PROBING THE INTERACTION OF HEPATITIS C VIRUS GLYCOPROTEINS WITH PUTATIVE RECEPTORS AND NEUTRALISING ANTIBODIES

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Abstract

Hepatitis C virus (HCV) is a hepatotropic blood-borne virus which causes chronic hepatitis in the majority of cases and represents a global health burden. In order for HCV to enter cells, proteins on the surface of the virus must interact and bind to receptors on target cells. HCV surface molecules involved with receptor binding, and cellular entry, as well as immune escape, are the glycoproteins E1 and E2. The cellular receptors SRBI, CD81, CLDNs and most recently occludin have been shown to facilitate HCV entry into hepatocytes. Several conservative regions on E1E2 have, through substitution mutagenesis, proven to be important for receptor binding and antibody neutralisation. We aimed to characterise one discontinuous region, amino acid residues 611, 613-619 and 621, and its role in the interaction with CD81 by single alanine substitution mutagenesis. Mutant plasmids were transfected into HEK 293FT cells and assessed for protein expression and binding by conformation-sensitive, CD81-inhibiting antibodies. Also, to investigate whether a conformational change of the E1E2 occurs upon SRB1 binding, rendering the CD81 binding domains accessible, two assays have been compared. A plate based experiment, exclusive of SRBI and a cell based assay, including SRBI was designed to examine the antigenic exposure of the CD81 binding regions to targeting monoclonal antibodies. Additionally, Huh-7 cells expressing different levels of SRBI were used to investigate whether some HCVpp isolates rely on high SRB1 levels for infectivity and sensitivity to neutralising antibodies. These studies were performed to help elucidate the

regions and residues important in HCV E1E2: receptor interaction and their interplay with each other and with neutralising antibodies.

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aa	Amino acid
AII	Angiotensin II
ALT	Alanine aminotransferase
AP	Alkaline Phosphatase
APO E	Apolipoprotein E
APOC-I	Apolipoprotein C-I
AR	Antigenic Region
BSA	Bovine serum albumin
CHO	Chinese Hamster Ovary
CLDN	Claudin
DAA	Direct- acting antiviral
DC-SIGN	Dendritic cell-specific Intercellular adhesion molecule-grabbing Non-Integrin
DNA	Deoxyribonucleic acid
E1	Envelope protein 1
E2	Envelope protein 2
EDTA	Ethylenediamenetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmatic Reticulum
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocynate
FRET	Fluorescence resonance energy transfer
GAG	Glycosaminoglycans
GlcNAc	N-acetylglucosamine
GNA	Galanthus nivalis agglutinin
HCV	Hepatitis C Virus
HCVcc	Hepatitis C virus cell culture
HCVpp	Hepatitis C virus pseudo particle
HDL	High density lipoprotein

HEK 293	Human embryonic kidney cells
HmAb	Human monoclonal antibody
HRP	Horse radish peroxidase
HS	Heparin Sulphate
Huh-7	Human hepatoma 7 cell line
HVR1	Hyper variable region 1
IC50	Maximum inhibitory concentration of 50 percent
IRES	Internal Ribosome Entry Site
JFH1	Japanese Fulminant Hepatitis clone 1
LB	Lysogeny Broth
LDL	Low density lipoprotein
LDLr	Low density lipid receptor
L-SIGN	Liver/Lymph node-specific Intercellular adhesion molecule-3-grabbing Integrin
mRNA	messenger RNA
NOB	Neutralisation of Binding
NS	Non structural
OCLN	Occludin
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEG-IFNα	Pegylated Interferon α
PNPP	Para-nitrophenyl phosphatise
RBV	Ribavirin
RNA	Ribonucleic acid
Rpm	revolutions per minute
RVR	Rapid Virological Response
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sE2	Soluble form of envelope protein 2
SIN	Sindbis virus
SOC	Standard of care
SRBI	Scavenger receptor class B type 1
SVR	Sustained Virological Response
TGF-β1	Transforming growth factor β1

Th1	T-helper cells 1
TJ	Tight Junction
TM	Transmembrane
TMB	'3,3',5,5' Tetramethylbezidine
Treg	T regulatory
VEE	Venezuelan equine encephalitis virus
VLDL	Very low density lipoprotein
WB	Western blot

1　General Introduction

1.1　HCV structure

1.1.1　Genome and proteins

Hepatitis C virus, a member of the hepacivirus genera of the Flaviviridae family is a small, enveloped ssRNA virus with six different genotypes and over 70 subtypes (Lavillette, Tarr et al. 2005). The virus genome consists of a 3000 amino acid long polypeptide which encodes the six non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B together with the three structural proteins; core, and envelope proteins E1 and E2. Most of the non-structural proteins are cleaved from the polyprotein by NS2 and NS3 proteases and helicase enzymes. It is believed that NS4A acts as a co-factor for NS3 (Lin, Thomson et al. 1995) and studies have shown that these two proteins are highly important for the virus in its effort to elude the host immune system (Lin, Lacoste et al. 2006).

The function of NS4B is yet to be fully understood however is suggested to participate in the formation of replication complexes. NS5B is the RNA dependent RNA polymerase, (Behrens, Tomei et al. 1996) an enzyme that replicates the viral genome and NS5A is possibly thought to be involved in virus replication and also a key factor in avoiding the immune system (Macdonald and Harris 2004). The core protein functions to form a nucleocapsid and package the virus RNA genome. E1 and E2 are glycoproteins on the viral envelope which are essential to virus attachment and entry

(Lavillette, Tarr et al. 2005). Finally, p7 is a small protein that is believed to create ion channels in the membrane to allow permeability and excretion (Lloyd, Jagger et al. 2007) All HCV- proteins have proven to be located in the cytoplasm apart from NS3, which have been found both in the cytoplasm and the nucleus (Melen, Fagerlund et al. 2004).
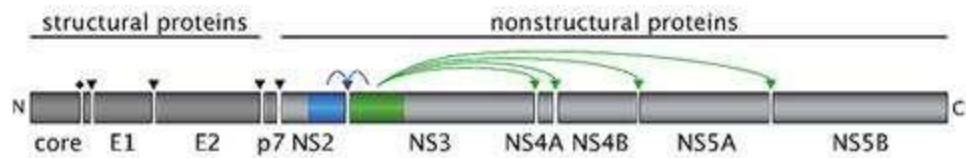
Figure 1.1. HCV polyprotein: Schematic of the HCV genome with the structural and non structural proteins.
The arrows demonstrates cleavage of the non-structural proteins by NS2 and NS3 proteases. (Lorenz et al. Crystal structure of the catalytical domain of the Hepatitis C virus NS2-3 Protease-2007).

1.1.2 HCV glycoproteins E1E2

Several published studies have reported HCV glycoproteins to be essential for cell entry (Voisset and Dubuisson 2004). These surface proteins bind cellular receptors as well as initiating fusion between the viral envelope and the cellular membrane at an initial stage of viral replication. The characterization and the antigenic structure of HCV envelope glycoproteins were first presented in 1993 (Ralston, Thudium et al. 1993) and since then it has been established that E1 (polyprotein residues 192 -383) and E2 (residues 384-746) (Flint, Thomas et al. 1999) form heterodimers that are attached to the viral lipid surface and consist of a N-terminal ectodomain and a hydrophobic C-terminal domain. HCV glycoproteins are thought to be class II fusion proteins, located at the endoplasmic reticulum (ER) (Voisset and Dubuisson 2004). During the formation of E1E2 the N-terminus of E1 is directed towards the ER lumen by a

signal peptide (Dubuisson, Penin et al. 2002). The C-terminal of the E1 transmembrane (TM) domain is located towards the ER lumen to permit E2 to relocate. The TM domain of E1 forms a hairpin loop before a signal peptidase cleaves between E1 and E2 and the C-terminus of E1 TM domain is redirected back towards the cytosol. By doing so, the hydrophobic TM end of E1 and E2 is not only suggested to contribute to the stability of the glycoprotein on the membrane but also to ER retention, heterodimerization, budding and virus entry. The ectodomains are highly modified through N-linked glycosylation. In fact, E1 and E2 present several glycosylation sites which are considered to be of great importance to the folding and conformation of the proteins (Voisset and Dubuisson 2004; Lavillette, Tarr et al. 2005).

1.1.2.1 HVR1

The hyper variable region 1, HVR1, is located on the N-terminus of E2 and consists of 27 amino acids. This sequence is highly prone to amino acid substitutions and is subjected to positive selection pressure applied by anti-HCV immune responses (Penin, Combet et al. 2001), explaining its strong variability across HCV isolates and genotypes. The function of HVR1 is not yet known, however its location on the surface of the glycoproteins has implied its participation in viral entry and therefore it is not believed that HVR1 is randomly variable. Penin et al proposed that HVR1 is always present in the HCV strains that infect humans, implying that the strains containing the full-length HVR1 holds a stronger survival advantage over the strains lacking intact HVR1. Regardless of variable amino acid substitutions, the chemophysical structure and conformation of HVR1 is conserved. HVR1 has

shown to be a basic stretch with the positive charged amino acids placed at specific conserved positions indicating the possibility of interaction with negatively charged molecules that might play a role in the virus entry process, such as the viral receptors and other molecules, for example lipids, GAGs (glycosaminoglycans) and other proteins (Dubuisson, Helle et al. 2008)

1.2 HCV lifecycle and replication

The HCV virion is approximately 70 nm in size and mainly consists of the core protein and the two envelope glycoproteins E1 and E2. The glycoproteins are believed to be attached to a host cell-derived, double-layered lipid envelope which shields a nucleocapsid. The nucleocapsid consists of core and the viral RNA genome (Moradpour, Penin et al. 2007).

Once the virus has entered the hepatocyte, the genome is released and uncoated in the cytoplasm as cap-independent, IRES- (internal ribosome entry site) mediated translation occurs. The polyprotein is thereafter processed by host-, as well as viral proteases to generate functional structural and non-structural HCV proteins. NS3, NS4A, NS4B, NS5A and NS5B gather into a vesicular structure called the membranous web, where it is thought that viral replication occurs and newly positive stranded genomes are generated. The HCV genome is assembled and packaged into new, mature virions before release from the cell (Fields Virology2007; Rice 2011).
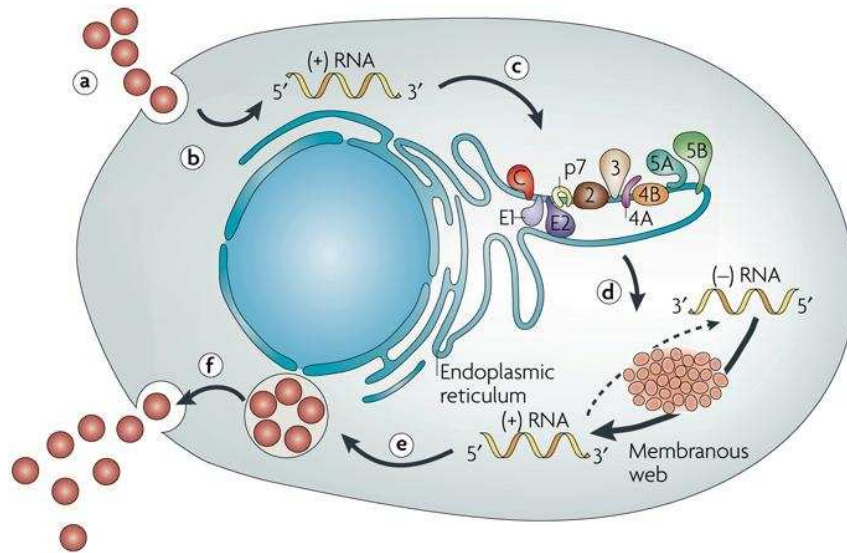
Figure 1.2: HCV life cycle
Once the virus has entered the hepatocyte, b) the genome is released and uncoated in the cytoplasm as cap-independent, IRES- (internal ribosome entry site) mediated occurs. The polyprotein is thereafter processed c) by host-, as well as viral proteases.The non-structural proteins gather into a vesicular structure called the membranous web d) where the viral replication occurs and newly positive stranded genomes are generated. The HCV genome is assembled and packaged e) into new, mature virions before released from the cell f) (Mouradpour, D 2007-Replication of Hepatitis C virus)

1.3 Prevalence and Epidemiology

It is estimated that 170 million people around the world are infected with Hepatitis C virus (HCV) (WHO 1999). Of those, 60-85% fail to clear the infection (Hoofnagle 2002; Lehmann, Meyer et al. 2004; van den Berg, Grady et al. 2011) and become chronic carriers of the virus. The prevalence reports of HCV infection depends highly on seroprevalence studies. These are cross-sectional studies that normally rely on results from blood donors or patients with chronic liver disease that does not fully represent the region in which they inhabit. It would be preferable to collect data from population-based studies;

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however such studies have been proven difficult to carry out in certain parts of the world (Shepard, Finelli et al. 2005).

In some global areas such as China, Indonesia, Thailand, Vietnam and certain areas of Africa the incidence of HCV is high. Most prevalence reports from the developing country are rough estimates with high variability. This is due to the fact that there are usually less data available to strengthen postulations regarding HCV infection in the third world countries. This wide scope of prevalence is noticeable when evaluating the estimates from highly populated developing countries. (PopulationReferenceBureau 2004) It is reported that China, a nation with a fifth of the world's inhabitants, has a seroprevalence of 3.2% (Xia, Liua et al. 1996). A community –based study reports a prevalence of 0.9% in India (Chowdhury, Santra et al. 2003) whereas in Indonesia HCV infection has an occurrence of 2.1%; however this number is based on a study of voluntary blood donors (Sulaiman, Julitasari. et al. 1995). Countries with the lowest reported prevalence rates include northern and western Europe, industrialised nations in North America, and Australia (WHO 1999). The HCV infection rate in Germany is believed to be 0.6 % (Palitzsch, Hottentrager et al. 1999), in Canada it is 0.8 % (Zou, Tepper et al. 2000) in France it is 1.1 % (Desenclos 2000) and in Australia it is 1.1 % (Law, Dore et al. 2003). In Pakistan, the reported data suggests that the prevalence rate ranges between 2.4 %-6.5 % (Luby, Qamruddin et al. 1997; Mujeeb, Shahab et al. 2000; Sultana, Qazilbash et al. 2000; Khattak, Salamat et al. 2002). Egypt, with a population close to 73 million people, has the highest HCV prevalence in the world with 22 % of the population infected (Frank, Mohamed et al. 2000).
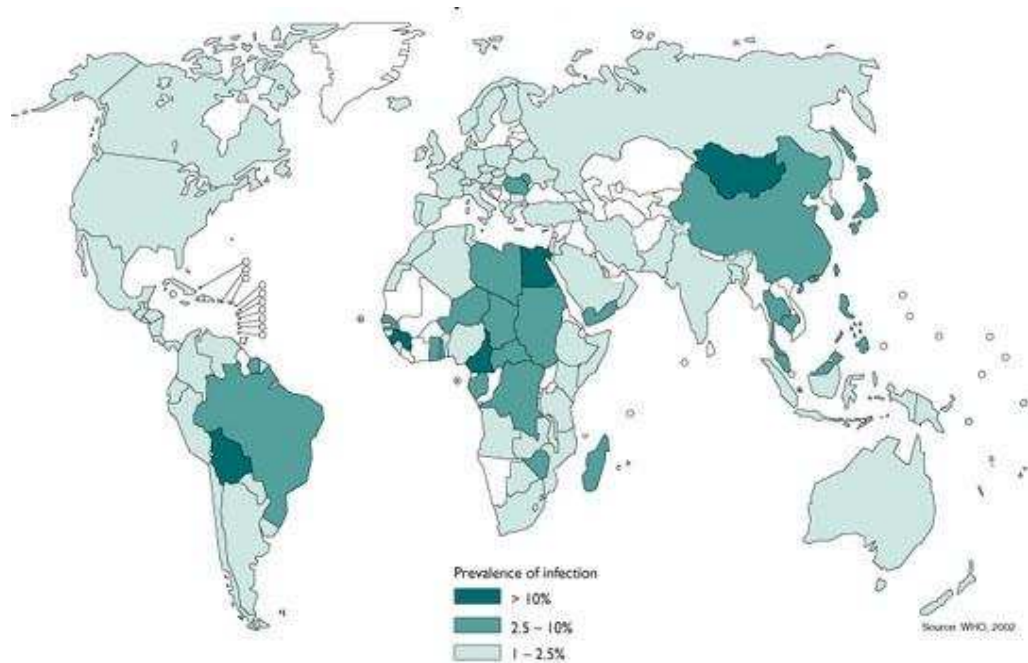
Figure 1.3: The prevalence of Hepatitis C virus worldwide (WHO 2002).

1.3.1 Transmission routes

The most efficient transmission route of Hepatitis C Virus is through large exposures to blood, e.g. blood transfusion and organ transplants, however, intravenous drug use has been shown shown to be the dominant risk factor worldwide (Centers for Disease Control and Prevention, 1998). There are reported cases of occupational, parenteral and sexual HCV transmission, however these modes of transmission are much less efficient and would therefore not be considered as the main sources for new incidences of HCV infection, despite of population size or country (Centers for Disease Control and Prevention, 1998; Terrault 2002). Moreover, there is evidence of environmental HCV transmission sources such as reported cases of re-usage of contaminated needles and syringes, medication vials and infusion bags,

however these are more likely to occur in under-developed countries (Hagan, Thiede et al. 2001; Williams, Perz et al. 2004).

1.4   Immune responses to Hepatitis C infections

It is believed that the evasion of the host immune responses enables HCV to persist in the host (Heim 2009; Thimme, Binder et al. 2011). There are different mechanisms in which the virus is able to interfere with both the innate and adaptive immune system.

1.4.1.1   HCV and the innate immunity

Interferons (IFNs) are cytokines that are important in the antiviral defence of the innate immunity. IFNs can be divided into three groups; type I IFNs, type II IFNs and type III IFNs (Pestka, Krause et al. 2004). In response to invading viruses, cells produce IFN-αs and IFN-β, belonging to the group of type I interferons. As most viruses consist of components originated from the host, these IFNs have evolved to detect viral genomes and nucleic acid. Two pathways that act to detect viral genomes are the toll-like receptor (TLR) dependent pathway (Iwasaki and Medzhitov 2004; Akira, Uematsu et al. 2006) and the cytosolic pathway that includes the interaction of viral RNA and the RNA helicases retinoic acid inducible gene-1(RIG-1) as well as the melanoma differentiation antigen 5 (MDA5) (Yoneyama and Fujita 2007). The activation of TLRs leads to the induction of a signalling cascade that engages NF-KB and a range of interferon regulatory factors (IRFs) both crucial transcription factors (Heim 2009).

The initiation of the type I IFN cytosolic pathway begins with the recognition of viral 5'triphosphate RNA as well as dsRNA by RIG-I and MDA5. This will trigger a conformational change of RIG-I and MDA5 which consequently will lead to the binding of the essential adaptor in the cytosolic pathway, MAVS (Cardif/IPS-1/VISA) (Meylan, Curran et al. 2005; Seth, Sun et al. 2005). This interaction results in the activation of IRF3 and NF-κβ, which subsequently bind to the IFN-β gene promoter to induce transcrption of the gene and secretion of IFN-β. Upon secretion, IFN-β binds to IFNAR, the interferon alpha receptor, which will activate the JAK/STAT pathway. This activation will result in a vast and efficient antiviral state of the infected cells and the adjacent area (Heim 2009). HCV NS3/4A has shown to interfere with the RIG-I and the TLR3 pathways by cleaving and subsequent inactivating MAVS and TRIF, (Li, Foy et al. 2005; Meylan, Curran et al. 2005) two essential adaptor proteins in these pathways. This interference which leads to the inactivation of these pathways could be one of the causes of viral persistence. However, there is evidence which show that HCV can enhance the IFN system despite the inactivation of MAVS and TRIF in the liver (Bigger, Brasky et al. 2001).

Type I interferons bind to the same interferon alpha/beta receptor (IFNAR). This receptor has two subunits, IFNAR1 and IFNAR2c which will individually bind a specific member of the Jak (Janus kinase) family (Heim 2009). IFNAR1 binds tyrosine kinase 2 and IFNAR2c binds to Jak1. Following the binding of type I IFN to IFNAR1 and IFNAR2c, TYK2 and Jak1 will initiate an activation process that involves the tyrosine phosphorylation of receptors and signal transducer and activator of transcription (STAT) 1, STAT2 and STAT3.

STAT1 and STAT2 together with IRF9, another transcription factor, make up interferon stimulated gene factor 3 (ISGF3) that on the promoter site, binds to interferon stimulated response element (ISREs) to induce the transcription of the numerous genes that are able to generate an antiviral state (Darnell, Kerr et al. 1994; Darnell 1997).

In attempts to evade eradication, HCV core is thought to up-regulate suppressor of cytokine signalling (SOCS) 3 proteins. SOCS can regulate the Jak/STAT signalling by a negative feedback loop. Another mechanism by which HCV is proposed to bypass the immune responses is via a proteasome-dependent degradation of STAT1 (Lin, Choe et al. 2005).

A published paper showed a reduced expression of STAT3 in the livers of chronically infected patients. The same group also noticed HCV hindered IFN-α induced phosphorylation of STAT1, STAT2 and STAT3 (Larrea, Aldabe et al. 2006).

Protein kinase R (PKR) is an interferon stimulated gene with broad antiviral effects. PKR phosphorylates eukariotic initiation factor 2a (eIF2a) to subsequently hinder the translation of various cellular and viral mRNAs. HCV NS5A has shown to interfere with the activation of PKR in cell culture experiments (Gale, Korth et al. 1997). Moreover, HCV E2 glycoprotein is able to bind PKR at the eIF2a binding site and subsequently hinder the inhibitory role of PKR on the translation process (Taylor, Shi et al. 1999).

1.4.1.2 HCV and T-cell response

As previously mentioned, a robust cellular immune response is believed to be the key behind the clearance of HCV infections. The alteration of HCV epitopes is a way of escaping recognition by neutralising antibodies and T-cells. Existing reports demonstrates the function of $CD4^+$ and $CD8^+$ T cells in HCV disease outcome (Diepolder, Zachoval et al. 1995; Lechner, Wong et al. 2000; Thimme, Oldach et al. 2001). In HCV infection, $CD8^+$ T-cells act as effector cells that regulate the immune response to the virus. $CD4^+$ T-cells helps to maintain the $CD8^+$ T-cell response and have been proven to have a crucial role in the long-term management of the infection (Shoukry, Grakoui et al. 2003; Klenerman and Hill 2005). Programmed cell death receptor, PD-1 has been noticed to increase on the $CD4^+$ and the $CD8^+$ T-cells in chronic infection. This observation has been linked to the T-cell exhaustion noticed in chronically infected patients (Wherry, Blattman et al. 2003; Radziewicz, Ibegbu et al. 2007; Kasprowicz, Schulze ZurWiesch et al. 2008).

1.4.1.2.1 CD4+ T-cell response

$CD4^+$ T-cells have important roles in the cellular and the humoral immune response which include the activation of antigen presenting cells, dispensing co-stimulatory signals to B-cells and aid in the maintenance of the $CD8^+$ T-cell response. Isolated $CD4^+$ T-cells from livers of chimpanzees acutely infected with HCV were shown to recognise and proliferate in response to HCV proteins. In contrast, these cells were absent in chimpanzees with chronic infection (Thimme, Bukh et al. 2002). Moreover; a published study which

included the depletion of CD4$^+$ T-cells from a chimpanzee that had been able to clear the virus and the re-infection of HCV resulted in chronic low level viremia in the same chimp. However, the liver exhibited a strong CD8+ T-cell response (Grakoui, Shoukry et al. 2003).

 A vigorous CD4+ T-cell response has shown to play a role in spontaneous clearance of HCV (Gerlach, Diepolder et al. 1999; Lechner, Wong et al. 2000; Rosen, Miner et al. 2002). The specificity of T- cells, have in reports, been demonstrated to be of importance as CD4$^+$ T-cells obtained from patients which have been able to clear infection, proved to recognise 10 of the 37 confirmed HCV epitopes, while the CD4$^+$ T-cells from chronically infected individuals showed to recognise only one (Day, Lauer et al. 2002). An additional paper examined the effect of CD4$^+$ T-cell response in the clearance of HCV infection and found that in a robust, broad and Th1 adjusted CD4$^+$ T-cell response were present in individuals that had been able to resolve infection. In contrast, weak and poor response was observed in patients that progressed to chronicity (Urbani, Amadei et al. 2006).

1.4.1.2.2 CD8+ T-cell response

CD8$^+$ T- cells utilises two mechanisms in the antiviral defence process. The first mechanism involves the abolition of infected cells with the help of perforin and granzymes whereas the second one involves the secretion of antiviral cytokines, such as IFNγ and TNF-α. As with the CD4$^+$ T-cells, CD8$^+$ T-cells have been shown to play an important role in clearing the virus. A correlation has been observed with elevated levels of virus specific CD8$^+$ T-

cells in the liver and a transient clearance of HCV RNA (Cooper, Erickson et al. 1999; Thimme, Bukh et al. 2002). Existing studies have shown that chimpanzees which exhibited poor CD8$^+$ T-cell responses progressed to chronic infection. Additionally, findings from a published report show that when CD8$^+$ T- cells were depleted from animals with resolved infection, upon re-infection, the animals developed long-term viremia regardless of the presence of memory CD4$^+$ T-cells. Moreover; 6 weeks following re-infection, when the levels of CD8$^+$ T-cell expression in the liver was restored, the viral replication was discontinued (Shoukry, Grakoui et al. 2003).

The capacity of CD8$^+$ T cells to recognise various epitopes amongst the HCV non-structural and structural proteins have been linked to viral clearance (Cucchiarini, Kammer et al. 2000; Lechner, Wong et al. 2000; Thimme, Bukh et al. 2002; Cox, Mosbruger et al. 2005). T-cells recognising few epitopes have been noticed in patients with persistent infection (Lechner, Wong et al. 2000; Lauer, Barnes et al. 2004).

One T-cell epitope specifically has shown to be of importance in clearance of HCV infection. Following a study involving a cohort of patients with HCV infection, identified a correlation between an epitope shielded by the HLA-B*27 allele, and clearance of the virus. This epitope, within the HCV NS5b protein, was still targeted by CD8$^+$ T-cells in five out of six patients that were able to clear the virus nearly 30 years previously. Alteration to this epitope by amino acid substitution resulted in HCV chronicity (Neumann-Haefelin, McKiernan et al. 2006).

The cause behind the impairment of $CD4^+$ and CD^+8 T- cell responses in chronic infection is not fully known, however it has been proposed T-cell exhaustion and viral escape from T-cell recognition are involved mechanisms. Viral escape refers to the viral mutations which occur in the T-cell epitopes upon T-cell- driven selective pressure. T- cells are, however, capable of recognising autologous HCV, therefore this mechanism alone is not a sufficient explanation to the poor T-cell response seen in chronic infection (Halliday, Klenerman et al. 2011).

1.4.1.3 Humoral immune response

Within the first trimester of Hepatitis C infection, circulating antibodies become detectable in the infected host (Schmilovitz-Weiss, Levy et al. 1993). An association was observed between the eradication of HCV and a robust production of high-titer of cross- neutralising antibodies (Pestka, Zeisel et al. 2007).

In contrast, chronic infections are also able to present a high- titer of neutralising antibodies, however, in these cases, neutralising antibodies alone are not sufficient to control the infection (Bartosch, Bukh et al. 2003). The HCV E1E2 proteins show the greatest genetic diversity on the virus and is therefore more likely that these genetic discrepancies in the E1E2 proteins of quasispecies that permits the virus to evade the targeting neutralising antibodies (von Hahn, Yoon et al. 2007).

There is evidence of various additional ways HCV utilised to escape the antibody response. Such ways include the direct cell-to-cell transfer (Timpe,

Stamataki et al. 2008), the induction of certain antibodies that hinder the neutralising antibodies and the protective glycosylation of neutralising epitopes on the E1E2 glycoproteins

1.5 Clinical Features

Hepatitis C virus is primarily a hepatotropic virus; however low levels of viral RNA has been detected in B- and T-cells. The disease tends to manifest in a variety of symptoms ranging from mild hepatitis to cirrhosis, and in very severe cases hepatocellullar carcinoma (Diedrich 2006).

