Investigation of the Molecular Mechanisms That Determine Isolate-Specific, Antibody-Mediated Neutralization of HCV infectivity

by

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Abstract

It is estimated that there are about 1.75 million new Hepatitis C Virus (HCV) infections per year worldwide and around 20% will develop liver cirrhosis or liver cancer if left untreated. Direct acting antiviral drugs are available for the treatment of HCV with success rates of over 90%. But these treatments are expensive and cured patients can still be reinfected. To eliminate HCV, a prophylactic vaccine is needed. One of the major challenges in the development of a vaccine is the genetic diversity of the virus. Currently, there are 7 major genotypes and hundreds of subtypes. A global vaccine needs to be effective against all HCV genotypes. Our laboratory is developing an adjuvanted vaccine comprising recombinant E1/E2 viral envelope glycoprotein and non-structural protein components designed to elicit cross-neutralizing antibodies along with broad cross-reactive T cell responses against HCV. Previous data shows that the 1a E1/E2 glycoprotein component can elicit broad cross-neutralizing antibodies in humans and animals. However, variation is seen in the effectiveness of these antibodies to neutralize different HCV genotypes. The 1a E1/E2 vaccine-induced antisera showed strong homologous neutralization activity against genotype 1a H77c virus, while exhibiting significant differences in neutralizing activity against two closely related isolates of HCV genotype 2a, the J6 and JFH-1 strains.

E1 and E2 glycoprotein domains were swapped between the resistant J6 and sensitive JFH strains to determine the location of this differential neutralization sensitivity. Exchanges of variant amino acids in the E2 glycoprotein of these two HCV genotype 2a viruses were then conducted systematically to determine if

specific amino acids were important for conferring this differential neutralization sensitivity. In addition, the role of the N-terminal hypervariable region 1 (HVR1) of the E2 protein was investigated as a determinant of this isolate-specific neutralization. Recombinant J6 viruses with the HVR1 deleted or replaced with HVR1 from JFH-1, 1a H77 or 3a S52 were created. These recombinant viruses were then tested for their neutralization sensitivity to 1a E1/E2 antisera and to broadly neutralizing monoclonal antibodies. The role of the HVR1 in interaction with the entry receptors cluster of differentiation 81 (CD81) and scavenger receptor class B type 1 (SR-B1) was also investigated.

While HVR1 was shown to be mediating this isolate-specific neutralization, interestingly, our vaccine antisera does not appear to target the HVR1 of either of the genotype 2a viruses implying that HVR1 has an indirect effect. Other data indicates that HVR1 is mediating exposure of antibody binding sites of broadly neutralizing monoclonal antibodies. Additionally, HVR1 is not directly impacting the CD81 binding domain, but mediates isolate specific interactions with the SR-B1 receptor. Together, my data provides new information on the mechanisms of differential neutralization and contributes towards the design of a better vaccine antigen or antigen cocktail capable of expanding and optimizing the breadth of cross-genotype protection.

Preface

Figure 3.4 was created by Dr. Holly Freedman at my request.

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List of Abbreviations

3D	Three Dimensional
Δ	Deleted
°C	Degrees Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
µs	Micro second
%	Percent
‘	Prime
ANOVA	Analysis of Variance
Apo	Apolipoprotein
AR	Antigenic Region
BSA	Bovine Serum Albumin
cc	Cell Culture
CD	Cluster of Differentiation
CLDN1	Claudin 1
cm	Centimeter
CO_2	Carbon Dioxide
C-terminus	Carboxyl-Terminus
DAA	Direct Acting Antiviral
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molucule-3-Grabbing Non-Integrin
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
E	Envelope Glycoprotein
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EphA2	Ephrin Receptor A2
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
Fc	Fragment Crystallizable
GAG	Glycosaminoglycan
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HDL	High-Density Lipoprotein
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
Huh	Human Hepatoma
HVR1	Hypervariable Region 1
HVR2	Hypervariable Region 2

IC50	Half Maximal Inhibitor Concentration
IFN	Interferon
IgVR	Intergenotypic Variable Region
IRES	Internal Ribosome Entry Site
JFH	Japanese Fulminant Hepatitis
kb	Kilo-Base pair
kDa	Kilodalton
KV	Kilovolt
LB	Luria Broth
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LEL	Large Extracellular Loop
LVP	Lipo-Viral Particle
L-SIGN	Liver/Lymph Node-Specific Intercellular Adhesion Molicule-3-Grabbing Integrin
mg	Milligram
miR	Micro RNA
ml	Millilitre
M	Molar
mM	Millimolar
NANBH	Non-A, Non-B Hepatitis
ND	No data
nluc	Nano Luciferase
nm	Nanometer
NPC1L1	Niemann-Pick C1-Like 1 Transporter
NS	Non-Structural
ns	Not significant
N-terminus	Amino-Terminus
NTPase	Nucleoside-Triphosphatase
OCLN	Occludin
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Potential Hydrogen
RIG-I	Retinoic Acid Inducible Gene 1
pp	Pseudo Particle
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
sE2	Soluble Envelope Protein E2
SEC14L2	Sec14-Like Protein 2
SR-B1	Scavenger Receptor Class B Type 1
T-cell	T lymphocyte
TCID	Tissue Culture Infectivity Dose
TfR1	Transferrin Receptor 1
TLR	Toll-Like Receptor
TMB	Tetramethylbenzidine

V	Volt
VLDL	Very Low-Density Lipoprotein
WT	Wild Type

Chapter 1: Introduction

1.1 History of viral hepatitis

Hepatitis is an inflammatory liver disease characterized by symptoms such as abdominal pain, jaundice, fatigue, loss of appetite and changes to urine and stool color (http://www.who.int/features/qa/76/en/ and https://www.healthline.com/health/hepatitis#symptoms). Hepatitis can be classified as non-viral or viral. Non-viral hepatitis has a variety of causes including other infections such as Weil's disease (leptospira bacterial infection), and non-infectious etiologies like exposure to toxic substances (eg., drugs and alcohol), or autoimmune disorders (http://www.who.int/features/qa/76/en/, (1, 2)). Hepatitis viruses was first recognized in the 1930's to be a cause of hepatitis and was observed to result from exposure to human serum used in the production of vaccines (1). Viral hepatitis is the most common cause of hepatitis worldwide (http://www.who.int/features/qa/76/en/). There are five main hepatitis viruses denoted by: A, B, C, D and E. Of particular concern are the hepatitis A, B and C viruses (HAV, HBV and HCV, respectively), which are capable of causing serious, and in the cases of HBV and HCV, chronic disease. HAV and HBV were first described in the 1960's and 1970's and tests to detect HAV and HBV infections were developed shortly after (3–5). It then became apparent that most of the cases of hepatitis contracted through blood transfusions were caused by a virus that was neither HAV nor HBV, giving rise to the name "Non-A, Non-B" hepatitis (NANBH) for the unknown virus (5, 6).

1.2 Hepatitis C virus

1.2.1 HCV research background. In 1989, a positive-stranded ribonucleic acid (RNA) virus was identified with a genome of about 10 000 nucleotides that could be recognized by antibodies in the serum of patients infected with NANBH (6).

This virus was given the name HCV and subsequently an assay was developed to screen for HCV antibodies in infected patients (7).

The study of HCV early after discovery was hindered by the lack of an efficient cell culture model (8). The first step towards the development of an *in vitro* system for HCV replication was the establishment of a sub-genomic non-structural (NS)3-NS5B replicon system in Huh7 human hepatoma cells (9, 10). This replicon system allowed for the study of HCV replication and the testing of potential therapeutic agents that can target HCV replication (8). To assist with the study of HCV entry and neutralizing antibodies, the HCV pseudo-particle (HCVpp) system was developed (11). Shortly after the devolvement of the HCVpp system it was discovered that a full-length 2a isolate of Japanese fulminant hepatitis (JFH) was capable of replicating in Huh7 human hepatoma cells (12). These discoveries have revolutionized the HCV research field allowing for the study of the full HCV life cycle using cell culture models and led to discoveries relating to the HCV proteins, replication strategy, as well as advances in treatments and vaccine research (8, 10). Building upon the initial discovery that JFH-1 virus replicates in Huh7 cells, it was subsequently demonstrated that replacement of the core to NS2 region of the JFH-1 virus with the core to NS2 proteins from all seven genotypes resulted in virus that was viable *in vitro* (13, 14). This cell culture system is still the most commonly used model for HCV research today. Another advance that has aided the cell culture model of HCV is the improved permissiveness of Huh7.5 cells over the Huh7 cell line. Huh7.5 cells are a subset of the Huh7 cell line that have defective interferon (IFN) signaling and support a more robust HCV infection (8, 10). Recently, it has been discovered that the addition of a host protein involved in lipid binding, SEC14-Like Protein 2 (SEC14L2), facilitated the replication in Huh7.5 cells of full length HCV genotypes as well as virus isolated from patients without the need for adaptive mutations (15).

1.2.2 HCV overview. HCV is a single stranded positive sense RNA virus from the *Hepacivirus* genus of the *Flaviviridae* family (8). HCV is a ~50-70 nanometers (nm) (8, 16) enveloped virion composed of inner core protein capsid bound to the

HCV genome and surrounded by a host-derived lipid membrane imbedded with the envelope glycoprotein (E)1 and E2 (8). To form what is termed as the HCV lipo-viral particle (LVP), virions associate with very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoprotein (HDL) in both patient sera and cell culture (16–18). Apolipoproteins (Apo) A1, B, C1, and E have been shown to associate with lipo-viral particles and participate in viral assembly and entry into host cells (17–23). The virus primarily infects hepatocytes and has a high replicative capacity, producing 10^{12} virions per day (24). HCV establishes a chronic infection in 70-80 percent (%) of infected individuals and remains a global health burden today despite newly available therapeutic drugs (8, 24).

1.2.3 HCV Viral Proteins. HCV has 9.6 kilo-base pair (kb) genome with uncapped 5 prime (') and 3' untranslated regions that are highly structured (8, 24). The 5' non-coding region contains an internal ribosome entry site (IRES) to facilitate translation of the viral proteins and the structure of this region is essential for translation (25). The genome also contains an open reading frame that encodes a polyprotein, which is post-translationally processed into 10 viral proteins by both viral and host proteases (8). The genome organization of HCV visualized in Figure 1.1 shows that there are three structural proteins (core, E1 and E2), p7 and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). Along with the genomic RNA, the three structural proteins form the virion, while the non-structural proteins facilitate viral replication and assembly (8).

1.2.3.1 Structural Proteins. Core protein is a 21 kilodalton (KDa) protein that contains two domains (24). Domain 1 contains basic amino acids that allows the protein to interact with genomic RNA (26). Domain 2 is a hydrophobic α-helix domain that allows core to interact with lipid droplets and the endoplasmic reticulum (ER) membrane (27, 28). The core protein's main function is binding and encapsulating HCV RNA (28). Core protein has been shown to interact with lipid droplets within cells and reorganizes these lipid droplets to facilitate viral assembly (29). Core protein is also implicated in modulating a number of host

cellular factors relating to lipid metabolism, tumor suppression and immune responses that can lead to liver fibrosis and hepatocellular carcinoma (HCC) (30).

The envelope glycoproteins E1 and E2 are glycosylated transmembrane proteins primarily involved in viral entry (31). Both envelope proteins have significant glycosylation, with 5-6 sites in E1 and 9-11 sites in E2 depending on the genotype (32). The E1 and E2 proteins contain regions predicted to be involved in low-potential hydrogen (pH) fusion and they are theorized to perform this function together (33). E1 and E2 together form a heterodimer currently predicted to form trimers of this heterodimer on the surface of the virion (34). E1 has also been shown to modulate E2 binding to entry receptors and is critical for HCV interactions with Claudin1 (CLDN1) (35, 36). The structure of E1 remains poorly studied, with one study suggesting it contains a β-hairpin and an α-helix region, and is capable of dimerizing with itself (37). Another study suggested that hydrophobic residues within E1 are involved in apolipoprotein binding (38).

The E2 protein contains three domains within the core region and an α-helix transmembrane domain, as well as three variable regions (hypervariable region 1 (HVR1), HVR2 and intergenotypic variable region (IgVR)) (39, 40). The E2 protein is directly involved in interactions with the entry receptors Human scavenger receptor class B type 1 (SR-B1) (41) and cluster of differentiation 81 (CD81) (42). E2 has been shown to bind directly to CD81 (42) and specific amino acid residues critical for E2 binding to CD81 have been identified (39, 40, 43–46). These CD81 binding regions are highly conserved between HCV genotypes and the binding of E2 with CD81 can be blocked by antibodies that target these regions (40, 47). E2 protein interactions with SR-B1 are complex; involving an interplay between lipoprotein interaction and the HVR1 of the E2 protein (41, 48–50). Initial attachment is via lipoprotein/SR-B1 interaction with a subsequent HVR1 dependent interaction with E2 protein (48). HDL is also involved in infection enhancement dependent on both the SR-B1 receptor and the presence of HVR1 on the virus (49). HVR1 is a 27 amino acid sequence at the Amino (N)-terminus of E2 with high sequence diversity among isolates and has been proposed to be immunodominant during natural HCV infection (46). HVR1 rapidly evolves under

immune pressure, and this rapid evolution leads to the production of antibodies targeting HVR1 that tend to be strain specific (46, 51). Although, HVR1 has such high sequence diversity, there is evidence of amino acid charge conservation indicating a conserved function of HVR1 (52). Virus with the HVR1 deleted has been shown to be viable in cell culture (53, 54) as well as in the chimpanzee model (55), and has allowed in-depth studies on the function of HVR1. In addition to interactions with SR-B1, HVR1 is also involved in protection of the virus from neutralizing antibodies, as well as modulating lipoprotein interactions (23, 50, 54, 56).

1.2.3.2 P7. While p7 is not required for viral replication *in vitro*, it is critical for the assembly and release of virions both i*n vitro* and *in vivo* (57, 58). P7 has also been shown to act as an ion channel and is therefore theorized to be a member of the viroporin family (59, 60). However, P7 still remains a poorly understood protein.

1.2.3.3 Non-Structural Proteins. The first non-structural protein, NS2, encodes a cysteine protease in the carboxyl (C)-terminal portion that functions to cleave the NS2 and NS3 junction and is enhanced by the presences of the N-terminal portion of the NS3 protein (61). The N-terminal portion of the NS2 protein is a transmembrane domain with three segments (62, 63). The crystal structure of the NS2 protein indicates that it forms a dimer with two active sites comprised of residues from both monomers (64). NS2 has also been shown to interact with many of the other HCV proteins and is theorized to play a role in viral assembly (65).

The NS3 protein, in addition to its interactions with NS2, forms a complex with the NS4A protein (65). The N-terminal portion of the NS3 functions as a serine protease and the C-terminal portion has nucleoside-triphosphatase (NTPase) / RNA helicase activity (66). The small 54 amino acid NS4A is a cofactor for the NS3 serine protease and together they are responsible for cleaving the downstream HCV non-structural proteins. The NS3 protein also acts as an NTPase / RNA helicase to unwind both double stranded and structured single stranded HCV RNA during replication.

NS4B protein contains four transmembrane domains, and two α-helix domains on both the N-terminal and C-terminal ends (67, 68). NS4B is involved in the formation of the double membrane vesicles of the membranous web that serves as the replication complex for HCV (67–70). The N-terminal and C-terminal ends of the NS4B protein are able to self-interact and this interaction is important for the formation of the membranous web (67). In a recent paper the membranous web was shown to contain discrete compartments for replication and assembly (71). Furthermore, the authors demonstrated that HCV utilizes the host nuclear transport machinery to selectively allow movement of molecules into the membranous web and exclude the pattern recognition receptors to prevent immune recognition of viral RNA. In addition to the formation of the membranous web, NS4B is involved in viral assembly, is able to bind HCV RNA and has been suggested to exhibit NTPase activity (72–74). However, NSB4 functions other than the formation of the membranous web have been poorly studied.

NS5A protein has an N-terminal membrane anchor and three structural domains (75). Domains 1 and 2 of NS5A are involved in RNA replication and domain 3 is involved in virion assembly (76–79). Domain 3 of NS5A has been shown to be important for NS5A localization to lipid droplets, the proposed site of virion assembly, along with the core protein during viral assembly (77). NS5A has also been shown to modulate the response to IFN-α therapy *in vitro* with the observation that mutations within NS5A correlate with the response to interferon therapy (80–83). However, the role of mutations within NS5A remains controversial as the association of mutations with favorable IFN treatment outcome appears to be genotype specific (83).

NS5B is an RNA-dependent RNA polymerase that synthesizes both the negative-strand and the genomic positive-strand HCV RNA (84, 85). The NS5B protein contains an N-terminal catalytic site and a C-terminal membrane anchor (84–86).

The NS3, NS4A, NS4B, NS5A and NS5B non-structural proteins are also involved in oncogenesis by modulating a number of cell cycle, cell growth and cell survival pathways, and can stimulate liver fibrosis (30).

1.3 HCV Entry and life cycle.

HCV entry is visualized in Figure 1.2. HCV entry into host cells is a complex process involving both the envelope proteins, lipoproteins present on the viral particle and a number of cell surface proteins and receptors (31, 87). Initial attachment of HCV virions to the cell surface is made through interactions with heparan sulfate glycosaminoglycans (GAG) mediated by binding with Apo E and HCV envelope proteins (88, 89). The low-density lipoprotein receptor (LDLR) also interacts via Apo E during early attachment of viral particles (90, 91). Virions are then able to bind with the SR-B1 receptor through stepwise interactions with lipoproteins and HCV E2 protein (48, 49). Binding to SR-B1 is thought to allow subsequent binding of the E2 protein directly to the CD81 receptor (31, 42). CD81 is also involved in the recruitment of epidermal growth factor receptor (EGFR) (92, 93). Interaction with CD81 primes the envelope proteins for low-pH-dependent fusion and activates cellular signaling that allows the virion to be trafficked to the tight junctions (93–95). At the tight junction, CD81 bound to HCV forms a complex with CLDN1 (96, 97). Occludin (OCLN) is a required late entry receptor, however the exact interactions of OCLN with the HCV receptor complex remains elusive (93, 98–100). The HCV receptor complex then enters the cell via clathrin-mediated endocytosis that is dependent on the presence of EGFR and OCLN (93, 101). Other entry factors, while they do not directly interact with HCV receptor complexes, have also been identified to be important during viral entry, such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and liver/lymph node-specific intercellular adhesion molecule-3grabbing integrin (L-SIGN) (24), ephrin receptor A2 (EphA2) (92), transferrin receptor 1 (TfR1) (102), Niemann-Pick C1-like 1 transporter (NPC1L1) (103), and CD36 (104).

HCV replication, assembly and release are visualized in Figure 1.3. The E1 and E2 proteins fuse with the endosome membrane in a pH-dependent manner and the nucleocapsid is released into the cytoplasm (33, 105). The genome is uncoated and released into the cytosol (87). The IRES within the 5’ end of the RNA allows

HCV's cap-independent translation and synthesis of the HCV polyprotein (25). A liver-specific micro RNA (miR), miR-122, binds to the 5' non-coding region and promotes viral replication and translation (106, 107). The polyprotein is processed into the three structural proteins and seven non-structural proteins, as described above, on the ER membrane (24, 87). Replication takes place in the membranous web, which is formed by rearrangement of the ER membrane by the NS4B protein (67, 68, 70, 71). Replication involves the co-operation of the NS5B, NS5A and NS3 proteins as well as host factors such as cyclophilin A and miR-122 to generate the positive-strand RNA genome for packaging into new virions (24, 87). Core and NS5A protein interactions with RNA and lipid droplets allow the formation of the nucleocapsid. Immature virions then bud through the ER where they become encased by a lipid membrane imbedded with the E1 and E2 envelope proteins and exit the cell via the golgi through the VLDL secretory pathway (87, 108). Alternatively, particles can be spread by cell-to-cell transmission through a process that is still poorly understood but was found to involve the lipid transfer function of SR-B1 (109, 110).

1.3.1 Essential entry receptors. The SR-B1 is highly expressed in the liver and selectively imports cholesterol into cells (111, 112). SR-B1 has N- and C-terminal cytoplasmic domains connected by an extracellular loop (112). The extracellular loop of SR-B1 is able to bind HDL and LDL, and facilitates lipid exchange at the plasma membrane. After initial low affinity binding to GAG and LDLR, HCV interacts with SR-B1 in a multistep process (48). Initial attachment of HCV to SR-B1 is mediated through interactions with the lipoprotein components of the virion. Subsequently, the lipid transfer function of SR-B1 allows "access" for E2 binding to CD81 and potentially alters the formation of the tetraspanin web, the network of receptor interaction at the cell surface during entry (48, 113). A third function of the SR-B1 also involves the lipid transfer of HDL and additionally requires the presence of the N-terminal HVR1 of the E2 protein (48, 110). This third interaction is thought to enhance infectivity. Reduced dependency on SR-B1 has been shown for some mutations within the E2 protein (114–116) as well as deleted HVR1 virus

(49, 50, 54) and the virus responds to neutralization by anti-SR-B1 antibodies in a genotype-specific manner (13). For the JFH-1 virus genotype, the SR-B1 has been shown to be interchangeable for entry with the LDLR (117). These findings highlight a potentially genotype-specific role the SR-B1 is playing in HCV entry and warrants further study.

CD81 has four transmembrane domains and both a small and a large extracellular loop (118). It is involved in several cellular processes including cell activation, proliferation, motility, metastasis and cell fusion (119). CD81 has been identified as a critical receptor for the entry of HCV and is known to bind directly to the E2 protein (42). Specific residues within CD81 Large Extracellular Loop (LEL) have been identified that bind to specific residues within the E2 protein (45, 118). E2 protein binding with CD81 primes the envelope proteins for low-pH dependent fusion, and activates cell signaling that leads to the recruitment of actin and subsequent trafficking of the HCV receptor complex to the tight junctions (31, 94, 95).

CLDN1 and OCLN interact with the HCV receptor complex at the tight junction. Similar to CD81, CLDN1 has four transmembrane domains and both a small and large extracellular loop (31). The large extra cellular loop of CLDN1 is important for binding with CD81 in the HCV receptor complex (96, 97). OCLN also has four transmembrane domains and two extracellular loops (31). OCLN's second extracellular loop has been shown to be important for the entry of HCV, though specific interactions of the HCV receptor complex with OCLN remains poorly understood (31, 98, 120). OCLN is also able to facilitate clathrin-mediated endocytosis of the HCV particle (93, 101).

1.4 HCV epidemiology and pathogenesis.

There remain about 62-79 million chronically infected individuals worldwide and 1.75 million new infections of HCV are estimated to occur each year ((87), http://www.who.int/news-room/fact-sheets/detail/hepatitis-c). The countries with the highest prevalence of HCV infection include Egypt, China,

Mongolia, Russia, Nigeria, Pakistan and India and they account for over half of the total worldwide infections (87, 121). A significant source of infection in these countries is due to contaminated blood products and unsafe medical practices. In Europe and North America, however, the majority of infections are among intravenous drug users (122). HCV is responsible for about 400 000 deaths per year and is the cause of about 25% of HCC. Untreated HCV infection can lead to progressive liver fibrosis and cirrhosis, and eventually HCC (30, 87). HCV promotes the formation of HCC through both indirect sustained chronic inflammation and directly through viral protein interactions with a variety of host factors that control the cell cycle and anticancer immunity.

