti-CD81,μg/ml]
W
Δ
B
*
ns
*
ns
Neutralization (%)
HCVpp:
W
Δ
W
Δ
Vaccine:
WT
ΔHVR1

Figure A.11 Neutralizing activity of (A) mAbs or (B) vaccinated mouse antisera against H77 HCVpp WT or H77 HCVpp lacking HVR1.
(A) Increasing concentrations of anti-E2 mAbs (H77.16 and AR3B) or anti-CD81 antibody were tested for neutralizing activity against HCVpp containing or lacking the HVR1 region. Of note, the H77 HCVpp ΔHVR1 virus contains the adaptive mutations H261R and Q444R described in a previous publication (Prentoe et al. 2014). Neutralizing activity was normalized to the luciferase activity level observed with no antibody added. This experiment was performed in three independent experiments in triplicate. The means from three independent experiments are shown and the error bars represent standard error. (B) Antisera from mice vaccinated with H77 rE1E2 WT or H77 rE1E2 ΔHVR1 were tested at a dilution of 1:100 against H77 HCVpp WT and H77 HCVpp ΔHVR1. Neutralizing activity of post-immunized antisera (final bleed) were normalized to the luciferase activity level observed with pre-immunization sera. This experiment was performed in three independent experiments in triplicate. The means from three independent experiments are shown and the error bars represent standard error. Statistical analysis (one-way ANOVA, Kruskal-Wallis, and Dunn's multiple comparisons test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$. This figure was generated by Dr. John Lok Man Law (Law et al. 2018).

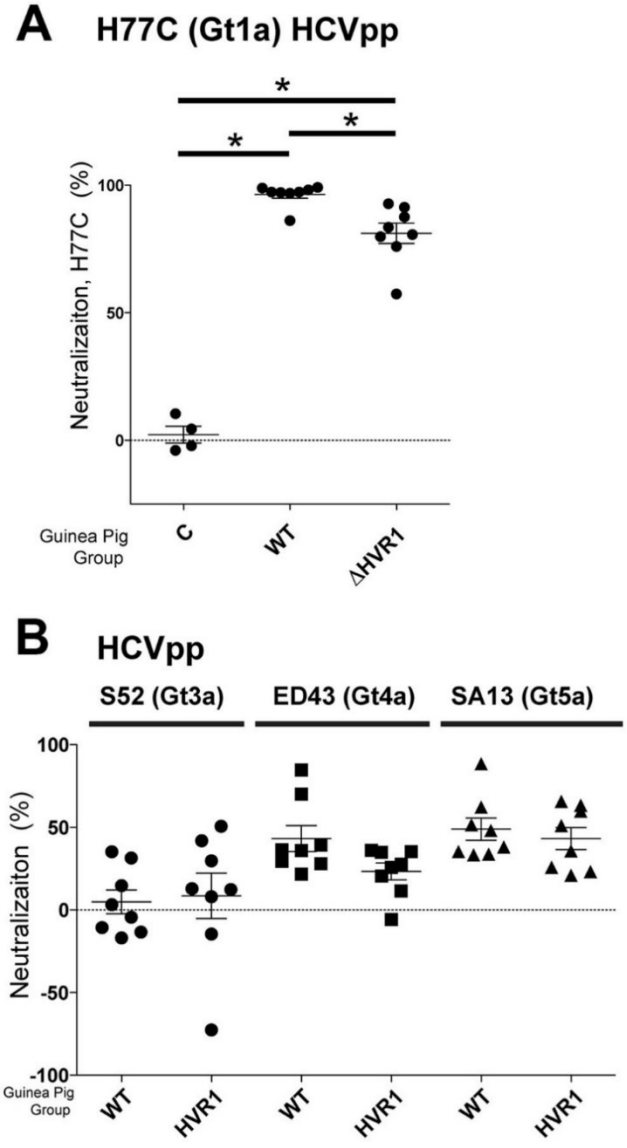
A
H77C (Gt1a) HCVpp
*
*
*
Neutralizaiton, H77C (%)
100
50
0
Guinea Pig Group
C
WT
ΔHVR1
B
HCVpp
S52 (Gt3a)
ED43 (Gt4a)
SA13 (Gt5a)
Neutralizaiton (%)
100
50
0
-50
-100
Guinea Pig Group
WT
HVR1
WT
HVR1
WT
HVR1

Figure A.12 Neutralizing activity against H77 HCVpp by antisera from guinea pigs immunized with H77 rE1E2 WT and H77 rE1E2 ΔHVR1.
Pre-immunization and post-immunized (final bleed) antisera (1:100) from three groups of guinea pigs (sham control (C), H77 rE1E2 WT (WT), H77 rE1E2 ΔHVR1 (ΔHVR1)) were tested for neutralizing activity against HCVpp pseudotyped with E1E2 from **(A)** the H77 (gt1a) strain or **(B)** the S52 (gt3a), ED43 (gt4a), and SA13 (gt5a) strains. HCVpp entry was quantitated by luciferase activity in ell extracts as described in **Chapter 2**. Neutralizing activity was normalized to the luciferase activity level observed with no antibody added. This experiment was performed in three independent experiments in triplicate. The means from three independent experiments are shown and the error bars represent standard error. Statistical analysis (one-way ANOVA, Kruskal-Wallis, and Dunn's multiple comparisons test) was done using Prism 7 (GraphPad Software, Inc.) and statistically significant differences were highlighted. (*) designates $P<0.05$, (**) designates $P<0.01$, and (***) $P<0.001$. This figure was generated by Dr. John Lok Man Law (Law et al. 2018).