1.5.1 Acute HCV infection

The definition of acute HCV infection refers to a new occurrence of viremia that converts a negative blood status to a HCV-RNA positive status. The acute phase of HCV infection falls into the initial six months after exposure where spontaneous clearance is achievable (Thomas and Seeff 2005). Acute stage of infection usually is asymptomatic however; some patients have exhibited symptoms that include nausea, malaise, and fatigue. These symptoms are common with acute Hepatitis A and or B and can only be distinguished by specific clinical viral tests. Viral RNA can be detected in acute HCV infections, within one to two weeks of exposure to the virus. Seroconversion, the occurrence of HCV specific antibodies, usually develops during the window period of infection, which is two to six months after exposure. Anti-HCV testing is therefore un-reliable in the early detection of acute infections;

consequently HCV-RNA assessments are preferable in the identification of acute HCV infections (Chung 2005; Thomas and Seeff 2005; Kamal 2008).
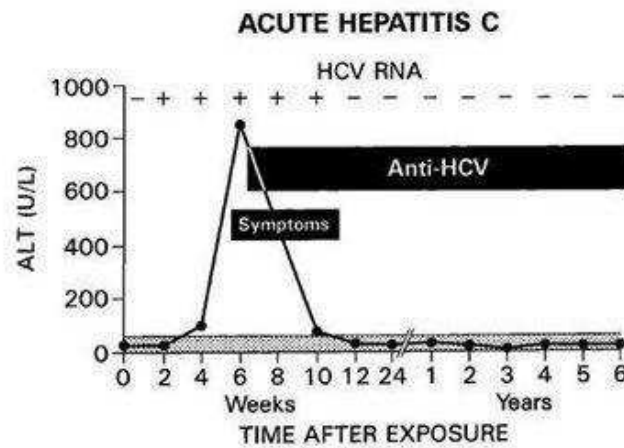
Figure 1.4: Schematic of anti-HCV antibody response in acute HCV infection (Hoofnagle 1997).

Patients that have proven able to clear HCV infection at the acute phase account for approximately 15 % to 40 % of acutely infected individuals. They are usually Caucasian with noticeable symptoms such as jaundice, as spontaneous clearance of acute HCV infection is associated with a robust immune response that often can result in liver inflammation, as cytotoxic T-lymphocytes destroy infected hepatocytes (Thimme, Oldach et al. 2001). Acutely infected individuals generally demonstrate a lower peak of viremia (Thomas, Astemborski et al. 2000; Lehmann, Meyer et al. 2004; Kamal, Fouly et al. 2006). Compared to genotype 1 infections, individuals infected with genotype 3 virus are more probable to clear the virus (Lehmann, Meyer et al. 2004).

The reasons behind spontaneous clearance of Hepatitis C in the acute phase is not completely understood, however a strong initial CD8+ T cell response has

been associated with natural viral eradication (Thimme, Oldach et al. 2001). A study, conducted in 2001on five healthcare workers that had been subjected to infectious needle sticks, showed that four progressed into chronic disease as one spontaneously cleared the virus. The healthcare worker who cleared the virus displayed an immune response which initially entailed an extended period of acute hepatitis with $CD38^+$ IFN-γ^- $CD8^+$ T cell response to the virus and a decrease in viremia. This was followed by a strong $CD4^+$T cell response as the $CD8^+$ T cells transformed into $CD38^-$ which in response to the virus began to produce IFN-γ. Consequently, an immediate 100.000-fold decrease in viremia was observed exclusive of disease activity.

Two of the four individuals who continued to develop persistent infection were not able to generate a sufficient T-cell response to HCV while the remaining two initially appeared to produce a strong T-cell response which proved to diminish.

1.5.2 Chronic Infection

Eighty five percent of all acute HCV infections will advance into persistent disease (WHO 1999) and of these, 20 to 30 % will continue to develop hepatic fibrosis and subsequently cirrhosis (Powell, Edwards-Smith et al. 2000). Chronic HCV patients tend to show a slight elevated alanine aminotransferase (ALT) levels, which might continue to fluctuate for the duration of the disease, and although the HCV-RNA is lower than that of the acute phase, a continuous viremia persists (Thomas, Astemborski et al. 2000; Lehmann, Meyer et al. 2004; Kamal, Fouly et al. 2006). In chronic HCV infections, the progression of

liver disease differs. Some patients develop cirrhosis of the liver within 1-2 years of exposure while in others, the disease progression is slower, delaying cirrhosis over several decades (Hoofnagle 1997). As such, chronic HCV infection will vary in course, ranging from severe (rapid progressive) to moderate (slow progressive) to mild (non progressive). The specific determinants of disease outcome has not been fully identified, however, studies have confirmed that the genotype and the vast genetic heterogeneity of the virus together with numerous host factors such as; age, immunodeficiency, sex and race can affect the disease progression. External factors such as alcohol consumption and smoking have also shown to accelerate liver fibrosis and disease outcome (Seeff 2002).

Cytokine response to tissue injury is a dominant factor in the progression of liver fibrosis. A study was published in 2000 investigated the host factors in disease outcome of 128 chronically infected patients. The study looked at the individual gene expression of the TGF-β1, IL10 and TNF-α genes in these patients (Powell, Edwards-Smith et al. 2000). Transforming growth factor β 1, (TGF-β1), is a pro-fibrogenic cytokine that helps activate stellate cells in the liver to produce extracellular matrix. In chronic HCV patients, elevated TGF-β1 mRNA levels in the liver are detected compared to healthy individuals (Castilla, Prieto et al. 1991). Angiotensin II (AII) has been suggested to enhance the accumulation of extracellular matrix (Noble and Border 1997). It is also implied that AII can aid the production of TGF-β1in fibrosis of the heart and kidneys. This study found a statistically significant correlation between a high producing TGF-β1and AII producing genotypes and the advancement of

progressive liver fibrosis. Most patients with chronic HCV rarely suffer from symptoms, however it does occur. The symptoms are often mild and non-specific to the infection but include fatigue, anorexia, nausea, pain in the right upper quadrant, dark urine and possible itching (Hoofnagle 1997). The appearance of symptoms is not, however, a direct reflection of liver disease; although once cirrhosis develops they tend to be more collective. Moreover, it is not reliable to predict disease prognosis by symptoms.
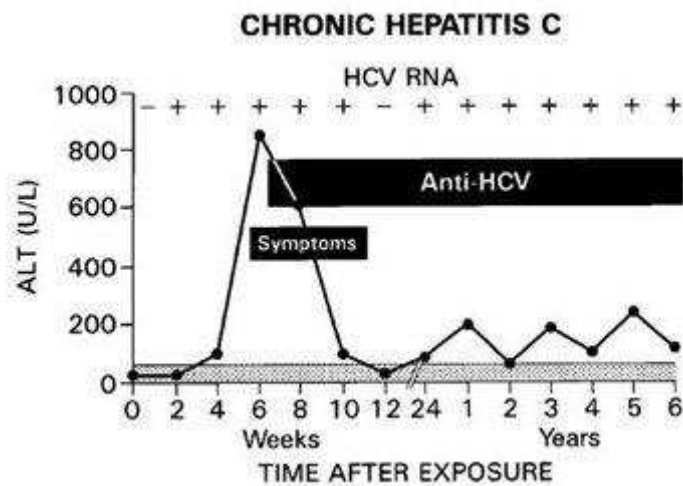
Figure 1.5: Schematic of anti-HCV antibody response in chronic HCV infection (Hoofnagle 1997).

Hepatitis C virus has developed certain means to evade the host innate and adaptive immune systems in order to sustain a persistent infection (see previous paragraphs for details). These evasion mechanisms involve, amongst other factors, inhibiting the production of type 1 interferons (Foy 2005; Li, Foy et al. 2005), hinder natural killer cells (Crotta 2002; Tseng 2002), generating various cytotoxic T-lymphocyte escape mutants (Erickson 2001) as well as neutralising antibodies targeting the E2 glycoprotein (Kato, Sekiya et al. 1993; Farci, Shimoda et al. 2000). It is also thought that HCV masks from

neutralising antibodies behind lipoproteins by binding them during cell entry (Thomssen, Bonk et al. 1992).

1.6 Current Treatment

With the current combined treatment of pegylated interferon α (PEG-IFN α) and ribavirin (RBV), it is observed that 50 % of patients infected with genotype 1 are able to clear the virus as well as 80 % of individuals that are infected with HCV genotype 2 and 3 (Fried, Shiffman et al. 2002). Side effects of this 48-72 week treatment do occur and include pancytopenia (reduction in red and white blood cells as well platelets), flu-like symptoms and depression. As these side effects can be severe, it could lead to premature termination of therapy. Not all chronically infected patients respond to treatment, and it has been shown that ethnic origin is associated with treatment outcome as Caucasians are much more likely to clear the virus compared to individual with African heritage.

A genome-wide association study was published in 2009 that identified a genetic polymorphism, adjacent to the IL28b gene, which is suggested to be linked with response to treatment. 1667 treatment-naive American patients (with varying ethnic backgrounds), chronically infected with HCV genotype 1, were included in the study that revealed a polymorphism, located on chromosome 19. This polymorphism, rs12979860 was shown to be significantly linked to sustained virological response (SVR) in all groups included in the study (Ge, Fellay et al. 2009).

1.6.1 Potential antivirals

1.6.1.1 Protease inhibitors

NS3, the non-structural protein that is responsible for cleavage of the polyprotein has proven to be an attractive anti-viral therapeutic target. NS3 inhibitors are generally designed to target the catalytic site of the protein, in order to subsequently inactivate the protease activity.

Telaprevir is a NS3/4A protease inhibitor that is currently being tried as a new direct anti-viral (DAA) drug. Two companies, Vertex (Cambridge, MA, USA) and Tibotec (Mechelen, Belgium) are at present developing this potential anti-HCV drug. Telaprevir was administered to treatment naive patients in trials which were randomised, double blind, placebo controlled phase II trials. Prove-1 took place in USA, and Prove-2 was conducted in Europe. In these trials, Telaprevir was administered, together with PEG-IFN-α2a and Ribivirin, for 12 weeks (Hezode, Forestier et al. 2009; McHutchison, Everson et al. 2009).

The results obtained from both Prove-1 and Prove-2 studies showed that although treatment with telaprevir achieved RVR in the patient groups that received the drug, the addition of RBV is crucial to obtain high SVR rates and also to reduce the risk of relapse. The side effects associated with telaprevir include anaemia, gastrointestinal discomfort and skin rashes. In the Prove-1 study, 13% of the patients which received telaprevir terminated the treatment due to side effects. This was only observed with 3% of the individuals belonging to the SOC control group.

A 65% SVR rate was observed in patients receiving treatment consisting of telaprevir, PEG-IFN and RBV for 12 weeks followed by the additional 12 weeks of only PEG-IFN and RBV.

Moreover; in an additional phase III, randomised, double blind, placebo-controlled study telaprevir together with PEG-IFN-α and RBV was administered to patients infected with genotype 1 virus and whom initially had failed to response to standard treatment consisting of PEG-IFN-α and RBV. The SVR rates for the relapsers receiving telaprevir for 12 weeks together with PEG-IFN and RBV followed by only PEG-IFN and RBV for an additional 36 weeks, was 83% compared to 24% of the control group that only received current combination therapy. This study included 662 patients with chronic HCV genotype 1 infection that had failed to response to IFN- based treatment (McHutchison, Manns et al. 2010).

Boceprevir, another protease inhibitor, acts by inhibiting the HCV NS3/4A protein. It is being developed by Schering Plough-MSD (Kenilworth, NJ, USA) (Asselah and Marcellin 2011). Boceprevir was assessed in a phase II study which included treatment naive patients infected with genotype 1 virus. The finding of the phase II study showed that boceprevir in combination of standard treatment showed an increase of SVR response after 12 weeks of treatment compared to the control group. The SVR rates doubled after 48 weeks compared to the current standard of treatment. Side effects with boceprevir have shown to include fatigue, anaemia, nausea and headache. Anaemia was seen in most patients receiving boceprevir, however treatment was seldom terminated (Kwo, Lawitz et al. 2010).

In a separate phase III international study, boceprevir was evaluated in 938 non- Black and 159 Black patient naive individuals infected with genotype 1 virus (Poordad, McCone et al. 2010). Briefly; boceprevir displayed an enhancement of SVR was seen in both non-Black (67% compared to 40% in the SOC control) and Black populations (53% compared to 23% in the SOC control), when boceprevir was administered together with PEG-IFN-α2b and RBV for 44 weeks. Boceprevir in combination with current SOC showed an enhanced SVR in treatment naive HCV genotype 1 infected individuals.

Patients infected with HCV genotype 1 and who failed to respond to current SOC treatment showed to achieve SVR rates up to 67% with current standard combination therapy together with boceprevir for 24 weeks. The control group achieved 21% SVR with standard combination therapy for 48 weeks (Bacon, Gordon et al. 2010).

1.6.1.2   Polymerase Inhibitors

NS5B, the RNA dependent polymerase, functions to produce a complementary minus -strand from the genome template in order to consequently generate a genomic positive strand RNA. There are two types of NS5B inhibitors, nucleoside polymerase inhibitors and non-nucleoside polymerase inhibitors. Nucleoside polymerase inhibitors cause chain termination by hindering the active site directly (Asselah and Marcellin 2011). The latter agent functions by allosteric hindrance to subsequently alter the conformation of the protein (Manns, Foster et al. 2007). Published data have confirmed that NS5B

inhibitors does reduce HCV RNA in serum (Olsen 2007), however most of these anti-viral drugs are currently on hold due to safety concerns.

1.6.1.3 Interferon λ

PEG-IFN-λ, also known as IL29, belongs to the type III IFN family and is currently being evaluated as potential antiviral agent against Hepatitis C infection (Asselah and Marcellin 2011). A phase II study by ZymoGenetics in Seattle, USA, is currently ongoing including nearly 600 individuals infected with HCV genotypes 1, 2, 3 and 4. These individuals will be divided into three groups of nearly 150 patients in each group. Each group will be administered different dosage of PEG-IFN-λ to evaluate the safety and antiviral efficiency of each dosage and compared to PEG-IFN-α2b. PEG-IFN-λ or PEG-IFN-α2b will be administered subcutaneously each week for 48 weeks in individuals infected with genotypes 1 and 4 and for 24 weeks in patients infected with genotype 2 or 3. In addition to these, the individuals included in this study will also receive ribavirin on a daily basis (Muir, Shiffman et al. 2010).

1.6.1.4 Combinations of direct-acting antivirals

Combination therapy of direct-acting antivirals are currently being studied and developed. This antiviral strategy aims to decrease resistance, increase the antiviral efficiency as well as control toxicity.

The initial study of combined DAAs involved the oral administration of either RG7227/ danaprevir (an NS3/4A protease inhibitor) and RG7128 (a nucleoside polymerase inhibitor) or with respective placebos to 87 individuals with genotype 1 HCV infection. These DAAs was administered for 13 days. The

antiviral suppression that resulted from this combination had a vast effect. The viral RNA was reduced by five logs and of those patients that were administered the highest dosage of RG7227, 900 mg twice daily, and RG7128,1000 mg twice daily, 88% showed undetectable HCV-RNA levels. During this trial, there was no sign of resistance to any of the DAAs and no serious side effects were reported. Both treatment naive patients and patients that failed to respond to initial SOC therapy responded to the DAA combination treatment with similar antiviral effects.

Currently, various studies are in progress investigating the antiviral efficiency of combination DAAs in treatment naive patients that include one protease inhibitor and one polymerase inhibitor (Gane, Roberts et al. 2010)

There are ongoing studies that are looking into the effects of combining direct acting antivirals at present (Lok, Gardiner et al. 2010; Zeuzem, Asselah et al. 2010; Zeuzem, Buggisch et al. 2010).

1.6.2 Vaccine strategies

As previously mentioned, a rapid, robust T cell response is suggested to be vital in clearing HCV infection. An optimal vaccine should be able to elicit T-cells responses without conflicting hepatic damage. Due to the chronic nature of the virus, the production of a potent vaccine against HCV is necessary. However, there are some obstacles challenging the developments, such as the vast heterogeneity of the virus as well as the failed attempts to propagate high quantities of HCV in in vitro (Berzofsky, Ahlers et al. 2004). However, advances are being made in the HCV vaccine field.

Most vaccine approaches have previously been studied in animals, mice and primates and very few of those have continued to develop into human studies.

There are difficulties with determining which region of the virus the vaccine should target. The E1E2 has proved to be important for viral entry and a vaccine which is capable of inducing HCV specific neutralising antibody response might be an attractive option. Although, studies have shown that induced HCV E1E2 antibodies will protect primates against viral challenge, this was only observed with homologous HCV strains (Farci, Shimoda et al. 1996). However, in contrast, recent chimpanzee studies with a recombinant E1E2 (E1E2/ MF59C.1) vaccine have shown to elicit both humoral and cell-mediated defence against viral infection from heterologous strains without serious side effects (Houghton and Abrignani 2005; Houghton 2011). The outcome of a completed Phase I study is yet to be published (Frey, Houghton et al. 2010).

Moreover; in the SCID mouse model (mouse transplanted with a human liver) antibodies have shown to be able to cross react between HCV genotypes (Law, Maruyama et al. 2008). Additionally, the current desirable vaccine should be able to elicit immune responses to HCV E1E2, either in combination with other agents or alone, and control initial stages of infection.

As the core protein is highly conserved both between and within HCV genotypes it is therefore an attractive choice of antigen. However, studies have failed to prove a robust T-cell response using HCV core, as it has been demonstrated that the core protein can hinder innate and adaptive responses in

HCV infection (Large, Kittlesen et al. 1999; Sarobe, Lasarte et al. 2003). Moreover; the T-cell responses against the core protein have proven to fail to control HCV replication (Semmo, Barnes et al. 2005). Although a robust anti-core T -cell response can be achieved in small animals, a heterologous prime-boost vaccine in primates failed to exhibit a response against when vaccinated with core, E1E2 and NS3.

In HCV vaccine development four types of vaccines have proceeded to human clinical trials. These include recombinant proteins vaccines, peptide vaccines, DNA vaccines and vector vaccines.

Recombinant protein vaccines are generated by expressing the desired protein(s) in yeast, bacteria or mammalian cells. The theory behind the use of recombinant proteins for vaccine purposes is to elicit a robust, adequate immune response to a limited amount of HCV epitopes. The uses of recombinant proteins in HCV vaccine approaches are currently being studied (Halliday, Klenerman et al. 2011). In a current phase II trial, a core-NS3 fusion protein (GI5005) have been assessed as potential vaccine candidate administered in combination with standard therapy to 66 chronic patients infected with genotype 1 virus. The patients received 12 weeks of standard therapy alone, followed by a weekly dose of GI5005 for five weeks and additional monthly doses for two months. Although this study showed an eleveted SVR in patients with IFN L3 alleles (Pockros, Jacobson et al. 2010), there are no evidence of elicited immune responses as of yet (Halliday, Klenerman et al. 2011). Moreover; there has been evidence of anti-core immune responses in humans when vaccinated with a recombinant HCV core

protein adjuvanted with saponin, cholesterol and phospholipid (Drane, Maraskovsky et al. 2009). This molecule is named ISOMATRIX and is currently being assessed in a phase I trial of 30 healthy volunteers. Eight individuals that received 50 mg, which was the highest dose of the trial, presented a strong antibody response to core, however only two of them showed detectable levels of T-cells. This study is ongoing.

Peptides are also used in attempts to elicit HCV specific T-cell responses in vaccine approaches. The vaccine peptide(s) are presented to the T- cell receptors by HLA molecules and there are only few HCV peptide based vaccines undergoing investigation. It has been noticced that the T-cell response from these vaccines are not sufficient enough to control infection as the peptide vaccines are HLA specific and therefore coverage is limited to a minority of the population. Moreover, as opposed to initiate a HCV counter immune response some peptides might actually promote a viral tolerance of Treg (regulatory T) and effector cells (Kaneko, Moriyama et al. 1997).

A current peptide vaccine, IC41 is undergoing clinical trials as a potential anti-HCV vaccine. IC41 which is a combination of five synthetic peptides from core, NS3 and NS4 HCV proteins that are conserved between genotype 1 and 2. IC41 is adjuvanted with poly-L-arginine and contains five HLA A2-retricted $CD8^+$ T-cell epitopes and three $CD4^+$ epitopes.

Recently, 50 patients with chronic HCV infection received intradermal (Firbas, Boehm et al. 2010) administration of IC41 twice a week. After four months, a considerable decrease in viral load was noticed and the T-cell response was

greater compared to previous monthly administration of IC41. There is an ongoing subsequent study that is investigating the anti-viral effects of IC41 in combination with a novel broad-spectrum anti-viral drug, nitazoxanide (Rossignol 2009; Rossignol, Elfert et al. 2009).

There have been attempts to develop a qualified DNA vaccine in the HCV field; however it remains a difficult task.

In 2009, a study that took place in Cuba administered CICGB-230, a combined plasmid consisting of HCV core/E1E2 antigens and the recombinant core protein Co.120 (Alvarez-Lajonchere, Shoukry et al. 2009) to 15 patients chronically infected with HCV genotype 1 and who had previously failed to respond to the available standard HCV therapy. The vaccine was administered on a monthly basis, intramuscularly, for aduration of six months. The outcome of this study showed a general poor T-cell response as soon as one month following the final vaccine administration. Only one patient exhibited a reduction in viral load ($>1 \log_{10}$).

An additional study was conducted in 2009, which investigated a DNA vaccine expressing NS3/4A (Sällberg, Frelin et al. 2009). This study utilised the method of electroporation in attempts to increase the immunogenicity when administered intramuscularly, also in order to increase the T-cell response and to permit sufficient DNA expression, the codons were subjected to vast modification. Twelve treatment naive patients infected with HCV genotype 1 and displayed low levels of viremia (<800.000 IU/ml) were administrated monthly doses of DNA vaccine for the duration of four months. Some of the

obtained results show that 65% of the patients that were administered the highest level of dosage displayed a decrease in viral load over 0.5 $\log_{10}$. This decrease was maintained for two to ten week post final vaccination. Three of these patients also exhibited an associated T-cell response. No serious side effects were reported with any of these two DNA vaccine studies.

Vaccines employing viral vector in order to effectively deliver HCV RNA is currently an attractive method in vaccine development. Initial studies have reported that adenoviral vectors are able to reduce high HCV viremia in the early stage of infection and also elicit a HCV specific T-cell response. As a beneficial attribute, these types of vaccines does not harbour a HLA restricted antigen and therefore could generate a breadth of viral epitopes (Halliday, Klenerman et al. 2011).

TG4040 is a vaccine that employs the modified vaccinia Ankara (MVA) poxivirus strain to express NS3/4/5B. This vaccine was assessed in an open-label, multicenter, dose-escalation study that included 15 chronically HCV infected patients which were administered the vaccine once a week for three weeks. Nine out of these patients continued to receive a final fourth injection after six months. The outcome of this trial showed a 0.5 to 1.4 $\log_{10}$ reduction in viral load was noticed in six patients which also exhibited an associated robust $CD8^+$ T-cell response. Further plans for this vaccine include a phase II study that will investigate the effect of TG4040 in combination with current standard therapy (Halliday, Klenerman et al. 2011).

There is currently another ongoing study that employs the beneficial properties of adenoviral vectors. Thirty six healthy volunteers are administered this vaccine containing HCV non- structural proteins NS3 to NS5B. These vaccine vectors have been subjected to mutagenesis to knock out replication ability and the polymerase activity of the non structural proteins. Also, to avoid any pre-existing immunity to adenoviruses, the origin of the chosen adenoviral vectors included in this study is not usually exposed to humans, AD6 and the simian adenoviral vector AdCh3. The initial reports from this study revealed that immunisation with AD6 resulted in a high immune response in these healthy individuals following the initial injection. Future studies will include HCV infected patients (Barnes, Folgori et al. 2009).

1.7   *Postulated Hepatitis C Virus entry*

Following host-cell receptor binding, HCV internalize and fuse with the endolysosomal membranes, the low pH in the endolysosomal compartments trigger a conformational change of the glycoproteins, exposing a fusion peptide (Lindenbach and Rice 2005) (Flint, Thomas et al. 1999). This fusion peptide disrupts and enables fusion between the membranes before the virus releases it single stranded RNA genome into the cytoplasm. There, the mRNA is translated into viral proteins and used as a template for RNA replication. Virions are formed in the endoplasmatic reticulum (ER) before they are secreted out, ready to infect other cells.

1.7.1 Host cell interactions involved in viral entry

1.7.1.1 *Receptors*

1.7.1.1.1 CD81

HCV entry is suggested to occur through a clathrin-dependent pathway, requiring low pH and being temperature dependent (Evans, von Hahn et al. 2007). This entry process has been repeatedly demonstrated to be dependent on engagement of the CD81 molecule on the surface of susceptible cells (Evans, von Hahn et al. 2007).

With the exception of platelets and red blood cells CD81, is expressed in all human cells. It is believed to play a role in several cell functions such as cell adhesion, morphology, proliferation and differentiation, and cell activation. By screening a cDNA library using recombinant E2 as a probe, Pileri et al identified CD81 as a putative Hepatitis C receptor in 1998 (Pileri, Uematsu et al. 1998). CD81, a tetraspanin, consists of a small and a large extracellular loop with four transmembrane domains (Owsianka, Timms et al. 2006). The large extracellular loop has been demonstrated to permit binding to the ectodomain of soluble E2 (Diedrich 2006). The large extracellular loop is conserved between humans and chimpanzees, the only species that are susceptible to HCV-infection (Pileri, Uematsu et al. 1998). Bartosch et al (2003) investigated the role of CD81 and another HCV receptor, SR-B1, in HCV entry. CD81 was identified as necessary for infection. However, co-expression of CD81 together with SR-B1 and low density lipid receptor (LDLr) in hepatocarcinoma cells enhanced infectivity, supporting the

hypothesis that CD81 alone is not sufficient to allow infection (Bartosch, Vitelli et al. 2003). Reports of CD81 polymorphism being a determinant in HCV host variety have been published. Different CD81 proteins derived from various species was studied to investigate their ability to bind sE2 and allow HCVpp infection (Flint, von Hahn et al. 2006). Human, monkey (chimpanzee, African green monkey and tamarin) and rodent (mouse, hamster and rat) CD81 were individually compared in experiments that showed the human- and to a lower extent, monkey CD81 did bind sE2 and also inhibited HCVpp infectivity. Rodents are not naturally susceptible to Hepatitis C infection, and accordingly rodent CD81 did not interact with sE2 nor inhibit HCVpp infectivity supporting the theory that CD81 could be one critical factor in the species restriction of HCV infection. However there could be other factors involved also since transgenic mice expressing human CD81 was nonetheless resistant to HCV infection, concluding that CD81 alone cannot be a determinant of HCV species restriction.

The importance of the interaction of HCV to CD81 has been demonstrated by antibody neutralisation of the interaction. Many human antibodies generated in natural infection have been isolated that compete CD81 binding (Burioni, Plaisant et al. 1998; Allander, Drakenberg et al. 2000; Hadlock, Lanford et al. 2000) and neutralise entry in a CD81-blocking manner (Bartosch, Vitelli et al. 2003; Logvinoff, Major et al. 2004; Meunier, Engle et al. 2005; Schofield, Bartosch et al. 2005; Pestka, Zeisel et al. 2007). Understanding the epitopes involved in these antibody interactions is of great importance to antibody therapeutics for HCV, and also to the understanding of the structural biology

of the HCV-CD81 interaction. Although some anti-E2 antibodies have been mapped (Petit, Jolivet-Reynaud et al. 2003; Tarr, Owsianka et al. 2006) the majority of human antibodies map to conformation sensitive epitopes that cannot be simply delineated, however mapping of these determinants is underway (Owsianka, Timms et al. 2006). Elucidation of the immunogenic regions of the HCV glycoproteins remains a significant challenge.

1.7.1.1.2 SRBI

Human scavenger receptor class B type 1 (SRBI) is a multiligand receptor expressed on liver cells and on steroidogenic tissue (Diedrich 2006). It consists of a 509 amino acid glycosylated protein located on the surface of the cell (Lobo, Huerta et al. 2001) (Acton, Scherer et al. 1994). SRBI is made up by two cytoplasmic domains and two membrane domains with a large extracellular domain in between. It is also reported to have nine N-glycosylation sites (Fidge 1999). Human SR-B1 binds to numerous lipoproteins such as high density lipoprotein (HDL), low-density lipoproteins (LDL), and very low density lipoproteins (VLDL).

In binding experiments, it has been difficult to show any direct interaction between SR-B1 and the full-length E1E2 heterodimer. However, when a truncated form of soluble E2 is used in the assays, a specific binding to SRBI is clearly observed (Scarselli, Ansuini et al. 2002) (Barth, Schafer et al. 2003). These findings support a potential theory that, in vitro, the binding domain of SR-B1 might not be able to access E2 in the form of a heterodimer.

SRBI is implicated in binding hypervariable region 1 (HVR1) of E2, as E2 lacking HVR1 does not bind to the receptor. Additionally, antibodies against HVR1 compete with SR-B1 for E2 binding (Scarselli, Ansuini et al. 2002).

High density lipoprotein, HDL, is the natural ligand of SRBI in the liver and on steroidogenic cells (Voisset and Dubuisson 2004). The function of HDL is to attach and remove free cholesterol from peripheral blood cells, transport it back to the liver, via SRBI, where it will be excreted (Yancy, Provenzale et al. 2001).