HCV is a highly diverse virus with seven major genotypes and 67 characterized subtypes (Figure 1.4) (8, 123–125). Genotypes 1 and 3 are the most common worldwide and together account for more than 75% of all HCV infections. HCV displays greater sequence diversity than even human immunodeficiency virus (HIV); and genotypes can differ by up to 30% in nucleotide sequence and subtypes differ by up to 15% (8, 126). Even HCV isolates within the same subtype can have sequence variance of up to 10%. This large sequence variation in HCV has been an ongoing challenge in the treatment and development of vaccines and direct acting antivirals (DAA) for HCV.

Acute clinical presentation of HCV infection is mild and usually asymptomatic, but acute infection leads to chronic HCV infection in about 75-85% of cases (87). The progression of disease usually occurs over several decades during which patients may experience fatigue, weight loss, and muscle, joint and abdominal pain. Due to the mild clinical presentation of early infection, patients often remain undiagnosed until the more serious clinical complications of cirrhosis become evident. Early treatments for HCV infection have consisted of pegylated-interferon combined with ribavirin, however, with a multitude of side effects and variable response rates this was not an ideal treatment (127). The discovery of DAA has improved the outcome in HCV treatment dramatically, with over 90% cure rates after a short course of oral drug administration comprising DAA targeting the HCV polymerase, NS5A and NS3 proteins. However, hurdles remain in the

treatment of HCV with DAA. The drugs remain expensive and are inaccessible to much of the population that needs them most (122). Recent research has indicated the emergence of DAA resistant mutations and highlighted the natural poorer response of patients infected with genotype 3a (128, 129). However, research is advancing the available DAA treatment regimens to overcome the reduced response of genotype 3a (130). Additionally, cured patients do not develop protective immune responses, leaving them unprotected from reinfection (131). Patients cured with DAA but with advanced fibrosis and cirrhosis at the time of treatment also remain at elevated risk for the development of HCC (132–134). Therefore, a vaccine to prevent HCV is still urgently needed.

1.5 Natural immune response to HCV.

Both the innate and adaptive immune systems respond to HCV infection (135). Initial HCV infection stimulates the production of type 1 IFN when the viral RNA is recognized by toll-like receptors (TLRs) (136) and retinoic acid inducible gene 1 (RIG-I) (137). Induction of an IFN response leads to an antiviral state within infected cells and up-regulates the subsequent adaptive immune response (135). The activation of the adaptive immune system by HCV leads to both cellular and humoral immune responses.

1.5.1 Cellular immune response. The importance of an adaptive T lymphocyte (T-cell) response in the clearance of acute infection has been demonstrated in many studies for both humans and chimpanzees (138–143). In the absence of effective $CD4^+$ and $CD8^+$ T-cell responses against HCV the virus is able to establish a chronic infection. This has been shown experimentally in chimpanzees where depletion of $CD4^+$ and $CD8^+$ T-cells resulted in HCV infection progressing to chronicity (142, 143). In clinical studies it has been observed that patients who fail to clear an acute infection have impaired $CD4^+$ and $CD8^+$ T-cell responses (138, 139). HCV escape from the T-cell response is mediated by both viral escape mutations as well as T-cell exhaustion (135).

1.5.2 Humoral immune response. Shortly after the discovery of HCV, it was recognized that antibodies could be detected in the blood from infected patients and an enzyme immunoassay could be used as a diagnostic test as well as to improve the safety of blood transfusions (7). Early studies suggested that the neutralizing antibody response to natural infection was insufficient to protect from reinfection (144–146), but later studies subsequently showed neutralizing antibodies to be protective (147, 148). This discrepancy was resolved with the development of the HCVpp infectious cell culture system (11), and the development of an assay for neutralizing antibodies. Using this HCVpp system, it was shown that neutralizing antibodies from chronically infected patients and chimpanzees could neutralize infection of homologous virus and showed some cross-reactivity to a heterologous subtype in cell culture (147). Subsequently, it has been shown that HCV antibodies are able to passively prevent infection in the chimpanzee and liver chimeric mouse models (150, 151).

The antibody response to HCV was further characterized by the isolation of neutralizing monoclonal antibodies from infected patients (47). Subsets of these antibodies have been shown to neutralize a broad range of both homologous and heterologous HCV genotypes and to target regions throughout the E1 and E2 proteins. The most effective of these antibodies target conserved regions within the E2 protein that are known to be involved in CD81 interactions (152–154). Additionally, two strongly cross-neutralizing antibodies antigenic region (AR)4A and AR5A that target discontinuous epitopes within E1 and E2 have been described (154). However, HCV is still able to evade these neutralizing antibody responses. There have been a large number of studies reporting on the role of mutations in the E1 and E2 proteins that allow escape from one or more of the monoclonal antibodies (Reviewed in (47)). In some cases, these mutations have also been shown to alter virus interactions with entry receptors CD81 and SR-B1 (115, 155).

In addition to mutation of neutralizing epitopes, HCV has evolved a variety of other mechanisms to escape neutralizing antibodies (156). HCV utilizes the immunodominant and easy mutable capabilities of HVR1 as an immune decoy in natural infections. HVR1 mutation is driven by the presence of neutralizing

antibodies and mutations in HVR1 are not detected in HCV infected patients who are deficient in immunoglobulin (157). Since HVR1 is non-essential for virus infection, the virus tolerates mutations in HVR1 (55). Therefore, antibodies that target HVR1 tend to be isolate-specific as the virus rapidly accumulates mutations in HVR1 that allow escape from these antibodies, while the antibodies remain able to only neutralize the unmutated isolate (51, 157–160). The glycosylation of the E2 protein is also able to hinder the binding of neutralizing antibodies (161). Removal of specific glycans in E2 results in increased sensitivity to neutralization indicating that glycosylation plays a role in protecting the virus from neutralizing antibodies. HCV association with lipids also protects the virus from neutralizing antibodies. Virus that is associated with lipids and lipoproteins is more resistant to neutralizing antibodies than virus lacking lipid components (162). Moreover, HCV can avoid exposure to circulating neutralizing antibodies by trafficking through tight junctions to infect neighboring cells (163, 164). This cell-to-cell transmission of HCV is facilitated by the presence of SR-B1, and thus viruses with a decreased dependency on SR-B1 are more sensitive to neutralization (114, 115, 165). Lastly, non-neutralizing antibodies can interfere with the ability of neutralizing antibodies to bind the virus. When non-neutralizing antibodies are depleted from patient sera, the remaining neutralizing antibodies are somewhat more effective in their ability to prevent infection of HCV in vitro (166). Antibodies targeting the HVR1 are also able to interfere with the binding of broadly neutralizing antibodies that recognize epitopes downstream of HVR1 by sterically hindering access of these neutralizing antibodies (167).

1.6 Vaccine development

Despite the viruses' ability to escape the natural immune response, there is hope for the development of an effective vaccine. Shortly after the discovery of HCV, an E1/E2 glycoprotein vaccine was tested in chimpanzees and the elicited immune response was able to prevent infection of homologous virus challenge (168). Additionally, while this vaccine was unable to prevent acute infection of a

heterologous strain, there was a significant decrease in the rate of chronic infections post vaccination and elicited antibodies capable of neutralizing a diverse range of HCV genotypes *in vitro* (169, 170).

There have been a number of other potential strategies for envelope glycoprotein vaccination developed including E1/E2 deoxyribonucleic acid (DNA) vaccination (171), HCV virus-like particles containing the three structural proteins (172), whole inactivated virus (173), soluble E2 protein (174), and modified E1/E2 proteins (175, 176). Additionally, a prime-boost immunization strategy involving a defective chimpanzee adenovirus vector and a defective modified vaccinia ankora vector both expressing NS3 through NS5B is currently undergoing phase II efficacy trials in humans (to be completed July 2018) (177). This strategy was based on a DNA vaccine tested in chimpanzees with mixed results such that, acute infection rates were reduced but effects on chronicity rates were difficult to determine due to resolution of infection in non-vaccinated controls (177, 178). Promising data has been obtained in humans using the full length E1/E2 glycoprotein vaccine (179). This first generation recombinant 1a E1/E2 glycoprotein vaccine has been proven safe in humans (180) and was shown to elicit antibody responses capable of neutralizing all genotypes of HCV in *vitro* (179). However, there is variation in the effectiveness across genotypes with strong neutralization against viruses of genotypes 1, 4, 5 and 6, but reduced effectiveness against genotypes 2, 3 and 7. This same 1a E1/E2 vaccine antigen has been further characterized by the vaccination of goats (45). Vaccine antisera was generated in goats due to the limited supply of the human antisera from the phase 1 clinical trial and the large quantities of antisera still needed to research the antibody response elicited by 1a E1/E2 vaccination. The goat antisera produced polyclonal antibodies that showed a similar profile of neutralization of the seven HCV genotypes seen in humans and competed for binding to E2 proteins with known broadly neutralizing monoclonal antibodies (45). This indicated that 1a E1/E2 vaccination elicited antibodies that targeted conserved epitopes known to be targets of broadly neutralizing monoclonal antibodies. Currently, improvements in the GMP manufacturing process and immunogenicity of this vaccine candidate are being undertaken at the University of

Alberta. Addition of a fragment crystallizable (Fc) Immunoglobulin tag has greatly improved the purification process of the E1/E2 glycoproteins and will allow the proteins to be purified in a large-scale manufacturing process (181). It is also likely that a combination of both a cross-neutralizing antibody response as well as a broad T-cell response will be required to optimally prevent HCV infection. As such, antigens encoding strong T-cell epitopes within the non-structural proteins will be included along with the E1/E2 glycoprotein component for upcoming vaccine trials in humans.

1.7 Current study and hypothesis.

This project utilizes the recombinant 1a E1/E2 vaccine-induced antiserum from vaccinated goats (45) to investigate the molecular determinants of the observed variation in neutralization sensitivity between HCV genotypes and to explore the potential underlying mechanisms that are mediating this variation. My data indicates that two highly related isolates of HCV genotype 2a, J6 and JFH-1, respond differently to neutralization by 1a E1/E2 vaccine-antisera. J6 is relatively resistant to neutralization while JFH-1 is very sensitive. Investigating the molecular determinants of the differential neutralization sensitivity between these two highly related isolates will shed light on the potential determinates of genotype-specific neutralization sensitivity observed using our vaccine-antisera (179).

It was hypothesized that the JFH-1 isolate is neutralized by vaccine-elicited antibodies targeting conserved epitopes within the envelope glycoproteins, while these epitopes are mutated or less reactive in the J6 isolate. There were four main aims of this study:

1. Determine if glycoprotein E1 or E2 was mediating the differential neutralization.
2. Investigate the role of variant amino acids within the glycoproteins between JFH-1 and J6 isolates in differential neutralization.
3. Determine the role of the E2 HVR1 in differential neutralization.

4. Hypothesize a possible mechanism to explain the differential neutralization observed between J6 and JFH-1.

This thesis will show that the E2 glycoprotein largely mediates the observed differential neutralization, with the E2 HVR1 being a major determinant in the isolate-specific neutralization sensitivity between these two genotype 2a isolates. Surprisingly, HVR1 appears to be acting indirectly to mediate this differential neutralization by modulating exposure of cross-neutralizing epitopes within the envelope glycoproteins. I have shown that HVR1 of J6 virus has enhanced interaction with SR-B1 which could account for its observed resistance to neutralization. Further work will explore the underlying mechanism. This data will help in the design of a more effective vaccine candidate and contribute to the understanding of the complex interactions of HCV with entry receptors.

1.8 Figures and tables

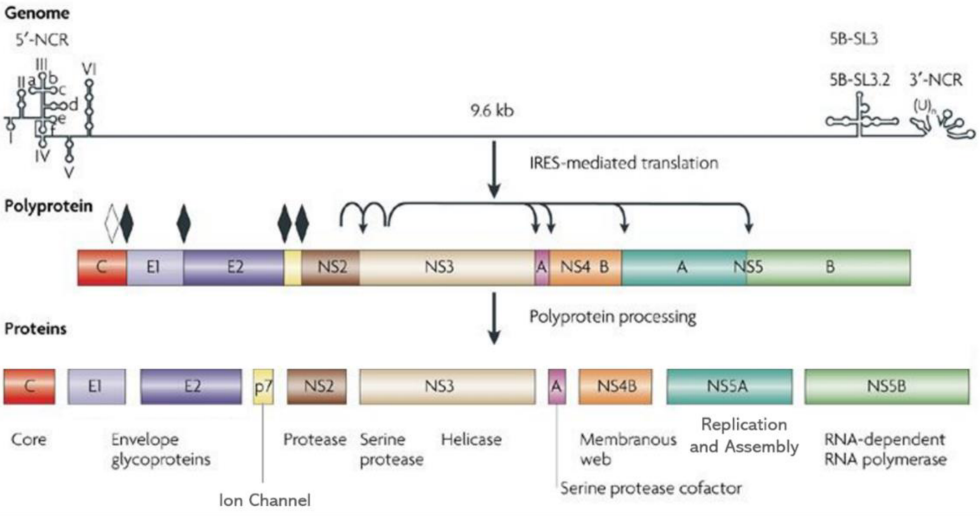

Figure 1.1. Genome organization of HCV proteins. HCV contains 5' and 3' untranslated regions that are highly structured and an open reading frame that encodes 10 viral proteins that are post translationally processed by host (indicated by diamonds) and viral proteases (arrows). Figure is adapted from the figure presented in Moradpour et al. (24) with permission from the publisher.

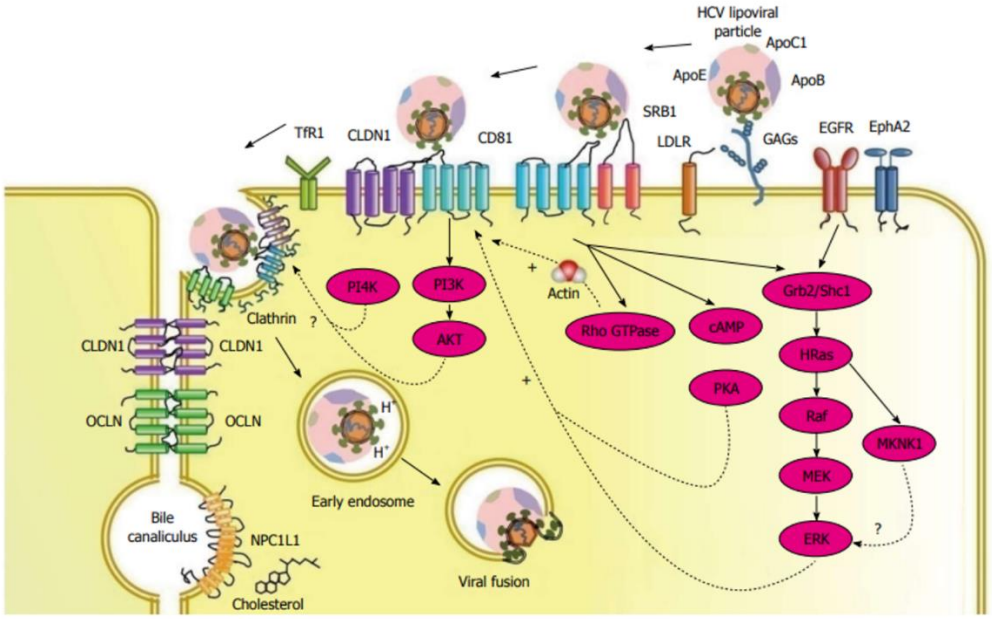

Figure 1.2. HCV entry. HCV LVP interact with GAGs and LDLR. HCV particles interact with SR-B1 through both lipoproteins and direct E2 interaction. Subsequently, HCV E2 protein binds CD81, which induces a signaling cascade that recruits actin to the cell surface and allows the HCV receptor complex to traffic to the tight junction. The HCV receptor complex interacts with CLDN1 and OCLN at the tight junction and enters the cell via clathrin-mediated endocytosis. Once inside the endosome, dropping pH induces fusion and release of the viral genome into the cytosol. Figure is adapted from Zhu et al. (31) with permission from the publisher.

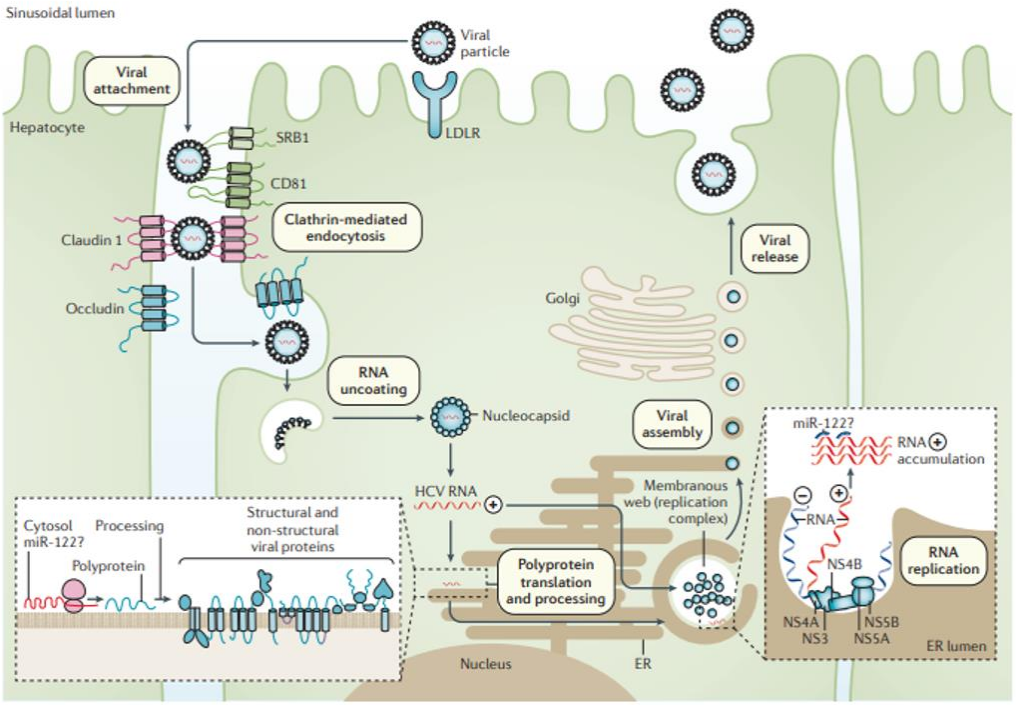

Figure 1.3. HCV life cycle. HCV enters the cell as described in Figure 1.2 and viral RNA is released into the cytosol. HCV RNA binds microRNA-122 and is translated into a polyprotein that is post-translationally processed into the structural and non-structural proteins. The structural and non-structural proteins localize to the ER membrane and the NS4B protein induces the formation of the membranous web replication complex. HCV RNA replication is facilitated by the NS5B RNA dependent RNA polymerase. Viral assembly occurs on the surface of lipid droplets through RNA binding with NS5A and core protein localized to these lipid droplets. Immature virions bud through the ER and are enveloped in host membrane imbedded with the E1 and E2 proteins. Virions are then trafficked through the golgi by the VLDL secretory pathway and released. Figure is adapted from Manns et al. (87) with permission from the publisher.

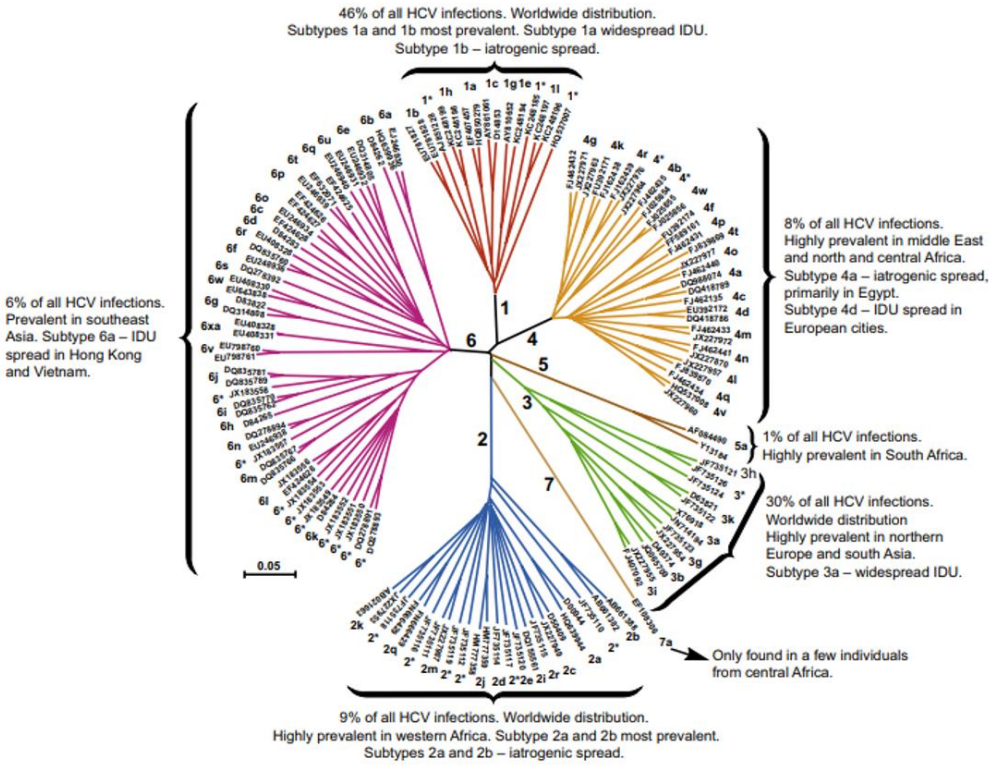

Figure 1.4. Phylogenetic classification of HCV. HCV contains 7 genotypes with 67 subtypes. Genotypes differ by up to 30% in nucleotide sequence and subtypes by up to 15% in nucleotide sequence. Worldwide prevalence is indicated as % of all HCV infections. Figure is adapted from Bukh (8) with permission from the publisher.

Chapter 2: Materials and Methods

2.1 Cell culture.

Huh7.5 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10%, heat-inactivated fetal bovine serum (FBS) (Sigma Aldrich), 0.1 millimolar (mM) nonessential amino acids (Invitrogen) 100 units/milliliter (ml) penicillin and 100 micrograms (µg)/ml streptomycin (Invitrogen) in an incubator supplemented with 5% carbon dioxide (CO_2) at 37 degrees Celsius (°C). Cells were propagated in T175 flasks (Greiner-Bio One) and passaged every three to four days when they reached ~80% confluency.

Cells were seeded into 96 well plates (Sarstedt) for use in the neutralization assays as follows. Plates were coated with poly-L-lysine (Trevigen) for 10 minutes at 37°C. Poly-L-lysine was removed and 100 microliters (µl) of Huh7.5 cells were added at a concentration of $1.0x10^5$ cells/ml (10 000 cells/well). Cells were placed in a 5% CO_2 incubator at 37°C overnight for use in neutralization assays the following day.

2.2 Antibodies and antiserum.

The HCV detection antibody mouse anti-NS5A (9E10) (provided by Charlie Rice and Tim Tellinghuisen) and envelope protein specific monoclonal antibodies H77.16 (Michael Diamond), AR1B, AR2A, AR3A, AR4A and AR5A (provided by Mansun Law) have been described previously (14, 154, 182, 183). H77.16, AR1B, AR2A, AR3A, AR4A and AR5A, as well as polyclonal Rabbit anti-SR-B1 (Abcam) and monoclonal mouse anti-CD81 antibody (BD Biosciences) were used to neutralize wild type (WT) and recombinant cell culture (cc) HCV.