In the context of HCVpp, it has been established that HDL facilitates HCVpp entry via SRBI. Voisset et al conducted experiments in 2004 to investigate the role of HDL in HCV entry and discovered that although the virus particles did not need to attach HDL to bind to the receptor, HCVpp infectivity increased by three- fold in the presence of HDLs. This enhancement is most probably not a result of a direct contact between HDL and E2 but would involve the lipid-transfer function of SRBI (Dreux and Cosset 2007). Furthermore, by silencing SRBI expression on the target cells, this enhancement of HDL-mediated HCVpp entry was distinctly reduced, suggesting that HDL is highly involved in HCV entry through SRBI interaction.

During the lipid transfer mediated by SRBI, the lipid membrane of the cell locally increases in cholesterol content rendering a possible reorganisation of the membrane and also might cause the HCV/ receptor complex to internalise and/or lead the virus to fuse with the cellular membrane (Dreux and Cosset 2007). Apolipoprotein C-I (ApoC-I), a subcomponent of HDL, is a 57 amino

acid long plasma protein that circulates in the peripheral blood together with HDL and with very low density protein (VLDL). It has been suggested that ApoC-I is the component responsible for observed enhancement of infection with HCVpp as it is believed to be recruited to the surface of the virus after the interaction between, HCV, HDL and SRBI to mediate fusion between the two membranes and enable virus entry. High levels of ApoC-I in experiments with HCVpp have demonstrated a decrease in infectivity, most probably due to a disruption of the viral envelope implying that only certain levels of ApoC-I is beneficial at the viral cell surface to facilitate fusion and subsequently HCV entry (Dreux, Boson et al. 2007).

1.7.1.1.3 CLDNs

Claudin (CLDN) proteins belong to a family of proteins that are involved in the formation of tight junctions (TJ) on liver cells and epithelial cells. They are engaged in mediating the permeability of endothelial and epithelial cells and also serve to adjust the cell polarity. Apart from the fact that the polymerisation of the CLDNs is important to form tight junctions, additional information regarding the molecular purpose and organisation of TJs is yet to be revealed. It is known that CLDNs interact in the plasma membrane on an individual cell or between opposing cells through their intracellular loops. Furthermore, many tight junctions has been shown to be main receptors for a variety of viruses, such as coxsackievirus receptor for coxsackievirus and adenovirus receptor for adenoviruses (Harris, Farquhar et al. 2008).The involvement of CLDN-1 in HCV entry was recently demonstrated as

expression of CLDN-1 on HEK 293T cells increased the infectivity different genotypes of HCVpp by a hundred fold (Evans, von Hahn et al. 2007). Functional analysis to study the interaction between HCV glycoproteins and CLDN-1 has shown to be difficult. Apart from possible low assay sensitivity, these observations can also mean that HCV E1E2 interact with cellular receptors sequentially in a cooperative manner. Fluorescence resonance energy transfer (FRET) was conducted in a study to investigate the interaction between fluorescently tagged CD81 and CLDN-1 expressed in different cell lines using receptor-specific antibodies attached to hepatocytes. The study demonstrated an association between a subpopulation of CD81 and CLDN-1. The authors of the paper subjected Huh-7 cells to HCV infection and neutralising anti-CD81 monoclonal antibody and noticed a modulation of homotypic (CD81-CD81) and heterotypic (CD81-CLDN-1) coreceptor protein complexes. The FRET frequency of CD81-CD81 coreceptor association decreased but the frequency of CD81-CLDN-1 protein complex did not; indicating possible differences in antigenic and functional properties of homotypic and heterotypic coreceptor protein complexes involved in HCV entry (Harris, Farquhar et al. 2008).

An American group published a paper in 2008 describing CLDN-1, -6 and -9 as cofactors for HCV(Meertens, Bertaux et al. 2008) CLDN-1, -2, -4, -6 and -9 was expressed in human CD81 positive endothelial cells to study HCVpp infectivity. HCV E1E2 derived from different genotypes was included in the experiments that confirmed CLDN-1 being a cofactor in HCV entry, and also

showed CLDN-6 and -9 permitting HCVpp entry using E1E2 from various genotypes, however not to the same extent as CLDN-1.

1.7.1.1.4 Occludin

A new cellular receptor has recently been proven to have a significant role in the HCV viral entry (Ploss, Evans et al. 2009). Human occludin, (OCLN), with its four transmembrane domains, is a tight-junction protein found in the tight-junction complex of polarised cells. There, it seems to act as a modulator of paracellular permeability and cell adhesion. Ploss et al recently found that the overexpression of occludin on HCVpp uninfectious murine cells allowed infection of HCVpp. When overexpressed together with the other HCV-receptors on uninfectable human cells, occludin seemed to enhance HCVpp uptake and when silenced on permissive cells, HCVpp- and HCVcc infection was noticeably impaired. Murine SRB1 and CLDN-1 have in studies shown that they can enhance HCVpp uptake as well as human proteins. However, only occludin and CD81 of human origin permit HCVpp uptake. As mentioned previously in this chapter, HCV is solely capable of infecting hepatocytes of human and chimpanzee origin. Moreover; it is proven, as with CD81, that the second extracellular loop of occludin holds the species-specific determinants of entry (Ploss, Evans et al. 2009).

1.7.1.2 Other molecules involved in HCV entry

1.7.1.2.1 LDL, LDLR and VLDL

Thommsen et al reported in 1992 that HCV also interacts with low density lipoprotein (LDL) and very low density lipoprotein (VLDL) present in human serum. The low density lipoprotein receptor (LDLR) is suggested to aid virus uptake, since the amount of expressed LDLR on cultured cells have been shown to correlate with the uptake of patient derived HCV by these cells (Agnello, Abel et al. 1999). Since HCVpp are produced in HEK 293T cells, a non-hepatic cell line, experiments with LDLR in the context of pseudo particles, are constricted (Bartosch, Dubuisson et al. 2003). Therefore, HCVcc, constructed in a hepatic cell line, would be a more appropriate model to study the involvement of LDLR in HCV uptake. ApoE, a subcomponent of VLDL, has previously proven to be engaged in the virion assembly process (Chang, Jiang et al. 2007) when ApoE and E2, in a membrane flotation analysis, showed to be present in the same sample fraction confirming the conclusion that VLDL and HCV virions form in the same intracellular compartment (unpublished data, Chang and Luo). In addition to those data; Chang et al also claimed in 2007 that ApoE is required for pseudo particle infectivity by showing that apoE-specific monoclonal antibodies neutralised HCVpp infection by 80% in a dose dependent manner (Chang, Jiang et al. 2007).

1.7.1.2.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) have also been proposed to be involved in facilitating HCV entry (Basu, Beyene et al. 2004), (Barth, Schnober et al. 2006) (Basu, Kanda et al. 2007). GAGs are linear polysaccharides bound to proteins present on various cell surfaces distributed throughout the human body and the main interaction with HCV is thought to occur via the viral glycoprotein E2 (von Hahn and Rice 2008).Heparan sulphates (HS) are very highly sulphated GAGs that exist in various compositions between different types of species and cells types and are formed by disaccharide chains in various repetitions of GlcA (glycoronic acid) and GlcNAc (N-acetylglucosamine)(Barth, Schnober et al. 2006). HS have proven to play important roles in binding E2, as studies have shown that HCVpp infectivity increased in the presence of heparin, a highly sulphated HS. Moreover, E2 showed reduced binding to desulphated heparin in the study and also a loss in HCVpp infectivity was noticed when targets cells were treated with glycosidases to remove the cell surface of GAGs. These combined results support the conclusion that sulphated GAGs are important in the HCV entry process (Basu, Kanda et al. 2007). Since GAGs are well distributed on cells throughout the body, their affinity for E2 is probably lower then the primary cellular receptors for HCV. Therefore it can be assumed that GAGs function to sustain the virus on the surface of the cell long enough for it to interact with the main HCV receptors, such as SRBI, CD81 and CLDNs (von Hahn and Rice 2008)

1.7.1.2.3 DC-SIGN and L-SIGN

Belonging to the C-type lectin family, DC-SIGN and L-SIGN are homotetrameric type II membrane proteins (Geijtenbeek and van Kooyk 2003) that consist of an oligomerized calcium-dependent carbohydrate- recognition domain as well as a membrane-proximal heptad-repeat region. The carbohydrate- recognition domain recognises carbohydrates on pathogens (Koppel, van Gisbergen et al. 2005) and is thought to bind HCV via high-mannose N- glycans on E2. Although studies with HCVpp has shown that virus particles bind to cells that express these molecules(Gardner, Durso et al. 2003), (Pohlmann, Zhang et al. 2003), (Lozach, Lortat-Jacob et al. 2003)DC-SIGN and L-SIGN are not receptors on hepatocytes. DC-SIGN is expressed on dendritic cells and L-SIGN is expressed in sinusoidal cells in the liver and lymph nodes (Koppel, van Gisbergen et al. 2005).

DC-SIGN acts as an antigen receptor that internalises and processes the bound antigen before presenting it to T-cells. It also functions as an adhesion receptor on dentritic cells and is important for the interaction with T-cells and epithelial cells (Engering, Van Vliet et al. 2002).

L-SIGN is suggested to have a role in the interaction with activated T-cells, by allowing the activated T-cell access to the liver and lymphnodes (Bashirova, Geijtenbeek et al. 2001), however the exact function is not known.

These proteins are implied to have a role in establishing persistent HCV infection through modulating dendritic cell functions and by internalising the

virus before transporting it to the liver (Cormier, Durso et al. 2004; Lozach, Amara et al. 2004).

Combining years of data obtained for other flaviviridae, appropriate assumptions have been made regarding Hepatitis C interactions with cellular receptors in the entry process. It is believed that prior to entering, the virus together with lipoproteins attach to GAGs and/or LDL receptors located on the target cell surface (Cocquerel, Voisset et al. 2006). After binding, HCV interacts with SRBI and CD81, then, as a late part of entry, the virus presumably interacts with either claudin 1, 6 or 9 prior to entering the cell through clatherin- mediated endocytosis (Blanchard, Belouzard et al. 2006).
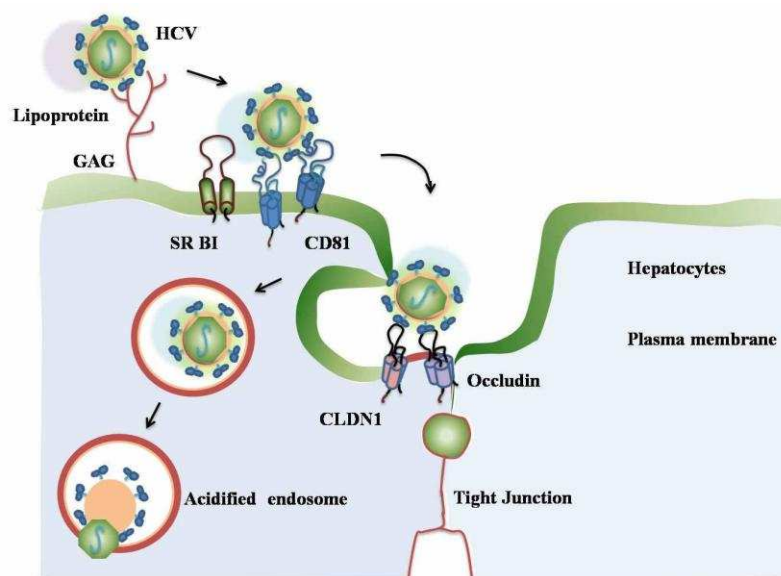
Figure 1.6: Postulated Hepatitis C virus entry.
HCV is suggested to be associated with apoliporoteins as it initially interacts with low-density lipoprotein receptor as well as glucosaminoglycans on the surface of the cell. Interaction with numerous host cell receptors are then required for the virion to enter the cell. These include initial binding to CD81 and Scavenger receptor class B type 1 (SRBI), followed by the interaction of the tight junction proteins Claudin 1 and Occludin. These cascades finally lead to the endocytosis of HCV in an acidified endosome prior to the release of the viral nucleocapsid. (http://chemeng.iisc.ernet.in/labone/pranesh.html).

1.8 HCV in vitro entry models

1.8.1.1 HCV replicon system

Even though several attempts had been made infect cell culture with live viral inoculum, it has never proven successful. While HCV replication was noticed in hepatoma-, B- and T-cell lines, primary hepatocytes derived from both humans and chimpanzees as well peripheral blood monocytes, the replication was in-efficient and could not be detected by any other means than highly sensitive RT-PCR assays. Therefore this system is not sufficient to study the specific details of HCV replication (Bartenschlager 2001).

In 1999, Lohmann et al published a report which described the development of a successful replicating HCV replicon model system (Lohmann 1999). This model was based on the knowledge that flaviviruses and pestiviruses does not involve structural proteins in successful replication (Bartenschlager 2001).

The replicon consists of a consensus Con1 cDNA which derived from a genotype 1b chronic infected liver. The replicon is costructed with HCV 5'NTR together with the initial twelve codons of the capsid protein, fused with neomycin phophotransferase, selective marker gene, which when expressed will present a resistance to G418, a cytotoxic agent. The functional replicon also holds the IRES from the encephalomyocarditis virus which helps control the translation of the HCV non-structural genes. Finally, the HCV 3'NTR is also included in the replicon structure. Once transfected into permissive Huh7 cells and selected for by G418, resistant cell colonies displayed replicons that

was able to generate high RNA levels of 1000-5000 copies of positive stranded HCV RNA per cell (Blight and Norgard 2006).

Following sequence analysis, adaptive mutation were found in the non-structural regions of of the Con1 replicon, however not in the 5' or the 3'NTR regions which are highly conserved amongst HCV isolates.

These mutations in the non-structural regions (NS4A, NS5A and NS5B) were shown to improve RNA replication in the permissive Huh7 cells.

The replicon system was for a long time restricted to Con1 replication, however recently other 1B isolates have proven functional, moreover, replicons generated from the HCV-N isolate does not seem to rely on adaptive mutations for effective replication (Blight and Norgard 2006).

A replicon derived from HCV genotype 1a has not been proven functional in this model until a chimeric version of the H77 -Con1 strain was developed (Lanford, Guerra et al. 2003). The H77 strain is generally associated with high viremia during the acute phase of infection; it requires a minimum of two adaptive mutations in order to replicate with efficiency in this model system.

The JFH1 isolate, a HCV genotype 2a, derived from a Japanese patient suffering from a fulminant hepatitis is the only non-genotype 1 isolate which has proven that will efficiently replicate in the replicon system (Kato, Date et al. 2003).

1.8.1.2 HCV cell culture system

In 2005, Wakita et al, were successful in isolating an HCV strain from a Japanese patient with fulminant hepatitis, extracting the full-length genome and replicating it in a hepatoma cell line (Wakita, Pietschmann et al. 2005). This system, JFH-1 HCVcc, is the only model of HCV that permits investigation of the entire HCV life cycle; entry, replication, assembly and release (Lindenbach, Evans et al. 2005). HCVcc model is the current gold-standard for investigating HCV entry.

1.8.1.3 HCV pseudo particle system

Recently, the HCV pseudo particle model (HCVpp) was developed to investigate HCV entry (Voisset and Dubuisson 2004). HCVpp are engineered viral particles consisting of a retrovirus-derived capsid enveloped by a lipid bilayer. Present within the envelope are HCV E1 and E2 gene products, which confer entry of the pseudoparticle into susceptible cells. To quantify the number of infected cells, a reporter gene, such as luciferase or green fluorescent protein, is also included between the two long terminal repeats of the retrovirus genome, allowing packaging of the RNA and delivery of the gene to an infected cell. Constructing the pseudo particles requires transfection of human embryo kidney (HEK) 293T cells with these expression vectors (Voisset and Dubuisson 2004). After the production of the particles, they are used to infect Huh-7 hepatocarcinoma cells. The number of infected cells can then be determined by the amount of expressed luciferase in the Huh-7 cells. Although the HCVpp- system has transformed HCV-research in many positive ways, it also lacks many important details that are linked with HCV-infectivity

in vivo (Diedrich 2006). Hepatitis C virus is bound to lipoproteins that have shown to facilitate the infectivity of the virus. Since pseudo particles are generated in HEK293 cells, which does not produce lipoproteins, HCVpp are not bound to lipoproteins. Native HCV virions assemble at the endoplasmatic reticulum (ER) in hepatocytes. Regarding HCVpp, it has previously been reported that pseudo particles assembles at the plasma membrane (Bartosch, Dubuisson et al. 2003), highlighting an additional dissimilarity to HCV entry in vivo. Moreover, in an article from 2003, Sandrin et al found HCVpp E1 and E2 present in intracellular vesicles and concluded that apart from the cell surface, assembly of the pseudo particles mainly occur inside the cell within the endocytic pathway, rather than at the plasma membrane (Sandrin, Russell et al. 2003).

1.9 Neutralising antibodies to HCV envelope protein 2

Numerous antibodies derived from HCV infected patients have proven to neutralise HCV infectivity. Following the isolation of human monoclonal antibodies (HmAbs) targeting HCV E2 of several genotypes, it was established that they are able to recognise certain conformational epitopes on E2 (Hadlock, Lanford et al. 2000; Owsianka, Timms et al. 2006; Johansson, Voisset et al. 2007). One approach to investigate whether monoclonal antibodies or serum posses the ability to prevent E2 binding to receptors is to use plate-based ELISA or cell binding assays known as neutralisation of binding (NOB) assays (Hadlock, Lanford et al. 2000). With the recent development of the HCVpp and HCVcc systems it is possible to investigate the effect of HmAbs on viral

neutralisation. The minimal concentration of an antibody / serum required to inhibit 50 % of interaction with target protein (IC50) is determined before the antibody qualifies as being inhibitory or neutralising. Hadlock et al (2000) generated a group of human monoclonal antibodies from B cells derived from an asymptomatic HCV infected patient that also presented with a high NOB serum titre (Hadlock, Lanford et al. 2000). The epitopes of these antibodies have been mapped to three immunogenic regions on HCV E2 (Keck, Li et al. 2005). The immunogenic regions designated domain A; which contained the epitopes recognised by the monoclonal antibodies CBH-4B,-4D and -4G; domain B, recognised by CBH-2,-5, -8C, -8E and -11, and domain C, targeted by CBH-7. Antibodies belonging to domain A did not exhibit neutralising activity in HCVpp nor did they have NOB activity, whilst domain B antibodies and CBH-7 from domain C have NOB activity. Various E1E2 clones representing all six genotypes proven functional in HCVpp assay were included in this study (Keck, Li et al. 2005). Using HCVpp supplemented with E1E2 from the major genotypes they showed that the antibodies that exhibited the broadest neutralising abilities were CBH-2, CBH-5 and CBH-7. CBH-2 managed to neutralise genotype 2b and 4 by nearly 50 % and genotype 2a, 5 and 6 by 70% however failed to neutralise genotype 3 and 1a H77 clone. CBH-5 was found to be broadly reactive as it neutralised all genotypes and reduced HCVpp infectivity by at least 40%. CBH-7 presented a 70% neutralising effect on genotype 1a of the H strain, a 40% neutralisation of genotype 1b, 2a and 2b and a 20% neutralisation of genotype 4. The NOB titres for these HmAbs correlated with their neutralising profile except for CBH-7 that was able to bind to genotype 6 E1E2 however failed to neutralise infectious pseudo

particles. The authors of this study also attempted to map the residues on E2 critical for the binding of HmAbs (Keck, Li et al. 2005). They found that five residues; G523, P525, G530, D535 and the glycosylated N540 were important in the CBH-5 binding, as point mutation of these residues abrogated binding. Four of these sites; G523, P525, G530, D535 are conserved across all six of HCV genotypes and three; G523, G530 and D535 are implicated in CD81-binding. This shows that CBH-5 competes for the same binding site as CD81. It also explains the high conservation of these residues as alteration would compromise CD81-binding and consequently viral entry (Owsianka, Tarr et al. 2008).

Law et al also produced a paper combining data from studies made with human monoclonal antibodies recognising discontinuous antigenic regions on E2. The antibodies were tested for interaction with CD81, E1E2 represented by all six genotypes; and virus neutralisation in the HCVpp and HCVcc systems (Law, Maruyama et al. 2008). AR (antigenic region), 1- specific antibodies, AR1A and AR1B, that did not recognise E1E2 from genotype 2 exhibited the ability to interact with genotype 1 E1E2, however failed to neutralise infectious virions. These results can be explained by assuming that the immunogenic regions within AR1 are perhaps non-neutralising epitopes. Antibodies that recognise the antigenic region 3 showed reactivity to both genotypes 1 and 2 at low IC_{50} and had neutralising activities in both HCVpp using E1E2 from different genotypes and JFH-1 E1E2 in the HCVcc assay; suggesting that the epitopes within AR3 are conserved. The AR3 targeting antibodies, AR3A, -B,-C and –D, are therefore considered to be broadly

neutralising across HCV genotypes and has been suggested to be potentially useful in the defence against infection by HCV quasispecies. AR1A and all of the AR3-specific antibodies blocked H77 E1E2-CD81 interaction. The epitopes in AR3 have been mapped by competition assays to the CD81-binding region of E2 explaining the CD81-blocking ability (Law, Maruyama et al. 2008).

Two additional neutralising HmAbs were identified by Johansson et al in 2007 (Johansson, Voisset et al. 2007). The HmAbs; 1:7 and A8 were included in NOB assays, HCVpp and HCVcc neutralising assays as well as CD81- binding experiments. The results obtained from these studies showed that HmAbs 1:7 and A8 succeeded to neutralise both HCVpp harbouring E1E2 from all six HCV genotypes and JFH-1 HCVcc at IC_{50} of 60ng/ml and 560ng/ml respectively. 1:7 also presented an affinity to H77 E1E2 that was ten times greater than that of A8. The residues on E2 involved in binding 1:7 and A8 were also mapped and encompassed the same residues recognised by the previously reported broadly neutralising antibodies. Since 1:7 and A8 display different E1E2 affinity patterns across the different genotypes, the authors suggest that other amino acid residues or slight structural variations of the epitopes might be involved in determining binding (Johansson, Voisset et al. 2007).

AP33, a linear monoclonal mouse antibody has shown to neutralise HCV infectivity in the pseudo particle assay carrying E2 of different genotypes (Owsianka, Tarr et al. 2005; Tarr, Owsianka et al. 2007), The AP33 has also shown to be potently capable of blocking CD81 interaction. The epitope on E2

AP33 binds to have been mapped to residues 412-423 and this region is highly conserved across the main HCV genotypes (Owsianka, Tarr et al. 2005)

Recently, a report was published describing four monoclonal antibodies recognising human SRBI (Catanese, Graziani et al. 2007). Two of them; 3D5 and 6B8 were generated in mice whilst C11 and C167 originate from phage libraries. All four showed to bind to SRBI expressed on Chinese hamster ovary (CHO) cells thereby blocking sE2-SRBI binding. The blocking ability of these MAbs seemed comparable to their affinity to sE2, with MAb C167 binding with highest affinity, followed by 3D5, C11 and 6B8 that displayed the weakest affinity to binding. All four recognised conformation-dependent epitopes on SRBI and three of them, C167, 3D5 and C11, reportedly cross-competed for the same or closely located regions. Furthermore, C167 and 3D5 also proved to inhibit HCV infection of human hepatoma cells in the HCVcc assay. The specific residues involved in binding these MAbs to SRBI are yet to be mapped (Catanese, Graziani et al. 2007).

HCV broadly neutralising antibodies is suggested to be of clinical significance in two highly relevant areas. First, to immunise liver transplant patients with these antibodies would be advantageous in order to reduce the incidence of graft re-infection (Schofield, Bartosch et al. 2005). They could also be of clinical importance for prophylactic purposes after accidental exposure to the virus, i.e. following needle sticks (Schofield, Bartosch et al. 2005; Owsianka, Tarr et al. 2008).

2 Defining the role of amino acid residues 611, 613-619 and 621, within HCV glycoprotein E2, in CD81 interaction

2.1 Background

The CD81-binding sites are thought to be located on discontinuous regions, solely on the HCV E2 protein (Yagnik, Lahm et al. 2000; Owsianka, Clayton et al. 2001; Hsu, Zhang et al. 2003; Roccasecca, Ansuini et al. 2003). Initial mapping of the specific regions of E2 involved in this interaction was performed by inhibition of E2 binding, antibody competition (Flint, Maidens et al. 1999; Owsianka, Clayton et al. 2001; Clayton, Owsianka et al. 2002), as well as using glycoprotein chimeras from isolates that demonstrated different binding abilities to CD81 (Patel, Wood et al. 2000). The outcome of some of the antibody-mapping studies identified several CD81 contact residues on E2 which included aa412-423 (Owsianka, Clayton et al. 2001), aa432-447 (Clayton, Owsianka et al. 2002), aa480-493 (Flint, Maidens et al. 1999), aa528-535 (Owsianka, Clayton et al. 2001) and aa544-551 (Flint, Maidens et al. 1999). The E2 amino acid residues 530 and 535 are highly conserved residues amongst isolates and have shown to be a common target for broadly neutralising antibodies (Owsianka, Timms et al. 2006; Tarr, Owsianka et al. 2006; Johansson, Voisset et al. 2007).

In 1999, Flint et al observed that the monoclonal antibody 6/41a, which targets the amino acid residues 480-493, blocked E2-CD81 binding and therefore

stated that aa480-493 on E2 could potentially be CD81 contact residues (Flint, Maidens et al. 1999). Owsianka et al designed a study that, through mutagenesis, further identified critical residues on E2 important in CD81 binding. The region comprised of amino acids 480-493 was also included in their investigation. However; amino acid substitution in this region did not result in loss of CD81 binding in this study (Owsianka, Timms et al. 2006). Findings from another previous study by the same authors, showed that mAb 6/41 was not able to block CD81-E2 interaction (Owsianka, Clayton et al. 2001). The combined results of these studies contest the involvement of E2 amino acid residues 480-493 in CD81 binding. The discrepancies in results from these studies may be explained by the fact that Flint et al included a truncated form of E2 in their work, while Owsianka used full length E1E2. The exposure of antigenic regions may differ with various forms of E2, explaining the inconsistencies in these findings.

Three regions have, through amino acid substitution, been designated and recognised as CD81 binding regions on E2 (Drummer, Boo et al. 2006; Owsianka, Timms et al. 2006; Rothwangl, Manicassamy et al. 2008). The first region spans from polyprotein position 474-492, the second region consists of amino acids 522-551, and the third binding region ranges from amino acid positions 612-619.

In 2008, a study was conducted where individual alanine substitutions were made on the first CD81 binding region at conserved residues across several HCV genotypes. The authors came to the conclusion that although the mutant proteins did show reduced HCVpp infectivity, the first binding region (amino

acid residues 474-492) is not critical in CD81 binding since the alanine mutants bound soluble CD81 as well as the wild type E2 (Rothwangl, Manicassamy et al. 2008), thus concurring with the findings of Owsianka et al (Owsianka, Timms et al. 2006).

The specific contact residues involved in the second CD81 binding region have been elucidated (Owsianka, Timms et al. 2006). Using site-directed mutagenesis of conserved amino acids identified across many functional E1E2 clones, the ability of these mutants to bind to CD81 and confer infectivity to HCVpp was assessed. The chosen regions were selected based on previous published reports on antibody reactivity and protein experiments that suggest their likely involvement in E2-CD81 binding. Thirty-seven E2 mutants were included in the study. The regions studied across these isolates were amino acid residues 412-424, 474-495 and 520-550 and between them 27 conserved residues were reported. This identified five amino acids that are potentially critical to the CD81 interaction: W420, Y527, Y529, G530 and D535. Drummer et al investigated the importance of the amino acid residues of region 436-443 (GWLAGLFY) in a study published in 2006. Mutant E1E2 virus particles showed a decrease ability to infect Huh 7, indicating that this region is important in viral entry. Moreover, amino acid residues 437,438, 441 and 442 were identified as crucial CD81 contact residues as amino acid substitution at those sites resulted in complete loss of CD81 binding (Drummer, Boo et al. 2006).

In addition to these residues already demonstrated to be important in CD81 binding, Roccasecca et al (2002) investigated a further binding site on E2; a

region encompassing amino acid residues 612-620. This region is suggested to be highly conserved across several HCV isolates (Roccasecca, Ansuini et al. 2003). These amino acids were simultaneously mutated from (YRLWHY) to (SAASAS) by site-directed mutagenesis. The mutated protein showed similar protein expression to the wild type protein, but CD81 binding was significantly reduced. Rothwangl et al conducted a similar study to investigate the individual importance of the residues within the third CD81 binding region. They presented comparable results as Roccasecca et al after performing single alanine substitution by site directed mutagenesis on amino acids 612-619. All mutants, excluding Y615A, lost their ability to bind CD81. The authors of this study claim the possibility that these residues play a critical role in binding CD81 during the entry process. However, this study did not assess the global conformation following the performed mutagenesis. The authors used AR3A a human monoclonal antibody that binds to the CD81 contact residues on E2 to verify that the CD81 binding regions were intact subsequent to the substitution experiments.