Antiserum from a goat (G757) immunized with recombinant E1E2 derived from the genotype 1a HCV-1 strain was described previously (45). Sera was heat inactivated at 56°C for 30 minutes prior to use in neutralization assays.

2.3 HCV plasmids.

Genotype 2a JC1-NS5A-nluc (referred to as J6 WT in this study) is a chimeric virus composed of the J6 genome (nucleotides 341-3430, encoding core to NS2 proteins) and the JFH-1 genome (nucleotides 1 to 340 and 3431 to 9679, 3' and 5' untranslated regions and NS3 to NS5B proteins) (14). This virus contains a Nano luciferase (nluc) reporter gene within domain III of the NS5A protein and was provided by Michael Beard and described in (184). Genotype 2a JFH-1-NS5A-nluc (referred to as JFH-1 WT in this study) was generated by replacing the fragment between the *Avr*II and *Sna*BI restriction sites (nucleotides 3867 to 8450) in the cell culture adapted JFHrr virus (provided by Dr. Rodney Russell and described in (185)) with the same *Avr*II and *Sna*BI fragment from the JC1-NS5A-nluc virus that contains the nluc gene. Fragments were ligated together using T4 DNA ligase (New England Biosciences) as described below.

To generate E1 or E2 hybrid virus constructs, polymerase chain reaction (PCR) was used to generate fragments of the J6 or JFH-1 E1 and E2 proteins. Fragments were created with overlapping sequences comprising the restriction cut sites of *Cla*I (at nucleotides 709 and 3931) for J6-JFH-1 E1 and J6-JFH-1 E2 constructs and, *Fps*I and *Avr*II cut sites (at nucleotides 11177 and 3867) in JFH-1-J6 E2 construct using the primers outlined in Table 2.1. Fragments were ligated together using T4 DNA ligase (New England Biosciences) as described below.

JFH-1 E2 single amino acid variants and HVR1 deleted (Δ) constructs in J6 WT virus were created using the QuikChange II site-directed mutagenesis kit (Agilent) as described in the manufacturers protocol with primers outlined in Table 2.2. For constructs that required multiple mutations that did not fit in one primer, constructs were sequentially created for each mutation using the previously constructed mutant as the template for the PCR reaction and the procedure was repeated until the desired mutations were achieved.

A construct containing all the JFH-1 E2 amino acid variants in the J6 WT virus was created using infusion cloning methods described below with a synthetic gBlock gene fragment (Integrated DNA Technologies) for the J6 E2 that contained

all the JFH-1 variant amino acids. Infusion cloning was also used to generate the HVR1 hybrid viruses with a gBlock gene fragment (Integrated DNA Technologies) created for 1a H77 HVR1, 3a S52 HVR1 and JFH-1 HVR1 to clone into J6 WT virus or J6 HVR1 to clone into JFH-1 WT virus. Primers and gBlock gene fragment sequences can be seen in Table 2.3.

2.4 Molecular cloning techniques.

2.4.1 Ligation. Plasmids were digested with desired restriction enzymes described above for 1 hour at 37°C. Fragments were then extracted after agarose gel electrophoresis for the vector band (larger fragment) and insert band (smaller fragment) and purified from the gel using the NucleoSpin Gel and PCR Clean-up kit (Machry-Nagel). Ligation reactions were set up using T4 DNA ligase (New England Biosciences). Ligation products were electroporated into DH5α electrocompetent *Escherichia coli* (*E. coli)* cells using 0.1 centimeter (cm) gap cuvettes, and 1.8 kilovolts (KV) for 5 seconds. Transformed cells were plated on ampicillin (0.1 milligram (mg)/ml) supplemented Luria broth (LB) agar plates and grown overnight at 37°C. Single colonies were then selected and grown in ampicillin (0.1 mg/ml) supplemented LB media overnight at 37°C shaking at 200 revolutions per minute (rpm). Miniprep DNA was isolated using the NucleoSpin Plasmid kit (Machry-Nagel) following the manufacturers protocol. Isolated plasmids were verified by Sanger DNA sequencing (The Molecular Biology Service Unit – University of Alberta). Selected positive clones were then streaked on ampicillin (0.1 mg/ml) LB agar plates and grown overnight at 37°C. The following day a colony was selected and grown in 200 ml ampicillin (0.1 mg/ml) supplemented LB media overnight at 37°C shaking at 200 rpm, and DNA was isolated from 200 ml cultures using a Highspeed Maxiprep kit (Qiagen) following the manufactures protocol to create stock plasmids.

2.4.2 Infusion cloning. PCR conditions for the creation of the fragments for use in infusion cloning are as follows: 95°C for 5 minutes, 30 cycles of 95°C for 30

seconds then 50°C for 30 seconds then 72°C for 30 seconds, 72°C for 5 minutes and 4°C for 30 minutes. PCR fragments were gel purified using the NucleoSpin Gel and PCR Clean-up kit (Machry-Nagel). Infusion cloning was then performed according to the manufacturers protocol (Takara Bio). Briefly, desired PCR generated fragments or gBlock gene fragments were combined and incubated at 50°C for 15 minutes with 2 µl of enzyme mix. 2.5 µl of infusion reaction was then transformed into 30 µl Stellar chemically competent cells (Takara Bio) by heat shocking for 45 seconds at 42°C followed by 2 minutes on ice. Cells were allowed to recover for 30 minutes at 37°C before plating on LB amp plates. Stock plasmid DNA was grown and sequenced as described above.

2.4.3 Site-directed mutagenesis. Plasmids created by site-directed mutagenesis were generated using the following PCR conditions: 95°C for 1 minute, 18 cycles of 95°C for 50 seconds, then 60°C for 50 seconds, then 68°C for 13.5 minutes, 68°C for 7 minutes, and 4°C for 30 minutes. Following PCR amplification, constructs were treated with *Dpn*I restriction enzyme for 1 hour at 37°C and transformed into XL-10 Gold ultracompetent cells (Agilent) by heat shocking for 45 seconds at 42°C followed by 2 minutes on ice. Stock plasmid DNA was grown and sequenced as described above.

2.5 HCVcc generation.

HCV plasmids were linearized with either *mlu*I (for J6 based plasmids) or *Sbf* I (for JFH-1 based plasmids) and RNA was transcribed using the T7 RiboMAX RNA production system (Promega). The resulting RNA was purified using the RNeasy mini kit (Qiagen) and quantified with the Nano-drop 2000 spectrophotometer (Thermo Fisher Scientific). HCV RNA was electroporated into Huh7.5 cells as previously described (179). Briefly, 5 µg of RNA was electroporated into $4x10^6$ Huh7.5 cells using the ElectroSquare Porator ECM 830 (BTX) set to 860 Volts (V), 5 pulses, 99 micro seconds (µs), 1.1 second interval, high voltage setting and 2 mm gap cuvettes Cells were allowed to recover at room

temperature for 10 minutes and then plated on p150 dishes. Cells were incubated in a 5% CO_2 incubator at 37°C. Supernatant virus was collected and filtered with 0.22 micrometer (μm) filters at 72 and 120 hours post electroporation. Virus was then aliquoted and stored at -80°C. The tissue culture infectivity dose $(TCID)_{50}$/ml of the virus was calculated as described previously (14). Briefly, Huh7.5 cells were plated on 96 well plates and infected with 100 μl of 10-fold serial dilutions of virus overnight and then virus was replaced with fresh growth media. After 48 hours, cells were fixed in methanol (Fisher Scientific) and stained for HCV infected cells using 9E10 NS5A specific antibody. Positive wells were scored by the presence of at least 1 stained foci. $TCID_{50}$/ml was calculated using the Reed & Muench calculation method (186).

2.6 Neutralization assay.

The neutralization assay protocol was performed similarly to previously described methods with modifications noted below (179). Briefly, diluted sera or monoclonal antibody was added to HCVcc diluted to 500 $TCID_{50}$/ml and the mixture was incubated for 1 hour at 37°C. Virus/antibody mixture was then added to Huh7.5 cells plated on 96 well plates for 6 hours followed by replacement with fresh growth medium. At 48 hours post infection, cells were lysed in Nanoglo Luciferase assay buffer and substrate (Promega), instead of fixing cells in methanol. The luminescence was measured using the EnSpire 2300 multilabel reader (Perkin-Elmer). The percent neutralization was calculated by subtracting the treatment signal from the virus only wells signal and dividing by the total possible infection (virus only well) with the following formula: $\frac{(Pre-sera\ signal)-(post-sera\ signal)}{(pre-sera\ signal)} * 100$. Percent neutralization was plotted in Graphpad Prism 7 software. Half maximal inhibitor concentration (IC50) values were determined by finding the nonlinear regression of a variable slope using the software in Graphpad Prism 7 software.

2.7 Peptide binding ELISA.

Biotinylated peptides of amino acids 384 to 411 (the 27 amino acids of the HVR1) of genotype 1a H77, genotype 2a J6, genotype 2a JFH-1 and amino acids 412 to 443 (the amino acids directly downstream of HVR1) of J6 and JFH were synthesized by GL Biochem. Peptides were added to neutravidin coated 96 well plates at 0.5 µg/well for 1 hour. Plates were then blocked with 5% bovine serum albumin (BSA) for 1 hour. The pre-sera or post vaccination polyclonal E1/E2 antisera from goat 757 was added to wells in 3-fold serial dilutions starting with 1/50 and incubated for 1 hour. Horseradish peroxidase (HRP) conjugated anti-goat antibody (Santa Cruz Biotechnology) was used to detect goat antisera binding to peptides. Plates were washed with 0.1% Tween-20 (Fisher Scientific) in phosphate buffered saline (PBS) between each addition. Plates were developed using tetramethylbenzidine (TMB) substrate (Mandel Scientific), and the reaction was stopped after 4.5 minutes by adding 1 molar (M) phosphoric acid (Sigma Aldrich). The EnSpire 2300 multilabel reader (Perkin-Elmer) was used to record absorbance values for optical density (OD)450 minus background OD570. Background absorbance values for negative controls were subtracted from experimental wells and data was plotted using Graphpad Prism 7 software.

2.8 Entry receptor binding ELISA.

Plates were coated with biotinylated HVR1 peptides described above at 1 µg/well. Plates were blocked in 5% BSA for 1 hour. Then recombinant Fc tagged SR-B1 (Fc-SR-B1) protein (Abcam) was added to wells in a 2-fold serial dilution starting with 1 µg/ml concentration. An anti-Fc HRP conjugated antibody (Jackson Immuno Research) was used to detect binding of the Fc-SR-B1 protein to wells. Plates were washed with 0.1% Tween-20 (Fisher Scientific) in PBS between each addition. Plates were developed using TMB substrate (Mandel Scientific), and the reaction was stopped after 4.5 minutes by adding 1M phosphoric acid (Sigma Aldrich). The EnSpire 2300 multilabel reader (Perkin-Elmer) was used to record

absorbance values for OD450 minus background OD570. Data was plotted in Graphpad Prism 7 software. The ability of human Fc protein (Abcam) alone to bind to biotinylated peptides was tested following the same protocol described above.

2.9 Receptor competition assay.

The LEL of CD81 was provide by provided by Dr. Joe Marcotrigiano. It is purified from *E. coli* as a GST-tagged fusion protein. The tag is subsequently removed by digestion with PreScission protease as previously described in (187). The protein was pre-incubated with 500 $TCID_{50}$/ml HCVcc in 5-fold serial dilutions starting with 10 μg/ml. The protein/virus mixture was then added to Huh7.5 cells for 6 hours before changing to fresh growth medium. CD81-LEL loop inhibition of virus infection was measured 48 hours post-infection by measuring luminescence and calculating percent inhibition as described above for the neutralization assay protocol.

2.10 Statistical Analysis.

Statistical analysis was one-way analysis of variance (ANOVA) or two-way ANOVA where appropriate. ANOVA was followed with a Dunnett's multiple comparison test to compare significance between groups. All analysis was performed using Graphpad Prism 7 software. P-values less than (<) 0.05 were considered statistically significant.

2.11 Figures and tables.

Table 2.1. Primers used for the creation of E1/E2 chimeric of J6 and JFH-1 virus.

Construct	Fragment	Orientation	Sequence (5' – 3')
J6-JFH-1 E1	J6 core	Forward (JJ9)	GCAACGTGGGTAAGGTCATCG
		Reverse (JJ5)	CTTCACCTGGGCAGCGGAGACCGGGGTGG
	JFH-1 E1	Forward (JJ6)	CCGGTCTCCGCTGCCCAGGTGAAGAATACC
		Reverse (JJ8)	CAGTATGGGTGCGCGCGTCCACCCCAGCGG
	J6 E2	Forward (JJ7)	GGGGTGGACGCGCGCACCCATACTGTTGGGG
		Reverse (JJ10)	GTCTCAACGGGGATGAAATCG
J6-JFH-1 E2	J6 Core / E1	Forward (JJ9)	GCAACGTGGGTAAGGTCATCG
		Reverse (JJ1)	GTGGTGGTGCCCGCGTCCACCCCGGCGGCC
	JFH-1 E2	Forward (JJ2)	GGGGTGGACGCGGGCACCACCACCGTTGG
		Reverse (JJ3)	CTCTAGTGCTGCTTCGGCCTGGCCC
	J6 Downstream	Forward (JJ4)	GGCCAGGCCGAAGCAGCACTAGAGAAGCTGG
		Reverse (JJ10)	GTCTCAACGGGGATGAAATCG
JFH-1-J6 E1	JFH-1 core	Forward (JJ36)	CGCCAGTTAATAGTTTGCGCAACG
		Reverse (JJ20)	CTTCACTTCGGCAGCAGAGACCGGAACGG
	J6 E1	Forward (JJ18)	CCGGTCTCTGCTGCCGAAGTGAAGAACATCAGTACC
		Reverse (JJ21)	GGTGGTGGTGCCCGCGTCCACCCCGGCGG
	JFH-1 E2	Forward (JJ19)	GGGGTGGACGCGGGCACCACCACCGTTGG
		Reverse (JJ27)	GCCCAACGACGTGGCCCCTAGG
JFH-1-J6 E2	JFH-1 core/E1	Forward (JJ36)	CGCCAGTTAATAGTTTGCGCAACG
		Reverse (JJ24)	CAGTATGGGTGCGCGCGTCCACCCCAGCG
	J6 E2	Forward (JJ22)	GGGGTGGACGCGCGCACCCATACTGTTGG
		Reverse (JJ25)	CTTCTCCAATGCTGCTTCGGCCTGGCCC
	JFH-1 Downstream	Forward (JJ23)	CAGGCCGAAGCAGCATTGGAGAAGTTGG
		Reverse (JJ27)	GCCCAACGACGTGGCCCCTAGG

Red text indicates sequence homologous to J6 and blue text indicates sequence homologous to JFH-1.

Table 2.2. Primers used for the creation of JFH-1 variant amino acid mutations in J6 virus.

Construct	Orientation	Sequence (5′ – 3′)
J6-M405H/K410N	Forward (JJ64)	GCTTATTTGACCACGGCCCCAGGCAGAACATCCAGCTCGTTAACACC
	Reverse (JJ65)	GGTGTTAACGAGCTGGATGTTCTGCCTGGGGCCGTGGTCAAATAAGC
J6-V414I	Forward (JJ90)	GCAGAAAATCCAGCTCATTAACACCAATGGCAGC
	Reverse (JJ91)	GCTGCCATTGGTGTTAATGAGCTGGATTTTCTGC
J6-I438L/S446R	Forward (JJ48)	GCACACCGGCTTTCTCGCGTCTCTGTTCTACACCCACCGCTTCAACTCG
	Reverse (JJ49)	CGAGTTGAAGCGGTGGGTGTAGAACAGAGACGCGAGAAAGCCGGTGTGC
J6-M456L	Forward (JJ66)	CGTCAGGATGTCCCGAACGCCTGTCCGCCTGCC
	Reverse (JJ67)	GGCAGGCGGACAGGCGTTCGGGACATCCTGACG
J6-Q493P	Forward (JJ68)	GCACTACCCACCAAGGCCGTGTGGCGTGGTCTCCG
	Reverse (JJ69)	CGGAGACCACGCCACACGGCCTTGGTGGGTAGTGC
J6-T594A	Forward (JJ70)	GGAAGCATCCTGATGCCACTTACCTCAAATGC
	Reverse (JJ71)	GCATTTGAGGTAAGTGGCATCAGGATGCTTCC
J6-S498P/T501S	Forward (JJ52)	CCAAGGCAGTGTGGCGTGGTCCCGCGAAGAGTGTGTGTGGCCC
	Reverse (JJ53)	GGGCCACACACACTCTTCGCGGGGACCACGCCACACTGCCTTGG
J6-S558T/Y560F	Forward (JJ54)	GGCTGCACGTGGATGAACTCTACTGGCTTCACCAAGACTTGCGG
	Reverse (JJ55)	CCGCAAGTCTTGGTGAAGCCAGTAGAGTTCATCCACGTGCAGCC
J6-I611V	Forward (JJ74)	CGCCAAGGTGCCTGGTCGACTACCCCTACAGGCTCTGG
	Reverse (JJ75)	CCAGAGCCTGTAGGGGTAGTCGACCAGGCACCTTGGCG
J6-N653D	Forward (JJ76)	CGTGGGGATCGTTGCGACTTGGAGGACAGAGACAG
	Reverse (JJ77)	CTGTCTCTGTCCTCCAAGTCGCAACGATCCCCACG
J6-F700Y/L708I	Forward (JJ78)	CGTACAATACATGTATGGCCTATCACCTGCCATCACAAAATACATCG
	Reverse (JJ79)	CGATGTATTTTGTGATGGCAGGTGATAGGCCATACATGTATTGTACG
J6-I719V	Forward (JJ80)	GGGAGTGGGTAGTACTCTTATTCCTGC
	Reverse (JJ81)	GCAGGAATAAGAGTACTACCCACTCCC

Red text indicates sequence homologous to J6 and blue text indicates sequence change to create the JFH-1 amino acid substitution. Constructs are color coded according to their location in Figure 3.4. Colored constructs primers were sequentially used to construct mutant as indicated in Figure 3.5.

Table 2.3. Primers and gBlock gene fragments used for the creation of J6 HVR1 modified constructs.

Construct		Orientation	Sequence (5′ – 3′)
J6-ΔHVR1		Forward (JJ16)	GGGTGGACGCGATCCAGCTCGTTAACACCAATGG
		Reverse (JJ17)	CGAGCTGGATCGCGTCCACCCCGGCGGCCAACAGAAGG
J6-JFH-1 HVR1		gBlock	CCTTCTGTTGGCCGCCGGGGTGGACGCGGGCACCACCACCGTTGGAGG CGCTGTTGCACGTTCCACCAACGTGATTGCCGGCGTGTTCAGCCATGGC CCTCAGCAGAACATCCAGCTCGTTAACACCAATGGCAGCT
J6-1a HVR1		gBlock	CCTTCTGTTGGCCGCCGGGGTGGACGCGGAAACCCACGTCACCGGGGG AAATGCCGGCCGCACCACGGCTGGGCTTGTTGGTCTCCTTACACCAGGC GCCAAGCAGAACATCCAGCTCGTTAACACCAATGGCAGCT
J6-3a HVR1		gBlock	CCTTCTGTTGGCCGCCGGGGTGGACGCGGAAACATATGTCACCGGTGG CAGTGTAGCTCATAGTGCCAGAGGGTTAACTAGCCTTTTTAGTATGGGCG CCAAGCAGAAAATCCAGCTCGTTAACACCAATGGCAGCT
JFH-1-J6 HVR1		gBlock	CCTTCTGCTGGCCGCTGGGGTGGACGCGCGCACCCATACTGTTGGGGG TTCTGCCGCGCAGACCACCGGGCGCCTCACCAGCTTATTTGACATGGGC CCCAGGCAGAAAATTCAGCTCATTAACACCAACGGCAGTT
J6 E1 or E2 fragments for HVR1 swaps	J6 E1	Forward (JJ9)	GCAACGTGGGTAAGGTCATCG
		Reverse (JJ51)	CCCCGGCGGCCAACAGAAGGATGACAACG
	J6 E2	Forward (JJ50)	CGTTAACACCAATGGCAGCTGGCACATCAACC
		Reverse (JJ10)	GTCTCAACGGGGATGAAATCG
JFH-1 E1 or E2 fragments for HVR1 swap	JFH-1 E1	Forward (JJ99)	CCTCACGCAGGGTCTGCGGACGCACATCG
		Reverse (JJ82)	CCCAGCGGCCAGCAGAAGGATGACAATGACC
	JFH-1 E2	Forward (JJ73)	CATTAACACCAGCGGCAGTTGGCACATCAACC
		Reverse (JJ100)	CGAGTGTCTCAACGGGGATGAAATCG

Red text indicates sequence homologous to J6, blue text indicates sequence homologous to JFH-1, purple text indicates sequence homologous to H77 (1a) and orange text indicates sequence homologous to S52 (3a).

Chapter 3: Results

3.1 Results

3.1.1 A major determinant in neutralization sensitivity between J6 and JFH-1 isolates is located in the E2 protein. The 1a E1/E2 glycoprotein vaccine raised antibodies in goats that specifically neutralize HCV infection (45). The goat antisera was also shown to compete for binding to the E1 and E2 proteins with known broadly neutralizing antibodies that target specific epitopes within the E1 and E2 proteins. Previously, our lab has observed a difference in neutralization sensitivity between two closely related HCV genotype 2a virus strains, the J6 and JFH-1. Since the vaccine antigen elicited antibodies targeting the E1 and E2 proteins, it was likely that the difference in neutralization sensitivity between J6 and JFH-1 was located in the E1 and/or E2 proteins. To investigate if the main determinants of neutralization were located within the E1 and/or E2 protein, the E1 or E2 proteins were exchanged between the relatively resistant J6 virus and the relatively sensitive JFH-1 virus (the recombinant virus constructs are shown in Figure 3.1). These recombinant HCVcc viruses were then tested for their neutralization sensitivity to the 1a E1/E2 vaccine antisera and their sensitivity were compared to the WT J6 and JFH-1 viruses (Figure 3.2). Similar to previous data from our lab, the J6 WT virus showed a resistant phenotype where only about 10% of the virus infectivity was neutralized, while the JFH-1 virus showed a sensitive phenotype with about 64% of the virus infectivity neutralized. J6 virus with the E1 protein from JFH-1 (J6-JFH-1 E1) did not show a significant increase in neutralization sensitivity over the resistant J6 WT virus (Figure 3.2) or the JFH-1 WT virus (Supplementary Figure 1). However, the J6 virus with the E2 protein from JFH-1 (J6-JFH-1 E2) did show a significant increase in neutralization sensitivity compared to the J6 WT virus (Figure 3.2). Conversely, replacement of the JFH-1 E2 with the E2 protein from J6 (JFH-1-J6 E2) reduced neutralization sensitivity to the level of the J6 WT virus (Figure 3.2). Replacement of the JFH-1 E1 with the E1 protein from J6 (JFH-1-J6 E1) construct was not viable in cell

culture. The E2 protein from JFH-1 was able to confer sensitivity to the J6 virus while the E2 protein from the J6 virus was able to confer resistance to the JFH-1 virus. These results indicate that the E2 protein is a major determinant of the isolate-specific neutralization sensitivity between the J6 and JFH-1 HCV isolates.

3.1.2 Variant amino acids throughout the E2 are not responsible for the differential neutralization between J6 and JFH-1. A potential explanation for the influence of the E2 protein on the isolate-specific neutralization sensitivity could be due to variant amino acids between J6 and JFH-1 throughout the E2 protein. Previous research by others has demonstrated that point mutations in the E2 protein can result in resistance to broadly neutralizing monoclonal antibodies and patient sera (114, 155, 188–191). Since single amino acid mutations can have such a dramatic effect on the neutralization by monoclonal antibodies, it was possible that amino acid differences between J6 and JFH-1 could be responsible for the observed difference in neutralization sensitivity. It was hypothesized then, that substituting variant amino acids from the JFH-1 E2 into the J6 virus would result in sensitivity of the J6 WT virus to polyclonal 1a E1/E2 vaccine antisera.

Variant amino acids between J6 and JFH-1 were selected by a comparison of the E2 amino acid sequences. There are 49 variant amino acids between J6 and JFH-1, however I wanted to narrow down the number of amino acids that are potentially important for neutralization sensitivity. Therefore, I compared the sequence of the J6 and JFH-1 isolates to that of the known sensitive 1a H77 strain that is homologous to the vaccine antigen (Figure 3.3) (45). I hypothesized that some of the amino acids within the E2 protein that are divergent between the resistant J6 strain and the sensitive strains, H77 and JFH-1, would affect neutralization sensitivity. Amino acids that were identical between the H77 and JFH-1 strains but variant in the J6 virus were selected for further analysis. Three additional amino acids positions were selected, two from the HVR1 region and one from domain 1 (the 32 amino acids directly downstream of HVR1 as described in Douam et al. (35)), at the suggestion of Dr. Holly Freedman due to their influence on the E2 structure based on their importance in the E2 computational model (39,

40). Amino acids at positions 405 and 410 were suggested to influence the structure of the C-terminal portion of HVR1 and amino acid position 446 was exposed on the surface within the CD81 binding site. Using this method 17 variant amino acid residues were identified for further study (Table 3.1). Then, in collaboration with Dr. Freedman, these variant residues were grouped according to their location in the known core structure of the E2 protein (Figure 3.5) (34, 39, 40). Groups of the variant amino acid that were close in proximity in the E2 protein structure from the JFH-1 virus were then introduced together into the J6 virus using site directed mutagenesis. In addition, a construct was also created where all 17 variant amino acids from the JFH-1 virus were introduced together into the J6 virus. All constructs were tested for their neutralization sensitivity to the 1a E1/E2 vaccine antisera. A comparison of their sensitivities relative to J6 and JFH-1 WT parental viruses at a 1/100 dilution of antisera is presented in Figure 3.6. None of the groups of mutations showed any significant change in neutralization sensitivity from the J6 WT virus. Two of the mutation groups (J6-M456L/Q493P/T594A and J6-I611V) were not viable in cell culture (yellow and grey). While the replacement of all 17 variant residues together was viable in cell culture, it similarly failed to show any significant change in neutralization sensitivity from the J6 WT virus (pale blue). Interestingly, J6-M405H/K410N virus (dark blue), while not significant, showed the greatest increase in neutralization sensitivity over J6 WT virus. Since no significant increase in neutralization sensitivity from that of the J6 WT virus was observed for any of the variant amino acid substitutions, my data indicates the variant amino acids investigated between J6 and JFH-1 are likely not responsible for the difference in neutralization sensitivity to 1a E1/E2 antisera.

3.1.3 HVR1 is a major determinant of differential neutralization sensitivity between J6 and JFH-1. Another aspect of the E2 protein that has been shown to be involved in sensitivity to broadly neutralizing monoclonal antibodies is the HVR1 region (amino acids 384-410). Previous studies have shown that deleting HVR1 results in an increased sensitivity to monoclonal antibodies as well as a decrease in the genotype variation to neutralization by monoclonal antibodies (50,

54, 56). Therefore, the role of the HVR1 in the isolate-specific neutralization sensitivity of the J6 and JFH-1 viruses was investigated. If HVR1 was responsible for the difference in neutralization sensitivity between the J6 and JFH-1 virus, then chimeric constructs containing the HVR1 sequence from the other isolate would have altered neutralization sensitivity to 1a E1/E2 antisera. Chimeric constructs were created within the J6 or JFH-1 virus background that had the HVR1 replaced with the equivalent HVR1 sequence from JFH-1 or J6, respectively. Additionally, I created a J6 construct that had the HVR1 sequence deleted to allow direct comparison of my data to that of the literature. The chimeric and deleted HVR1 constructs visualized in Figure 3.6. These chimeric viruses were then tested for their neutralization sensitivity to 1a E1/E2 antisera. First, the effect of replacement of the HVR1 in J6 for the JFH-1 HVR1 was investigated. Recombinant J6-JFH-1 HVR1 virus showed a significant increased sensitivity to the 1a E1/E2 antisera as compared to J6 WT virus (Figure 3.7 A green line compared to red line). Deletion of the HVR1 from the resistant J6 virus resulted in a hypersensitive phenotype to our 1a E1/E2 antisera compared to J6 WT virus (Figure 3.7 A dark blue line). Interestingly, the J6-JFH-1 HVR1 recombinant virus is still about 10 times more resistant to neutralization than the J6-ΔHVR1 virus from a comparison of IC50 values (1/745.2 for J6-JFH-1 HVR1 compared to 1/7088 for J6-ΔHVR1). Conversely, replacement of JFH-1 HVR1 with the J6 HVR1 within the JFH-1 virus backbone showed a statistically significant (p-value of 0.0042) reduction in the neutralization sensitivity at the highest dilution (1/100) for the chimeric virus compared to JFH-1 WT virus (Figure 3.7 B brown line). A comparison of the statistical difference in neutralization sensitivity from the J6 WT virus for all chimeric viruses at each dilution is presented in Supplementary Figure 2. Since HVR1 alters the sensitivity of WT J6 and JFH-1 virus to 1a E1/E2 antisera, these data strongly indicate that HVR1 is a major determinant of isolate-specific neutralization sensitivity. Additionally, deletion of HVR1 rendering the virus hypersensitive to neutralization by 1a E1/E2 antisera, is consistent with previously published data showing that deletion of HVR1 renders the virus hypersensitive to neutralization by monoclonal antibodies and reduces genotype-specific differences

in neutralization sensitivity (56). Again, this is indicating that HVR1 is influencing the neutralization sensitivity to 1a E1/E2 antisera.

Since my data has indicated that HVR1 is influencing isolate-specific differences in neutralization sensitivity and previously it was shown that HVR1 influences genotype-specific differences in neutralization sensitivity (56), I investigated if my findings regarding the influence of HVR1 on the 2a isolate-specific neutralization sensitivity to 1a E1/E2 antisera could be extended to differential neutralization of other more variant genotypes (45, 179). If HVR1 is also influencing genotype-specific neutralization sensitivity to 1a E1/E2 antisera, then J6 chimeric virus with HVR1 from different genotypes should show neutralization sensitivity similar to that of the genotype of origin of the HVR1 sequence. I investigated if chimeric viruses containing HVR1 from the known sensitive 1a H77 strain and the known resistant 3a S52 strain that were introduced into the J6 virus showed alteration in neutralization sensitivity to 1a E1/E2 antisera compared to the J6 WT virus. The J6-3a HVR1 virus remained resistant to neutralization (Figure 3.7 C orange line), but surprisingly the J6-1a HVR1 virus also showed resistance to neutralization (Figure 3.7 C purple line). Since the resistance of the J6-1a HVR1 chimeric virus was surprising, I further characterized the incorporation of the 1a HVR1 into the J6-1a HVR1 construct to confirm that 1a H77 HVR1 could still be recognized within the context of the J6 WT virus. I found that it could still be neutralized with a 1a H77 HVR1-specific monoclonal antibody, H77.16 (described in Sabo et al. (182)), confirming that the HVR1 in this recombinant virus was correctly incorporated and able to be recognized by specific antibodies (Figure 3.8).

Overall, since the HVR1 sequence is sufficient to influence isolate-specific neutralizations sensitivity between J6 and JFH-1 virus, HVR1 is likely a major determinant in neutralization sensitivity to 1a E1/E2 antisera, however the surprising resistance of the J6-1a HVR1 virus indicates that HVR1's influence on neutralization sensitivity might also be influenced by interaction of the E1 and/or E2 proteins with the isolate-specific HVR1.

3.1.4 HVR1 of heterologous genotypes is not a direct target of polyclonal antibodies elicited by the 1a E1/E2 vaccine. One potential explanation for HVR1's influence on the isolate-specific neutralization sensitivity of the J6 and JFH-1 viruses could be antibodies in the antisera directly target the HVR1. It is known that antibodies can be elicited by the HVR1 and that these antibodies are capable of neutralizing HCV infection (46, 158). Since antibodies can be raised to the HVR1 sequence, it was possible that antibodies within the 1a E1/E2 antisera target the HVR1 of JFH-1 but not J6. I therefore wanted to test if antibodies in the antisera were able to recognize the HVR1 sequence of JFH-1 but not J6. I predicted that if HVR1 specific-antibodies in the 1a E1/E2 antisera neutralize JFH-1 virus but not J6, then antisera should show binding to an HVR1 peptide of JFH-1 but not to that of J6. To test this, I performed a peptide enzyme-linked immunosorbent assay (ELISA) that measured the binding of 1a E1/E2 antisera to peptides of the HVR1 of H77, J6 and JFH-1 as well as peptides of the 31 amino acids immediately downstream of HVR1 for J6 and JFH-1. The region immediately downstream of HVR1 is known to contain many neutralizing epitopes for broadly neutralizing monoclonal antibodies (47) and our antisera is known to contain antibodies that target these epitopes (45).

Binding of antisera to homologous 1a H77 HVR1 peptide was clearly detected, but none or very little binding was observed to JFH-1 or J6 HVR1 peptides, respectively (Figure 3.9). Antisera was able to bind to the amino acid region immediately downstream of HVR1 in both the J6 and JFH-1 strains. Statistical difference in binding was calculated for all peptides at each dilution compared to the scramble control peptide (Supplementary Figure 3). These data indicate that vaccine-induced antibodies are not directly targeting JFH-1 HVR1 but are able to recognize conserved epitopes downstream of HVR1. The observed differential neutralization between the J6 and JFH-1 strains is therefore unlikely to be a consequence of cross-reacting antibodies directly targeting HVR1 of JFH-1 but not J6.

3.1.5 HVR1 influences monoclonal antibody binding in a genotype-specific manner. Since HVR1 is not directly targeting the HVR1 of JFH-1 to cause the observed differential neutralization sensitivity, it is possible that HVR1 is able to indirectly influence neutralization sensitivity. Previously, it was shown that HVR1 is influencing genotype-specific neutralization sensitivity to monoclonal antibodies that target diverse epitopes throughout the E1 and E2 proteins (56). Additionally, as the 1a E1/E2 antisera was shown (45) to contain antibodies that target similar epitopes to the antibodies used in the Prentoe et al. (56) study, it was of interest to determine if HVR1 is influencing the isolate-specific exposure of certain neutralizing epitopes or if HVR1 is having a more global influence on the exposure of antibody epitopes. If HVR1 is globally influencing the exposure of neutralizing epitopes, then J6 virus lacking HVR1 or containing the HVR1 from the sensitive virus should show greater sensitivity to many different neutralizing monoclonal antibodies that target epitopes throughout E1 and E2. To characterize the exposure of neutralizing epitopes of the J6 E2 protein without HVR1 (J6-ΔHVR1) or with HVR1 substitutions from the other isolate/genotypes (J6-JFH-1 HVR1, J6-1a HVR1, and J6-3a HVR1), their sensitivities to neutralization by a panel of monoclonal antibodies were tested. A panel of monoclonal antibodies was selected that contained antibodies that bind to a diverse range of epitopes throughout the E2 protein (AR1B, AR2A and AR3A), as well as conformational epitopes requiring amino acid residues within both E1 and E2 (AR4A and AR5A) (154, 183). AR1B is a non-neutralizing antibody that targets the non-neutralizing face of the E2 protein and was included as a control as it was not expected to neutralize either J6 or JFH-1 virus (40, 154). AR2A is an 1a isolate-specific neutralizing antibody that targets the glycan face of the E2 protein. AR3A is a broadly neutralizing antibody that targets a region that overlaps with CD81 binding within the E2 protein. AR4A and AR5A are also broadly neutralizing antibodies but target discontinuous epitopes requiring both the E1 and E2 proteins (183). J6 WT and chimeric viruses were tested for neutralization by AR1B, AR2A, AR3A, AR4A and AR5A monoclonal antibodies and IC50 values were calculated to determine if HVR1 was increasing antibody recognition of any of these epitopes in an isolate-specific

manner or if HVR1 was globally influencing the exposure of all these antibody binding epitopes. For AR1B no neutralization was observed for any of the viruses tested, as expected, since this antibody is known to be non-neutralizing against HCVcc (154, 183) (Figure 3.10 A). For AR2A the J6-ΔHVR1 virus was sensitive to neutralization and the J6-JFH-1 HVR1 virus showed some sensitivity at the highest dilutions (Figure 3.10 B) indicating that AR2A, despite being a 1a isolate-specific antibody can recognize J6 virus when it contains JFH-1 HVR1 or has no HVR1. For AR3A, AR4A and AR5A a shared pattern of neutralization sensitivity was observed (Figure 3.10 C, D and E). J6-ΔHVR1 virus was hypersensitive to all three antibodies. While J6-JFH-1 HVR1 virus was sensitive to neutralization by all three antibodies, it was about 10 times more resistant to neutralization than J6-ΔHVR1 by comparison of IC50 values. J6-1a HVR1 showed sensitivity to all three antibodies at higher concentrations. J6 WT virus showed sensitivity to AR3A and AR4A at the highest concentration. J6-3a HVR1 remained resistant to all monoclonal antibodies tested indicating that HVR1 is influencing the sensitivity to AR3A, AR4A and AR5A of chimeric HCVcc in an isolate-specific manner that depends on the isolate of origin of the HVR1 sequence. IC50 values were calculated and are shown in Table 3.2. Statistical comparison of all viruses to J6 WT at each dilution for each antibody are presented in Supplementary Figure 4. Both J6-ΔHVR1 and J6-JFH-1 HVR1 virus showed a strongly significant increase in neutralization sensitivity over WT virus for AR2A, AR3A, AR4A and AR5A. For the J6-1a HVR1 and J6-3a HVR1 viruses no significant difference was observed from J6 WT for any antibody except AR4A. For AR4A J6-1a HVR1 showed a significant increase in sensitivity for the 10 μg/ml dilution and the J6-3a HVR1 virus showed a significant decreased neutralization sensitivity at the 50 μg/ml dilution only. Together, these results indicate that HVR1 has a broad effect on various neutralizing epitopes and this effect is dependent on the genotype of the HVR1 present.

3.1.6 The influence of HVR1 on binding to entry receptors CD81 and SR-B1.
Previously, it's been shown that in addition to increased sensitivity to broadly

neutralizing antibodies, virus lacking the HVR1 binds to CD81 better than WT virus, indicating that HVR1 is important for protecting the CD81 binding site from neutralizing antibodies (192). Since the HVR1 from J6 virus containing the HVR1 from the JFH-1 virus is sensitive to neutralization by both 1a E1/E2 antisera and a monoclonal antibody known to interfere with CD81 binding (AR3A) (154), it is possible that the JFH-1 HVR1 is not able to protect the CD81 binding site from neutralizing antibodies. Therefore, the effect of HVR1 on the ability of virus to bind with the CD81 was investigated. If HVR1 was modulating an increased binding to the CD81 receptor then preincubation with CD81-LEL should result in greater inhibition of infection for HVR1 deleted J6 virus and J6-JFH-1 HVR1 virus compared to J6 WT virus. An experiment was designed that utilized the portion of CD81 known to bind with the E2 protein, CD81-LEL, in a similar manner to the neutralization assay described in the materials and methods. J6 WT, deleted HVR1 and J6-JFH-1 HVR1 viruses were pre-incubated with CD81-LEL before infecting cells. Percent inhibition was calculated. All three viruses were inhibited by preincubation with CD81-LEL at higher concentrations, with the J6-JFH-1 HVR1 virus showing a slight resistance to inhibition with an IC50 value of 4.91 compared to an IC50 of 1.15 for J6 WT virus, however, none of the viruses tested significantly differed from each other in their response at any of the dilutions (Figure 3.11 A). This indicates that HVR1 was not modulating an increased exposure of the CD81 binding site within the J6 virus.

Despite increased binding of deleted HVR1 virus seen in Bankwitz et al. (192), the authors did not see a difference in WT versus HVR1 deleted virus in their requirement for CD81 to enter cells. This was shown by blocking the CD81 receptor with a monoclonal antibody that targets CD81. I therefore wanted to investigate if HVR1 was influencing the requirement of CD81 for entry for J6 and JFH-1 virus differentially. If HVR1 was influencing the requirement of CD81 for virus entry in an isolate-specific manner, then HVR1 chimeric virus would display an altered inhibition by anti-CD81 compared to the WT virus. The inhibition of virus entry by an anti-CD81 antibody was tested for WT and recombinant HVR1 viruses (Figure 3.11 B). The inhibition of virus entry by anti-CD81 was not

significantly different between J6 WT and JFH-1 WT or between J6 WT and either J6-JFH-1 HVR1 or J6-ΔHVR1. Surprisingly, JFH-1-J6 HVR1 virus was significantly less inhibited by anti-CD81 compared to J6 WT, although this virus was not significantly different from the JFH-1 WT virus. Overall, since there were no significant differences between WT and HVR1 chimeric viruses in their inhibition by both CD81-LEL and anti-CD81, these data suggest that HVR1 is not playing a major role in interaction with CD81.

HVR1 has been identified to directly influence HCV/SR-B1 interaction and HDL mediated infection enhancement (48–50). Additionally, these studies have shown that virus lacking the HVR1 in addition to increased sensitivity to neutralization also show decreased dependence on the SR-B1 for entry. Therefore, I investigated if HVR1 was influencing isolate-specific interaction with SR-B1 for the J6 and JFH-1 virus. Given HCV's complex interactions with SR-B1 in cell culture involving both the E2 protein and HDL (48), I first wanted to examine the interactions of HVR1 with SR-B1 directly without the influence of HDL. I designed a peptide binding ELISA that utilized biotinylated peptides of the HVR1 sequences from the 1a H77, 2a J6 and 2a JFH-1 isolates and a commercially available Fc tagged SR-B1 protein. The binding of SR-B1 protein to HVR1 from different isolates / genotypes therefore was then assessed. To confirm that the binding of the Fc-SR-B1 protein to HVR1 peptides was due to the SR-B1 protein and not the Fc tag, an Fc protein control was tested in a similar manner. Interestingly, SR-B1 protein specifically bound to the HVR1 of J6 and showed no binding to either H77 or JFH-1 HVR1 (Figure 3.12 A). The binding of Fc protein alone was negligible (Figure 3.12 B). Together, this indicates that SR-B1 is specifically interacting with J6 HVR1 but not H77 or JFH-1 HVR1.

Previously, it has been shown that despite the complicated nature of the HCV/SR-B1 interaction, antibodies against SR-B1 are able to block HCV infection (50, 182). Since I have shown that HVR1 is able to interact directly with SR-B1 in an isolate-specific manner, it is possible that HVR1 would be able to influence resistance to antibodies against SR-B1 in an isolate-specific manner as well. If HVR1 influences isolate-specific interactions of the J6 virus with SR-B1 in cell

culture, then an antibody blocking SR-B1/HCV interactions should inhibit entry of J6 WT virus more than that of J6-JFH-1 HVR1 or deleted HVR1 virus. The inhibition of entry of J6 WT and modified HVR1 viruses by a commercially available polyclonal SR-B1 antibody. J6 WT, J6-ΔHVR1 and J6-JFH-1 HVR1 virus all showed resistance to inhibition by anti-SR-B1 and no statistically significant difference in neutralization sensitivity at any of the dilutions (Figure 3.13). This polyclonal anti-SR-B1 antibody was not able to block isolate-specific interactions of HCV with SR-B1 in cell culture.

3.2 Figures and tables

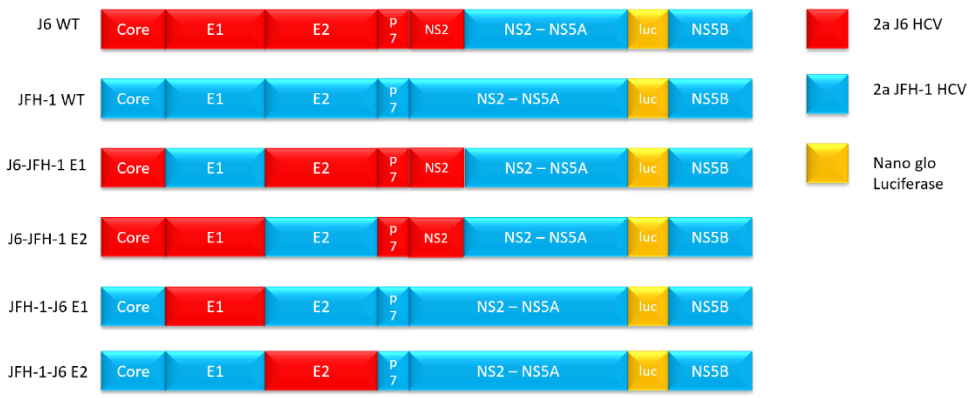

Figure 3.1. Schematic diagram of the recombinant E1 and E2 HCVcc virus genomes. All constructs contain the nonstructural proteins from the C-terminal portion of NS2 protein to the NS5B from the JFH-1 virus (as described in Wakita et al. (12) and Gottwein et al. (13)). Structural proteins from the core to NS2 protein are from either J6 (red), JFH-1 (blue) or a combination of as indicated. A nano-luciferase reporter gene was inserted within the domain III of NS5A for monitoring virus replication (yellow) (184).