In our study, site-directed mutagenesis across conserved JFH1 E2 residues 611, 613-619 and 621, (Figures 2.1 and 2.2), was performed to individually alter single amino acid (aa) residues to alanine, and determine which are important in protein expression and binding of monoclonal antibodies known to react with wild type JFH1 E1E2. Amino acid residues 612 (proline) and 620 (cysteine) were not included in this work. Residue 612 has shown to be variable across HCV isolates and genotypes and is therefore highly unlikely to have a great role in the CD81 interaction. The cysteine at residue 620 was not

subjected to mutagenesis as altering a cysteine by mutagenesis could possibly disrupt the over-all conformation of the glycoprotein (A.W Tarr, personal communications). These mutants were initially intended to be characterised in the HCVcc assay, (therefore produced in the JFH1 strain), as well as included in a CD81-binding assay to determine the binding abilities of these mutants to CD81. However, the CD81- binding assay could not be accurately performed due to experimental limitations and the HCVcc system was not established in the lab during the course of this work. For these reasons, it was decided to take a different approach in investigating the role of amino acid residues 611, 613-619 and 621 in CD81 interaction.

The main focus of this investigation was to study the effect of mutations in the third CD81 binding site of E1E2 in order to determine the roles of individual amino acids in protein expression and their potential interaction with neutralising antibodies targeting the CD81 binding site.
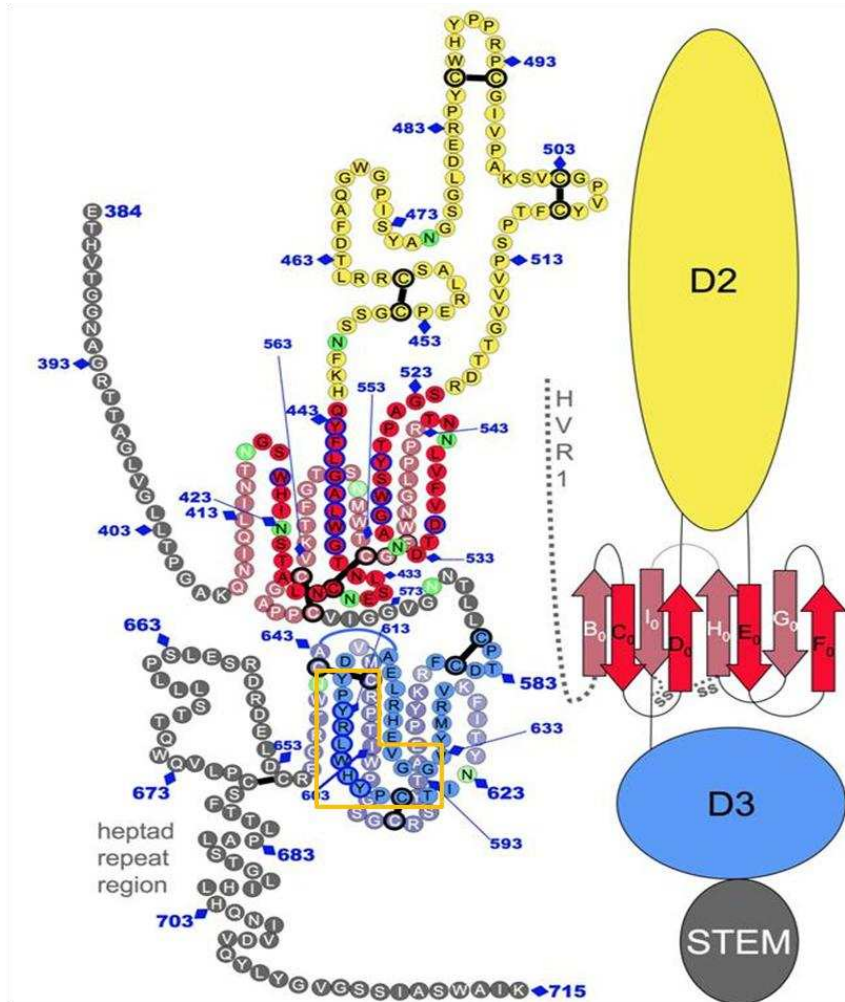
Figure 2.1: Schematic of HCV E2.
There is no published crystal structure of HCV envelope proteins E1E2 to date. Krey et al have proposed this structure, through antibody and disulfide mapping. The red middle region is implicated in CD81 and neutralising antibodies interaction. The grey 27 amino acid stretch on the left is HVR1which has been proven to bind SRBI and the orange highlighted area in the blue region marks the residues of the third CD81 site, subjected to mutagenesis in this study.
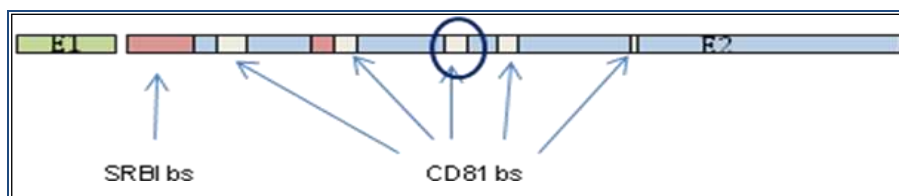
Figure 2.2: A simplified schematic of the E1E2 structure
The CD81 interacting regions are highlighted in grey where as HVR1 and HVR2 is marked in red. The circled region symbolizes the third CD81 binding site, the main focus of this study.

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2.2 Material and Methods

To generate mutant plasmids that would permit analysis of the properties of the individual amino acids in region 611-621 of the E2 protein, site directed mutagenesis was carried out to generate the panel of point mutated proteins shown in Table 2.1.

Table 2.1: Table of the individual polyprotein residues 611-621 subjected to alanine substitution by site directed mutagenesis
The left hand column shows the designated residues on the polyprotein, the middle column shows the amino acid substitution and the right hand column displays the aligned mutants to the wild type JFH1 E1E2 611-621 residues.

Residue	aa Substitution	YPYRLWHYPCT
Y611A	Tyrosine-Alanine	A----------
Y613A	Tyrosine-Alanine	--A--------
R614A	Arginine-Alanine	---A-------
L615A	Leucine-Alanine	----A------
W616A	Tryptophan-Alanine	-----A-----
H617A	Histidine-Alanine	------A----
Y618A	Tyrosine-Alanine	-------A---
P619A	Proline-Alanine	--------A--
T621A	Threonine-Alanine	----------A

The primers used in the mutagenesis reactions were designed to introduce single amino acid substitutions to specific residues in the JFH1 E1E2-phCMV vector (Table 2.2).

Table 2.2: Site directed mutagenesis primers of 611-621
Primers were designed in PrimerX software and used in the mutagenesis reaction. The highlighted bases introduce the desired mutation.Mutant plasmids were transfected into HEK 293FT cells and assessed for protein expression by SDS-PAGE and Western blot and binding interaction with conformation-sensitive, CD81-inhibiting antibodies in ELISA assays.

	Reverse: 5' ccagagtctgtaaggg*gc*gtggaccaggcactttg 3'	
JFH1 Y613A	Forward: 5' ctggtccactaccct*gc*cagactctggcattac 3'	
	Reverse: 5' gtaatgccagagtctg*gc*agggtagtggaccag 3'	
JFH1 R614A	Forward: 5' gtccactacccttac*gc*actctggcattacc 3'	
	Reverse: 5' ggtaatgccagagt*gc*gtaagggtagtggac 3'	
JFH1 L615A	Forward: 5' cactacccttacaga*gc*ctggcattacccctg 3'	
	Reverse: 5' caggggtaatgccag*gc*tctgtaagggtagtg 3'	
JFH1 W616A	Forward: 5'ctacccttacagactc*gcc*cattaacccctgcacag 3'	
	Reverse: 5' ctgtgcaggggtaatg*ggc*gaagtctgtaagggtag 3'	
JFH1 H617A	Forward: 5'cttacagactctgg*gcc*tacccctgcacagtc 3'	
	Reverse: 5'gactgtgcaggggta*ggc*ccagagtctgtaag 3'	
JFH1 Y618A	Forward: 5'cccttacagactctg*gca*tgcaccctgcacagtcaattttac 3'	
	Reverse:5'gtaaaattgactg*tgc*agggtgcatgccagagtctgtaaggg 3'	
JFH1 P619A	Forward: 5'cttacagactctggcattac*g*cctgcacagtcaattttac 3'	
	Reverse: 5'gtaaaattgactgtgcagg*c*ggtaatgccagagtctgtaag 3'	
JFH1T621A	Forward: 5'ctctggcattacccctgc*gcc*gtcaattttaccatcttc 3'	
	Reverse: 5'gaagatggtaaaattgac*ggc*gcaggggtaatgccagag 3'	

2.2.1 Site- directed mutagenesis

Mutagenesis reactions were prepared in a 50 μl volume and were set up as follows: 1× Phusion™ HF Buffer, 0.5U of Phusion™ HF polymerase (Finnzymes, Loughborough, UK), 3% DMSO (Finnzymes), 50 ng of template plasmid DNA (phCMV vector containing JFH1 full-length E1E2 or H77 sE2-pcDNA 3.1 D-TOPO vector), (Invitrogen, Paisley, UK) 125 ng of antisense primer (Table 2), 125 ng of sense primer (Table 2) (MWG/Eurofins, London, UK), 200 μM of each dNTP (Roche, Welwyn, UK). The reaction of was made up to a total volume of 50 μl using DNase and RNase-free water (Sigma-

Aldrich, Dorset, UK). Mutagenesis was achieved by thermal cycling using a PTC-100 thermocycler (Bio-Rad, Hemel Hempstead, UK) [Initial denaturation at 98 ^{o}C for 2 minutes, 25× 98 ^{o}C for 10 seconds, 55 ^{o}C for 15 seconds, 72 ^{o}C for six minutes]. The samples were then digested for two hours in 37°C with 0.5 U Dpn I (New England Biolabs, Hitchin, UK). Dpn I digest methylated bacterial DNA, and was used here to remove wild type template, leaving only mutagenesis products.

2.2.2 Gel electrophoresis

Size of mutated plasmids was confirmed via electrophoresis with an ethidium bromide-stained 1% agarose gel (Bioline, London, UK). All gels were made up at 1% (w/v) in 100 ml Tris-Acetate (40 μM, pH 8.3) (Sigma-Aldrich) EDTA (1 μM) (BDH, Lutterworth, UK) (TAE) Buffer (Sigma-Aldrich). After heating to dissolve agarose, and before pouring into casting tray, 5 μl of Ethidium Bromide (Fluka/Sigma-Aldrich) (1% w/v) was added. Gels were run in 600 ml of (TAE) Buffer and 30 μl of Ethidium Bromide (1% w/v). Gels were run at 90 V for 35 minutes. Gels were viewed using a UV trans-illuminator and the results recorded photographically.

2.2.3 Transformation in Top 10F E.coli strains

Five microlitres of mutated plasmid was added to 25 μl of OneShot Top10F E.coli (Invitrogen, Paisley, UK) and incubated on ice for ten minutes. The cells were then heat shocked in 42°C water for 30 seconds and 125 μl of Super Optimal broth with Catabolite repression (S.O.C) medium (Invitrogen) was

added and the samples were incubated in 37 ^{o}C for one hour. After incubation the transformed cells were spread on agar plates containing 100 μg/ml ampicillin sodium salt (Sigma-Aldrich) and incubated at 37 ^{o}C overnight. Bacterial colonies were inoculated in 3 ml of Lysogeny Broth (LB) (Sigma-Aldrich) containing 3 μg/ml ampicillin and incubated at 37°C overnight with shaking (225 rpm).

2.2.4 Plasmid purification (mini-prep) and quantification

Plasmids were purified from the bacterial cells using Qiaprep Miniprep Kit (Qiagen, Crawley, UK) according to the manufacturer's vacuum protocol and quantified by spectroscopy at 260 nm (Nanodrop Techonologies).

2.2.5 DNA sequencing

Approximately 150 ng of plasmid was used as template for sequencing, in a reaction containing 0.5 μl of BigDye (Applied Biosystems, Paisley, UK, Version 1.1), 3.2 pmol sequencing primer (phCMV_For or phCMV_Rev), 3.5 μl Better Buffer (Microzone, Haywards Heath, UK) and made up to a total of 10 μl using DNase and RNase-free water (Sigma-Aldrich). The reactions were run on a thermal cycler according to the following parameters: $25 \times$ 96 ^{o}C for 30 seconds, 50 ^{o}C for 10 seconds, and 60 ^{o}C for four minutes. To precipitate sequenced DNA, 50 μl 100 % ethanol, 2 μl sodium acetate (pH 5.2) and 2 μl EDTA (125 mM) and 10 μl H$_2$O (Sigma-Aldrich) was added and left at room temperature for one hour. Samples were then centrifuged at $18000 \times$ g for 30 minutes before 375 μl 70% ethanol was added to the pellet and re-spun at

$18000 \times$ g for 10 minutes. All samples were sequenced using ABI 3031 genetic analyser (Applied Biosystems) and the required mutations were confirmed using Chromas v2.23 and SeqMan computer software (DNASTAR, Madison, WI, USA).

2.2.6 Plasmid purification (midi-prep) and quantification

Plasmids were purified from bacterial cells using Qiaprep Midiprep Kit (Qiagen, Crawley, UK) according to the manufacturer's vacuum protocol and quantified spectroscopically at 260 nm (Nanodrop Techonologies) before use in cell transfection.

2.2.7 Production of JFH1 E1E2 mutants in HEK 293FT cells

HEK 293FT cells were transfected with Polyethylenimine (PEI; Fermentas, Loughborough, UK) and 6 μg of purified plasmid in 10 ml of Optimem (Gibco, Paisley, UK) media and incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 for six hours prior to changing the media to DMEM (Gibco) media containing 10% Foetal Bovine Serum (Biosera, Ringmer, UK) 1% Genetecin (Invitrogen) and 1% Non Essential Amino Acids (NEAA) (Invitrogen). The cells were incubated for an additional 48 hours at 37 ^{o}C and then lysed in lysis buffer containing 150 mM NaCl (Fisher Scientific, Loughborough, UK), 1% NP-40 (Sigma-Aldrich), 50 mM Tris (pH 7.4) (Sigma-Aldrich) and 1 mM EDTA and spun at 12.000 x g for 10 min to pellet the cell-debris. Lysates were collected and stored in -80 ^{o}C prior to usage.

2.2.8 SDS-PAGE and Western blot

In order to determine the presence of expressed mutant proteins, lysates were separated on sodium dodecyl sulfate polyacrylamide electrophoresis gel, (SDS-PAGE) (9% resolving gel and 5% stacking gel) for 1.5 hours at 150V. The proteins were transferred on to a nitrocellulose membrane and blocked in 50 ml of 5% milk in phosphate buffered saline (PBS: Oxoid, Cambridge, UK) and Tween 20 (a polysorbate surfactant) (Sigma-Aldrich) for one hour at room temperature. The membrane was then washed three times in PBS- 0.05% Tween before probed and incubated with 1:200 mAb AP33 for one hour. After the wash was repeated, polyclonal rabbit anti-mouse horseradish peroxidase was added in a dilution of 1:1000 to the membrane and incubated for one hour. Membrane was washed as before, and soaked in ECL-reagents (GE-Healthcare, Chalfont St. Giles, UK) for two minutes before developed on film (Kodak: Sigma-Aldrich) by exposing the film to the membrane for approximately three minutes before initially placing the film in 250 ml Kodak Developing solution (Sigma-Aldrich) for 30 seconds and then in 250 ml Kodak Fixing solution (Sigma-Aldrich) for an additional 30 seconds. The developed film was finally rinsed in 250 ml of water and dried for analysis.

2.2.9 Densitometry scan of the Western blot by AlphaDigiDoc 1201 software

The Western blot presenting the expressed JFH1 mutants, as well as the wild type protein, was analysed by AlphaEaseFC/ AlphaDigiDoc 1201 densitometry software (Alpha Innotech) to accurately quantify the expression of each sample. The results were analysed in Prism GraphPad version 4.

2.2.10 GNA capture ELISA of mutant proteins

Enzyme linked immunosorbent assay (ELISA) was performed to confirm a dose-dependent recognition of the mutant expressed proteins by the primary antibody, AP33. A 96-well Maxisorp microplate (Nunc, Roskilde, DK) was coated in 5 µg/ml of Galanthus nivalis agglutinin (GNA; Sigma-Aldrich) and incubated overnight at 4 ^{o}C. The wells were blocked with 5% milk in PBS-0.05% Tween for two hours before adding 50 µl of mutant E1E2, diluted in PBS- 0.05% Tween, in three-fold dilutions series and placed at room temperature for two hours. The plate was then washed three times in PBS-0.05% Tween and 50 µl of 1 µg/ml mAb AP33 was added to the wells and incubated for one hour at room temperature. After repeating the washes with PBS-0.05% Tween as above, the wells were incubated with 50 µl of 1:1000 rabbit anti-mouse IgG-Horse radish peroxidase (Sigma-Aldrich) in PBS-0.05% Tween. After three washes, 50 µl of 3,3',5,5' Tetramethylbenzidine (TMB; Sigma-Aldrich) substrate was added, the plate developed for a maximum of five minutes. As the available plate reader was not able to read at a wavelength of 620 nm (after the addition of TMB), the reaction was stopped with 25 µl of 2 M H_2SO_4 (Sigma-Aldrich) and plate was read at 450 nm (Multiskan EX plate reader: MTX Lab Systems, Vienna, Virginia, USA).

2.2.11 Binding of monoclonal antibodies to E1E2 mutants

ELISA was performed using a 1:6 dilution of the mutant E1E2 and JFH1 wild type E1E2. The proteins were again captured with 5 µg/ml GNA and detected with 10 µg/ml of mouse monoclonal antibody ALP98 and human monoclonal

antibodies, 1:7, AR3A, AR3B, AR3C and CBH4G. 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG in PBS-0.05% Tween was added to detect 1:7, AR3A, AR3B, AR3C and CBH4G as goat anti-mouse IgG-alkaline phosphatase was used to detect ALP98. Following three washes, para-nitrophenylphosphate (PNPP; Sigma-Aldrich,) substrate was added, the plate developed for a maximum of 20 minutes and read at 405 nm (Molecular Devices V max plate reader, Sunnyvale, CA, USA).

As alkaline phosphatase conjugated secondary antibody was used in this experiment due to evidently greater specificity and sensitivity than horse radish peroxidise conjugated secondary antibodies, PNPP substrate was included and the plate was read at a different wavelength to the prior experiment.

2.3 Results

2.3.1 Alanine scanning on the individual residues of the third CD81 binding site, 611-621

To elucidate the importance of each individual residue of the third CD81 binding site on E2, site-directed mutagenesis was performed on JFH1 E1E2 to introduce individual alanine residues in the region spanning amino acids 611-621. Sequencing of mutant plasmids confirmed successful alanine substitutions at the desired amino acid positions (Table 2.1).

2.3.2 Expression and Western blot analysis of JFH1 E1E2 611-621 mutant E2 glycoproteins

The mutant plasmids were transfected into HEK 293FT cells to express the desired proteins. The cell lysates containing the mutant E1E2 were run on SDS-PAGE and detected with an anti-E2 monoclonal antibody (AP33) in a Western blot to confirm the presence of expressed E1E2 proteins. The gel electrophoresis, (Figure 2.3), verified that the single alanine substitutions did not impair the expression of the mutant proteins as all the lysates contained detectable E2 glycoprotein at the predicted molecular weight.
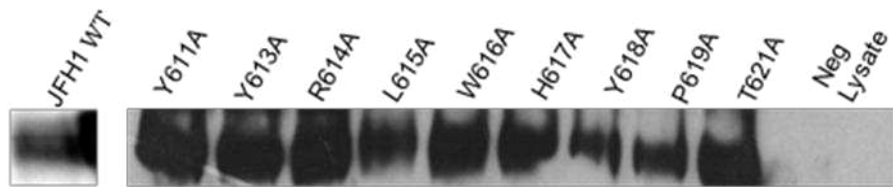
Figure 2.3: Western blot of the expressed JFH1 E1E2 mutant proteins.
Single alanine substitutions were introduced by site-directed mutagenesis at the individual residues of the third CD81 binding site on JFH1 E2, polyprotein residues 611, 613-619 and 621. The mutants were generated in the pcDNA 3.1 vector prior to transfection in Human Embryonic Kidney 293FT cells. The expressed proteins were separated on a polyacrylamide gel by SDS-PAGE and detected by mouse monoclonal antibody AP33 in a Western blot. An untransfected HEK293T lysate was included as negative control. Protein expression was verified by the Western blot. JFH1 E1E2 wild type was analysed on a separate gel due to limited space.

2.3.3 Densitometry analysis of the Western blot displaying the JFH1 mutants

To quantify the levels of expression in each individual protein sample, the Western blot was scanned and an Integrated Density value was calculated for each protein band by AlphaEaseFC/AlphaDigiDoc 1201 software. Background values were subtracted from each protein band and the results were analysed in Prism GraphPad software version 4.

The results obtained from the densitometry analysis (Figure 2.4) were generated based on the protein expression levels of the Western blot (Figure 2.3) and confirm that the site-directed mutagenesis did not impair protein expression; in fact, Figure 2.4 shows enhanced expression of the mutant proteins compared to the JFH1 wild type protein. This data indicates that single alanine substitution at these residues increase the translation efficiency in HEK 293FT cells.

66
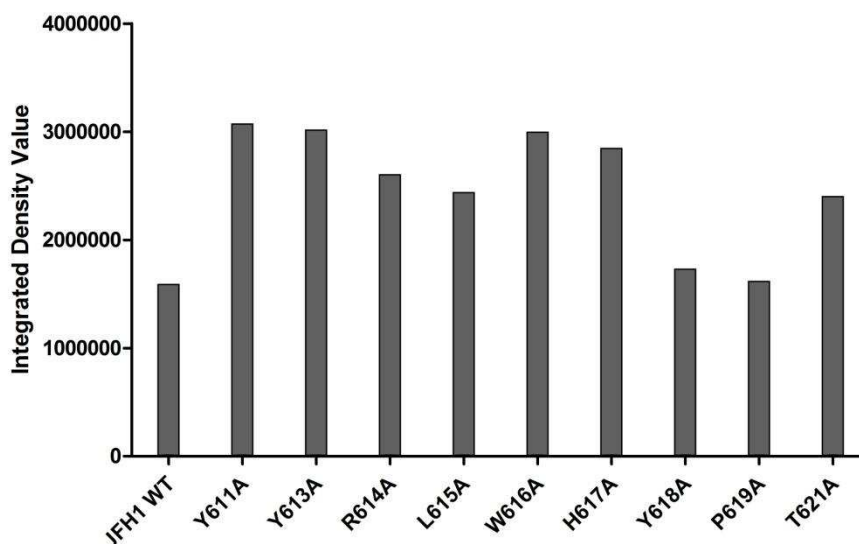
Figure 2.4: Densitometry analysis of the Western blot film displaying the expressed JFH1 proteins.

AlphaEaseFC/AlphaDigiDoc 1201 software was utilised to quantify the Integrated Density Value of each expressed protein sample. The background value was subtracted from each sample and the results were analysed in Prism GraphPad software version 4. Mutant proteins show higher expression levels compared to wild type JFH1.

2.3.4 GNA capture ELISA of mutant proteins

As a spectrophotometer will include a total protein count in the quantification and therefore not discriminate between protein aggregates and correctly formed E1E2 proteins, the outcome will result in an inaccurate quantification. Therefore, the lysates were analysed using a GNA capture ELISA and detected with the E2- specific linear mouse monoclonal antibody AP33. Titration curves were created to determine the maximum signal produced using the minimal amount of cell lysate. The preferred working concentration, a dilution of 1:6 of JFH1 WT and mutant protein was based on the graph below.
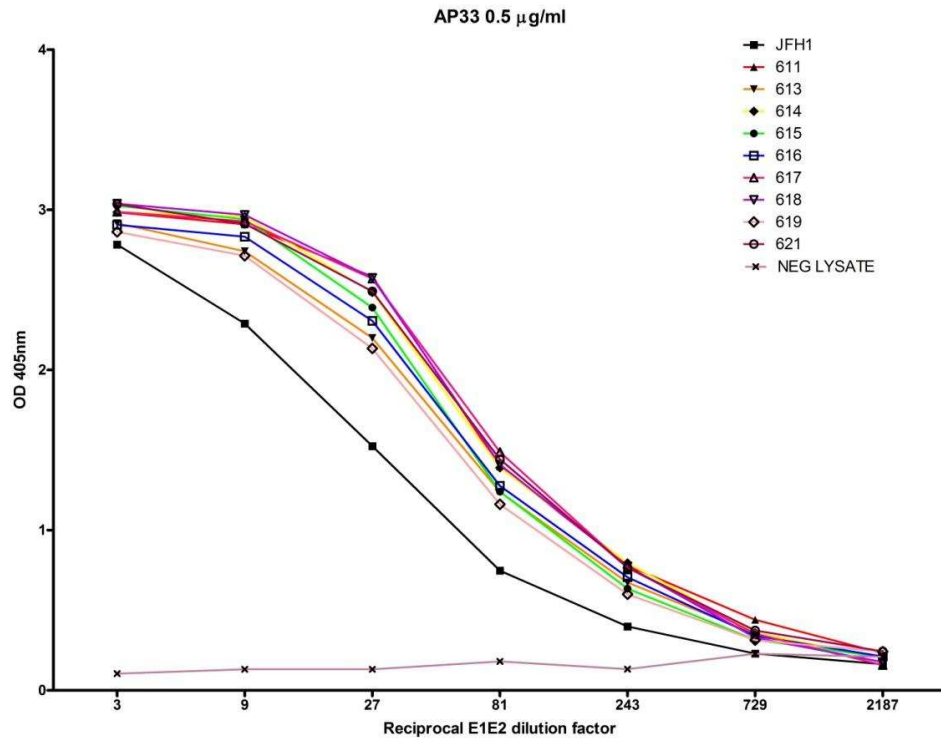
Figure 2.5: Titration graph of the JFH1 E1E2 mutant proteins.
An ELISA was performed by capturing E1E2 on 5 µg/ml GNA prior to detecting with 0.5 µg/ml monoclonal mouse antibody AP33. Anti-mouse Alkaline Phosphatase was added in a dilution of 1:1000 to the wells before the addition of PNPP substrate followed by 25 µl of 1M H_2SO_4 stop solution. The plate was read at an optical density of 450 nm and the results were plotted in Fig.4 using GraphPad Prism version 4. Negative lysate was included as a negative control. Based on the results of the graph, a dilution of 1:6 was determined as the working concentration of the E1E2 lysates.

2.3.5 ELISA assay with linear mouse monoclonal antibodies AP33 and ALP98

For an additional verification of similar JFH1 E1E2 expression levels between the lysates, one further ELISA experiment was performed. Two mouse monoclonal antibodies were included that target E2 at different linear epitopes; AP33 that binds polyprotein residues 412-424 and ALP98 targeting residues between 640-654. As both of these antibodies bind to E2 outside of the region subjected to site- directed mutagenesis in this work; they are expected to bind with comparable affinity to the JFH1 E1E2 lysates and therefore subsequently

should show relative protein amounts in samples. Briefly; JFH1 E1E2 proteins were added at a dilution of 1:6 to wells coated with GNA before detecting with mouse monoclonal antibodies AP33 and ALP98. Upon the addition of conjugated anti-mouse secondary antibody and substrate, the plate was read at an optical density of 405 nm and the results were plotted on a graph.

Figures 2.6A and B further confirms the expression of JFH1 E1E2 proteins by the linear mAbs AP33 and ALP98 respectively; moreover, a similar binding pattern to the JFH1 mutants can be observed.