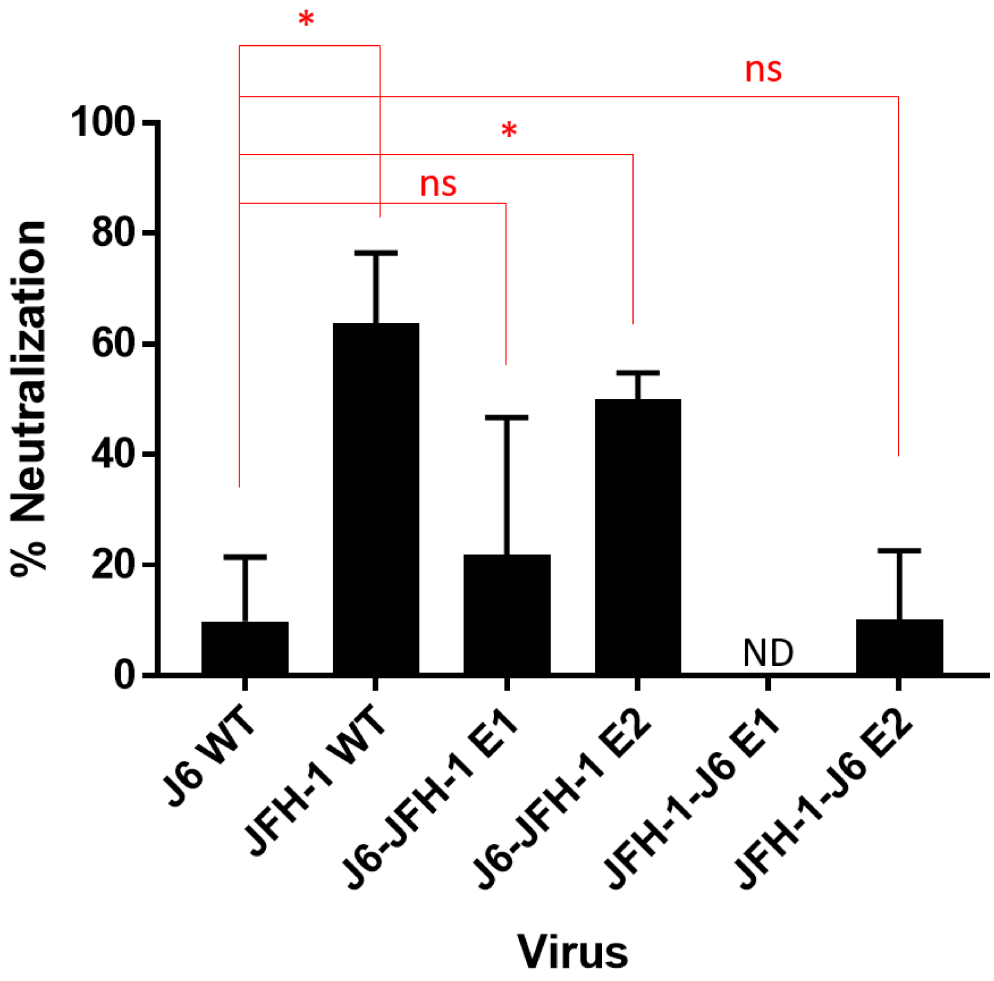

Figure 3.2. E2 determines the neutralization sensitivity of recombinant HCVcc. The ability of vaccine-induced antisera (1a E1/E2) to neutralize J6 WT, JFH-1 WT and recombinant forms of the J6 and JFH-1 viruses was tested. All HCVcc stocks were diluted to a titer of 500 $TCID_{50}$/ml and preincubated with heat inactivated antisera at a dilution of 1/100 and the mixture was then added to Huh 7.5 cells. The luminescence signal was read 48 hours after infection. The percent neutralization was normalized against the neutralization activity of pre-immunization sera. The error bars represent standard deviation of two independent experiments each performed with triplicate wells. Statistical significance was calculated using a one-way ANOVA followed by a Dunnett's multiple comparisons test to compare the means of each column to J6 WT using Graphpad Prism 7 software and significant differences from J6 WT is indicated above bars, while statistical differences from JFH-1 WT are indicated in Supplementary Figure 1. P- value < 0.05 is indicated by * and "ns" indicates no significance. ND indicates no data was obtained.

Table 3.1. Point mutations that are identical between the sensitive H77 and JHF-1 strains but variant in the resistant J6 strain.

Amino Acid Change in J6 to JFH-1
M405H
K410N
V414I
I438L
S446R
M456L
Q493P
S498P
T501S
S558T
Y560F
T594A
I611V
N653D
F700Y
L708I
I719V

Amino acid changes are shown from the J6 version to the JFH-1 version. Colors represent groups of mutations that were introduced together into the J6 WT virus based on their predicted proximal locations shown in Figure 3.4.

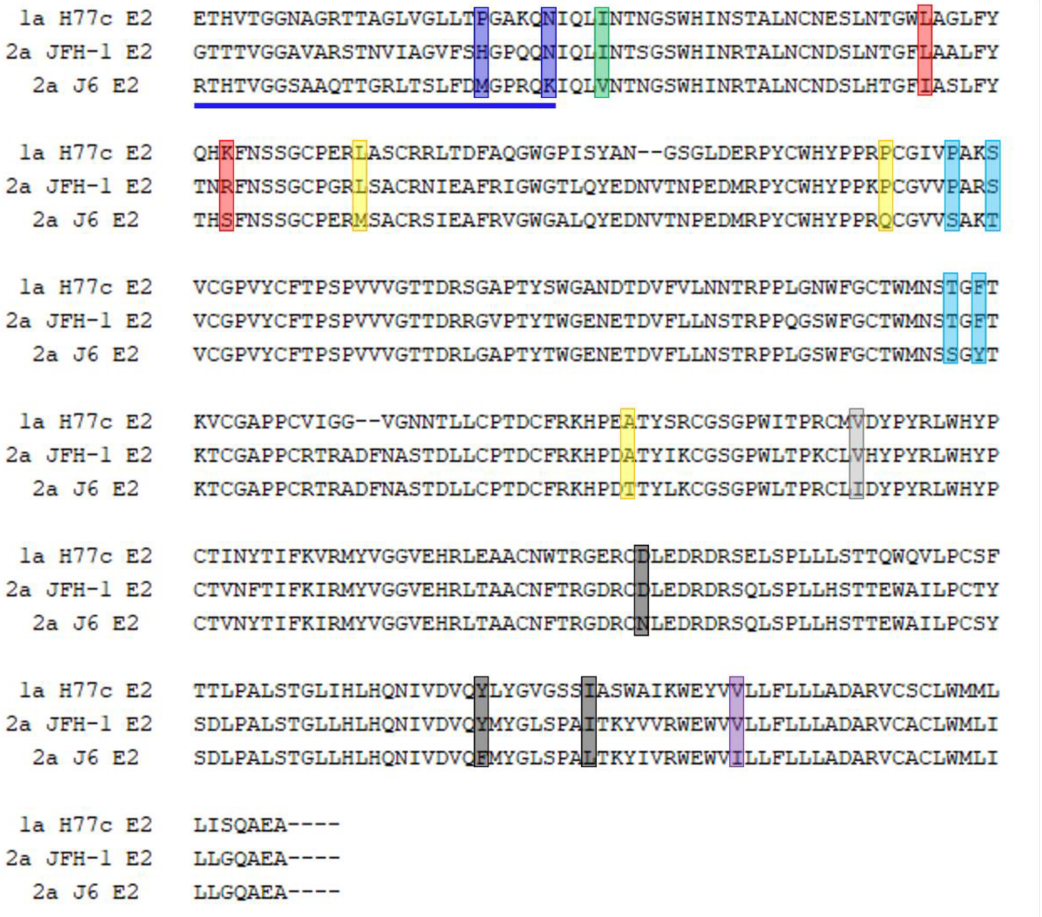

Figure 3.3. Amino Acid Sequence alignment of the H77, JFH-1 and J6 E2 protein sequence. Alignment was done using Geneious software based on the clustalW algorithm. The HVR1 sequence (amino acids 384-410) is underlined in blue. Amino acid positions that were identical between H77 and JFH-1 but differed in J6 are highlighted, along with three additional amino acids positions, two from the HVR1 region and one from domain 1 suggested by Dr. Holly Freedman (M405H, K410N and S446R). Colors represent mutation groups according to their location in the 3D structure presented in Figure 3.4.

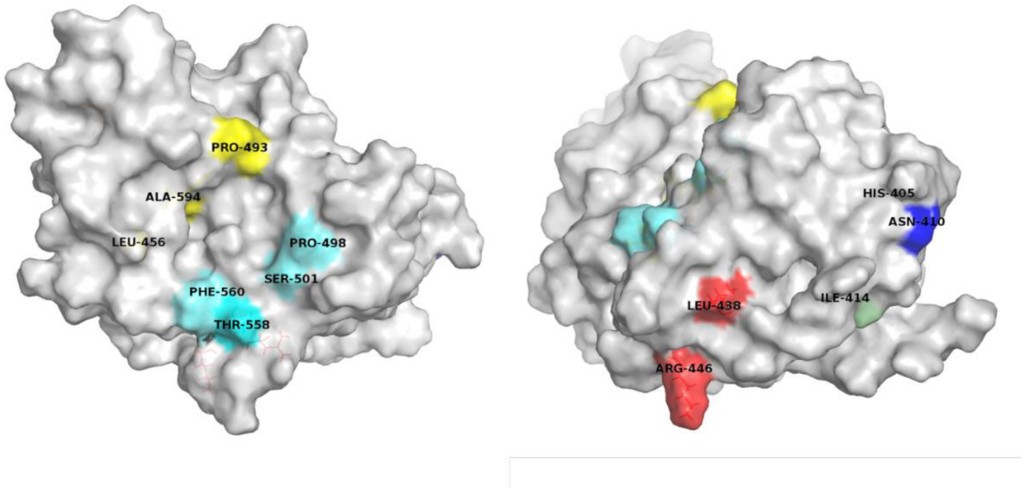

Figure 3.4. Variant E2 amino acid residues are highlighted in the solved crystal structure of the E2 core domain. Point mutations, listed in Table 3.2, that differ between J6 and JFH-1, but were identical between the sensitive H77 and JFH-1 viruses, along with three additional amino acids positions, two from the HVR1 region and one from domain 1 suggested of Dr. Holly Freedman (M405H, K410N and S446R) are shown. These mutations were grouped (various colors) based on their proximity within the proposed structure of the E2 protein and are shown in the colored regions of the 3-dimensional (D) structure. Mutations from amino acid 611 – 719 are located within the stem and transmembrane domains and are not located on this model. Figure was created by Dr. Holly Freedman at my request using the Pymol Molecular Graphics Program version 2.0.4 based on the published E2 structural models (39, 40).

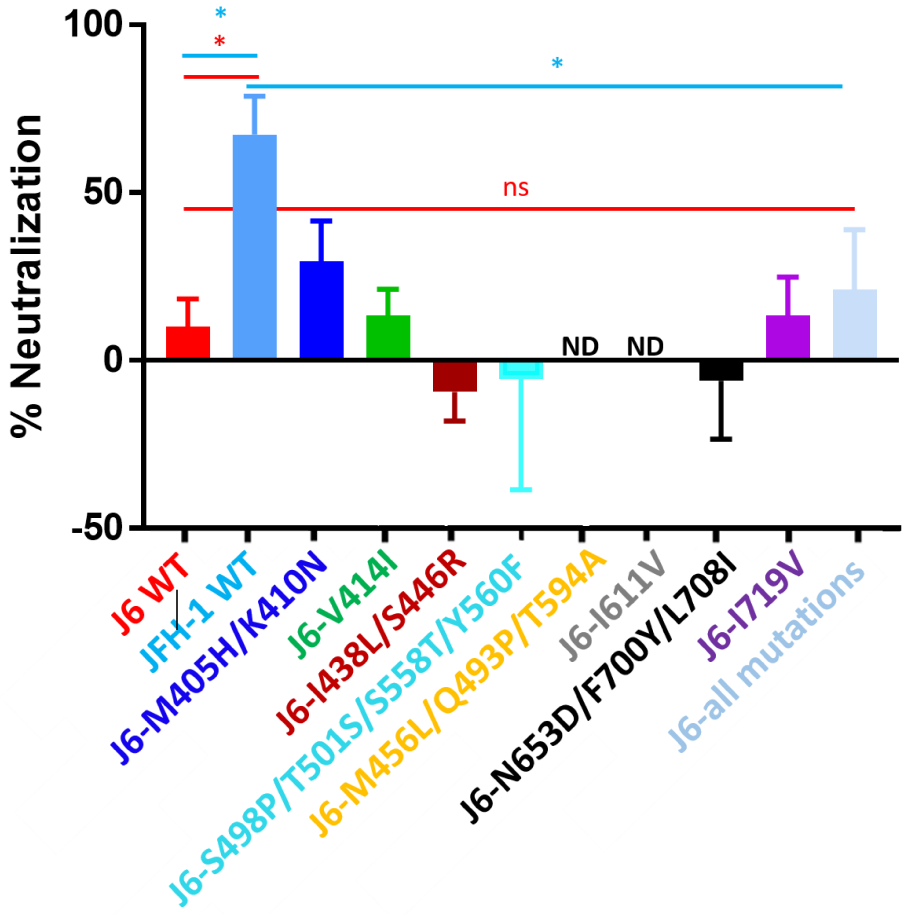

Figure 3.5. Variant amino acids within the E2 protein do not confer differential neutralization sensitivity between J6 and JFH-1 virus. Variant amino acid mutations from the sensitive JFH-1 WT virus were introduced into the resistant J6 WT virus using site directed mutagenesis. All HCVcc stocks were diluted to a titer of 500 $TCID_{50}$/ml for infection. Heat inactivated, pre and post 1a E1/E2 antisera was preincubated with HCVcc at a dilution of 1/100 and the mixture was added to Huh7.5 cells. The luminescence signal was read 48 hours after infection. The percent neutralization was normalized against neutralization activity of pre-immunization sera. The error bars represent the standard deviation of three independent experiments each performed within triplicate wells. One-way ANOVA followed by a Dunnett's multiple comparison test was calculated comparing the means of each column to the J6 WT or JFH-1 WT using Graphpad Prism 7 software. Significant differences from J6 WT are shown in red and significant differences from JFH-1 are shown in blue. P -value < 0.05 is indicated by * and "ns" indicates no significance. ND indicates no data was obtained.

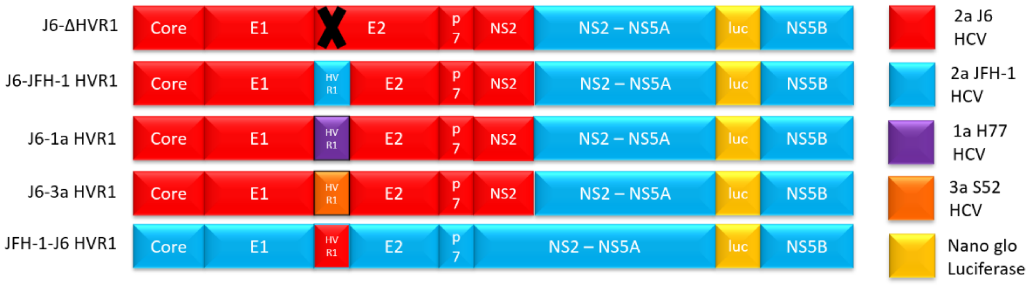

Figure 3.6. Schematic diagram of the recombinant HVR1 HCVcc virus genomes. All constructs contain the nonstructural proteins from the C-terminal portion of the NS2 protein to the NS5B from the JFH-1 virus (as described in Wakita et al. (12) and Gottwein et al. (13)). Structural proteins from the core to NS2 protein are from J6 (red). HVR1 sequences (amino acids 384-410) are from JFH-1 (blue), 1a H77 (purple), 3a S52 (orange) or deleted (denoted by "X"). All constructs contain a nano luciferase reporter gene located within domain III of the NS5A protein for detection of infection (yellow) (184).

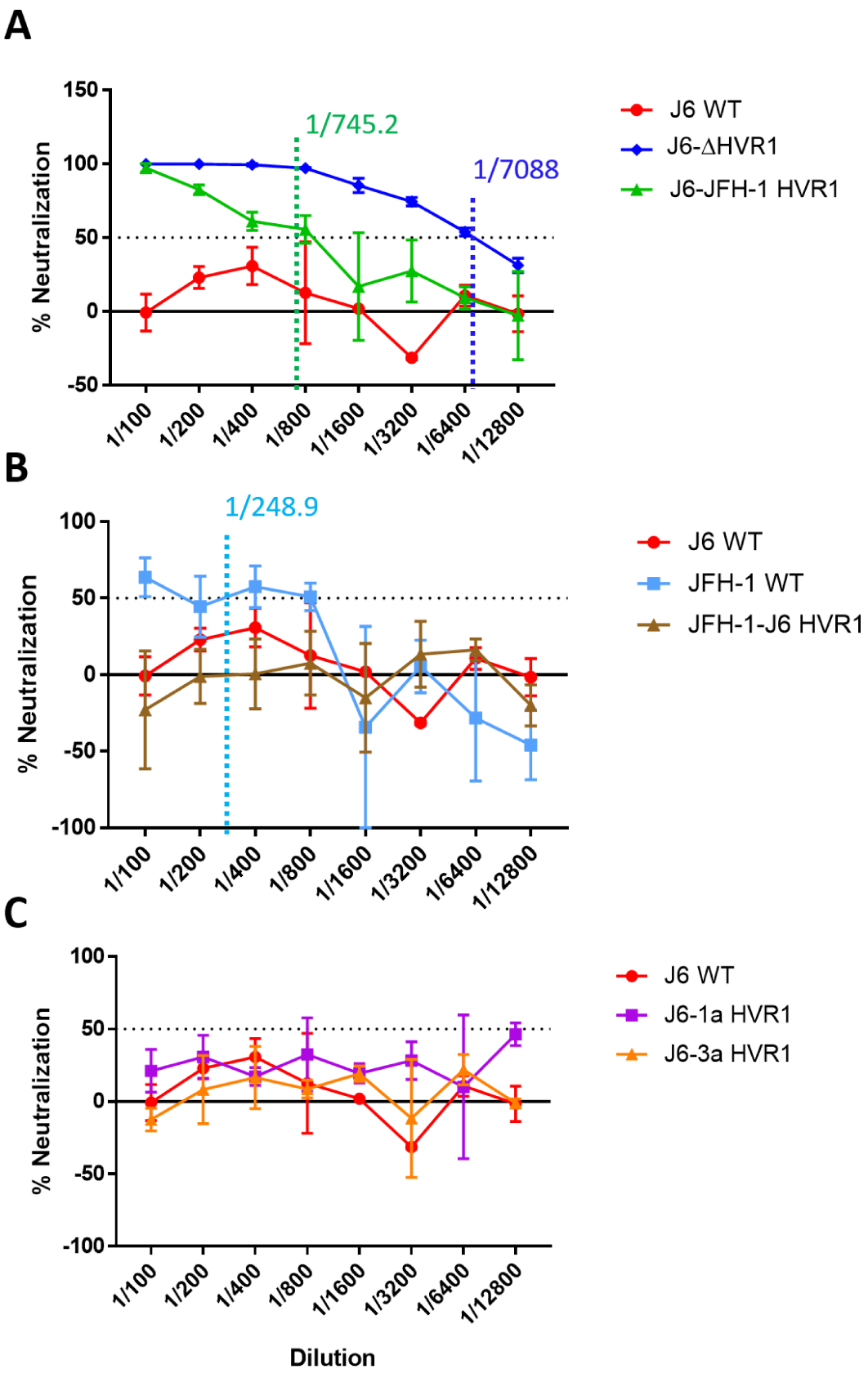
A
150
100
50
0
-50
% Neutralization
1/745.2
1/7088
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12800
J6 WT
J6-ΔHVR1
J6-JFH-1 HVR1
B
100
50
0
-50
-100
% Neutralization
1/248.9
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12800
J6 WT
JFH-1 WT
JFH-1-J6 HVR1
C
100
50
0
-50
-100
% Neutralization
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12800
Dilution
J6 WT
J6-1a HVR1
J6-3a HVR1

Figure 3.7. HVR1 is a determinant of isolate-specific neutralization sensitivity. All HCVcc stocks were diluted to a titer of 500 $TCID_{50}$/ml for infection. Two-fold diluted antisera (between 1/100 and 1/1600) was preincubated with various recombinant HVR1 HCVcc and mixtures were added to Huh 7.5 cells. The luminescence signal was read 48 hours post-infection. The percent neutralization was normalized against the observed neutralization activity of virus incubated with pre-immunization sera. The error bars represent the standard deviation of two independent experiments each performed with triplicate wells. The IC50 was calculated using Graphpad Prism 7 software by finding the non-linear regression of a variable slope. Statistical increase of JFH-1 WT, ΔHVR1 and HVR1 recombinant viruses from J6 WT was calculated in Graphpad Prism 7 software using a two-way ANOVA followed by a Dunnett's multiple comparison test and is presented in Supplementary Figure 2. **(A)** Neutralization sensitivity of J6 WT (red), J6-ΔHVR1 (dark blue) and J6-JFH-1 HVR1 (green) virus with our vaccine-induced antisera. IC50 values are indicated on the graph by a dotted line in the same color as the virus. IC50 for J6 WT could not be calculated as less than 50% neutralization was observed for all dilutions. **(B)** Neutralization sensitivity of JFH-1 WT (light blue) and JFH-J6 HVR1 (brown). IC50 values are indicated on the graph by a dotted line in the same color as the virus. IC50 for JFH-J6 HVR1 could not be calculated as less than 50% neutralization was observed for all dilutions. **(C)** Neutralization sensitivity of J6 WT (red), J6-1a HVR1 (purple) and J6-3a HVR1 (orange). IC50 values could not be calculated as less than 50% neutralization was observed for all dilutions.

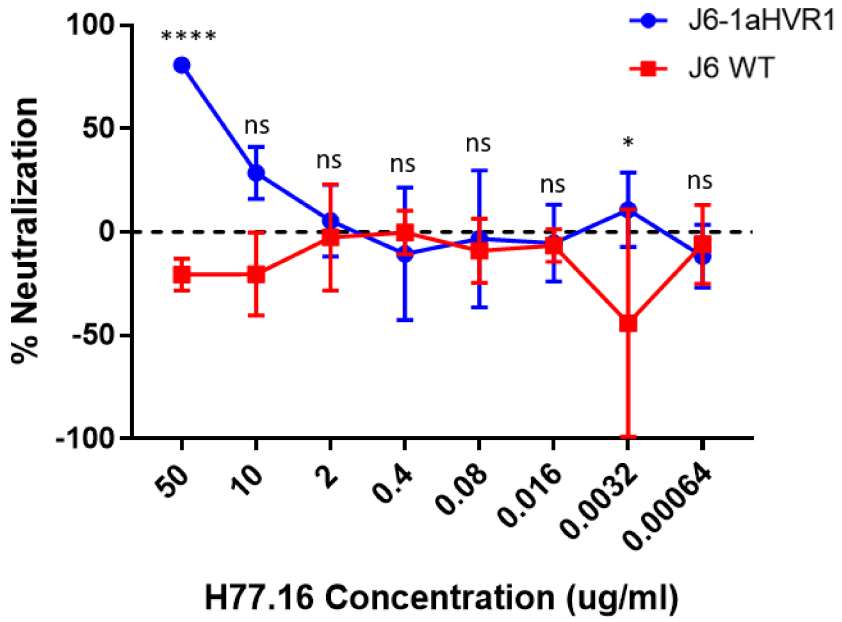

Figure 3.8. HVR1 specific antibody H77.16 neutralizes J6-1a HVR1 but not J6 WT virus. HCVcc diluted to 500 $TCID_{50}$/ml was pre-incubated with monoclonal H77.16 antibody starting with 50 µg/ml and subsequent 5-fold dilutions. The mixture was added to Huh7.5 cells. The luminescence signal was read 48 hours after infection, with increased luminescence indicating increased infection. The percent neutralization was normalized with neutralization activity of virus only wells. The error bars represent standard deviation of three independent experiments each performed within duplicate wells. Statistical difference of J6-1a HVR1 from J6 WT virus was calculated for each concentration using a two-way ANOVA followed by a Dunnett's multiple comparisons test. P-value <0.0001 is indicated by ****, p-value < 0.001 is indicated by ***, p-value < 0.01 is indicated by **, p-value < 0.05 is indicated by * and "ns" indicates no significance.