A)
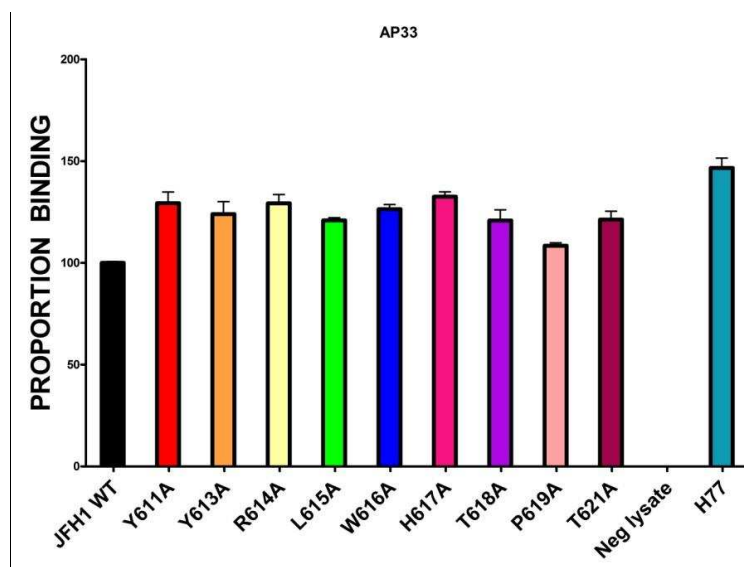
B)
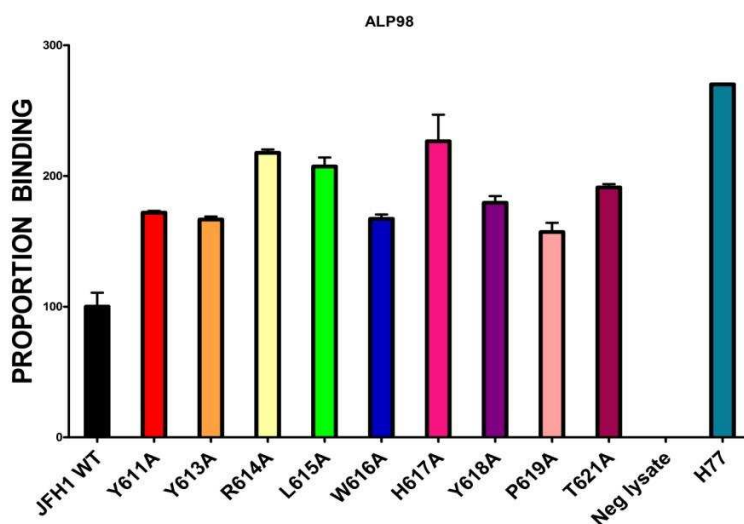
Figure 2.6:ELISA assay with JFH1 E1E2 mutants detected with linear monoclonal antibodies AP33 and ALP98.
1:6 JFH1 E1E2 mutant proteins diluted in PBS was captured by 5μg/ml of GNA and detected with 10 μg/ml linear mouse monoclonal antibodies, AP33 (A) and ALP98 (B). Following the addition of conjugated anti-mouse secondary antibody, PNPP substrate and 1M of H_2SO_4, the plate was read at an optical density of 405 nm and the results were plotted in a graph using GraphPad Prism Version 4. The Optic Density (OD) of the mutants were normalized to the OD of the JFH1 WT. Negative lysate acts as negative control and H77 E1E2 acts as primary antibody control in the assay. The data obtained from the graphs confirm the expression levels of the various mutant proteins as the two linear antibodies bind to the JFH1mutants respectively in a comparable fashion.

2.3.6 ELISA assay with CD81 inhibiting monoclonal antibodies

To assess the significance of the individual residues in the third CD81 binding site, the generated JFH1 E1E2 mutant proteins along with JFH1 wild type were included in an ELISA assay utilising four conformation sensitive human monoclonal antibodies; 1:7, AR3A, AR3B and AR3D (Allander, Drakenberg et al. 2000; Law, Maruyama et al. 2008). These CD81 inhibiting (Law, Maruyama et al. 2008) human mAbs target discontinuous epitopes on the HCV E2 glycoprotein and testing them against the JFH1 E1E2 mutants would disclose the importance of each amino acid residue in 611-621 -region in binding them. Figures 2.7A to D show bar graphs of each antibody targeting the mutants and the JFH1 WT protein. The mutant proteins Y611, R614, L615, H617, Y618, P619 and T621 showed reduce binding to all of the conformation-sensitive CD81 binding site antibodies, suggesting that either these residues were involved in antibody binding or that the introduced changes abrogated protein fold. By contrast, all mAbs showed a slight enhanced binding to mutant protein Y613A which indicates a possible conformational change due to the mutagenesis at that residue that exposes the epitopes to these antibodies. W616A showed a binding pattern comparable to the JFH1 wild type protein.
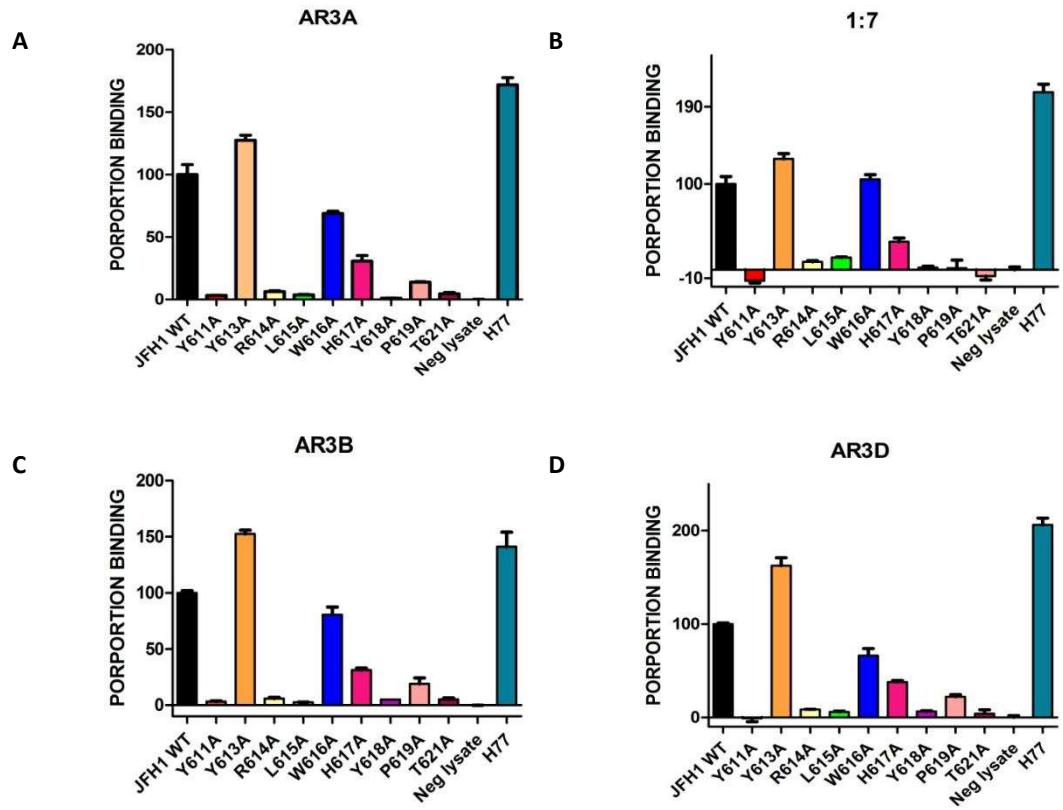
Figure 2.7: JFH1 E1E2 mutants detected by AR3A, 1:7, AR3B and AR3D.
1:6 JFH1 E1E2 mutant proteins diluted in PBS was captured by 5μg/ml of GNA and detected with human conformation sensitive monoclonal antibodies, AR3A (A), AR3B (B), AR3D (C) and 1:7 (D). Following the addition of conjugated anti-human secondary antibody, PNPP substrate and 1M of H2SO4, the plate was read at an optical density of 405 nm and the results were plotted in a graph using GraphPad Prism Version 4. The ODs of the mutants were normalized to the OD of the JFH1 WT. Negative lysate acts as negative control in the assay and H77 E1E2 acts as primary antibody control. The collective data obtained from these graphs indicate that Y611, R614, L615, H617, Y618, P619 and T621 possibly are crucial in binding these mAbs.

2.3.7 ELISA assay with a non-CD81 inhibiting, conformation sensitive monoclonal antibody

CBH4G is a conformational sensitive human monoclonal antibody that targets a discontinuous epitope (E2 residues unknown) on E2 outside of the CD81 binding regions (Hadlock, Lanford et al. 2000). Analysing CBH4G against the JFH1 E1E2 mutants will disclose whether the single alanine substitution has disrupted the overall conformation of E1E2 and subsequently rendered the proteins misfolded. This is crucial information since this will help interpretation of the data obtained for the CD81 binding site antibodies (Figure 2.7).

The mutant E2 proteinsY611, R614, L615, H617, Y618, P619 and T621 all displayed reduced recognition by the non-CD81bs antibody CBH4G, highlighting that all of these mutations probably disrupted the overall conformation of the E2 glycoprotein (Figure 2.8).

73
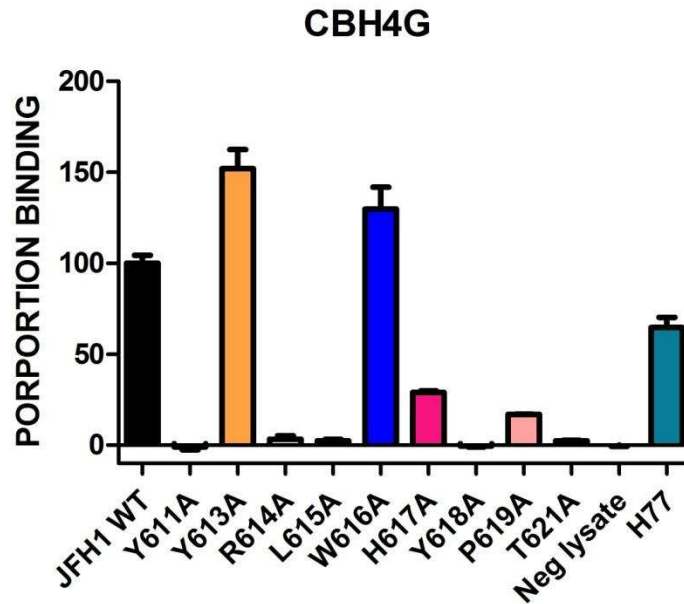
Figure 2.8: JFH1 E1E2 mutants detected by CBH4G in ELISA assay.

To investigate whether the site-directed mutagenesis disrupted the overall protein fold of E1E2, 1:6 JFH1 E1E2 mutant proteins (diluted in PBS) was captured by 5 µg/ml of GNA and detected with human conformation sensitive a monoclonal antibody CBH4G, which target E2 outside the CD81 contact residues. Following the addition of conjugated anti-human secondary antibody, PNPP substrate and 1M of H_2SO_4, the plate was read at an optical density of 450 nm and the results were plotted in a graph using GraphPad Prism Version 4. The ODs of the mutants were normalized to the OD of the JFH1 WT. Negative lysate acts as negative control and H77 E1E2 acts as primary antibody control. The results obtained from this experiment reveal that site-directed mutagenesis at Y611, R614, L615, H617, Y618, P619 and T621 disturbs the overall conformation of JFH1 E1E2 and that these residues are involved in the upholding of the overall protein fold.

2.4 Discussion

To understand the specific activity of broadly neutralising antibodies it is important to identify the receptor interactions which they block. Identification of specific contact residues on the surface of E2 directs generation of neutralising antibodies against these regions in an attempt to block the interaction between the glycoproteins and host receptors to subsequently hinder viral entry. Antibodies that are generated against these receptor binding sites and are successfully capable of neutralising the main genotypes of the virus are potentially attractive therapeutic agents; preferably in a liver transplant setting to prevent re-infection of the new liver. Since approximately thirty percent of liver transplanted HCV infected patients will develop cirrhosis of the liver within five years of the transplant, the demand for liver transplants are thought to increase dramatically (Schofield, Bartosch et al. 2005).

CD81 has been identified as one of the important receptors on cells that HCV glycoproteins bind to during the entry process (Pileri, Uematsu et al. 1998). Published reports have suggested that various discontinuous conserved regions of HCV glycoprotein E1E2 within and between different genotypes of the virus are involved in CD81 binding (Lavillette, Tarr et al. 2005; Owsianka, Timms et al. 2006). These regions have been mutated to reveal their importance in CD81 binding and other cellular receptors (Scarselli, Ansuini et al. 2002; Owsianka, Timms et al. 2006), as well as their ability to react with certain monoclonal antibodies derived from patients with acute and chronic Hepatitis C infection (Allander, Drakenberg et al. 2000; Law, Maruyama et al.

2008). One of these regions, aa613-618 on E2, has been implicated by mutation to be important in the interaction with CD81 (Roccasecca, Ansuini et al. 2003).

There are three antigenic regions on E2, AR1, AR2 and AR3 (Law, Maruyama et al. 2008). Anti-E2 antibodies, which target the identical CD81 binding regions, have shown to differ in neutralisation breadth in HCVpp and HCVcc assays (Johansson, Voisset et al. 2007; Keck, Li et al. 2008; Law, Maruyama et al. 2008; Edwards, Tarr et al. 2012) and can be divided into three groups, the broadly neutralising, non-neutralising and restricted neutralising antibodies. Collected data on the specificity and neutralisation ability of known E2 targeting antibodies show that the non-neutralising antibodies target AR1, while the broadly neutralising antibodies target AR3. AR2A, the only antibody targeting AR2, has been shown to neutralise specific isolates of HCV, and therefore, shows restricted neutralisation ability (Edwards, Tarr et al. 2012). The discontinuous human monoclonal antibodies AR3A, AR3D and 1:7 have all exhibited broad neutralisation ability (Johansson, Voisset et al. 2007; Law, Maruyama et al. 2008). Like all broadly neutralising E2 antibodies (Edwards, Tarr et al. 2012) these three antibodies also target AR3 (Law, Maruyama et al. 2008). The fact that some antibodies recognise the same E2 amino acid residues and block CD81 binding in a similar fashion, but display different neutralisation properties suggests that there are unidentified epitopes that are involved in the recognition of these antibodies. We included the AR3A, AR3D and 1:7 in our study with the prospects of disclosing such epitopes within the third CD81 binding region on E2.

We discovered, in this work, that site-directed mutagenesis in the third CD81 binding region, aa residues 611-619 and 621, disrupted the overall conformation of E1E2. Previously published studies on substitution analysis of the third CD81 binding region on E2 solely assessed the region subjected to mutagenesis as the antibody included in the experiment targets CD81 contact residues only (Rothwangl, Manicassamy et al. 2008). From these findings the authors conclude that these regions on E2 are important in binding CD81 (Roccasecca, Ansuini et al. 2003; Rothwangl, Manicassamy et al. 2008). However, as these studies lacked the appropriate experiments to confirm correct global protein folding following the mutagenesis, misfolded protein could be responsible for these results. In fact, in this study, three mutants did not bind to AR3A (the antibody used to confirm intact E2 structure). Rothwangl et al discuss these observations as due to a possible disruption in the epitope of AR3A or a potential misfolded E2 (Rothwangl, Manicassamy et al. 2008), but do not go on to differentiate between the two. The loss in CD81 binding observed with the mutants in these studies could be due to misfolded proteins, as correct conformation of E2 is known to be required for its interaction with CD81 (Flint, Maidens et al. 1999). AR3A is a human conformation sensitive monoclonal antibody that binds to residues 424, 525, 530 and 535 (Law, Maruyama et al. 2008), all of which have been implicated in CD81 contact (Owsianka, Timms et al. 2006). AR3A was used in the Rothwangl study to ensure that those specific residues, proven critical in CD81 interaction, were intact following the site-directed mutagenesis. However, the use of this antibody does not provide any information about the conformation of rest of the mutant E1E2 protein outside of the CD81 binding region tested.

Two of the mutants that did not bind to AR3A in the Rothwangl article, R614A and W616A, were included in our study. AR3A also showed a loss of binding to R614A in our experiments. In the case of W616A, abrogation was not complete as approximately 70% binding was noticed compared to the wild type protein. These discrepancies in results can be explained by either the difference of E1E2 genotypes or the antibody assays performed. JFH1 E1E2 (genotype 2) was included in our quantitative ELISA experiment while Rothwangl et al used H77 E1E2 (genotype 1) in a qualitative immunoprecipitation assay.

Owsianka et al published a paper in 2006 that characterised conserved regions on E2 that are implicated in CD81 binding. The authors performed alanine substitutions on the first and second CD81 binding regions on H77 E1E2. Although aa residues 611,613-619 and 621 on E2 was not included in that study, this work is similar to our study as a conformation sensitive mouse monoclonal antibody that binds outside of the CD81 binding regions was in fact used to confirm correct overall fold of the protein (Owsianka, Tarr et al. 2005). This antibody was not suited for our work as H53 does not recognise JFH1 E2, therefore CBH4G was selected instead. CBH4G was included as it is a human conformation sensitive monoclonal antibody targeting E2 outside of the CD81 binding sites (Hadlock, Lanford et al. 2000), disclosing the general structure of the JFH1 E1E2 proteins following mutagenesis. We produced the mutants in the JFH1 genotype as it was intended to include them in the HCVcc assay, however the assay could not be established while this work was performed. There were also extensive attempts to include the mutants in a

CD81-binding assay; however, as the control parameters showed unreliable results, the data from the assay could not be included in this work.

The combined results obtained from our study showed that single alanine substitutions at HCV polyprotein residues Y611A, R614A, L615A, H617A, Y618A, P619A and T621A enhanced the protein expression in HEK 293FT cells (compared to the wild type protein), but disrupted the native overall conformation of E2 and thereby the epitopes of CD81-targeting monoclonal antibodies.

All mutants showed an enhancement in protein expression levels compared to the wild type JFH1 protein, demonstrated by the Western Blot (Figure 2.3) and Densitometry analysis (Figure 2.4). This might imply that alanine substitutions at the residues of the third CD81 binding region may or may not enhance the translation efficiency in HEK 293FT cells. As these experiments were carried out in a cell line that would not normally harbour HCV, and have not yet been replicated in vitro in hepatoma cell lines, (e.g., Huh7 or Huh7.5 cells), nor in primary hepatocytes in vivo, it is difficult to assume whether such mutations would result in similar enhancement in protein expression in natural disease settings. Should the virus carry a mutation in natural infection would it benefit the translation efficiency of the infected cell?

Other potential explanations for the noticed enhanced mutant protein expression compared to the wild type protein might be the possible difference in antibody binding between the mutants and the wild type E1E2. The

mutations could introduce changes in the epitopes of these mAbs on the glycoproteins.

Most work involving substitution mutagenesis use alanine as the substitution preference as it is a small, non-polar, hydrophilic molecule. The alanine backbone contains a C-beta carbon that helps in adapting to conformational changes in protein structures (Betts and Russell 2003) just as well as other amino acids. Its side chain has proven to be non-reactive, and therefore is not often involved in protein function. These properties of alanine can account for favouring this amino acid in substitution mutagenesis. However, when employing this strategy to generate the mutant E2 proteins used in this study, substitution of various amino acids with alanine was found to have numerous effects on E2 expression and folding, probably due to the loss of critical properties (e.g. size, charge, presence of functional side chains, etc.) provided by the original amino acids at those sites. These effects are discussed in more detail below.

As JFH1 mutants Y611A and Y618A displayed a decrease in binding the conformational dependent monoclonal antibodies, this suggests that the effect of the substitution from Tyrosine (Y) to Alanine at position 611 and 618 was vast enough to disrupt the native conformation of the residues. By contrast, Y613A mutant protein seemed to bind the monoclonal antibodies better than the wild type protein. This may have been the result of a post mutation positional effect as the swap from Tyrosine to Alanine at residue 613 might have triggered a conformational change, exposing the epitopes and consequently enabling the antibodies to bind.

Some of the substitutions involved a change from amino acids with functional side chains to Alanine, for example, Proline to Alanine at position 619 (P619A). Proline connects into the protein backbone twice, introducing a turn. It is reasonable to assume, therefore, that the loss of Proline at this site would result in a loss of protein conformation.

Other substitutions in the third CD81 binding region performed in this work have also included the exchange of polar amino acids, for example Arginine (R), Threonine (T), Tyrosine (Y) and Histidine (H) to the non-polar amino acid Alanine. The loss of polarity at the specific residues could possibly explain the disruption of protein fold and subsequent loss of recognisable antibody epitopes.

While it may not be ideal to use a small, neutral amino acid such as Alanine to substitute bigger, charged amino acids in functional phenotypical experiments, it is the best option to date as the potential knock out effect caused by the substitution can aid the understanding of the protein functionality. However, it is of great importance to assess the overall conformation of the protein in order to authenticate any significant findings.

In conclusion, whilst the residues discussed above are suggested to be critical for CD81 binding, the findings of this research project support their importance in correct overall conformation of the E1E2 glycoprotein. Moreover, these results confirm that E2:CD81 interaction is conformation dependent.

3 Antigenic exposure of CD81 binding sites upon E2: SRBI interaction

3.1 Background

During the entry process it is proposed that HCV E2 initially binds to SRBI, prior to interacting with the other purposed HCV receptors CD81, Claudin-1 and Occludin. Initial findings that lend support to this theory included a study performed in 2006. Dreux et al investigated the role of HDL: SRBI interaction in HCV cell entry. The authors concluded that HDL and SRBI interaction lead to the endocytosis of SRBI bound HCVpps. Moreover, a time lag of approximately 60 minutes was noticed for the endocytosis of cell surface-bound virus particles. The authors of this study suggested that HDL: SRBI interaction potentially could increase the rate at which CD81 is recruited to bind virus and possibly aid internalization into the hepatocyte through a cholesterol dependent pathway (Dreux, Pietschmann et al. 2006). There is evidence that SRBI and CD81 act in cooperation in HCV infection (Kapadia, Barth et al. 2007). When Huh 7 cells were incubated with anti-CD81 and anti-SRBI antibodies at identical concentrations, a significantly greater inhibition of JFH1 infectivity was observed. Twenty five microlitres of anti-CD81 antibody or 1:50 dilution of anti-SRBI resulted in approximately 12- and 9-fold decrease, respectively, in JFH1 RNA levels; however, these antibodies

together accomplished a decrease in JFH1 RNA of over 100-fold suggesting that CD81 and SRBI cooperate in regulating HCV entry.

A subsequent article emerged when Evans et al in 2007 constructed a study which identified claudin-1 as a co-receptor in HCV late stage entry (Evans, von Hahn et al. 2007). The binding of HCVcc-derived virus to Chinese hamster ovary cells expressing SRBI, CD81 and claudin-1 separately was investigated. Of these cells, viral E2 binding was only observed with SRBI. These observations lead to the conclusion that HCV glycoproteins possibly required binding to SRBI prior to an eventual conformational change rendering the epitopes on E2 accessible for CD81 binding.

Grove et al designed a study in 2008 that investigated the effect of a mutation at residue G451R on JFH1 E2 in receptor dependency and sensitivity to neutralising antibodies in the HCVcc system. In their study, the authors found that this G451R JFH1 virus exhibited enhanced binding to CD81 as well as increased sensitivity to both patient-derived polyclonal IgG neutralization and mAb 3/11 (which targets the specific E2 residues at aa412-423; (Flint, Thomas et al. 1999)) compared to the wild type JFH1 virus. The results of this study indicated glycine-to-arginine substitution at E2 residue 451 caused an epitope exposure of the CD81 binding region (Grove, Nielsen et al. 2008). Moreover, published studies have shown that the deletion of HVR1, the SRBI binding site on E2, enhances the binding to CD81 (Roccasecca, Ansuini et al. 2003; Bankwitz, Steinmann et al. 2010) as well as reducing SRBI binding (Scarselli, Ansuini et al. 2002; Roccasecca, Ansuini et al. 2003). This suggests that HVR1 is, in the native conformation, possibly shielding the CD81 contact residues on

E2, and therefore, when SRBI binds to HVR1 on E2, the CD81 epitopes become exposed and arranged for CD81 binding.

This work investigates a potential conformational change of the glycoproteins following SRBI binding that renders the CD81 binding sites on E2 accessible to the CD81 receptor. To examine this theory further, a plate based assay was performed with monoclonal antibodies targeting the CD81 binding domains. Biotinylated anti-his-tag antibody served as a control to which the experiments are normalised. A cell-based SRBI binding assay was also performed, which included the same antibodies previously utilized in the plate based assay, to establish whether the presence and contact with SRBI exhibits an effect on the antigenic exposure of the CD81 binding regions.

By combining the results of these experiments, the accessibility of these binding domains can be investigated to either confirm or refute a theory of a potential conformational change upon E2:SRBI interaction, prior to CD81 binding (Figure 3.1).

A)

A)

B)

B)
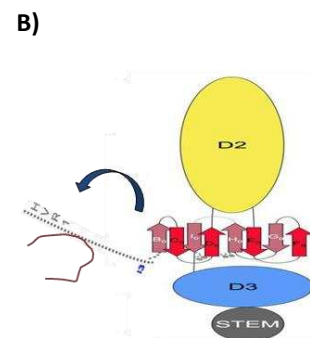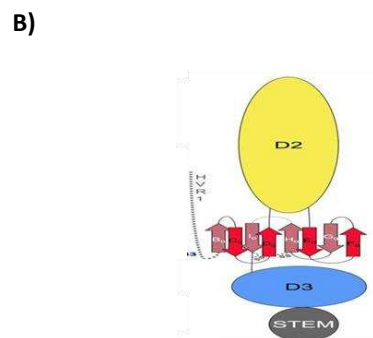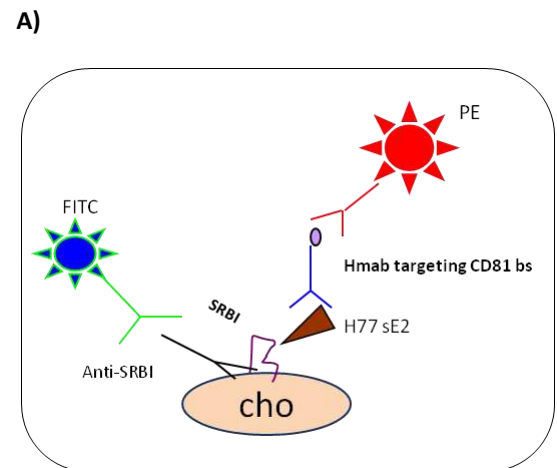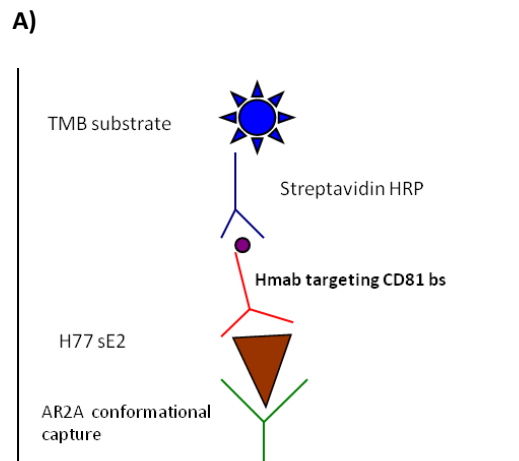
Figure 3.1: A) A schematic diagram of the plate based assay (ELISA) not including SRBI. AR2A was coated in a 96 well microtitre plate to capture the correctly folded H77 soluble E2.

AR2A was coated in a 96 well microtitre plate to capture the correctly folded H77 soluble E2. Biotinylated human monoclonal antibodies targeting CD81 binding sites was added prior to the addition of Streptavidin horse radish Peroxidase (HRP). The reaction was visualised using TMB before the plate was read at an Optical Density (OD) of 620 nm. B) A diagram of the expected form of E2 included in the experiment. The red and pink arrows represent the CD81 binding sites, yellow circle represents Domain 2 (D2) and Blue circle demonstrates Domain 3 on the E2 structure. The long stretch of amino acids on the left is HVR1, implicated in SRBI binding. It is, in this figure, showing the way HVR1 is suggested to shield the CD81 binding sites on E2.

Figure 3.2: A) A schematic of the cell based assay including SRBI. Chinese Hamster Ovary (CHO) cells were transfected with human Scavenger Receptor B Type 1 (SRBI).

Chinese Hamster Ovary (CHO) cells were transfected with human Scavenger Receptor B Type 1 (SRBI). H77 sE2 was added to the cells before detecting the SRBI with anti-human SRBI antibody and FITC-conjugated secondary antibody. The sE2 was detected with biotinylated human monoclonal antibodies targeting the CD81 binding sites and PE-conjugated secondary antibody. The binding was visualised by flow cytometry analysis. B) A diagram of the expected form of E2 included in the experiment. The red and pink arrows represent the CD81 binding sites, yellow circle represents Domain 2 (D2) and Blue circle demonstrates Domain 3 on the E2 structure. The long stretch of amino acids on the left is HVR1, implicated in SRBI binding. It is, in this figure, bound to SRBI to show the possible conformational change upon HVR1: SRBI interaction, exposing the CD81 binding sites.

3.2 Material and Methods

3.2.1 Production of H77 soluble E2 truncated at aa 661

The H77 sE2 is produced without the transmembrane residues (aa717-727), permitting secretion of the protein. HEK 293T cells were transfected with 3 ml of Optimem media (Invitrogen, Paisley, UK) containing 24 µg of H77 $sE2_{661}$ pcDNA 3.1 D-TOPO plasmid with 60 µl lipofectamine (Invitrogen), and incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 for 72 hours. Collected supernatant was spun at 250 x g for five minutes to remove non-adherent cells. To prevent the formation of disulphide bonds amongst free cysteines, and subsequently the development of protein aggregates, 20 mM of Iodoacetamide (Sigma-Aldrich, Dorset, UK) was added to harvested supernatant before short term storage at 4 ^{o}C.