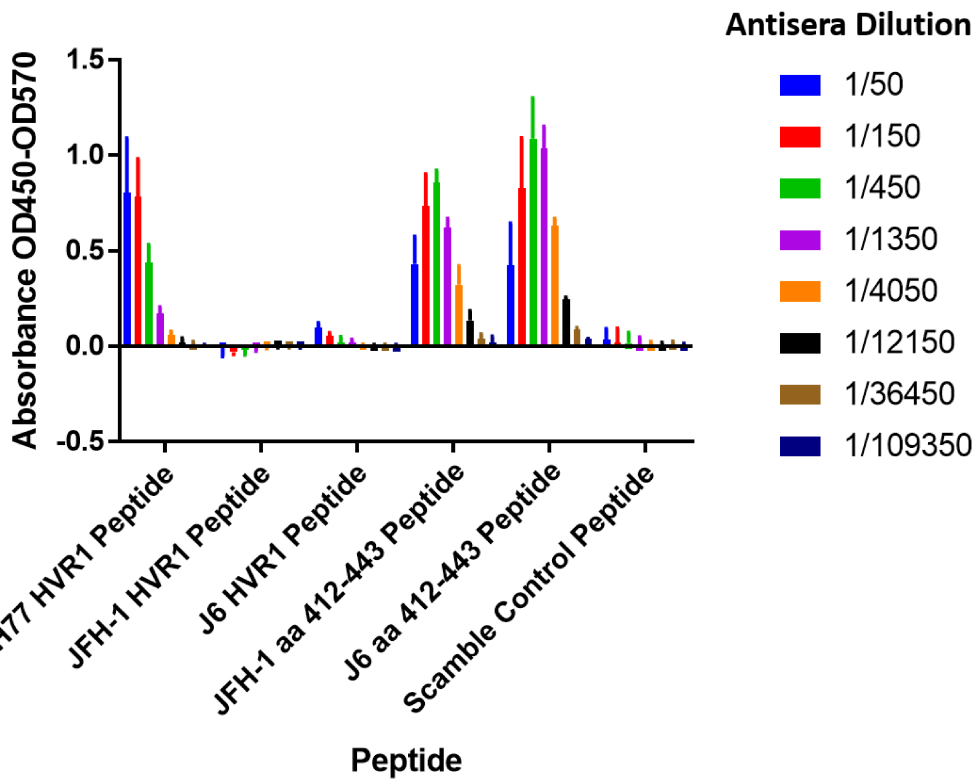

Figure 3.9. HVR1 of JFH-1 is not directly targeted by antibodies in 1a E1/E2 antisera. The binding of 1a E1/E2 antisera was assessed to peptides encoding the HVR1 of 1a H77, 2a JFH-1 and 2a J6 virus, as well as the 31 amino acids (aa 412-443) directly downstream of HVR1 of the JFH-1 virus. Biotinylated peptides were bound to wells coated with neutravidin and serial diluted pre- and post-vaccinated antisera from 1a E1/E2 vaccinated goat was added. Bound IgG was detected with an anti-goat HRP conjugated antibody. Absorbance was read at 570 nm and 450 nm. Background binding of pre-sera and post sera to no peptide control wells were subtracted and absorbance values were plotted in Graphpad Prism 7 software. Error bars represent standard deviation of three independent experiments each performed within duplicate wells. Statistical significance of the binding of 1a E1/E2 to each peptide was compared to the scramble control peptide at each dilution using a two-way ANOVA followed by a Dunnett's multiple comparison test in Graphpad Prism 7 software and is presented in Supplementary Figure 3.

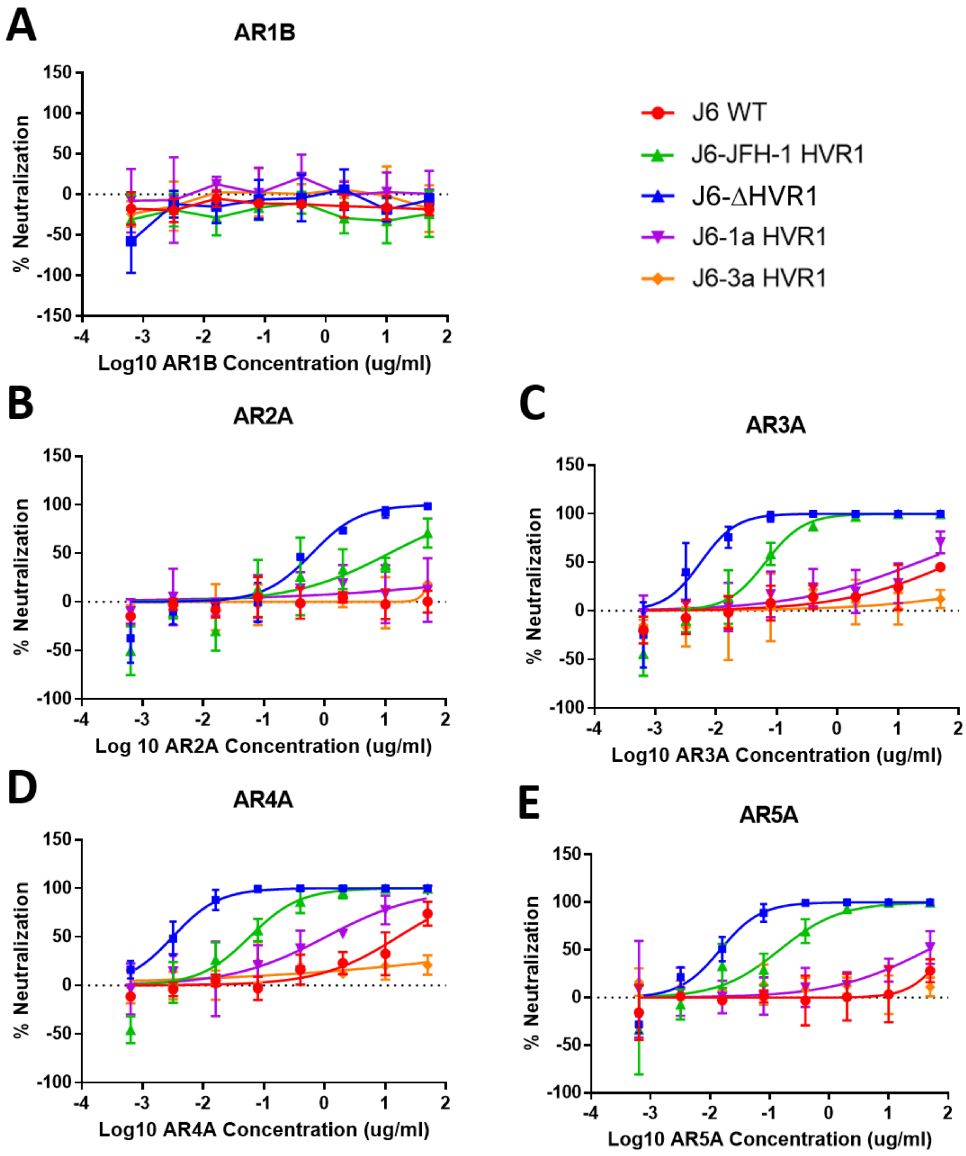

Figure 3.10. HVR1 modulates exposure of neutralizing antibody epitopes in a genotype specific manner. Broadly neutralizing monoclonal antibodies (AR1B **(A)**, AR2A **(B)**, AR3A **(C)**, AR4A **(D)** and AR5A **(E)**) were pre-incubated with 500 $TCID_{50}$/ml WT or modified HVR1 HCVcc and then mixture was used to infect Huh7.5 cells. The luminescence signal was read 48 hours post-infection. The percent neutralization was normalized with neutralization activity of virus without the addition of antibody. The error bars represent standard deviation of three independent experiments each performed within duplicate wells. IC50 values were calculated using Graphpad Prism 7 software by finding the non-linear regression of a variable slope. Statistical comparisons of all chimeric HVR1 virus to J6 WT for all antibodies was calculated with a two-way ANOVA followed by a Dunnett's multiple comparisons test using Graphpad Prism 7 software and is presented in Supplementary Figure 4.

Table 3.2. IC50 values for virus neutralized with a panel of monoclonal antibodies.

Antibody	J6 WT	J6-ΔHVR1	J6-JFH HVR1	J6-1a HVR1	J6-3a HVR1
AR1B	>50	>50	>50	>50	>50
AR2A	>50	0.622	11.67	>50	>50
AR3A	>50	0.006	0.066	21.72	>50
AR4A	16.26	0.003	0.058	1.052	>50
AR5A	>50	0.015	0.147	45.77	>50

IC50 values are given in μg/ml.

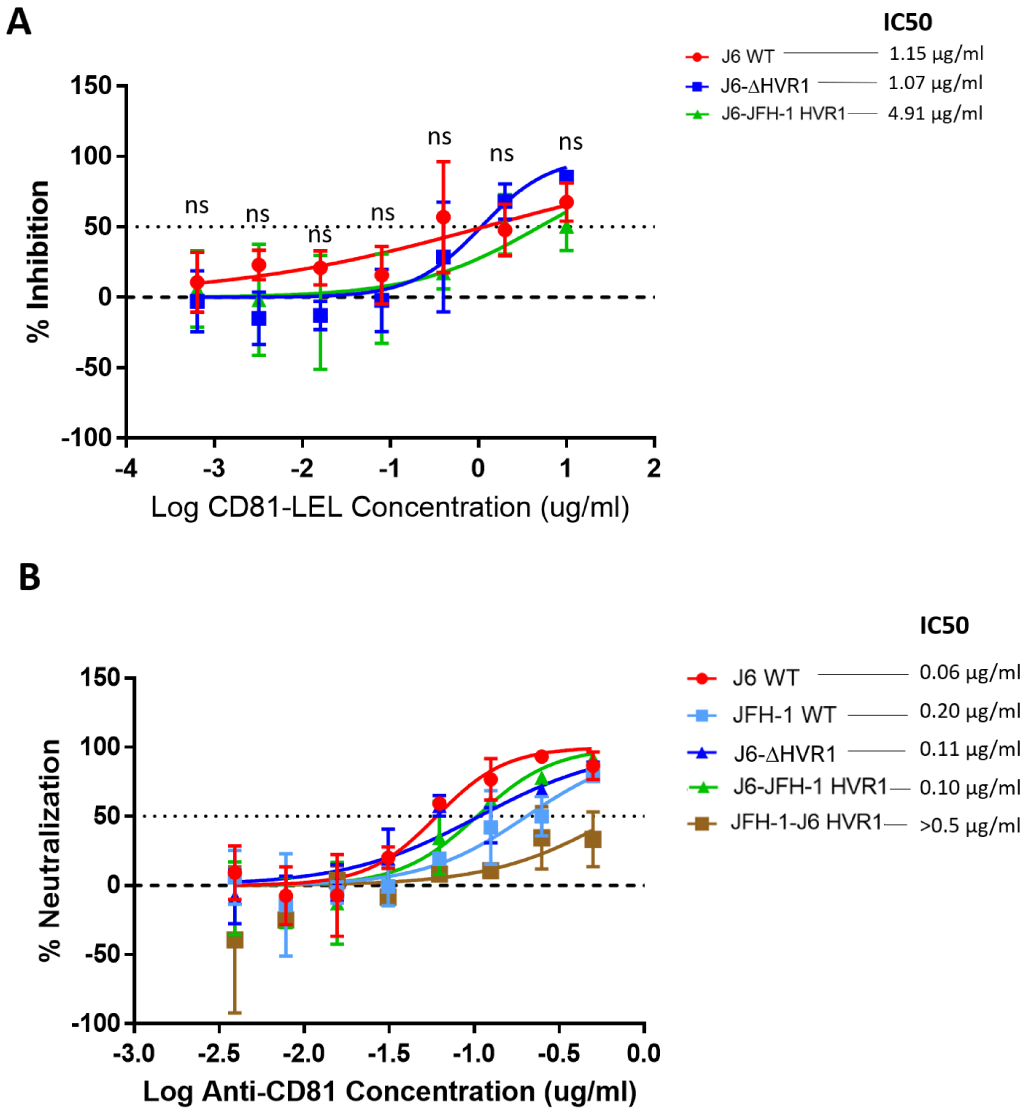
A
IC50
J6 WT 1.15 µg/ml
J6-ΔHVR1 1.07 µg/ml
J6-JFH-1 HVR1 4.91 µg/ml
% Inhibition
Log CD81-LEL Concentration (ug/ml)
ns
B
IC50
J6 WT 0.06 µg/ml
JFH-1 WT 0.20 µg/ml
J6-ΔHVR1 0.11 µg/ml
J6-JFH-1 HVR1 0.10 µg/ml
JFH-1-J6 HVR1 >0.5 µg/ml
% Neutralization
Log Anti-CD81 Concentration (ug/ml)

Figure 3.11. HVR1 modification of J6 virus does not impact interaction with the CD81. **(A)** CD81-LEL was pre-incubated with 500 $TCID_{50}$/ml J6WT or modified HVR1 HCVcc starting with 10 µg/ml and subsequently 5-fold diluted. CD81-LEL / HCVcc mixture was then added to Huh 7.5 cells. **(B)** Neutralization of various HVR1 recombinant virus by anti-CD81. HCVcc diluted to 500 $TCID_{50}$/ml was preincubated with 2-fold diluted anti-CD81 (starting with a concentration of 0.5 µg/ml) and then mixture was added to Huh 7.5 cells. Luminescence signal was detected 48 hours after infection. Percent inhibition for both CD81-LEL and anti-CD81 was normalized to virus only infection and plotted in Graphpad Prism 7 software. IC50 values were calculated using Graphpad Prism 7 software by finding the non-linear regression of a variable slope. Error bars represent the standard deviation of data from triplicate experiments each performed within duplicate **(A)** or triplicate **(B)** wells. Statistical difference between the viruses tested for each dilution were calculated using a two-way ANOVA followed by a Dunnett's multiple comparison test in Graphpad Prism 7 software. Luminescence signal was detected 48 hours post-infection. The statistical differences are presented in Supplementary Figure 5 **(B)** or indicated on the figure **(A)**.

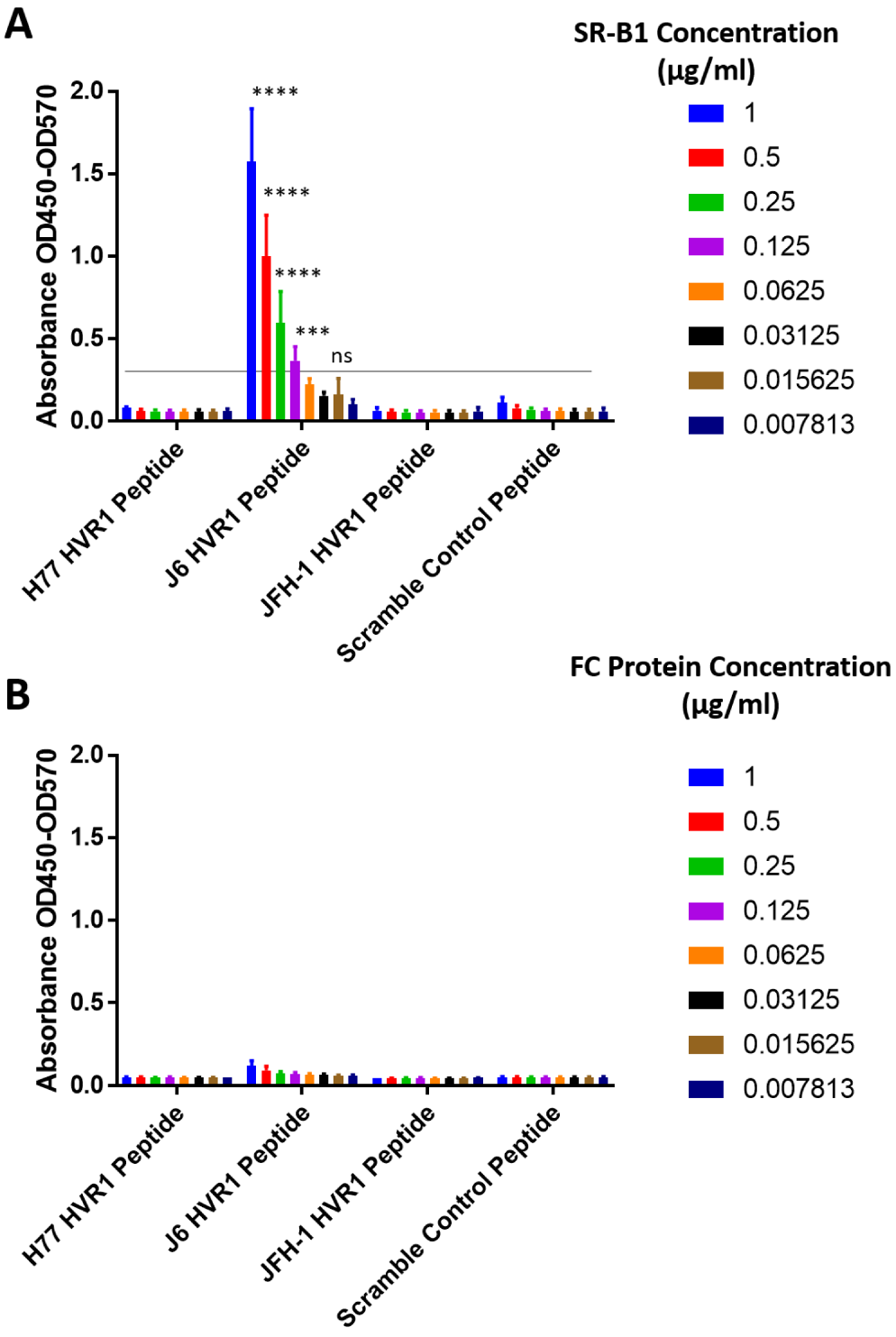
A
SR-B1 Concentration (µg/ml)
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
0.007813
Absorbance OD450-OD570
2.0
1.5
1.0
0.5
0.0
****
****
****
***
ns
H77 HVR1 Peptide
J6 HVR1 Peptide
JFH-1 HVR1 Peptide
Scramble Control Peptide
B
FC Protein Concentration (µg/ml)
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
0.007813
Absorbance OD450-OD570
2.0
1.5
1.0
0.5
0.0
H77 HVR1 Peptide
J6 HVR1 Peptide
JFH-1 HVR1 Peptide
Scramble Control Peptide

Figure 3.12. SR-B1 protein specifically binds J6 HVR1 but not H77 or JFH-1 HVR1. The binding of Fc-SR-B1 **(A)** and human Fc **(B)** proteins to peptides encoding the HVR1 of 1a H77, 2a JFH-1 and 2a J6 virus were assessed. Biotinylated peptides were bound to wells coated with neutravidin. Two-fold serial diluted Fc tagged SR-B1 protein was added to plate coated with peptides. Bound SR-B1 protein was detected with an HRP conjugated anti-Fc antibody. The absorbance (570 nm and 450 nm) was read. Error bars represent standard deviation from three independent experiments each performed within duplicate wells. Statistical differences from scramble control peptide were calculated with a two-way ANOVA using Graphpad Prism 7 software. P-value < 0.0001 is indicated by ****, p-value < 0.001 is indicated by ***, p-value < 0.01 is indicated by **, p-value < 0.05 is indicated by * and "ns" indicates no significance.

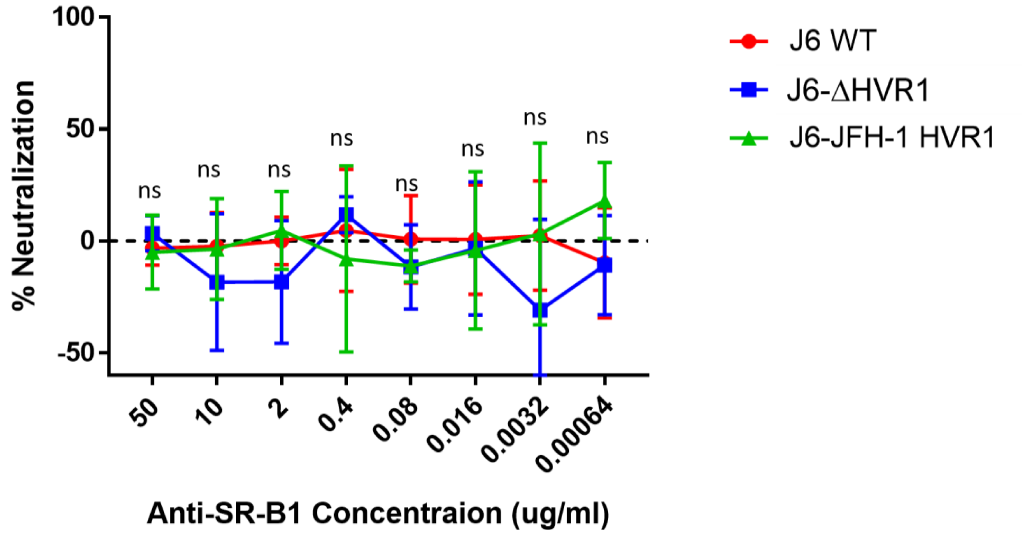

Figure 3.13. HVR1 modified J6 virus neutralization by anti-SR-B1 antibody. Decreasing concentrations of anti-SR-B1 (starting with 50 µg/ml) were pre-incubated with 500 $TCID_{50}$/ml HCVcc. HCVcc/antibody mixture was then added to Huh 7.5 cells. Luminescence signal was detected 48 hours post-infection. Percent inhibition was normalized against virus without addition of antibody. Data was plotted with Graphpad Prism 7 software. Error bars represent the standard deviation of data from three independent experiments each performed within duplicate wells. Statistical significance of the different viruses' neutralization sensitivity for each dilution was calculated with a two-way ANOVA using Graphpad Prism 7 software. No statistical differences were observed (ns).

Chapter 4: Discussion

4.1 Summary of results.

Neutralizing antibodies have been shown to be protective against HCV infection in chimpanzees and *in vitro* (147–149). An effective global prophylactic HCV vaccine will need to elicit antibodies capable of preventing infection from all HCV genotypes. Previous characterization of 1a E1/E2 antisera from vaccinated patients and animal models showed that there was variation in effectiveness of antibodies to neutralize the different HCV genotypes (45, 170, 179). In this thesis I investigated the molecular determinants of isolate-specific differences in neutralization sensitivity between two highly related HCV genotype 2a isolates, J6 and JFH-1, to the 1a E1/E2 glycoprotein vaccine-induced antisera.

The E1 and E2 proteins were investigated as potential determinants of neutralization sensitivity as it was reasonable to assume that the main difference would be due to alteration of raised antibody binding to E1 and/or E2 proteins used as antigens in the vaccine. Exchange of the E1 or E2 proteins between the sensitive JFH-1 WT virus into the resistant J6 WT virus reveals that a major determinant of differential neutralization sensitivity is attributed to the E2 protein. A sensitive phenotype could be conferred to resistant J6 WT virus with the E2 protein from JFH-1 and conversely the resistant phenotype could be conferred to the sensitive JFH-1 WT virus with the E2 protein from J6.