3.2.2 Nickel-agarose purification of soluble E2 supernatant

The expressed sE2 had been tagged with six histidines in order to facilitate purification with HisTrap HP affinity column (Amersham Bioscienses, Little Chalfont, UK). Histidines, as other amino acids, have the ability to covalently bind to metal. This function has been employed in the purification by AKTA PRIME (Amersham Bioscienses). A binding buffer containing 20 Mm NaH_2PO_4 (Sigma-Aldrich), 300 mM NaCl (Sigma-Aldrich) and 20 mM Immidazole (Sigma-Aldrich), pH 7.4 was used to pre-wash the column before commencing the purification. The sE2 supernatant, with added 150 Mm NaCl

(Sigma-Aldrich), was applied to the column. The HisTrap column was then washed with binding buffer and the purified protein was eluted at 100 mM immidazole (Sigma-Aldrich) by increasing step-wise gradient to a maximum of 500 mM immidazole in 20 mM NaH_2PO_4 (Sigm-Aldrich), 300 mM NaCl (Sigma-Aldrich), pH 7.4. All eluted fractions were collected for analysis.

3.2.3 SDS-PAGE and Western blot

In order to determine which fractions contained the purified protein, eluted fractions were separated on a sodium dodecyl sulfate polyacrylamide electrophoresis gel, (SDS-PAGE) (9% resolving gel and 5% stacking gel) for 1.5 hours at 150 V. The proteins were transferred on to a nitrocellulose membrane and blocked in 50 ml of 5% milk in phosphate buffered saline (PBS) (Oxoid, Hampshire, UK) and 0.05% Tween (Sigma-Aldrich) for one hour at room temperature. The membrane was then washed three times in PBS -0.05% Tween before probed and incubated with the mAb ALP33 at a dilution of 1:200 for one hour. After the wash was repeated, polyclonal rabbit anti-mouse horseradish peroxidase (Sigma-Aldrich) was added in a dilution of 1:1000 to the membrane and incubated for one hour. Membrane was washed as before, and soaked in ECL-reagents (GE-Healthcare, Chalfont St. Giles, UK) for two minutes before developed on film (Kodak: Sigma-Aldrich) by exposing the film to the membrane for approximately three minutes before initially placing the film in 250 ml Kodak Developing solution (Sigma-Aldrich) for 30 seconds and then in 250 ml Kodak Fixing solution (Sigma-Aldrich) for an additional 30 seconds. The developed film was finally rinsed in 250 ml of water and dried for analysis.

3.2.4 Concentration of H77 sE2661

Pooled fractions containing sE2 was concentrated using Amicon Ultra Centrifugal Filtering Devices (Millipore, Watford, UK) with a molecular weight cut-off at 30 kD. Briefly, 15 ml of purified fractions were centrifuged for five minutes at 4000 x g. Concentrated protein was recovered in approximately 1 ml of PBS. The sE2 was then quantified spectroscopically at 260 nm (Nanodrop Techonologies) before use in experiments.

3.2.5 Biotinylation of human monoclonal antibodies

Mouse monoclonal antibodies AP33 and ALP98 along with human monoclonal antibodies 1:7 and AR3A were biotinylated according to the EZ-Link Micro Sulfo –NHS- Biotinylation kit (Pierce, Cramlington, UK). Briefly, Sulfo-NHS-Biotin was added to approximately 50 µg of antibody. After 60 minutes incubation at room temperature the samples were spun in a column at 1000 x g for two minutes to remove the excess biotin. The antibodies were collected and stored in -20 $^{\circ}$C.

3.2.6 Titrations of sE2 protein captured by AR2A

Enzyme linked immunosorbent assay (ELISA) was performed to confirm capture of correctly folded protein by a conformation sensitive human monoclonal antibody, AR2A. Also, to verify a dose-dependent recognition of sE2 by the primary antibody, mouse monoclonal AP33 was used. A micro titre plate (Nunc Maxisorp, Roskilde, DK) was coated with 1 µg/ml AR2A and incubated overnight at 4 $^{\circ}$C. The wells were blocked with 5% milk in PBS-

0.05% Tween for two hours before adding 50 µl sE2 (stock concentration of 300 µg/ml), diluted in PBS-0.05% Tween, in three-fold dilutions series and placed at room temperature for two hours. The plate was then washed three times in PBS-0.05% Tween and 50 µl of 0.5 µg/ml biotinylated AP33 was added to the wells and incubated for one hour at room temperature. After repeating the washes with PBS-0.05% Tween as above, the wells were incubated for one hour with 50 µl of 1:1000 Streptavidin horseradish peroxidase (Sigma-Aldrich) in PBS-0.05% Tween. After thorough washing, 50 µl of TMB (Sigma-Aldrich) substrate was added and the plate developed and read at 620 nm (Molecular Devices V max plate reader, Sunnyvale, CA, USA).

3.2.7 Titrations of biotinylated HmAbs captured by AR2A (ELISA)

A 96- well microplate (Nunc Maxisorp) was coated with 1 µg/ml AR2A and incubated overnight at 4 ^{o}C. The wells were blocked with 5% milk-PBS 0.05% Tween for two hours before adding 50 µl sE2 at 6 µg/ml of H77 sE2 and placed at room temperature for two hours. The plate was washed three times with PBS- 0.05% Tween and detected with decreasing titrations (starting at 10 µg/ml and diluted five-fold thereafter) of biotinylated anti-his-tag antibody (Qiagen, Crawley, UK), biotinylated human monoclonal antibodies 1:7, AR3A, and mouse monoclonal antibodies AP33 and ALP98. 1:1000 dilution Streptavidin horseradish peroxidase (Sigma-Aldrich) in PBS-0.05% Tween was added to detect the primary antibodies and placed for one hour at room temperature. After thorough washing, 50 µl of TMB (Sigma-Aldrich)

substrate was added and the plate developed and read at 620 nm (Molecular Devices V max plate reader).

3.2.8 Human SRBI transfection of CHO cells

Chinese Hamster Ovary (CHO) cells were seeded in Ham's F-12 Nutrient media (Sigma-Aldrich) in six well dishes (Corning; Sigma-Aldrich) and transfected in 10 ml Optimem (Sigma-Aldrich) with 4 µg human SRBI DNA in pCDNA 3.1 vector and 10 µl Lipofectamine (Invitrogen) per well. The cells were then incubated for six hours before replacing the media with 10 ml of Ham's F-12. The cells were incubated for 24 hours at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 and then before harvested and stained.

3.2.9 Cell based SRBI staining assay (SRBI binding assay)

The CHO cells were washed three times with 1 ml PBA (PBS with 0.1% BSA (Sigma-Aldrich)) and centrifuged at 300 x g for five minutes to remove excess formaldehyde prior to SRBI staining. To permeabilise the cells previous to the addition of the intracellular anti-SRBI antibody, the cells were washed once with 1 ml of PBA buffer containing 40 µg/ml Saponin (PBA/ SAPONIN) (Sigma-Aldrich) before washed twice with 1 ml of PBA/SAPONIN buffer with 2% FCS (PBS/SAPONIN/FCS). Between washes the cells were centrifuged for five minutes at 300 x g. Five microlitres (1:1000 dilution) of anti-SRBI antibody (Abcam, Cambridge, UK) was added to the cells and incubated at 4 ^{o}C for one hour in the dark. The cells were thereafter washed twice as previously with 1 ml of PBA buffer and 1 ml of PBA/ Saponin/ FCS

respectively. Five microlitres (1:10 dilution) of polyclonal swine anti-rabbit FITC (Dako, Ely, UK) was added to the samples and incubated as previously. Excess antibody was removed by washing twice with PBA/ Saponin before fixed in 500 µl 0.05% formaldehyde. Samples were stored in 4 ^{o}C in the dark in preparation for analysis by flow cytometry on an Epics Altra (Beckman Coulter, High Wycombe, UK).

3.3 Results

3.3.1 Plate based ELISA assays of biotinylated monoclonal antibodies targeting CD81 binding sites on histidine-tagged H77 soluble HCV glycoprotein E2

To assess the binding affinity of the biotinylated monoclonal antibodies to histidine-tagged H77 soluble E2, ELISAs were performed. AR2A, a conformational sensitive non-neutralising human monoclonal antibody which targets E2 outside of the CD81 binding residues (Law, Maruyama et al. 2008; Edwards, Tarr et al. 2012), was included in the experiment to capture correctly folded soluble E2.The bound his-tagged soluble E2 was then detected using dilutions of biotinylated monoclonal antibodies (mAb); AP33 targets region 412-423 on E2, ALP98 binds to amino aa640-654, (both ALP98 and AP33 are linear mouse mAbs), AR3A which binds E2 residues 424, 436-447 and 523, 530,535, 538, 540 , 1:7 binds to residues 523, 529, 530, 535 (both are conformation sensitive mAbs) and anti-his-tag. The anti-his mAb acted as the positive control, against which all the other antibody reactivities were

normalized. AP33, 1:7 and AR3A all bind to putative CD81 contact residues; ALP98 targets E2 outside of the receptor binding regions.

A)

Bio-AP33

B)

anti-his-tag

C)

Bio-1:7
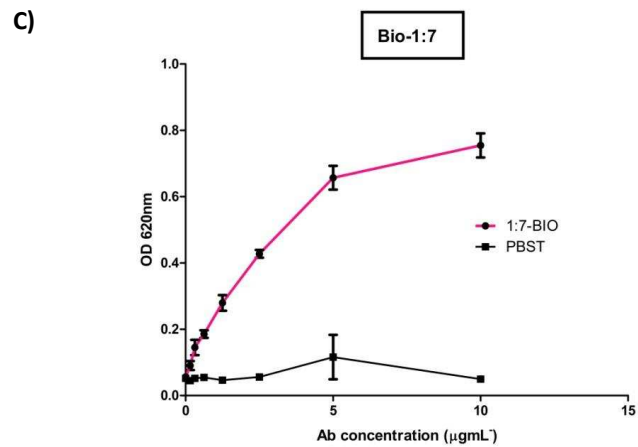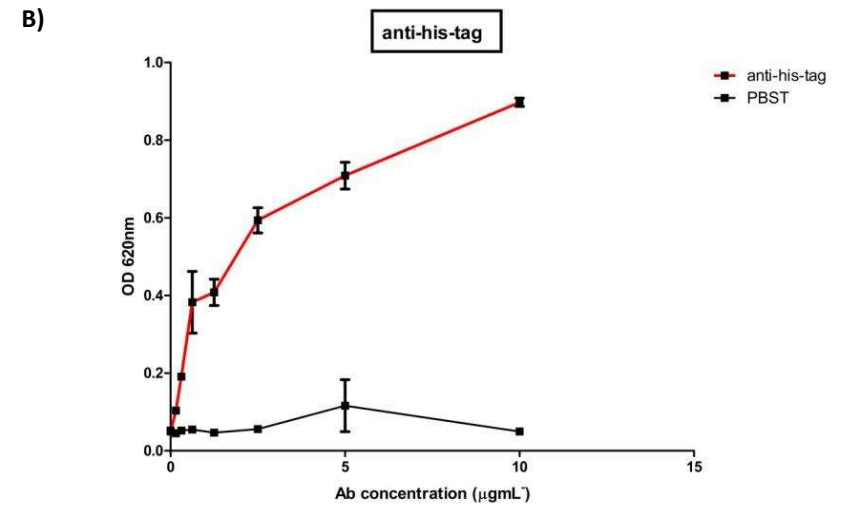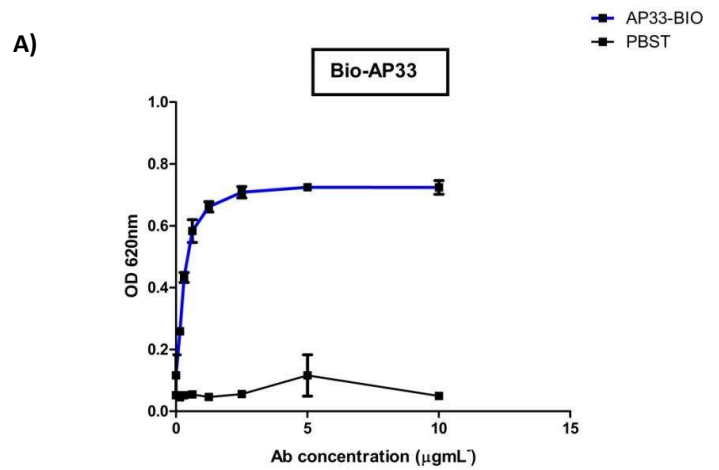
Figure 3.3: Titration curves of three of the antibodies bound to his-tagged H77soluble E2.

HCV E2 was captured by AR2A, a conformation sensitive human monoclonal antibody that targets E2 outside of the CD81 binding regions. The detecting biotinylated antibodies were added to 6 μg/ml sE2 ,at an initial concentration of 10 μg/ml and then continuously diluted in PBS-0.05% Tween in fivefold dilutions. The primary antibodies were detected with Streptavidin HRP at a dilution of 1:1000 before the assay was visualized with TMB and read at 620 nm. A) Anti-his-tag antibody was included as the positive control. B) Blue graph shows an example of a linear monoclonal antibody, biotinylated AP33 detecting his-tagged sE2. C) The red graph demonstrates an example of a conformation sensitive monoclonal antibody, biotinylated 1:7 detecting his-tagged sE2.The black line in each graph the negative reading of PBS-0.05% Tween.

3.3.2 Cell based SRBI assay with biotinylated monoclonal antibodies targeting CD81-binding sites on Histidine-tagged H77 soluble E2.

In order to determine whether the CD81 binding sites are exposed following SRBI-E2 interaction, a cell based SRBI assay was performed. Human SRBI was firstly transfected onto Chinese Hamster Ovary (CHO) cells. His-tagged H77 soluble E2 was added before detecting the SRBI- bound sE2 with the biotinylated monoclonal antibodies used in the previous plate based ELISA experiment at a concentration of 50 µg/ml. Streptavidin- PE was added to detect the biotinylated antibodies. To ensure the successful expression of human SRBI on the CHO cells, the cells were washed with buffer containing saponin to permeabilise the cell membrane allowing the intracellular anti-SRBI antibody to bind. Secondary antibody conjugated with FITC was added and the stained cells were fixed in 0.5% Formaldehyde before analysed by flow cytometry.

The SRBI-stained cells displayed approximately 80% positivity for total SRBI expression. Single secondary antibody stains were performed on the CHO cells to assess any potential background signal. Figures 3.4 and 3.5 show dot plots obtained from FACS results. Of the SRBI-positive cells, approximately 22% (PE MFI of 93), 32% (PE MFI of 47), 26% (PE MFI of 72), 33% (PE MFI of 73) and 22% (PE MFI of 48) stained with the anti-his antibody and the mAb AP33, mAb ALP98, mAb 1:7 and mAb AR3A, respectively.
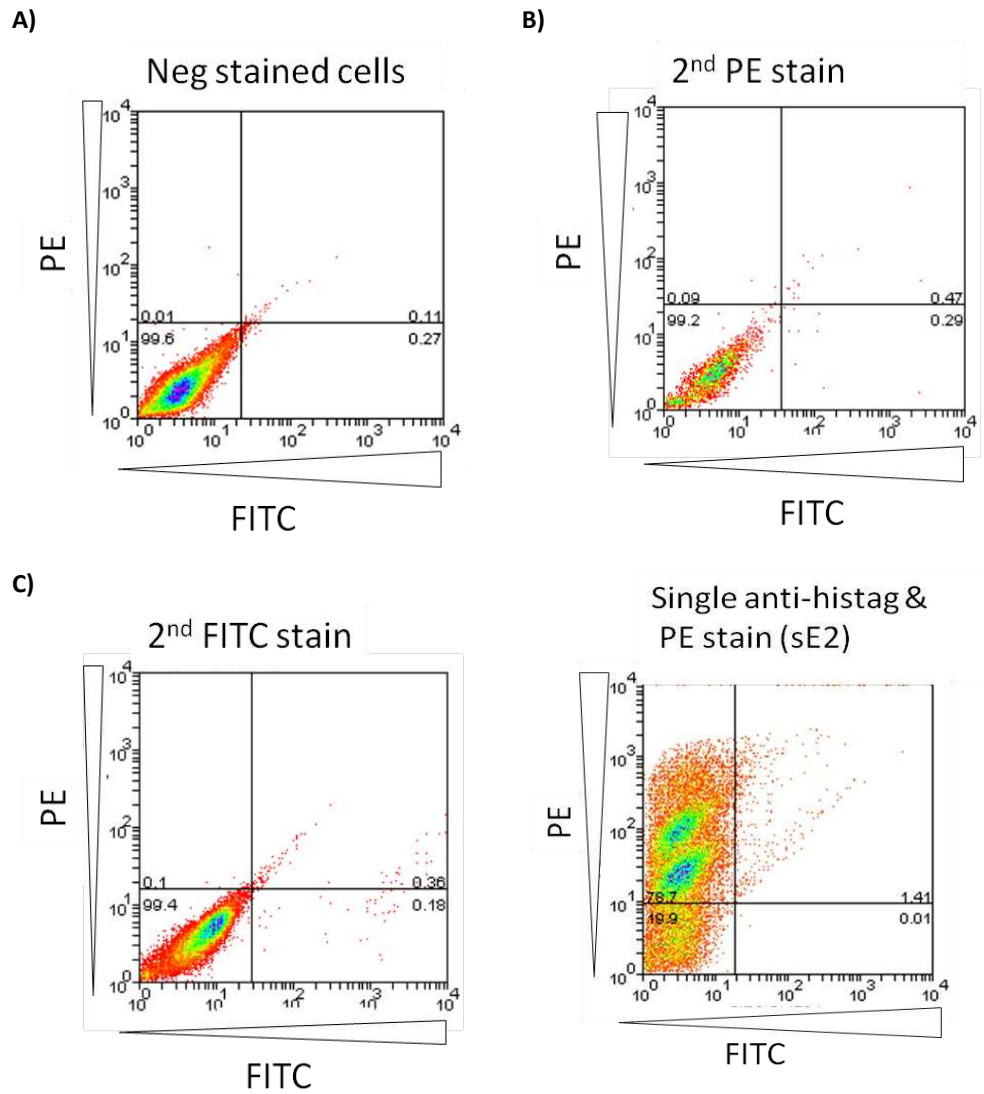
Figure 3.4: Flow cytometry graphs of the controls included in the cell based SRBI assay.
A) displays unstained Huh7 cells. B) and C) show cells stained singularly with PE- and FITC labeled secondary antibody respectively to assess any potential background signal. D) shows cells stained singularly with PE secondary antibody to assess any potential background signal.
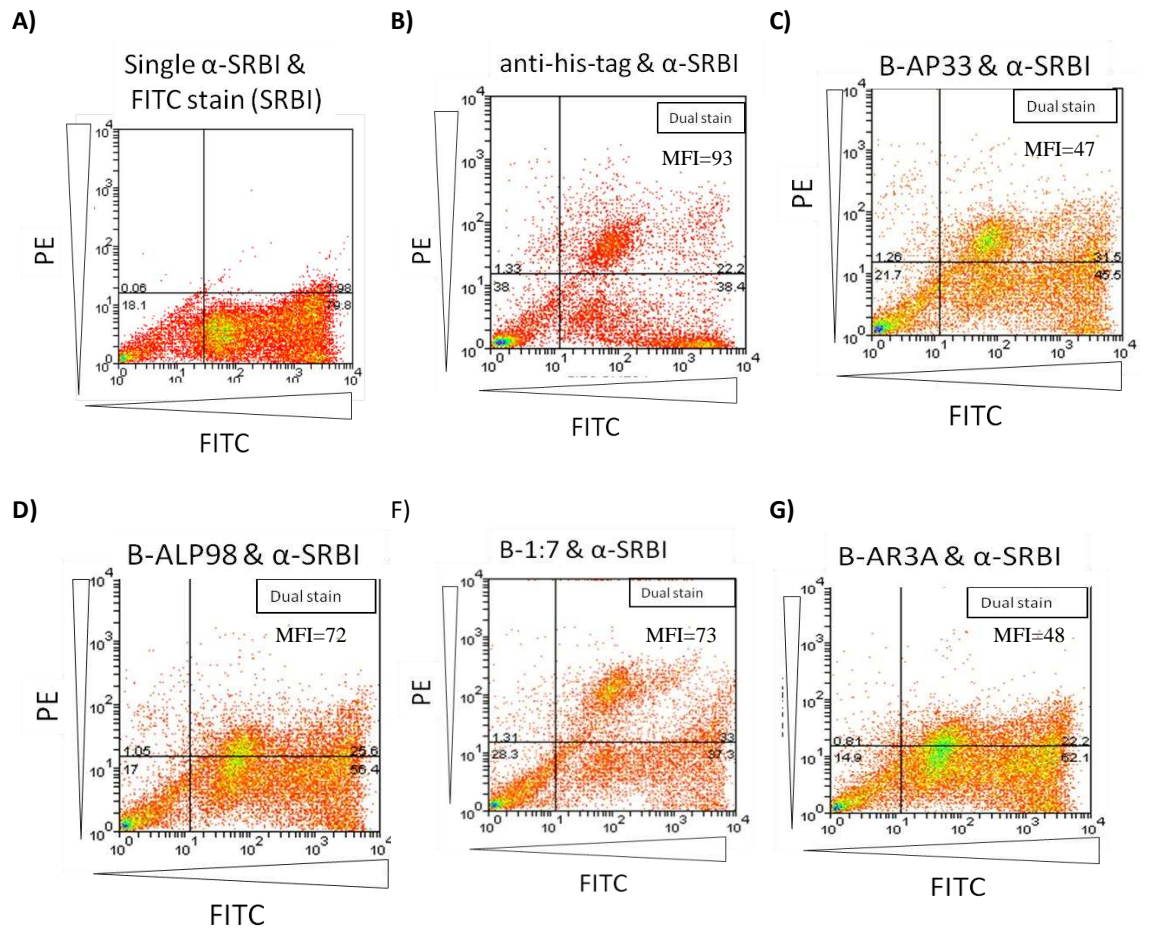
Figure 3.5: Flow cytometry graphs of the controls included in the cell based SRBI assay.

Flow cytometry graphs of the controls included in the cell based SRBI assay. A) and B) show cells stained singularly with PE- and FITC labeled secondary antibody respectively to assess any potential background signal. Dual stain of sE2 and SRBI was performed with the addition of secondary Streptavidin PE-labeled antibody and FITC conjugated secondary antibody respectively, prior to flow cytometry analysis. C)-G) The dual stain (right upper quadrants of the dot plots) illustrates the percentage of sE2 (detected by the biotinylated mAbs and Streptavidin PE) bound to SRBI (detected with the intracellular α-SRBI antibody and FITC conjugated antibody). The PE Mean Fluorescence Intensity of the sE2 bound to SRBI is presented on the right upper quadrant of each dot plot.

3.3.3 Regression analysis of the results obtained from the biotinylated monoclonal antibodies in plate based ELISA and the cell based SRBI assay

In order to obtain a comprehensive impression of the binding affinity of each antibody used in the experiments to His-tagged H77 soluble E2, Scatchard plots were created from the ELISA and FACS dilution curves. Scatchard plots are commonly used to linearise data from saturation binding curves to obtain binding constants and thereby used to calculate the affinity of a ligand with a protein. This plot is a method to establish the quantity of ligand-binding sites on a receptor and the respective affinities of each. The X-axis displays the specific binding of the ligand and the Y-axis shows the specific binding divided by free ligand concentration. From the graph, a value for Bmax and Kd are obtained whereby Bmax (X-intercept) is the maximum binding of the antibody to the protein and Kd (negative reciprocal of the curve) is the affinity constant. Bmax refers to the available binding events and Kd is the dissociation constant which reveals the affinity (strength of binding) between the receptor and its ligand (Kenakin 2009). Figures 3.6 to 3.10 displays the Scatchard plots of the biotinylated monoclonal antibodies bound to his-tagged H77 soluble E2 in the plate based ELISA assay and the cell based SRBI binding assay. Displayed on the graphs below are the Bmax and the Kd values of respective antibody obtained from the regression analysis. The Kd values are presented in units of µg/ml as well as nM (for a general ease of comparison) while the Bmax are presented as OD values at 620 nm (ELISA) and MFI values (cell based assay). The Kd values observed in the cell based assay were higher than those observed in the ELISA as demonstrated in Table

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3.1. This suggests that the antibody affinities to E2 were reduced for cell-bound protein in the SRBI binding assay, compared to plate-bound E2 in the ELISA. Additionally, 1:7 exhibited the weakest affinity to soluble E2 both in the plate based assay and the cell based assay, with Kd values of 2.770 µg/ml (1.8×10^{-2} nM) and 7.7 µg/ml (5×10^{-2} nM), respectively. 1:7 also exhibited the highest Bmax values of the E2-targeting antibodies in both assays at an OD of 0.9720 and an MFI value of 77 in the cell based assay. In contrast to 1:7, AR3A displayed the strongest affinity to plate bound E2 of all monoclonal antibodies targeting the CD81 binding regions, with a Kd value of 0.1290 µg/ml (8.6×10^{-4} nM). In the cell based assay, AR3A also proved to bind with high affinity (second to AP33) to SRBI-bound E2, with a Kd value of 1.1 µg/ml (7.4×10^{-3} nM). The Bmax value of AR3A was comparable to the rest of the conformational sensitive mAbs in the ELISA with an OD of 0.8039. However, AR3A displayed a lower Bmax in the cell based assay compared with the other human mAbs, with an MFI of 43. These results indicate that AR3A possesses less binding sites on SRBI-bound soluble E2 compared to 1:7 and interacts with greater affinity. AP33 proved to bind with the highest affinity to cell-bound E2, with a Kd value of approximately 1.0 µg/ml (6.6×10^{-3} nM). This linear mAb also exhibited the lowest Bmax with an MFI of 41 in the cell based assay. ALP98 exhibited lowest Bmax value of all antibodies in the ELISA with an OD of 0.2026 compared to the rest of the antibodies, suggesting that this antibody comprises less binding sites on plate-bound sE2 than the conformational sensitive mAbs targeting the CD81 contact residues. The Kd and Bmax values of all antibodies are summarized in Tables 3.1 and 3.2, respectively.

LINEAR mAb AP33: PLATE BASED ELISA
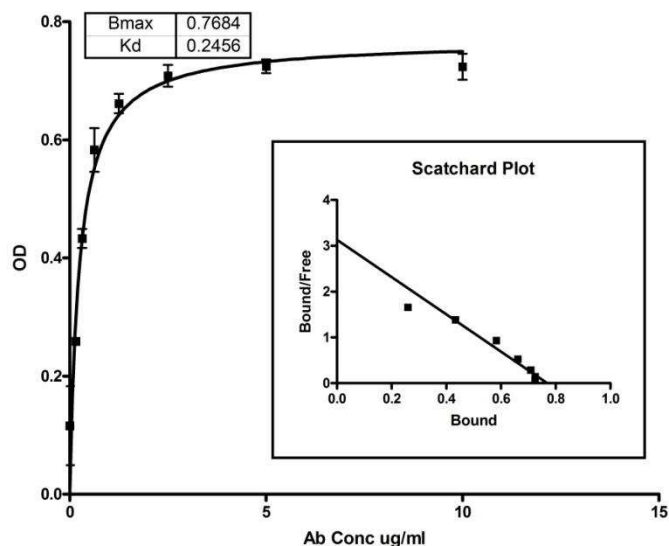
LINEAR mAb AP33: CELL BASED SRBI ASSAY
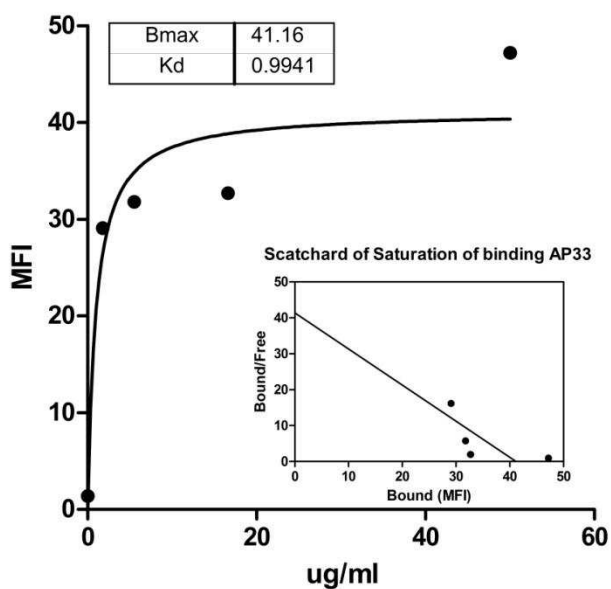
Figure 3.6: Scatchard plots of biotinylated linear mAb AP33 targeting histine –tagged H77 soluble E2 at the CD81 binding regions.

Bmax (X-intercept) is the maximum binding of the antibody to the protein presented as OD value at 620 nm in the plate based assay and MFI value in the cell based assay. The Kd (negative reciprocal of the curve) is the affinity constant presented in μg/ml.

ANTI-HIS-TAG: PLATE BASED ELISA
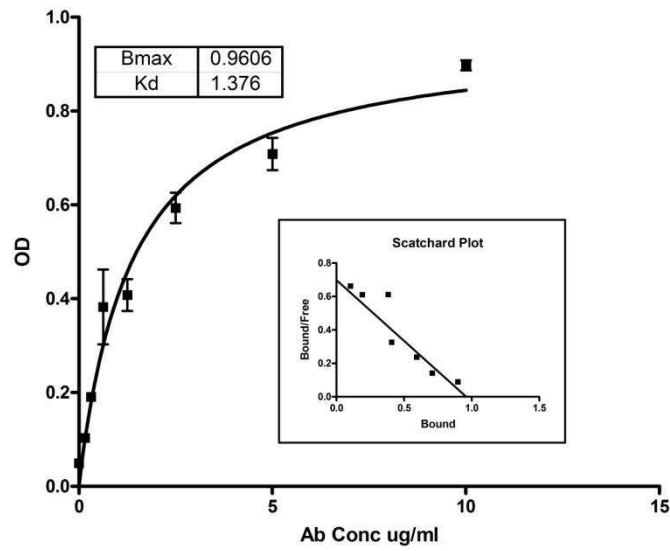
ANTI-HIS-TAG: CELL BASED SRBI- BINDING ASSAY
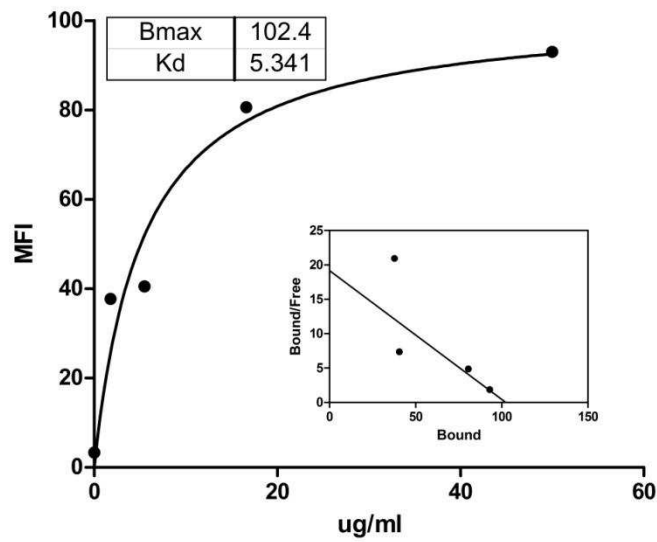
Figure 3.7: Scatchard plots of anti-his-tag antibody targeting histine –tagged H77 soluble E2. Bmax (X-intercept) is the maximum binding of the antibody to the protein presented as OD value at 620 nm in the plate based assay and MFI value in the cell based assay. The Kd (negative reciprocal of the curve) is the affinity constant presented in µg/ml.

ALP98: PLATE BASED ELISA ASSAY
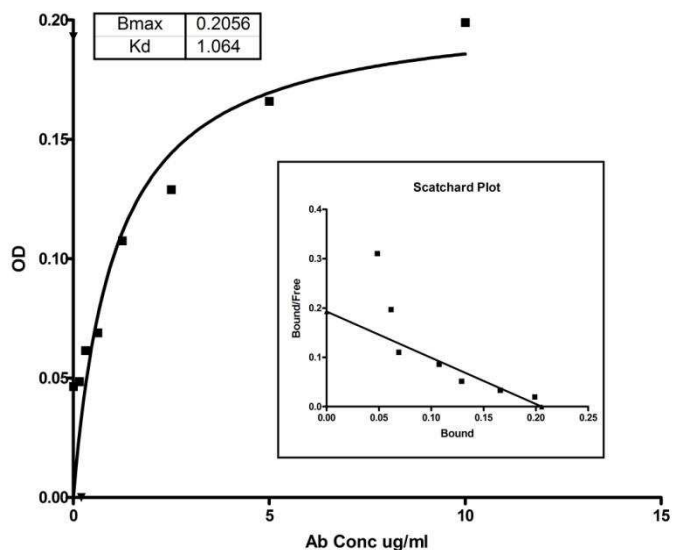
ALP98: CELL BASED SRBI-BINDING ASSAY
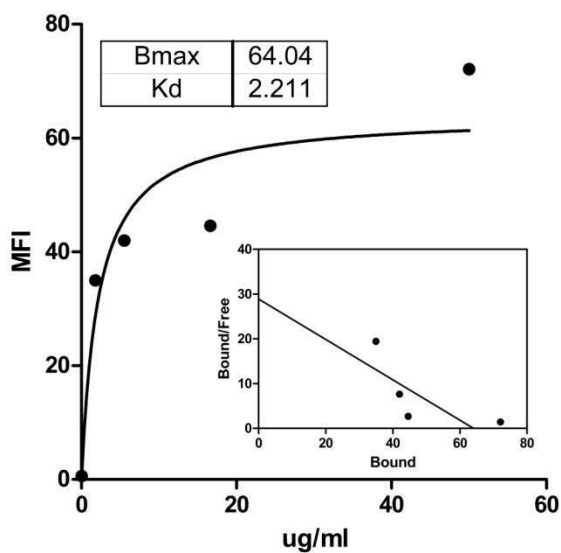
Figure 3.8: Scatchard plots of the linear mouse antibody ALP98, targeting histine –tagged H77 soluble E2 outside the CD81 binding regions.
Bmax (X-intercept) is the maximum binding of the antibody to the protein presented as OD value at 620 nm in the plate based assay and MFI value in the cell based assay. The Kd (negative reciprocal of the curve) is the affinity constant presented in µg/ml.

1:7: PLATE BASED ELISA
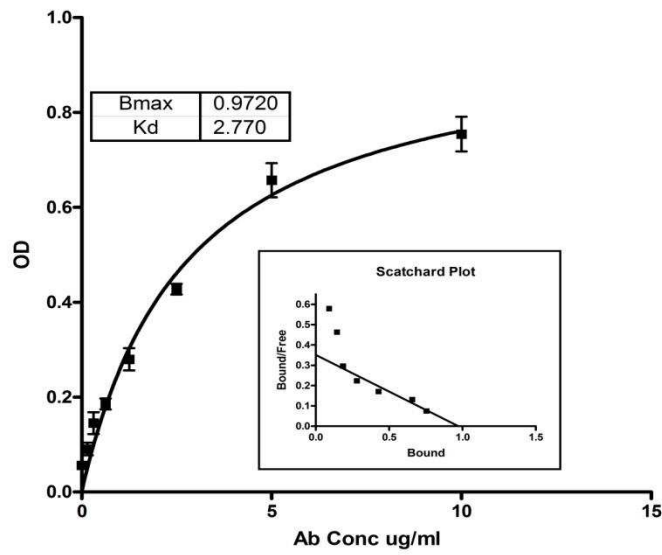
1:7: CELL BASED SRBI- BINDING ASSAY
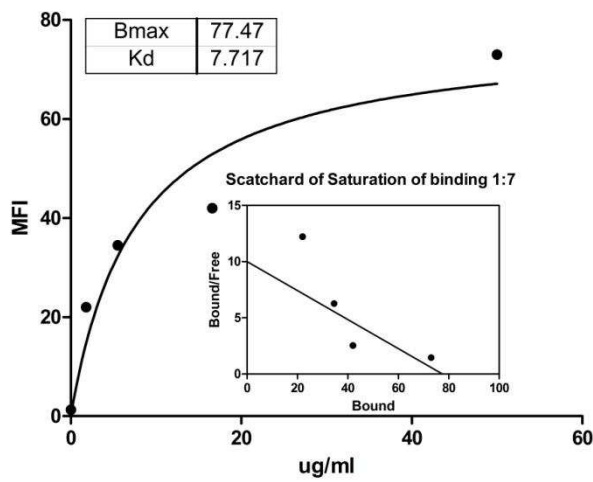
Figure 3.9: Scatchard plots of the conformation sensitive human antibody 1:7, targeting histine –tagged H77 soluble E2 at the CD81 binding regions.
Bmax (X-intercept) is the maximum binding of the antibody to the protein presented as OD value at 620 nm in the plate based assay and MFI value in the cell based assay. The Kd (negative reciprocal of the curve) is the affinity constant presented in µg/ml.

AR3A: PLATE BASED ELISA
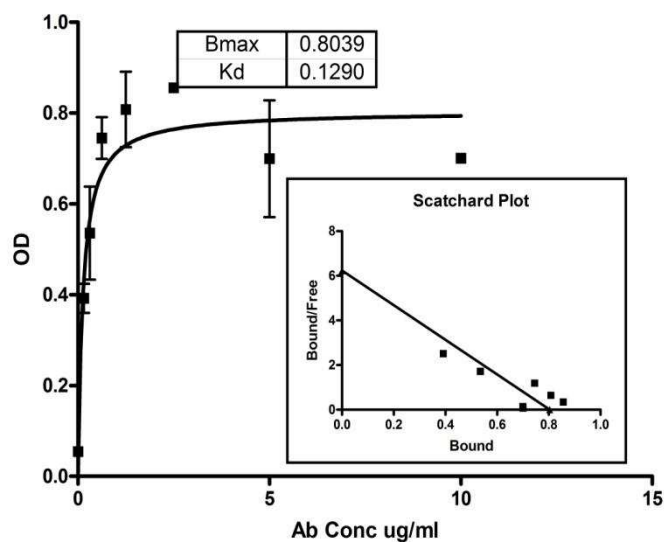
AR3A: CELL BASED SRBI-BINDING ASSAY
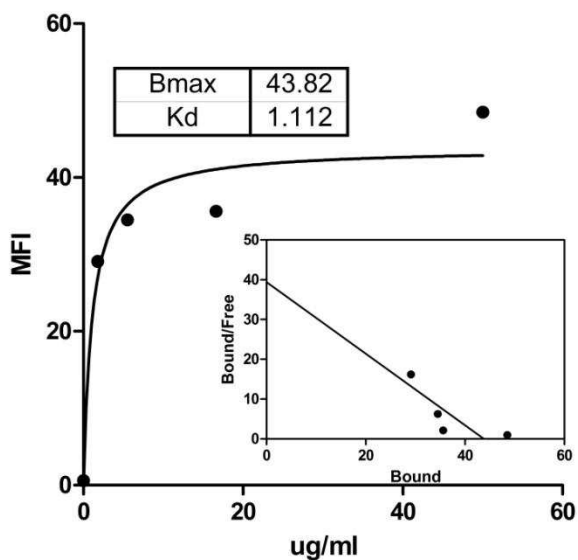
Figure 3.10: Scatchard plots of the conformation sensitive antibody AR3A, targeting histidine-tagged H77 soluble E2 at the CD81 binding regions.
Bmax (X-intercept) is the maximum binding of the antibody to the protein presented as OD value at 620 nm in the plate based assay and MFI value in the cell based assay. The Kd (negative reciprocal of the curve) is the affinity constant presented in µg/ml.

Table 3.1: Kd values obtained from the regression analysis of both the ELISA assay and the cell-based SRBI-binding assay.
The Kd value of each antibody in the cell binding assay was higher than the Kd value of the respective antibody in the plate based assay. The Kd concentrations were converted into Molarity by taking the molecular weight of IgG (150 kD) into account for the general ease of comparison.

Monoclonal Antibody	ELISA K_d (ug/ml)	ELISA K_d (nM)	SRBI assay K_d (ug/ml)	SRBI assay K_d (nM)
Anti-his-tag	1.3760	9.2×10^{-3}	5.3	3.5×10^{-2}
B-AP33	0.2560	1.7×10^{-3}	1.0	6.6×10^{-3}
B-ALP98	1.064	7×10^{-3}	2.2	1.5×10^{-2}
B-1:7	2.770	1.8×10^{-2}	7.7	5×10^{-2}
B-AR3A	0.1290	8.6×10^{-4}	1.1	7.4×10^{-3}

Table 3.2: Bmax values obtained from the regression analysis of both the ELISA assay and the cell-based SRBI-binding assay.

Monoclonal Antibody	ELISA Bmax (OD 620nm)	Cell assay Bmax (MFI)
Anti-his-tag	0.9606	102
B-AP33	0.7684	41
B-ALP98	0.2026	64
B-1:7	0.9720	77
B-AR3A	0.8039	44

3.3.4 Comparison of the Bmax obtained from the plate and cell based assays, normalized against anti-his-tag antibody

To further assess whether the accessibility of the monoclonal antibodies to histidine-tagged soluble E2 compared to anti-histidine-tag antibody, a comparison between the plate based and cell based assay was made. Using GraphPad Prism version 4.0, the Bmax values obtained from the respective assays were normalized against the value of anti-his antibody and the data was presented as percentage of binding to further investigate whether the presence of SRBI exposes the binding sites of the CD81-competing antibodies to soluble H77 E2 (Figure 3.11). The combined results reveal a change in overall

conformation of the cell-bound E2 as binding sites for the mAbs targeting the CD81 binding regions decrease. By comparison, the binding sites for ALP98, which, as mentioned previously, targets a linear epitope outside of the CD81-binding regions, increase on SRBI-bound E2 compared to plate-bound E2. These observations indicate that the antigenic regions on E2, targeted by AP33, 1:7 and AR3A are less exposed on SRBI-bound E2 compared to plate-bound E2 while the epitopes targeted by ALP98 become more exposed on SRBI-bound E2.
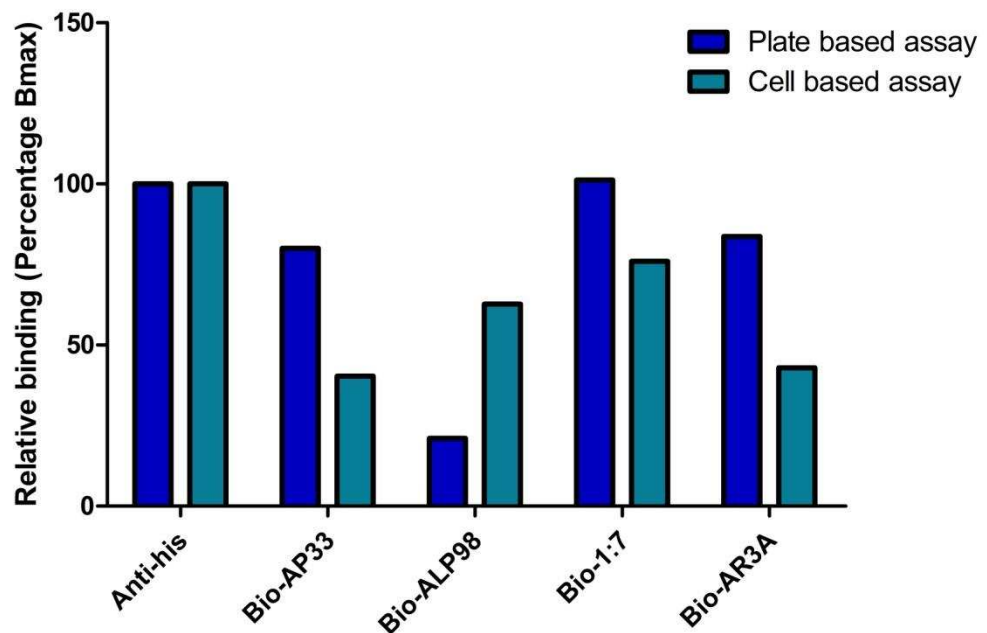
Figure 3.11: Bmax values obtained from both plate based and cell based assay, normalised to the values obtained from the anti-his-tag antibody.
The antibodies targeting the CD81 binidng sites, AP33, 1:7 and AR3A showed a decrease in binding to SRBI bound E2 in the cell based assay, suggesting a concealement of the antigenic epitopes of these mAbs. On the contrary, the E2 binding sites targeted by ALP98 (which targets a linear epitope on E2 outside of the CD81 binding sites) show enhanced exposure.

3.4 Discussion

The specific details around Hepatitis C virus entry into the host cell are currently not fully known. There have been many assumptions around the mechanisms of entry; however, with new emerging methods the details are now being elucidated. Results obtained from receptor binding assays in HCVcc experiments have led to the conclusion that the HCV glycoprotein E2 first engages with host cell receptor SRBI prior to CD81 contact (Evans, von Hahn et al. 2007). The tight junction proteins claudins and occludin are believed to be involved in late stage entry. A study conducted in 2008 found that a mutation (G451R) in JFH1 E2 presented a reduced dependency on SRBI for infectivity compared to the wild type virus particle. However, additional findings of the same study concluded that this mutant virus was more sensitive to neutralisation by human CD81LEL and that mutation from glycine to arginine on E2 aa residue 451 caused an exposure of the CD81 binding site on E2 (Grove, Nielsen et al. 2008). From these experimental outcomes it is concluded that glycine at position 451 on E2 is involved in the regulation of virus interaction with SRBI and CD81.

We aimed to determine that SRBI contact triggers a conformational change of HCV E2 that renders the CD81 binding sites more accessible to the receptor. To further investigate this, two assays with monoclonal antibodies that target the CD81 binding regions on HCV E2 were designed: a plate based ELISA assay without the involvement of human SRBI, and a cell based assay including human SRBI. Since there are limitations with E1E2 in the cell based

SRBI binding assay, we chose to produce histidine-tagged H77 soluble E2 to include in both assays. Full length E1E2 have not been successful in binding SRBI in the cell based SRBI binding assay as purity and concentration of the protein seem to be pivotal for a successful experiment. The production of E1E2 in a mammalian cell line generates quite high amounts of aggregates that compromise the purity of the protein sample. E1 and E2 are covalently linked and centrifugation (with size exclusion purification) can dislodge the proteins from each other. E1E2 form aggregates due to the hydrophobic properties of the transmembrane domains (Cocquerel, Wychowski et al. 2000), tagging as well as purification of the heterodimer has proven difficult. Also, the assay requires a high concentration of the glycoprotein to bind SRBI, and that has been proven difficult to achieve with E1E2 production, as with the presence of detergents E1E2 remain cell-associated and therefore not released into the supernatant. Experiments with virus particles have not been described as of yet and would technically be difficult due to low viral titres. Histidine- tagged soluble E2 was included in this work as the histidine-tag was exploited for purification of the protein. Once the protein was purified, it was concentrated and used at a higher yield in the experiment. The antibodies were biotinylated to keep the detection system with streptavidin equivalent between the assays. The binding data from the two assays were included in regression analysis where the Kd and Bmax values for each antibody were obtained. This analysis showed a reduced affinity of all antibodies to SRBI- bound E2 in the cell based assay compared to the plate-bound E2 in the ELISA, as the Kd values in the cell based assay were higher than those obtained in the plate based assay. Furthermore, the Bmax values of mAb of sE2 (normalised as a percentage of

the Bmax obtained with the anti-his-tag antibody) were also lower in the cell based assay compared to the plate based assay. This would indicate a concealment of the CD81 binding sites on E2. However the binding sites for ALP98 showed an enhancement of exposure in the cell based assay compared to the plate-based assay. These results appear to contradict the prevailing theory of an antigenic exposure of the CD81 binding sites on E2 post-SRBI binding.

In a study by Grove et al, patient derived polyclonal IgG sera and the mAb 3/11 were able to neutralise HCVcc JFH1 glycoprotein mutant G451R virus particle over ten-fold compared to the JFH1 wild type virus particle. However, when these antibodies were used to detect plate-bound sE2 in an ELISA, no difference in binding was observed. The authors concluded that discrepancies in epitope exposure of sE2 can occur (Grove, Nielsen et al. 2008). The results observed in our study may be a consequence of the difference in how sE2 is bound between the assays and consequently the difference in available binding sites for the mAbs included in these experiments.

The human conformational monoclonal antibodies 1:7 and AR3A target three known common amino acid residues on E2, aa523, 530 and 535. Our study reveals that these interactions occur with different affinities, as AR3A bind with high affinity and 1:7 bind with low affinity to E2. This discrepancy is probably due to variations in the hyper variable regions of these two antibodies.

The observation of decreased affinity of all the mAbs to cell-bound E2 might be explained by the difference in conditions to which the antibodies bind between the assays. The interaction occurs in a solution of room-temperatured PBS-0.05% Tween in the ELISA, however in chilled PBS buffer containing 0.1% BSA in the cell based assay. Discrepancies in buffer conditions, such as temperature and salt content can have an effect on the binding affinities between the antibodies and E2.

The experimental approach for this work is not optimal as these two assays are vastly different in the environmental settings of antibody and E2 binding. As mentioned in previous paragraphs, plate-bound sE2 and cell-bound sE2 are not spacially situated in a similar fashion, and might consequently expose antibody epitopes to different degrees. Although this work was normalised to the binding of anti-histag to the Histidine tag on sE2, the fundamental components of which these assays are based on may be overly dissimilar to justify a comparison. We adopted this experimental approach as it was the available choice at the time this work was performed.

Ideally, including a soluble form of CD81 and fullength E1E2 in the assays to replace the role of the human monoclonal antibodies and sE2 in the experiments would have been of interest as it would present a closer reflection of actual CD81:E1E2 binding. As mentioned previously, the difficulty in producing pure mammalian expressed E1E2 combined with the continuous technical difficulties with the CD81- binding assay in this study lead to the exclusion of these experiments from this study.

In future experiments; an additional potential method of trying our theory would be to express E1E2 on the surface of a cell line and investigate how either monoclonal antibodies targeting the CD81 binding sites or CD81 molecules bind in the absence and presence of E1E2-bound SRBI. The inclusion of additional antibodies that do not target the CD81 contact residues but other antigenic regions (Edwards, Tarr et al. 2012) in these experiments could generate more data explaining how the alteration in conformation following SRBI:E2 contact affects other antibody epitopes on E2.

Also, the addition of HDL has proven to enhance HCV infectivity both in the HCVpp (Bartosch, Verney et al. 2005; Voisset, Callens et al. 2005) and HCVcc assay (Dreux, Pietschmann et al. 2006). It is believed this enhancement is mediated by HVR1:SRBI interaction at the viral entry stage (Bartosch, Verney et al. 2005; Dreux, Pietschmann et al. 2006). It is proposed that the lipids act to protect the virus from circulating neutralising antibodies by shielding the CD81 binding sites (Grove, Nielsen et al. 2008). Lipids were not included in our study; however, investigating the exposure of the CD81 binding epitopes in the context of lipid would be advantageous.

The results presented in this study appear to indicate that interaction of E2 with SRBI leads to concealment of CD81-binding sites. This conclusion is based on the reduction in the number of such sites available for binding by the mAbs that target CD81-binding regions on SRBI-bound E2 as opposed to plate-bound E2. Only conformational sensitive antibodies appeared to be hindered by SRBI binding as the linear mAb ALP98 which binds to a region outside of the CD81 contact residues showed an increase in binding sites to SRBI-bound

E2. Finally, we conclude from this work that there is a concealment of the antigenic epitopes upon E2:SRBI interaction that is restricted to the CD81 binding sites.

4 SRBI-dependency of Hepatitis C E1E2 clones

4.1 Background

It is known that after HIV binds to CD4, its receptor on lymphocytes, the viral envelope glycoprotein, gp120, undergoes conformational changes exposing epitopes crucial for a later stage in viral entry (Sattentau and Moore 1991; Trkola, Dragic et al. 1996; Wu, Gerard et al. 1996). CD4 expression has been proven to regulate HIV entry. Viruses grown in vitro have presented a decreased dependency on CD4 as well as an enhanced sensitivity to neutralising antibodies (Platt, Wehrly et al. 1998; Dumonceaux, Chanel et al. 1999; Hoffman, LaBranche et al. 1999; Bannert, Schenten et al. 2000; Blish, Nguyen et al. 2008). Our theory is that SRB1 might act similarly to CD4 in HCV entry, where the HCV clones derived from infected patients that exhibit different levels of infectivity in the HCV pseudo particle assay possibly require additional accessible SRBI to facilitate cell entry.

In 2005, Lavilette et al investigated the importance of SRBI expression in HCV infectivity. SRBI was knocked down in Huh 7 cells using three siRNAs that target SRBI mRNA via a vesicular stomititis virus (VSV-G) HIV derived retroviral vector. The siRNA knockdown resulted in a 10-fold reduced SRBI expression compared to parental Huh 7cells. Patient derived E1E2 clones, representing genotypes 1 to 6, were incorporated into HCV pseudo particles and tested for infectivity in the SRBI knockdown Huh7 cells and parental

Huh7 cells. The results of these experiments showed the down-regulation of SRBI reduced the infectivity efficiency of all pseudo particles, albeit to varying degrees. For instance, glycoproteins derived from the 1a (H77) genotype showed highest dependency on SRBI as a ten-fold decrease in infectivity was observed in the SRBI-silenced Huh 7 cells. Genotype 2a derived HCVpp showed less dependency on SRBI as the down-regulation of the receptor only resulted in approximately 30% less infectivity compared to native Huh 7 cells. The combined data of this study suggests that different HCV E1E2 genotypes interact with SRBI with varying dependency (Lavillette, Tarr et al. 2005).

A separate study has shown that the over-expression of SRBI on Huh7.5 cell can enhance J6/JFH infectivity by three-fold compared to infection in wild type Huh 7.5 cells (Grove, Huby et al. 2007). The SRBI over-expression was achieved by transducing the Huh 7.5 with a lentiviral TRIP-vector package containing the human SRBI sequence. To verify that this observed increase of infectivity was not restricted to the J6/JFH HCV strain, the authors of this study conducted the same experiment with a JFH1 HCV strain. The infectivity of JFH1 increased approximately 18-fold compared to parental cells, suggesting that this enhancement in infectivity is not restricted to one HCV strain.

Huh 7 cells express SRBI, CD81, claudin and occludin on the cell surface, and all of these have proven a role in HCV entry (Pileri, Uematsu et al. 1998; Scarselli, Ansuini et al. 2002; Evans, von Hahn et al. 2007; Ploss, Evans et al. 2009). In a study performed to investigate the SRBI-CD81 co-relationship as

well as the role of lipid in the HCV entry process, the addition of the cholesterol-inhibiting drug, MβCD, to Huh 7 was shown to decrease the expression of CD81 on these cells by approximately 2.5-folds. On the contrary, the SRBI expression on Huh 7 increased by approximately 2.4-folds. The observed effects were reversed when cholesterol was once again added to the cells (Kapadia, Barth et al. 2007).

In our work, the expression of SRBI on Huh 7 cells appeared to be variable, with both high and low SRBI-expressing phenotypes observed. To investigate whether the level of SRBI expression could alter the infectivity of nine different HCV E1E2 clones, (all representing HCV genotype 1 to 3), which have previously shown to achieve varying infectivity abilities in the HCVpp assay, we aimed to achieve different levels of SRBI expression on Huh 7 cells and subsequently investigate whether different HCVpp isolates exhibit varying SRBI dependencies for entry.

4.2 Material and Methods

4.2.1 Extracellular SRBI staining on Huh 7 cells

To determine the level of SRBI expression on live Huh 7 cells an anti-SRBI antibody targeting the extra cellular domain of SRBI, C167 (kindly gifted by Alfredo Nicosia, Naples, Italy) was used. Upon harvesting, Huh 7 cells were washed with a buffer containing 1 x PBS, 10 mM HEPES (Sigma-Aldrich) and 0.2% BSA (Sigma-Aldrich) before centrifuged at 250 x g for five minutes. The cells were incubated with 550 ng/ml of C176 for one hour in room

temperature. The wash and centrifugation was repeated before 1:100 of biotinylated anti-human IgG4 mouse monoclonal antibody (BD Pharmingen, Oxford, UK) was added to the cells and incubated at room temperature for one hour. An additional wash and centrifugation was repeated prior to adding 1:100 of Streptavidin-R-PE (Qiagen, Crawley, UK) to the cells. The cells were thereafter sorted according to the level of expressed SRBI by flow cytometry, Epics Altra (Beckman Coulter, High Wycombe, UK).

4.2.2 Production of lentiviral human SRBI in HEK Lenti-x 293T cells

Lenti-X 293T cells were co-transfected in10 ml Optimem (Gibco) media with 6 µg of human SRBI in pTRIP vector, 2 µg pMD2G vector encompassing VSV-G envelope and 6 µg pCMV. ΔR8.74 (HIV-1 derived packaging vector) with 24 µl PEI (Fermentas, Loughborough, UK). Supernatant was collected following overnight incubation at 37 ^{o}C in a humidified atmosphere containing 5% CO_2, second collection after 48 hours and third collection after 72 hours. All collections were pooled and purified using Amicon Ultra-15 Centrifugal Filter Units 100 NMWL (Millipore, Watford, UK) by centrifuging at 2000 x g for 20 minutes. Virus stock was stored in -80 ^{o}C.

4.2.3 Lentiviral human SRBI transduction of wild type Huh 7 cells

Human hepatoma 7 (Huh 7) cells were seeded and grown in a T25 (Costar, London, UK) flask containing DMEM (Gibco, Paisley, UK) media, 10% FCS (Biosera, Ringmer, UK), 1% Penicillin/Streptomycin (Invitrogen, Paisley, UK) and 1% Non essential amino acids (Invitrogen). When 50-60% confluency was

obtained, 400 µl of concentrated virus stock was added together with 3 ml of growth medium. The transducted cells were incubated overnight at 37 $^{\circ}$C in a humidified atmosphere containing 5% CO_2 before adding five millilitres of growth media. The cells were then incubated for an additional 48 hours in 37 $^{\circ}$C. The cells were subsequently harvested and stained with intracellular antibody against SRBI (as described in the previous chapter) to ensure the over-expression of SRBI.