There are two possible explanations proposed for the importance of the E2 protein in the isolate specific neutralization sensitivity. The difference could be the result of variant amino acids between J6 and JFH-1 either individually or in combination as several studies have found that point mutation can result in altered sensitivity to neutralization (114, 155, 188–191). Alternatively, the HVR1 could be responsible as indicated in previous studies by other groups that found HVR1 to have an influence on the genotype-specific sensitivity to neutralization by monoclonal antibodies (49, 56). Investigation of variant amino acids throughout E2 that are identical between the sensitive strains H77 and JFH-1 but differ in J6

were found to not be responsible for the differences in neutralization sensitivity between the J6 and JFH-1 isolate. However, HVR1 had a striking influence on the isolate-specific neutralization sensitivity between J6 and JFH-1 by the 1a E1/E2 antisera leading to further investigations on the mechanisms of HVR1's influence on neutralization sensitivity.

Two potential mechanisms for the importance of HVR1 in isolate-specific neutralization sensitivity include: the direct recognition of HVR1 of JFH-1 but not J6 by neutralizing antibodies in the antisera, or an indirect influence of E2 protein interactions with antibodies and/or entry receptors. While our antisera recognized the homologous 1a H77 HVR1, antibodies were not shown to directly bind either J6 or JFH-1 HVR1, indicating HVR1 is likely acting indirectly. To investigate how HVR1 could be indirectly affecting neutralization sensitivity, the effect that HVR1 has on a variety of neutralizing epitopes was investigated using a panel of broadly neutralizing monoclonal antibodies targeting regions throughout the E2 protein. J6 virus with HVR1 deletion resulted in a general and substantial increase in neutralization by these antibodies and similarly replacement of J6 HVR1 with JFH-1 HVR1 conferred an increased neutralization sensitivity compared to J6 WT virus to AR2A, AR3A, AR4A and AR5A neutralizing monoclonal antibodies. Additionally, HVR1's influence on the virus interaction with the entry receptors CD81 and SR-B1 were investigated. Preincubation of J6 WT, J6-ΔHVR1 and J6-JFH-1 HVR1 HCVcc with CD81-LEL showed similar levels of inhibition of infection. The inhibition of infection by anti-CD81 was also not significantly different between J6 WT and JFH-1 WT or between J6 WT and either J6-ΔHVR1 or J6-JFH-1 HVR1. Surprisingly, JFH-1-J6 HVR1 was significantly less inhibited by anti-CD81 as compared with J6 WT, although for this virus there was not a significant difference in inhibition compared to JFH-1 WT virus. Overall, these data suggest that HVR1 is not playing a major role in interactions with the CD81.

Interestingly, it was found that the SR-B1 was able to specifically bind to HVR1 of J6 but not to HVR1 of JFH-1, suggesting that this interaction may be responsible for the differential neutralization. However, an antibody against SR-

B1 had no effect on the entry of either J6 WT, J6-ΔHVR1 or J6-JFH-1 HVR1 virus in contrast to previously published data with other anti-SR-B1 antibodies (50, 182).

4.2 The location of differential neutralization sensitivity between J6 and JFH-1.

My results indicate that the E2 protein is the major determinant of isolate-specific neutralization sensitivity to the 1a E1/E2 vaccine antisera. Exchange of the J6 E2 protein for the JFH-1 E2 is sufficient to confer sensitivity to the resistant J6 virus and vice versa. There have been several studies identifying mutations throughout E2 or deletion of HVR1 resulting in drastic changes to the sensitivity of the virus to neutralization by monoclonal antibodies (47, 49, 53, 56). The determinants of neutralization sensitivity located in the E2 proteins were then further investigated.

4.3 Variant Amino Acids throughout the E2 protein.

The E2 protein has been shown to be constantly under selective pressure from neutralizing antibodies both *in vivo* (193) and *in vitro* (114, 188, 190, 194–196). While specific point mutations of known antibody binding residues can result in resistance to neutralizing antibodies, it has also been shown that mutations outside of known antibody binding epitopes are also capable of conferring resistance to broadly neutralizing antibodies (47, 114).

To investigate the possibility that specific variant amino acids were responsible for the differential neutralization sensitivity between the J6 and JFH-1 isolates by polyclonal 1a E1/E2 antisera, 14 amino acid variants throughout the E2 protein that were identical between the sensitive 1a H77 and JFH-1 strains but which differed in the resistant J6 strain, as well as three amino acids (two from HVR1 and one from E2 domain 1), were investigated for their effect on the neutralization sensitivity of the J6 virus. When the amino acids from the sensitive JFH-1 virus were introduced into the J6 virus, no significant change from J6 WT virus in neutralization sensitivity was observed for any of the variant amino acid

groups (based on their proximity within the E2 structure) or when all 17 variant amino acids were combined. This was surprising given that many of these residues fall within known antibody binding epitopes (47). Of particular note was the variant at amino acid position 438. Mutations at position 438 have been associated with resistance either alone or in combination with other mutations for monoclonal antibodies that have amino acid 438 within their binding epitopes (155, 190, 195). However, I found that mutation at position 438, either in combination with a point mutation at position 446 or with all 16 other variants, did not significantly alter the J6 virus sensitivity to vaccine-induced antisera. There is a potential that amino acid position 438 in combination with mutations not identified by my selection criteria could also result in neutralization sensitivity to 1a E1/E2 antisera and further investigation of variant amino acids between J6 and JFH-1 might be warranted. Also, there are three additional amino acids that share charge conservation, but were not identical, between the sensitive H77 and JFH-1 virus but are variant in the J6 virus at amino acid positions 440, 471 and 522 that may be of potential interest for further study. However, given the lack of change in sensitivity of the J6 virus with all 17 variant amino acids, it is unlikely that mutations selected by this method are the cause for the major differences in neutralization sensitivity observed between the J6 and JFH-1 isolates, and therefore these additional variant amino acids were not investigated.

Two of the variant constructs, J6-M456L/Q493P/T594A and J6-I611V, were not viable in cell culture. One potential explanation for their inability to produce viable virus is suggested in a recent study where it was shown, through alanine scanning mutagenesis of the E1 and E2 proteins, that mutations between amino acids 490-650 are capable of interfering with the overall folding of the E1 and E2 proteins (197). It is interesting to note that these variant amino acids when present in combination with the other JFH-1 mutations listed in Table 3.1 did not result in decreased infectivity of the virus. Therefore, it is possible that some of the variant amino acids identified could be involved in compensating for the folding changes induced by the mutations in the 490-650 region. Mutations in this region have been shown to be viable when in combination with other mutations in E2 (188,

190), and it may be worthwhile in the future to investigate different clusters of variant amino acids other than those presented in the current study

4.4 The Role of HVR1 in isolate-specific neutralization sensitivity.

Deleting HVR1 renders the virus more sensitive to neutralizing antibodies (56), which suggests that the variation in HVR1 from different strains may contribute to their differential neutralization. Prentoe, et al. (56) found that the variation in neutralization sensitivity between 1a H77, 2a J6 and 3a S52 strains was reduced when the HVR1 region was removed. This is in agreement with a number of previous studies where the removal of the HVR1 increased the neutralization sensitivity of HCV to broadly neutralizing monoclonal antibodies, independent of genotype (49, 53, 56, 192, 194). Similarly, in the current study deletion of HVR1 from the resistant J6 virus resulted in hypersensitivity to neutralization by 1a E1/E2 vaccine-induced antisera (Figure 3.7 A). Exchanging J6 HVR1 for JFH-1 HVR1 renders the recombinant J6 virus sensitive to neutralization by vaccine-induced antisera (Figure 3.7 A). Conversely, resistance to vaccine-induced antisera was conferred to the JFH-1 virus by exchanging the HVR1 from the J6 virus for the JFH-1 HVR1 (Figure 3.7 B). This shows that HVR1 is a major determinant of isolate-specific differences in neutralization sensitivity between the J6 and JFH-1 isolates.

To confirm if these findings could be generalized at a genotype-specific level, J6 recombinant viruses with the HVR1 exchanged for 1a H77 and 3a S52 HVR1 were created. While I predicted that the J6-1a HVR1 virus would be sensitive to neutralization and the J6-3a HVR1 virus would be resistant, a resistant phenotype was seen for both recombinant viruses (Figures 3.7 C). The 1a HVR1 region in the context of the J6 virus was correctly recognized by 1a HVR1 specific antibody H77.16 (Figure 3.8), indicating that incorrect incorporation of the HVR1 was not the result of unexpected resistance of the J6-1a HVR1 virus. It is possible that the HVR1 of genotype 1a H77 and 3a S52 similar to that of WT J6 can somehow prevent exposure of neutralizing epitopes outside of HVR1 and render

the chimeric virus resistant to neutralization. Therefore, my results suggest a potential interaction of HVR1 with other domains within E1 and/or E2 that is genotype-dependent. Previously, it was shown that while J6-ΔHVR1 does not require adaptive mutations to propagate in cell culture, H77-ΔHVR1 or S52-ΔHVR1 do require adaptive mutations in E1 or E2 (54). This would suggest that HVR1 has a genotype specific effect that is dependent on the structure of the accompanying E1 and E2 proteins similar to my findings. This was further suggested in another study that exchanged portions of the E1 and E2 proteins between two isolates of the 1a genotype and showed differing infectivity and entry receptor usage (35). In combination with my data, this suggests that HVR1 has an effect on neutralization sensitivity that is dependent on the isolate-specific context of the E1 and E2 proteins and suggest there may be multiple mechanisms at play in the effect of HVR1 on neutralization sensitivity.

4.5 Mechanisms of HVR1 effect on neutralization sensitivity.

4.5.1 Direct antibody targeting of the HVR1. The ability of 1a E1/E2 vaccine-induced antisera to recognize HVR1 of homologous 1a H77 and heterologous 2a J6 or JFH-1 was assessed by ELISA. Antisera specifically bound to 1a H77 HVR1 but was unable to bind either 2a J6 or JFH-1 HVR1. Since antibodies to linear epitopes within HVR1 are able to neutralize viral infectivity (158, 161), this indicates that antisera is not directly targeting JFH-1 HVR1 to mediate neutralization of JFH-1 WT. However, this assay is limited in that it only detects antibodies that are binding to linear epitopes of HVR1 and the possibility of discontinuous epitopes involving the HVR1 of JFH-1 and other residues within E1 or E2 remains. Indeed, recognition of discontinuous epitopes involving HVR1 from 1a H77 and 2a J6 by vaccine-induced antibodies have been described (182). The authors show that both residues within HVR1 and E2 were necessary for efficient binding of two of the isolated neutralizing antibodies to virus.

Encouragingly for the development of the vaccine, peptides containing the 31 amino acids downstream of the HVR1 of both 2a J6 and JFH-1 were recognized

by 1a E1/E2 vaccine-induced antisera. This region of the E2 protein is known to contain conserved residues important for binding of many broadly neutralizing monoclonal antibodies (47). This agrees with previously published data from our lab showing that vaccine-induced antisera compete for binding with broadly neutralizing monoclonal antibodies that target this region (45). The lack of neutralization of the J6-1a HVR1 virus in combination with the data showing the vaccine antisera contains antibodies that target the HVR1 of 1a H77 indicates that the main neutralization seen against 1a H77 HCVcc in our labs previous experiments is not due to antibodies targeting the HVR1.

4.5.2 HVR1 mediated exposure of antibody binding epitopes. A panel of broadly neutralizing antibodies was used to determine if the deletion or exchange of HVR1 impacted the overall exposure of neutralizing epitopes. Deletion of HVR1 or substitution with JFH-1 HVR1 in the J6 virus resulted in increased sensitivity compared to the J6 WT virus for all neutralizing monoclonal antibodies tested (Figure 3.10 B, C, D, and E). In agreement with the literature regarding deletion of HVR1 (56), modification of HVR1 somehow exposes the epitopes of many broadly neutralizing monoclonal antibodies. Similar to the data obtained for the vaccine-induced antisera, J6-JFH-1 HVR1 is 10 times more resistant than the J6-ΔHVR1 to neutralization by monoclonal antibodies. J6-1a HVR1 mirrors the sensitivity phenotype of the J6 WT virus with the exception of a slight (but statistically significant) increase in sensitivity to AR4A at the second highest dilution. J6-3a HVR1 shows a strongly resistant phenotype to all antibodies tested. It is clear that HVR1 is impacting the neutralization sensitivity of a range of antibodies that target sites throughout the E2 protein (and even antibodies that target conformational epitopes that require both E1 and E2 (AR4A and AR5A) (154, 183). This indicates HVR1 is having a strong and general impact on epitope exposure. As was shown in (56), it is likely this increased neutralization sensitivity by both 1a E1/E2 vaccine-induced antisera and broadly neutralizing monoclonal antibodies is due to increased ability of antibodies to bind viral envelope proteins,

although this requires further testing to investigate the affinity of 1a E1/E2 vaccine-induced antibodies for the J6 WT versus HVR1 modified viruses.

4.5.3 HVR1's impact on CD81 interactions. Previous studies have shown that deletion of HVR1 has no effect on neutralization sensitivity to anti-CD81 (50, 54, 192) with both WT and ΔHVR1 virus being equally neutralized by various monoclonal anti-CD81 antibodies. In agreement with these previous studies, J6 WT, J6-ΔHVR1 and J6-JFH-1 HVR1 viruses were similarly neutralized by anti-CD81 (Figure 3.11 B). Of note, however, is the neutralization of JFH-1-J6 HVR1 virus. While, JFH-1 WT virus does not differ in its neutralization sensitivity to anti-CD81 from either J6 WT or the JFH-1-J6 HVR1 virus, the JFH-1-J6 HVR1 virus does significantly differ from the J6 WT virus for several dilutions. This indicates a potential role of HVR1 in the JFH-1 virus interaction with the CD81. There are two potential explanations for the JFH-1 differential interaction with CD81; first, that JFH-1 has a decreased reliance on CD81 for entry or, second, that JFH-1 has an increased affinity for CD81 that competes for binding with the anti-CD81 antibody. The first explanation that JFH-1 is less reliant on CD81 is unlikely given the established research in the field describing CD81 as an essential entry receptor and the recent study demonstrating that cells lacking CD81 are unable to support HCV infection of JFH-1 virus (117). The second explanation is supported by studies that show mutations in the JFH-1 E2 protein can result in increased binding to the LEL of CD81 (114, 115). If this is the case the combination of JFH-1 E1 and E2 proteins with the HVR1 of J6 seems to augment this increased affinity, possibly through increased exposure of CD81 binding sites, although the exact mechanism remains unknown.

While the J6 WT, J6-ΔHVR1 and J6-JFH-1 HVR1 viruses were not differentially neutralized by anti-CD81, it was previously shown that deletion of HVR1 from J6 virus increases the ability of the virus to bind CD81-LEL (192). The ability of J6 WT as well as J6-ΔHVR1 and J6-JFH-1 HVR1 virus to bind CD81-LEL was assessed. Contrary to what was previously shown, both J6 WT and J6-ΔHVR1 infection was similarly inhibited by CD81-LEL and while a small

increase in resistance was observed for J6-JFH HVR1 this difference was not significant (Figure 3.11 A). This indicates that for the J6 virus, the HVR1 is not impacting the affinity of the E2 protein in recognition of the CD81.

4.5.4. HVR1 isolate-specific interaction with SR-B1. Past research indicates there is a complex interaction involving HCV's usage of the SR-B1. The interaction of the HCV particle with SR-B1 has been shown to involve multiple steps (48). Lipoprotein interactions are involved in initial attachment of viral particles to the SR-B1. Subsequently, the HCV E2 protein interacts with SR-B1 in an HVR1 dependent manner and this interaction results in infection enhancement (48, 49). This infection enhancement function has been shown to be related to the presence of HDL and involves the lipid transfer function of the SR-B1 (49, 110). Additionally, deletion of the HVR1 results in loss of binding to SR-B1 when assayed by flow cytometry (50) and resistance to antibodies that target SR-B1 (50, 54, 192). An ELISA interrogating the ability of SR-B1 protein to bind peptides of the HVR1 from the J6, H77 and JFH-1 viruses shows that SR-B1 binds specifically to J6 HVR1 but not to, either the H77 or JFH-1 HVR1 peptides. This finding is striking and shows for the first time an isolate-specific direct interaction of HVR1 with SR-B1. It is possible that this additional interaction with HVR1 that occurs for the J6 virus is allowing an alteration of the kinetics of entry that allows the virus to enter the cell faster than the JFH-1 virus and thus is reducing the exposure time of the virus to neutralizing antibodies and results in a relative resistance to neutralization. Additionally, as has been suggested previously, J6 virus envelope proteins could be in a "shielded" conformation prior to binding SR-B1 and HVR1 specific interactions with SR-B1 result in a conformational change that then exposes these epitopes (198). The H77 and JFH-1 virus may be in an exposed conformation prior to SR-B1 binding and is thus more sensitive to neutralizing antibodies.

A commercial polyclonal anti-SR-B1 antibody that targets the 15 N-terminal amino acids of SR-B1 was used to compare the neutralization of J6 WT, J6-ΔHVR1 and J6-JFH HVR1 virus. A similar antibody was previously shown to

inhibit infection by both the H77 and J6 viruses (182). Unfortunately, the antibody used in the previous study was no longer available and no neutralizing activity was observed for the antibody used in this current assay (Figure 3.13). Interestingly, the authors of the previous study observe a difference in the sensitivity of H77 versus J6 to the anti-SR-B1 antibody used, such that H77 shows approximately 30% less inhibition than J6 virus (182). The poor neutralization seen for the commercial anti-SR-B1 antibody could be due to the antibody failing to prevent virus binding to critical SR-B1 residues, amino acids 70-87 and amino acid E210, previously shown to be essential for sE2 binding to SR-B1 (199). Future investigations would be warranted with a more effective antibody. A monoclonal anti-SR-B1 antibody produced in mice (C167) by (200), has been shown by a number of groups to strongly inhibit HCV infection (50, 54, 116, 200). C167 inhibits infection of HCV through blocking HDL binding to SR-B1 (200). This antibody could determine if HVR1 modified J6 virus are differentially utilizing HDL for SR-B1 entry. The genotype 2a JC1 isolate was shown to be sensitive to neutralization by C167, but JC1-ΔHVR1 virus was resistant (50). Interestingly, it has been shown that C167 has differential neutralization capacity against H77, J6 and S52 genotypes (54). It was shown that H77 and S52 are readily neutralized by C167 anti-SR-B1, while J6 is less sensitive to neutralization. Furthermore, upon deletion of HVR1, H77 and S52 become completely resistant to anti-SR-B1 but J6-ΔHVR1 remained similarly sensitive compared to WT virus. It was then theorized the discrepancy in their data from the previous report in (50) was from differences in 2a isolate used. It would be interesting to determine if there is also a difference between the J6 and JFH-1 isolates used in my thesis in their response to C167.

4.6 Conclusions.

Overall my data indicates that HVR1 is a major determinant of isolate-specific neutralization sensitivity between the J6 and JFH-1 viruses. Linear neutralizing epitopes within HVR1 from either J6 or JFH-1 are not a direct target of vaccine-induced antibodies. This indicates HVR1 is indirectly affecting the differential

neutralization sensitivity of these two highly related HCV strains. HVR1 affects the general overall accessibility of several known neutralizing epitopes and participates in isolate-specific interactions with entry receptors, as supported by my data and others (49, 50, 54, 56). J6 HVR1 was shown to directly interact with SR-B1, while my data also indicates that JFH-1 may have a greater affinity for CD81 than J6. A potential model that explains these findings in the context of the current literature is presented in Figures 4.1, 4.2 and 4.3. There are three prevailing explanations in the literature for HVR1's involvement in isolate specific neutralization response and interactions with SR-B1, however, they may not be mutually exclusive. One explanation that was recently proposed suggests HVR1 is capable of inducing a conformation change in the E2 protein that is able to "shield" neutralizing epitopes (56). It has been theorized that HVR1 binding to the SR-B1 receptor may induce a conformational change in the E2 protein that allows for subsequent binding to the CD81 (198). HVR1 could keep the virus in a "shielded" conformation until the virus binds with SR-B1, hence keeping these conserved residues involved in CD81 binding (that are also a target of broadly neutralizing antibodies) protected until the virus is primed to enter the cell (198). My findings extend this theory and suggest that if this mechanism is utilized by HCV it may vary depending on the isolate. J6 virus appears to require an HVR1 specific interaction with SR-B1, while H77 and JFH-1 virus do not. HVR1-SR-B1 interaction may induce a conformational change in the J6 virus that then allows further binding to CD81, with the J6 virus adopting a closed conformation prior to binding SR-B1 (Figure 4.1). Furthermore, the JFH-1 virus may utilize a modified version of this mechanism. To compensates for the lack of HVR1 binding SR-B1, JFH-1 has a partially exposed conformation, and the JFH-1 virus has evolved an increased affinity for CD81 through mutations in the E2 protein to compensate (Figure 4.2).

An alternate mechanism suggested by the literature involves the association of the virus with lipoproteins. Studies have shown that deletion of HVR1 results in altered density profiles of infectious particles from multiple genotypes and the enhancement of viral infectivity by HDL is related to the presence of HVR1 (49,

50, 53), suggesting an HVR1 dependent role for the association of lipoproteins with viral particles and an HVR1 dependent role of lipoproteins in the infectivity of HCV. The lipid transfer function of SR-B1 is involved in the enhancement of infection independent of E2/SR-B1 interactions (110). HDL enhancement is, however, dependent on the presence of HVR1 on viral particles (49). JFH-1 virus in particular has been shown to have a neutralization sensitivity dependent on the density (and theorized lipoprotein content) of infectious particles, where high density fractions are more sensitive to neutralization than low density fractions (53). This pattern was only observed for the JFH-1 virus and none of the other genotypes tested in that study. Additionally, JFH-1 virus was recently shown to have a lower buoyant density than J6 virus (116). The differential association with lipids could be an additional factor in the isolate specific neutralization sensitivity observed between J6 and JFH-1 virus dependent on the HVR1.

A third potential explanation could be the J6 HVR1 interaction with SR-B1 allows for an alteration of the kinetics of entry of the J6 virus, leading to increased kinetics of entry into hepatocytes (Figure 4.3). This would decrease the time the J6 virus is exposed to antibodies compared to JFH-1 and could potentially explain the relative resistance to neutralizing antibodies observed for the J6 virus.

4.7 Future Directions

The role of lipoproteins was not investigated in this study and given the evidence of HDL involvement in HCV interactions with SR-B1 cited above, it would be a logical next step to determine the role HDL plays in the isolate specific differences in neutralization sensitivity between J6 and JFH-1. To determine buoyant density of infectious virions, density gradient ultracentrifugation could be performed using methods described in several studies (48, 53, 114, 116, 192). Since Huh7.5 cells grown in FBS are known to be deficient in lipoprotein production, virus could be produced in the presence of human sera components as described in Steenbergen et al. (201). Density of infectious particles of both J6 and JFH-1 could then be compared, with lower density infectious peaks indicating

higher association with lipoproteins. In a recent paper the buoyant density of JFH-1 E2 mutations were compared to JFH-1 WT as well as J6 virus (116). While they found no difference in buoyant density of infectious particles of JFH-1 mutations compared to WT, their data indicates a difference in buoyant density of JFH-1 to J6. J6 has a slightly higher density of infectious particles. This could indicate a potential difference in lipoprotein association between J6 and JFH-1. HCV particles isolated from cells grown in human serum have been shown to have higher association with lipoproteins than particles grown in FBS (201). The difference observed in (116), could be augmented by using this system that is more physiologically relevant (201). Subsequently, the various densities of virus particles could be assessed for their sensitivity to neutralizing antibodies.

HVR1 modified virus interactions with SR-B1 could be investigated further. The comparative sensitivity of the J6 WT, J6-ΔHVR1 and J6-JFH-1 HVR1 viruses against the highly effective C167 antibody would give some insight into the HDL utilization of the recombinants, as C167 is known to inhibit HDL binding with SR-B1 (200). Additionally, while direct incorporation of ApoE has been confirmed to not be directly related to HVR1 (23, 54), the role of HVR1 has in the association of other apolipoproteins with HCV particles has been poorly investigated. These lipoproteins may account for the differences in infectious particle buoyant density observed when HVR1 is deleted from multiple genotypes (53).

While blocking SR-B1/HCV particle interaction is a way to probe the involvement of HVR1 on SR-B1 dependence, a cell line lacking the SR-B1 could be used to discover if J6 HVR1 recombinant virus has a decreased dependency on SR-B1 for cell entry. Recently an SR-B1 knock out cell line was developed and it was demonstrated that, for the JFH-1 virus, the SR-B1 and LDLR are performing redundant functions (117). Based on my results showing binding of SR-B1 to J6 HVR1 but not JFH-1 HVR1, it would be worthwhile to determine if the SR-B1 and LDLR redundancy observed was for JFH-1 virus only or if it extends to other isolates and genotypes as well.

The isolate specific binding of SR-B1 observed in this study could be extended to investigate if genotype specific differences in SR-B1 binding are also occurring. Peptides of the HVR1 regions from all HCV genotypes could be tested using the ELISA described in this study. Additionally, it has been suggested that SR-B1 may interact with E2 outside of HVR1 despite evidence that HVR1 is critical for SR-B1 binding (50, 116). This could be a factor that depends specifically on the isolate or genotype tested, since in Zuiani et al. (116) the genotype used was JFH-1 while in Bankwitz et al. (50) the JC1 isolate was used. My data indicates that HVR1 of J6 and JFH-1 differentially interact with SR-B1. It would be valuable to investigate if SR-B1 binding to amino acids throughout E2 is also genotype-specific. Peptides could be designed to map SR-B1 binding to the E2 protein for all seven genotypes and then tested via ELISA.

Additionally, it is likely that the determinants located in the E2 protein are interacting in a complex way in the specific context of the E1 and E2 proteins, such that both the E1 and E2 proteins function together in an isolate specific manner. J6-JFH-1 E2 virus did not show a sensitive phenotype to the same degree as the JFH-1 WT virus and so it is likely that E1 is also involved in the sensitivity of the JFH-1 virus. This has been shown to be the case in a study the investigated the importance of the isolate specific context of the E1 and E2 proteins in interactions with entry receptors for two 1a isolates (35). Similarly, resistance of the J6-1a HVR1 virus to 1a E1/E2 antisera when it was predicted to be sensitive to neutralization indicates a potential genotype-specific interaction of HVR1 with the E1 and/or E2 proteins that would be of interest to investigate further.

Lastly, as our lab is focused on the development of a global vaccine candidate capable of eliciting an effective antibody response against all seven genotypes, my results can be applied to the rational design of a second generation E1/E2 glycoprotein component. My data provides further justification for the need to include antigens from multiple genotypes in a single vaccine, as the antibody response elicited by just 1a E1/E2 shows a relative inability to neutralize virus containing either the 2a J6 and 3a S52 HVR1 despite having elicited antibodies that are capable of neutralizing virus lacking HVR1.

4.8 Figures and tables

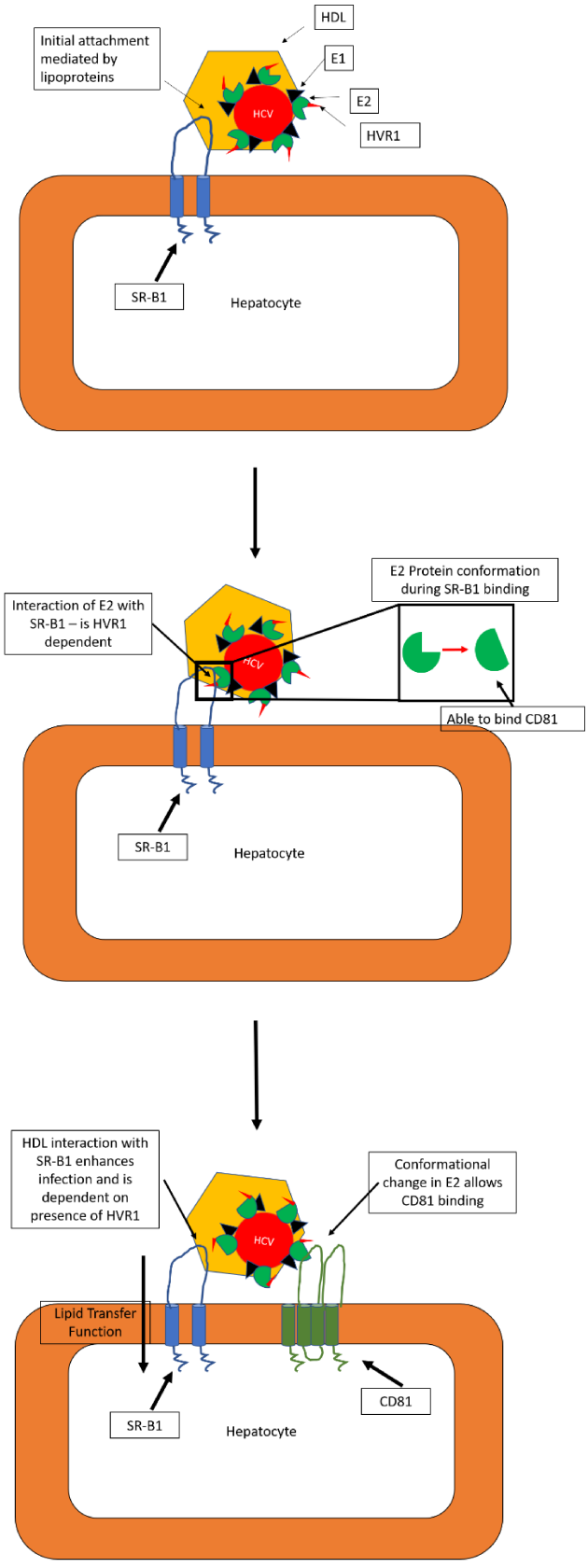

Figure 4.1. J6 isolate-specific E2 protein interaction with SR-B1. After initial attachment to the SR-B1 mediated by lipoproteins (48) there is an E2 specific interaction with the SR-B1 for the J6 virus that is dependent on HVR1 (48, 50). HVR1 binds directly to SR-B1 and this may mediate a conformational change that allows subsequent binding to the CD81 (50, 54, 197). Additionally, HDL facilitates entry enhancement by utilizing the lipid transfer function of SR-B1 dependent on the presence of HVR1 on viral particles (48, 49, 108).

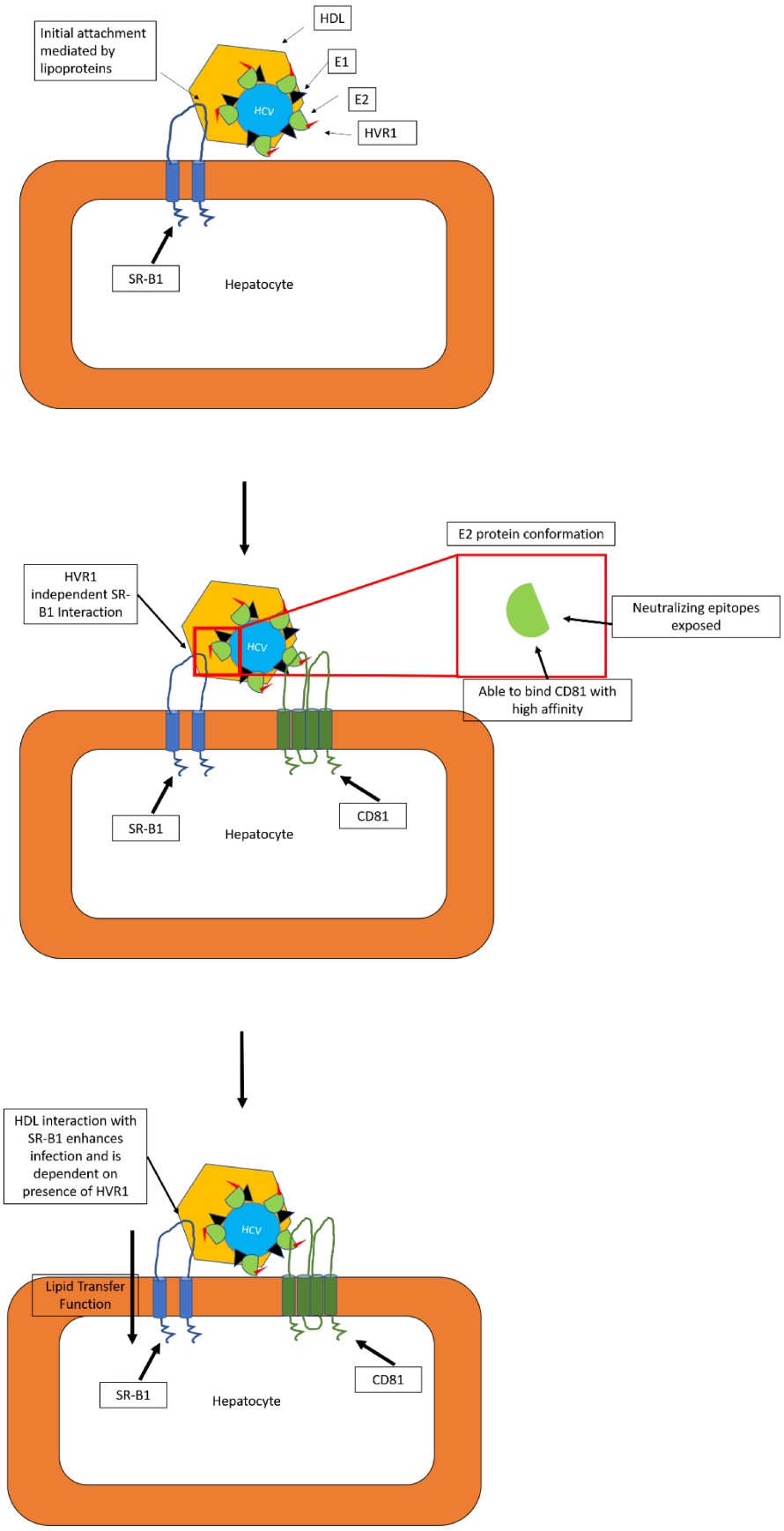
Initial attachment mediated by lipoproteins
HDL
E1
E2
HVR1
HCV
SR-B1
Hepatocyte
E2 protein conformation
HVR1 independent SR-B1 Interaction
Neutralizing epitopes exposed
Able to bind CD81 with high affinity
CD81
HDL interaction with SR-B1 enhances infection and is dependent on presence of HVR1
Lipid Transfer Function

Figure 4.2. JFH-1 isolate-specific interaction with SR-B1. Initial interaction with SR-B1 is facilitated through lipoproteins (48). HVR1 does not bind SR-B1 directly and E2 protein is able to bind CD81 without this prior interaction with SR-B1. The open conformation of the JFH-1 virus provides less protection from neutralizing antibodies and thus is sensitive to neutralization by monoclonal antibodies and vaccine antisera. As a compensation the JFH-1 virus has evolved a higher affinity for the CD81, although this is not as effective at the protection from antibodies as the J6 HVR1. Lastly, HDL facilitates entry enhancement by utilizing the lipid transfer function of SR-B1 dependent on the presence of HVR1 on viral particles (48, 49, 108).

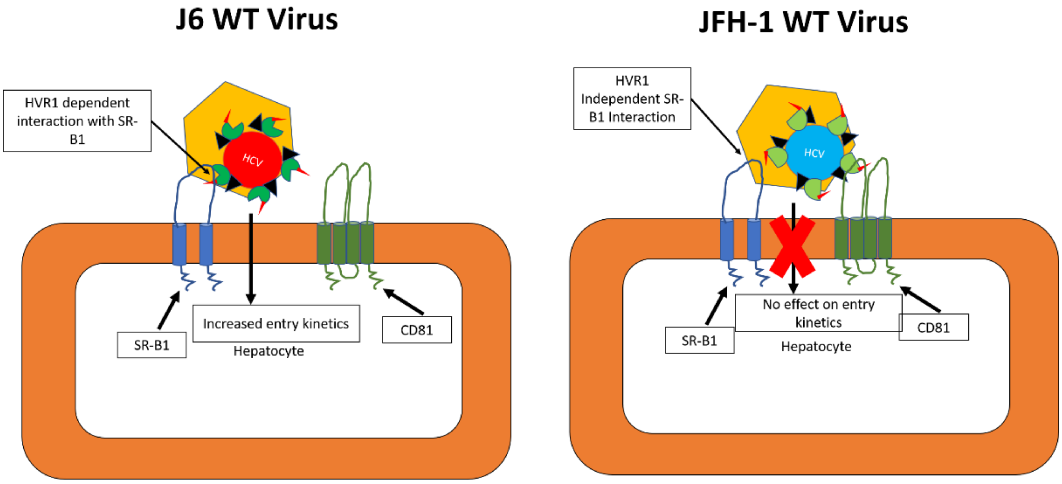

Figure 4.3. HVR1 specific interaction with SR-B1 results in altered entry kinetics for J6 virus. J6 virus HVR1 interacts with SR-B1, as shown in Figure 3.12, but JFH-1 HVR1 does not. This specific interaction with of HVR1 with SR-B1 for the J6 virus may result in altered kinetics of entry that allow the J6 virus entry into cells to proceed faster, thus decreasing the time the virus is exposed to neutralizing antibodies. While JFH-1 virus, which does not have this HVR1 specific interaction with SR-B1, has decreased entry kinetics compared to J6 virus and increased exposure time to neutralizing antibodies, thus increasing its sensitivity to neutralization.

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Appendix

Supplementary Figures

	Virus			
Dilution	J6-JFH-1 E1	J6-JFH-1 E2	JFH-1-J6 E1	JFH-1-J6 E2
1/100	ns	ns	ND	*

Supplementary Figure 1. Statistical significance of E1 and E2 chimeric virus compared to JFH-1 WT virus for neutralization by 1a E1/E2 antisera. The statistical significance of the difference in 1a E1/E2 neutralization was calculated with a one-way ANOVA using Graphpad Prism 7 software for the difference of each chimeric virus construct from WT virus. Statistically significant difference was denoted by "*'s" such that a p-value <0.0001 is indicated by ****, p-value < 0.001 is indicated by ***, p-value < 0.01 is indicated by **, p-value < 0.05 is indicated by * and "ns" indicates no significance. ND indicates no data available.

	Virus					
Dilution	JFH-1 WT	J6-ΔHVR1	J6-JFH-1 HVR1	J6-1a HVR1	J6-3a HVR1	JFH-1-J6 HVR1
1/100	*	****	****	ns	ns	ns
1/200	ns	****	***	ns	ns	ns
1/400	ns	***	ns	ns	ns	ns
1/800	ns	****	**	ns	ns	ns
1/1600	ns	****	ns	ns	ns	ns
1/3200	ns	****	***	ns	ns	ns
1/6400	ns	**	ns	ns	ns	ns
1/12800	ns	*	ns	ns	ns	ns

Supplementary Figure 2. Statistical significance of JFH-1 and HVR1 chimeric virus compared to J6 WT virus for neutralization by 1a E1/E2 antisera. The statistical significance of the difference in 1a E1/E2 neutralization was calculated with a two-way ANOVA using Graphpad Prism 7 software at each dilution in the 2-fold dilution series. Statistically significant difference was denoted by "*'s" such that a p-value <0.0001 is indicated by ****, p-value < 0.001 is indicated by ***, p-value < 0.01 is indicated by **, p-value < 0.05 is indicated by * and "ns" indicates no significance.

	Virus				
Dilution	H77 1a HVR1	JFH-1 HVR1	J6 HVR1	JFH-1 aa 412-443	J6 aa 412-443
1/50	****	ns	ns	****	****
1/150	****	ns	ns	****	****
1/450	****	ns	ns	****	****
1/1350	ns	ns	ns	****	****
1/4050	ns	ns	ns	***	****
1/12150	ns	ns	ns	ns	**
1/36450	ns	ns	ns	ns	ns
1/109350	ns	ns	ns	ns	ns

Supplementary Figure 3. Statistical significance of 1a E1/E2 antisera binding to various HVR1 peptides or peptides of J6 and JFH-1 amino acids 412-443 compared to scramble control peptide for all dilutions. The statistical significance of the difference in 1a E1/E2 binding was calculated with a two-way ANOVA using Graphpad Prism 7 software at each dilution in the 3-fold dilution series. Statistically significant difference was denoted by "*'s" such that a p-value <0.0001 is indicated by ****, p-value < 0.001 is indicated by ***, p-value < 0.01 is indicated by **, p-value < 0.05 is indicated by * and "ns" indicates no significance.

A AR1B

Dilution (µg/ml)	Virus			
	J6-ΔHVR1	J6-JFH HVR1	J6-1a HVR1	J6-3a HVR1
50	ns	ns	ns	ns
10	ns	ns	ns	ns
2	ns	ns	ns	ns
0.4	ns	ns	ns	ns
0.08	ns	ns	ns	ns
0.016	ns	ns	ns	ns
0.0032	ns	ns	ns	ns
0.00064	ns	ns	ns	ns

B AR2A

Dilution (µg/ml)	Virus			
	J6-ΔHVR1	J6-JFH HVR1	J6-1a HVR1	J6-3a HVR1
50	****	****	ns	ns
10	****	ns	ns	ns
2	***	ns	ns	ns
0.4	*	ns	ns	ns
0.08	ns	ns	ns	ns
0.016	ns	ns	ns	ns
0.0032	ns	ns	ns	ns
0.00064	ns	ns	ns	ns

C AR3A

Dilution (µg/ml)	Virus			
	J6-ΔHVR1	J6-JFH HVR1	J6-1a HVR1	J6-3a HVR1
50	**	**	ns	ns
10	****	****	ns	ns
2	****	****	ns	ns
0.4	****	****	ns	ns
0.08	****	**	ns	ns
0.016	****	ns	ns	ns
0.0032	*	ns	ns	ns
0.00064	ns	ns	ns	ns

D AR4A

Dilution (µg/ml)	Virus			
	J6-ΔHVR1	J6-JFH HVR1	J6-1a HVR1	J6-3a HVR1
50	ns	ns	ns	***
10	****	****	**	ns
2	****	****	ns	ns
0.4	****	****	ns	ns
0.08	****	****	ns	ns
0.016	****	ns	ns	ns
0.0032	***	ns	ns	ns
0.00064	ns	*	ns	ns

E AR5A

Dilution (µg/ml)	Virus			
	J6-ΔHVR1	J6-JFH HVR1	J6-1a HVR1	J6-3a HVR1
50	****	****	ns	ns
10	****	****	ns	ns
2	****	****	ns	ns
0.4	****	****	ns	ns
0.08	****	ns	ns	ns
0.016	**	ns	ns	ns
0.0032	ns	ns	ns	ns
0.00064	ns	ns	ns	ns

Supplementary Figure 4. Statistical comparison of HVR1 deleted or chimeric virus to J6 WT virus for neutralization by monoclonal antibodies. The statistical significance of the difference in neutralization sensitivity was calculated with a two-way ANOVA using Graphpad Prism 7 software for AR1B **(A)**, AR2A **(B)**, AR3A **(C)**, AR4A **(D)** and AR5A **(E)** at each dilution in the 5-fold dilution series. Statistically significant difference was denoted by "*'s" such that a p-value <0.0001 is indicated by ****, p-value < 0.001 is indicated by ***, p-value < 0.01 is indicated by **, p-value < 0.05 is indicated by * and "ns" indicates no significance.

A Comparison to J6 WT

	Virus			
Dilution	JFH-1 WT	J6-ΔHVR1	J6-JFH-1 HVR1	JFH-1-J6 HVR1
0.5	ns	ns	ns	*
0.25	ns	ns	ns	**
0.125	ns	ns	ns	**
0.0625	ns	ns	ns	*
0.03125	ns	ns	ns	ns
0.015625	ns	ns	ns	ns
0.007813	ns	ns	ns	ns
0.003906	ns	ns	ns	*

B Comparison to JFH-1 WT

	Virus			
Dilution	J6 WT	J6-ΔHVR1	J6-JFH-1 HVR1	JFH-1-J6 HVR1
0.5	ns	ns	ns	ns
0.25	ns	ns	ns	ns
0.125	ns	ns	ns	ns
0.0625	ns	ns	ns	ns
0.03125	ns	ns	ns	ns
0.015625	ns	ns	ns	ns
0.007813	ns	ns	ns	ns
0.003906	ns	ns	ns	ns

C Comparison to J6-ΔHVR1

	Virus			
Dilution	J6 WT	JFH-1 WT	J6-JFH-1 HVR1	JFH-1-J6 HVR1
0.5	ns	ns	ns	*
0.25	ns	ns	ns	ns
0.125	ns	ns	ns	ns
0.0625	ns	ns	ns	*
0.03125	ns	ns	ns	ns
0.015625	ns	ns	ns	ns
0.007813	ns	ns	ns	ns
0.003906	ns	ns	ns	ns

D Comparison to J6-JFH-1 HVR1

	Virus			
Dilution	J6 WT	JFH-1 WT	J6-ΔHVR1	JFH-1-J6 HVR1
0.5	ns	ns	ns	**
0.25	ns	ns	ns	ns
0.125	ns	ns	ns	*
0.0625	ns	ns	ns	ns
0.03125	ns	ns	ns	ns
0.015625	ns	ns	ns	ns
0.007813	ns	ns	ns	ns
0.003906	ns	ns	ns	ns

E Comparison to JFH-1-J6 HVR1

	Virus			
Dilution	J6 WT	JFH-1 WT	J6-JFH-1 HVR1	J6-ΔHVR1
0.5	*	ns	**	*
0.25	**	ns	ns	ns
0.125	**	ns	*	ns
0.0625	*	ns	ns	*
0.03125	ns	ns	ns	ns
0.015625	ns	ns	ns	ns
0.007813	ns	ns	ns	ns
0.003906	*	ns	ns	ns

Supplementary Figure 5. Statistical comparison of WT, ΔHVR1 or HVR1 chimeric virus to each other for neutralization by anti-CD81. The statistical significance of the difference in neutralization sensitivity was calculated with a two-way ANOVA using Graphpad Prism 7 software for J6 WT **(A)**, JFH-1 WT **(B)**, J6-ΔHVR1 **(C)**, J6-JFH-1 HVR1 **(D)** and JFH-1-J6 HVR1 **(E)** at each dilution in the 2-fold dilution series. Statistically significant difference was denoted by "*'s" such that a p-value <0.0001 is indicated by ****, p-value < 0.001 is indicated by ***, p-value < 0.01 is indicated by **, p-value < 0.05 is indicated by * and "ns" indicates no significance.