4.2.4 Lipid starvation of wild type Huh 7 cells

Wild type Huh 7 cells were grown in media containing DMEM (Gibco), 2% lipoprotein deficient Fetal Calf Serum (Sigma-Aldrich), 250 µM Mevalolactone (Sigma-Aldrich), 40 µM Mevastatin (Sigma-Aldrich) and 3 µg/ml of AcLDL (Invitrogen, Paisley, UK) for 18 days before harvesting and intra cellular staining for SRBI expression as described previously. This growth media (which later in this chapter will be referred to as MAC-media) was prepared containing Mevastatin, a statin that works to inhibit the rate-limiting enzyme 3-hydroxy-3 methylglutaryl-CoA (HMG-CoA) reductase in the cholesterol synthesis pathway (Roitelman, Olender et al. 1992). The inhibition of HMG-CoA reductase would, in the liver, lead to a down regulation of cholesterol synthesis, as well as an enhancement of the low density lipoprotein (LDL)-receptor synthesis. Mevalolactone, an additional ingredient to the media, is an organic compound involved in the steroid biosynthesis (Endo 1992). These components were included together with lipoprotein deficient Foetal Calf Serum (FCS) and acetylated low density lipoprotein (AcLDL) in the MAC-media.

4.2.5 Receptor analysis on wild type Huh 7 cells and SRBI knock down (B6) Huh 7 cells

Huh 7 cells were seeded in 6-well plate with media containing DMEM (Gibco), 1% non essential amino acids (Gibco) and 1% Penicillin/Streptomycin. Once confluent, the cells were harvested and centrifuged at 250 x g for five minutes to remove excess media. JS-81, (an anti-CD81 antibody) anti-claudin and anti-occludin primary antibodies were separately added to the cells at a concentration of 10 µg/ml. Following 30 minutes incubation at 4 ^{o}C, the cells were washed and centrifuged at 250 x g for five minutes in 1 ml buffer containing 1X PBS (Oxoid) and 2% FCS (Biosera). A Pcy5 -conjugated antibody was then added to the cells at a dilution of 1:10, diluted in wash buffer, and incubated for a further 30 minutes at 4 ^{o}C. The stained cells were washed and spun twice with PBA buffer before fixing in 500 µl 0.5% formaldehyde. Samples were stored at 4 ^{o}C in the dark prior to analysis by flow cytometry Epics Altra (Beckman Coulter).

4.2.6 HCV Pseudo Particle system

Human embryonic kidney cells (HEK) 293T cells were co-transfected in Optimem media (Gibco) with 2 µg of phCMV-5349 encompassing MLV gag-pol polyprotein, pTG-luc 126 vector carrying the firefly luciferase gene and pcDNA 3.1 E1E2, containing the full length HCV E1E2 glycoproteins. Nine patient derived E1E2 clones, all previously proven functional in the HCV pseudo particle system (Lavillette, Tarr et al. 2005) and representative of genotypes 1, 2 and 3, were included in this study; H77 (1a control strain), 1A20.8, 1B5.23, 1B12.16, 2A2.4, 2B1.1, 2B2.8, 3A13.6 and 3A.1.28. Twenty

four microlitres of PEI was added to the co-transfections before adding the mixture to the cells. The cells were incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 for 72 hours before supernatant was collected and filtered through a 0.45 µm filter (Sartorius, Aubagne, France).

Filtered supernatant was added to a one ml sucrose gradient of 20% sucrose (Sigma-Aldrich) in separate ultracentrifuge tubes. The supernatants containing the HCV pseudo particles were centrifuged at 107,000 x g for two hours at 4 ^{o}C. The pellet was re-suspended in 500 µl sterile PBS after the supernatant was removed and incubated at room temperature for one hour. Fifty microlitres of purified HCV pseudo particles together with 50 µl of growth medium was then added in triplicate to seeded wild type Huh 7 cells as well as SRBI knock out-Huh 7 cells in 96-well Optilux microtitre plates (BD, Oxford, UK). The plate was incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 for 72 hours.

The supernatant was removed following 72 hours of incubation and the HCVpp infected cells were lysed in 25 µl lysis buffer (Promega) before the addition of 50 µl of luciferase substrate (Promega) to each well. The 96-well plate was analysed by luminescence (FLUOstar OPTIMA; BMG Labtech, Aylesbury,UK).

4.2.7 SDS-PAGE and Western blot analysis of expressed E1E2 generated during HCV pseudo particle production

To assess whether the HEK 293T cells which generated the pseudo particles expressed the E1E2 proteins, the cells were lysed and SDS-PAGE as well as

Western blot was performed as previously described in Chapter 2.3.8. Briefly, the lysed supernatants were run on a 9% polyacrylamide gel before transferred onto a nitrocellulose membrane. The membrane was then probed with 1:200 AP33 and subsequently with polyclonal rabbit anti-mouse horseradish peroxidise at a dilution of 1:1000. The membrane was exposed onto a film before prepared and developed for analysis.

4.2.8 HCV pseudo particle neutralisation assay

HCV pseudo particles were produced as described in the previous paragraph. The HCV neutralising monoclonal antibodies AP33 and 1:7 were added at the point of infection at titrating concentrations starting at 5 µg/ml and thereafter in fivefold dilution series. The pseudo particle/monoclonal antibody (pp/mAb) complexes were incubated at room temperature for one hour before infecting wild type Huh 7 cells with 50 µl of sample in triplicate wells in a 96 microtitre plate (Optilux). Fifty microlitres of growth media was added to the cells and the 96-well plate was incubated at 37 ^{o}C for a period of 24 hours before a further addition of 150 µl of growth media. The cells were incubated for an additional 48 hours before being lysed in lysis buffer (Promega, Southampton, UK) and analysed by luminescence with the addition of luciferase substrate (Promega).

4.3 Results

4.3.1 Huh 7 cell sorting for high and low SRBI expressers

To investigate whether certain Hepatitis C virus clones, derived from infected patients, that had been proven infectious by the pseudo particle assay, could in fact be functional, however merely more reliant on the accessibility and quantity of SRBI, wild type human hepatoma 7 cells were stained for the expression of SRBI with an extra cellular anti-SRBI antibody, C167, and FITC conjugated secondary antibody prior to sorting a high respectively low expressing population by FACS analysis. The intention was to create two separate environments with high- and low levels of SRBI accessible to the HCV E1E2 clones at the time of pseudo particle infection to examine whether the different levels of SRBI available to the clones makes a difference in pseudo particle infectivity and also to determine if this was associated to neutralization sensitivity.

Following the sorting experiments, the cells were placed back into DMEM growth media and incubated at 37 $^{\circ}$C for further proliferation. Once the cells were confluent, they were stained again to ensure they had proliferated into the desired SRBI- expressing phenotypes. As Figure 4.1 shows, the cells that produced a fluorescent signal between 10^2 and 10^3 were designated high SRBI expressing Huh 7 cells and appear in the R2- region. The cells that produced a fluorescent signal between 10^1 and 10^2 were designated low SRBI expressers and fitted in the R1-region. Mean fluorescent intensity values for the cells are displayed alongside each region. Flow cytometry results showed that both

populations had reverted back to a mixed population of high and low SRBI

expressers, indicating that the expression level was not stable phenotype.

A) Non-stained cells

B) 2nd Ab PE

C) α- SRBI & PE — Low expressers, R2, R1

D) α- SRBI & PE — High expressers, R2, R1

E) α- SRBI & PE — Low expressers, R2, R1
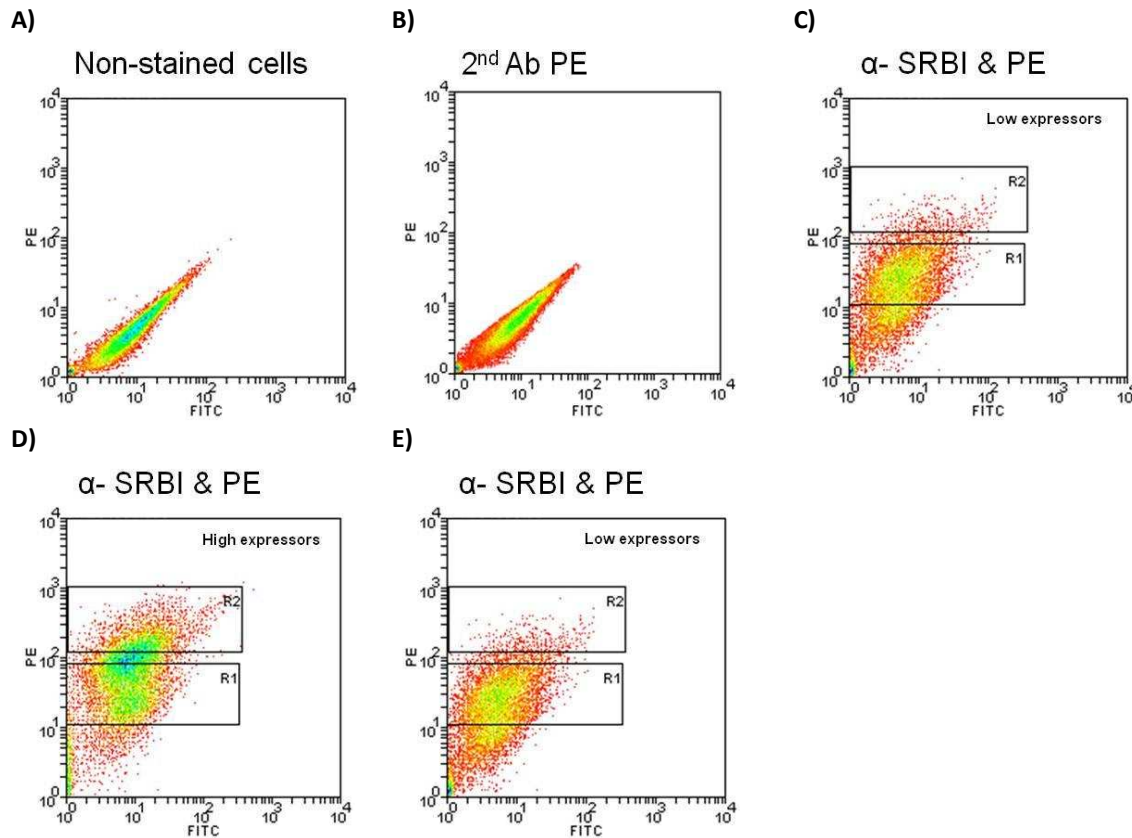
Figure 4.1: Huh 7 sorting for cell populations of high and low SRBI expressers.

Huh 7 cells were stained using CI67, an extracellular anti-SRBI antibody and PE-conjugated secondary antibody. The cells were analysed by flow cytometry and sorted with regards to SRBI expression. R1 contains the cells designated as low expressers while the cells in the R2 box were classified as high expressers. After cell sorting and collection, the cells were placed back in growth media to proliferate in a 6-well plate at 37 ^{o}C with 5% CO_2.Following proliferation, the cells were stained additionally to examine whether the Huh 7 cells had remained in the desired and sorted phenotypical state or reverted back to a population of mixed SRBI expressers. Figure 1 A-E shows dot plots of re-stained Huh 7 cells after initial sorting and expansion. 1A) represents non-stained cells, 1B) shows cells single stained with PE conjugated secondary antibody (background signal-control). Figure 1C) and 1D) exhibit sorted high and low – SRBI expressing cells. The cells that appear in the R2-region were considered high SRBI-expressers with a PE -fluorescent signal at 10^2-10^3. The cells that appear in the R1-region were considered low SRBI expressers with a PE- flouroscent signal of 10^1-10^2. As a control of the sorting, figure 1E, the low SRBI expressers were sorted and analysed through the flow cytometer an additional time to examine whether the cells re- appear in the R2-region to ensure the correct selection of the desired cell population by the flow cytometer. As 1E) demonstrates, the cells show to be the selected low SRBI expressers. The Mean Fluorescent Intensity (MFI) of each region is displayed on the graphs. Figure 1 C) and 1 D) reveal that the cells do revert back to a population of mixed SRBI-expressers after sorting and proliferation.

4.3.2 Transduction of Lentiviral human SRBI into Huh 7 cells

As the sorting experiment failed to produce two stable phenotypes of distinct populations of Huh 7 cells expressing high and low levels of SRBI, Lenti viral experiments were designed to over express SRBI on Huh 7 cells. Lenti-X 293T cells were co-transfected with human SRBI in pTRIP vector, pMD2G vector encompassing VSV-G envelope and pCMV. ΔR8.74, which is a HIV-1 derived packaging vector. To verify a successful transduction, the Green Fluorescent Protein (GFP) in a LV-CRE vector was separately included as a positive control alongside human SRBI in the experiment. Subsequent to overnight incubation at 37 ^{o}C, supernatant was collected, followed by the second collection after 48 hours and a third collection after 72 hours. The supernatants were pooled before the lentiviral stocks carrying human SRBI and GFP respectively were used to tranduce Huh 7 cells. Following 72 hours of incubation at 37 ^{o}C, the Huh 7 cells transduced with the human SRBI were harvested and stained for SRBI expression with an intracellular anti-SRBI antibody and a FITC conjugated secondary antibody prior to analysis by flow cytometry. The GFP-transduced cells were included in the analysis to verify a successful lentiviral delivery. Figure 4.2 displays the results of the flow cytometry. Although the GFP delivery was highly successful as the cells display a MFI of 4060, the cells transduced with human SRBI did not seem to express SRBI levels much greater than the wild type Huh 7 cells displaying MFI values of 128 and 102, respectively. These findings resulted in unsuccessful experiments rendered the experiment uninformative.

A) Non-stained Huh7 cells

B) 2nd Ab FITC

C)

SRBI

Mean Fluorescent Intensity (MFI):
102
4060
128
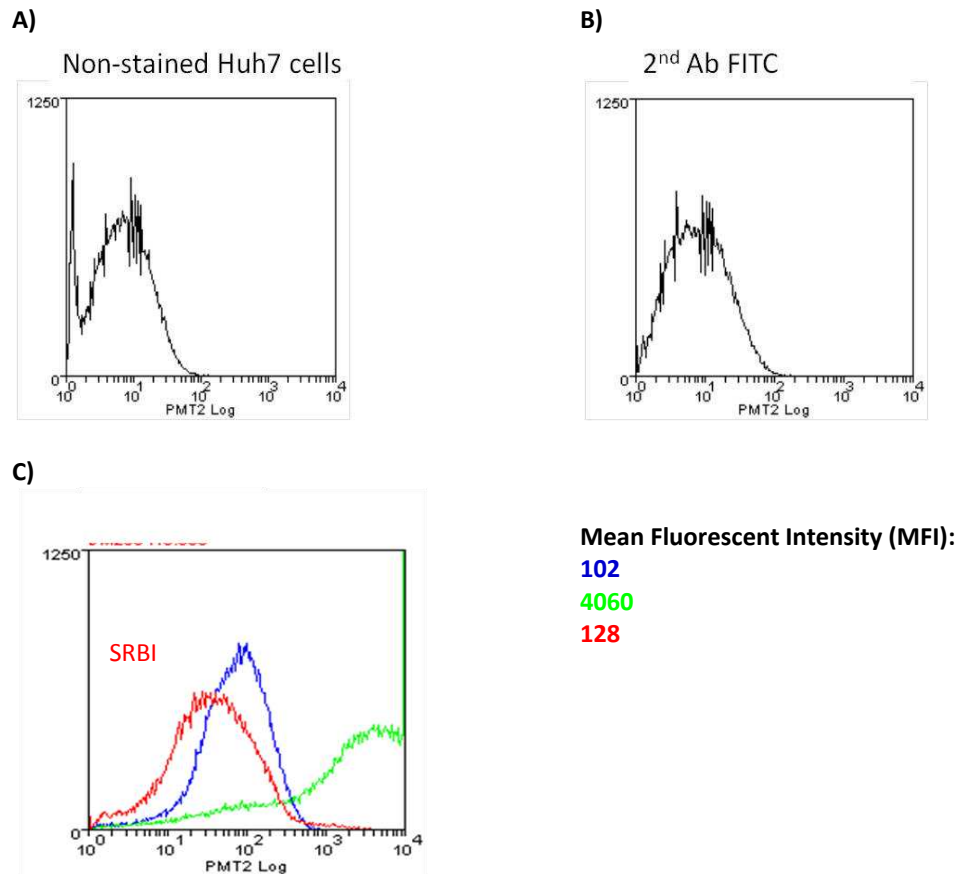
Figure 4.2: Lentiviral transduction of SRBI into Huh 7 cells.
Lentiviral stocks carrying human SRBI - and the green fluorescent protein (GFP) genes respectively were separately transduced into Huh 7 cells. The lentiviral stocks bearing the GFP gene were included in this assay as a transduction control by verifying the correct conditions for gene delivery. Following 72 hours of incubation at 37 ^{o}C, human SRBI transduced cells were stained with an intra cellular anti-SRBI antibody and a FITC- conjugated secondary antibody to ensure the over expression of the receptor, prior to flow cytometry analysis. Figure 4.2 displays the flow cytometry data with A) showing non-stained Huh 7 cells, B) a background signal control with a single stain of FITC- conjugated secondary antibody. C) Blue histogram represents stained un-transduced wild type Huh 7 cells, green histogram displays the GFP-transduced Huh 7 cells and red histogram represents the results of the human SRBI-transduced Huh 7 cells. As the un-transduced cells display the native SRBI expression on Huh 7 with a MFI value of 102, the lenti viral over expression of human SRBI proved to be unsuccessful, displaying a MFI value of 128. However, the lentiviral delivery of the GFP gene proved successful with a MFI value of 4060.

4.3.3 Lipid starvation of human hepatoma 7 cells induces stress related changes

As Huh 7 cells express SRBI natively, an experiment was designed to starve the Huh 7 cells of lipids, as SRBI is a lipid receptor, with the aim to drive the cells to upregulate the SRBI expression. Huh 7 cells were incubated with

MAC-media while control cells were placed in standard growth media containing DMEM, 10 % FCS, 1 % non essential amino acids and 1 % penicillin/streptomycin. The cells in MAC-media initially displayed a reduced growth rate compared to the Huh 7 cells in standard media. The cells in MAC-media also displayed granules on the cell surface, indicating stressed conditions. Following 18 days of proliferation in T75 flask with MAC-media, the cells were harvested and stained with an intra cellular anti-SRBI antibody and a FITC conjugated secondary antibody prior to flow cytometry analysis. Figure 4.3 displays the results obtained from the flow cytometry. The Huh 7 cells incubated in MAC media demonstrated a slight increase in SRBI expression as demonstrated by a shift of staining peak from an MFI of 77 for the control Huh 7 to 106 for the cells cultured in the MAC media (Figure 4.3C).

However, despite a slight difference in SRBI- expression levels, the Huh 7 cells cultured in MAC-media showed signs of stress, as indicated by a change in morphology – specifically granulation of the cell cytoplasm, and detachment from the plastic surface. In order to investigate the optimal environment in which the Huh 7 cells could proliferate and sustain a healthy state without compromising the SRBI-up regulation, 100 000 cells per well were seeded on a six well plate in MAC-media containing different concentration of lipids, ranging from 100 % - to 5 % lipid content. One well was seeded with Huh 7 cells in serum-free Optimem media to examine the behaviour of the Huh 7 cells in a serum-free environment. Figure 4.4 displays the appearance of the cells following 12 days of proliferation in MAC-media with titrating amount of

lipid. As shown, the cells display more signs of distress with a reduced amount of lipid content in the surrounding environment. For example, granulations appear immediately as the lipid content in the MAC-media reduces by 50 % (Figure 4.4B). As the lipid content decreased to 12.5 % and 5% respectively, numerous cells began to detach (Figures 4.4D and 4.4E). After 14 days of culture, the cells had developed an extra cellular matrix, which made subsequent harvesting difficult as he cells tended to adhere strongly to the plastic surface. As the cells proved impossible to collect, staining for SRBI-expression was not possible. The production of the extra cellular matrix, again, indicated a high level of stress, which rendered the cells impractical to include in any future experiments.

A) Neg stained cells

B) 2nd FITC Ab

C) Huh7 cells in MAC Media (green)

Mean Fluorescent Intensity (MFI):
77
106
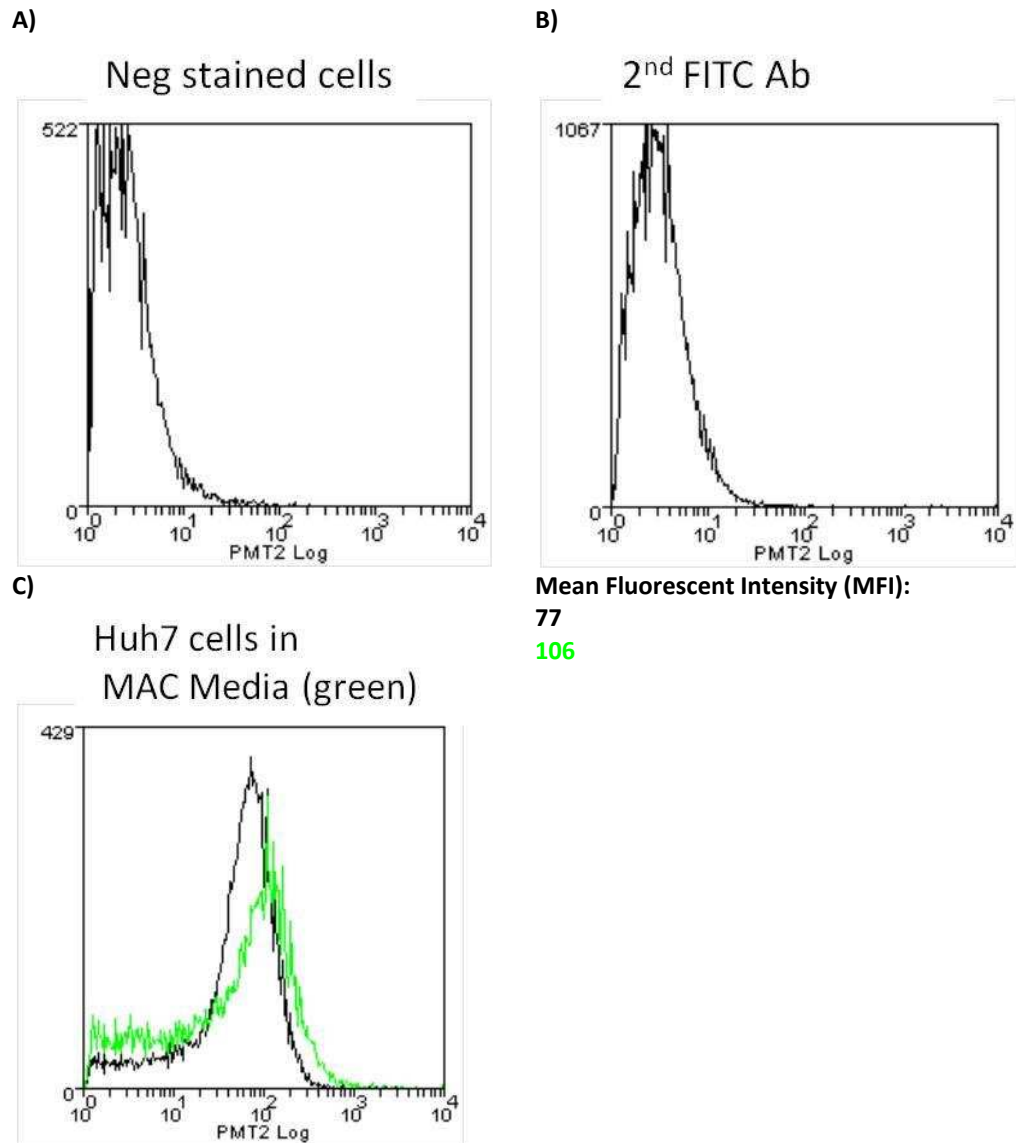
Figure 4.3: Flow cytometry analysis of the transduced Huh 7 cells.
Wild type Huh 7 cells were grown inT75 flask with media containing DMEM, 2% lipoprotein deficient Fetal Calf Serum, Mevalolactone, Mevastatin and AcLDL for 18 days prior to harvest and intra cellular staining for SRBI expression. A) displays non-stained Huh 7 cells, 3B) represents cells stained with secondary antibody to determine any potential background signal. C) displays the histogram of the cells grown in standard media (black line) and the cells grown in Mac-media (green line). As shown, the Huh 7 in Mac- media display a MFI value of 106, indicating a slight elevation in SRBI-expression compared to the cells expanded in standard media.

127
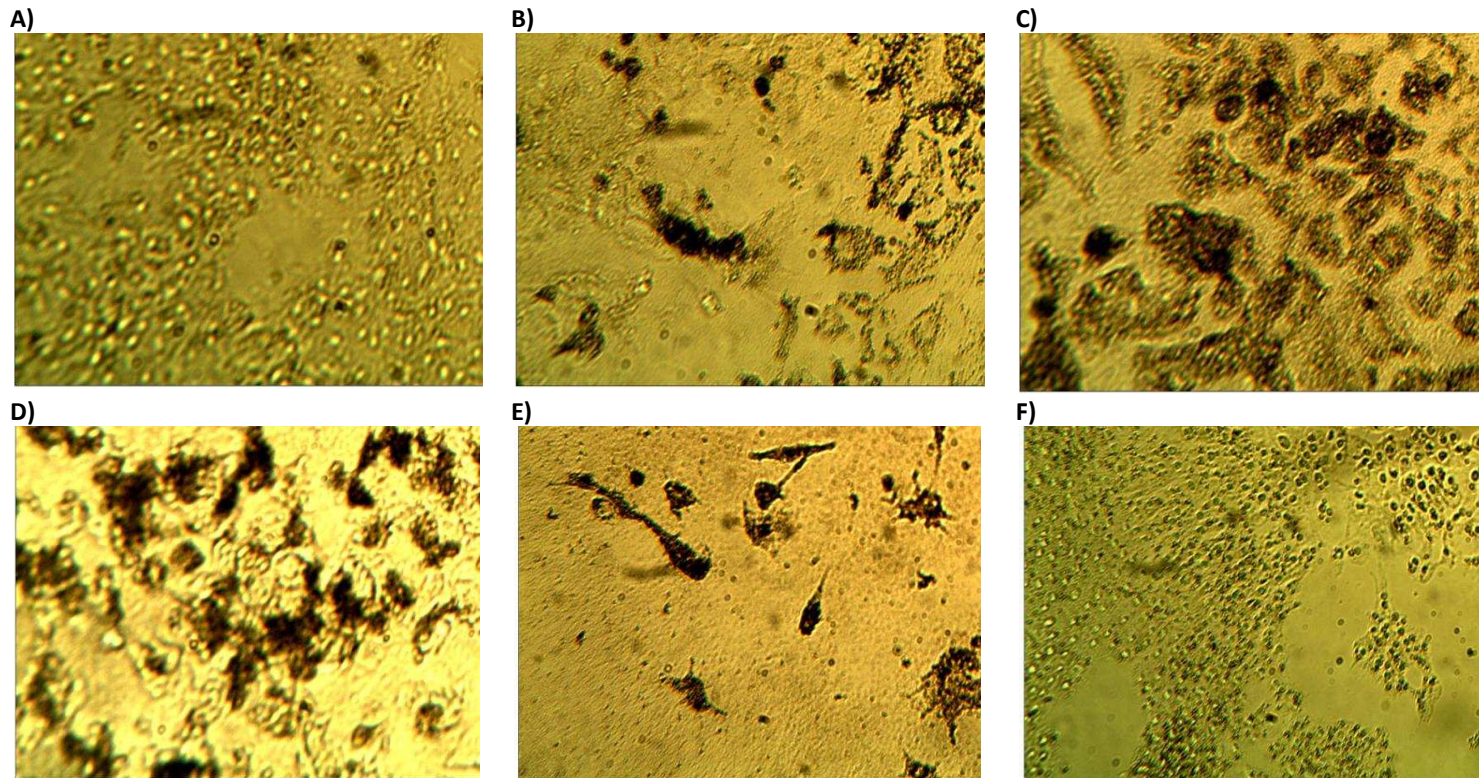
Figure 4.4: Huh 7 cells in MAC-media with titrating amount of lipid ranging from100 % to 5 %, following 12 days of proliferation (45 x magnification).
As shown, the cells display more signs of distress as the lipid content of the media reduces. Granulations become apparent immediately as the lipid content decreases 50 % (4B). At the lipid levels of 12.5 % and 5 %, numerous cells started to detach from the plastic surface and die (4D and 4E).

4.3.4 SRBI knock down Huh 7 cells show over 50 % reduction in SRBI expression compared to the wild type Huh 7 cells

As attempts to increase SRBI expression had proven unsuccessful, the next approach was to assess whether or not the role of SRBI in HCV entry could be studied using cells that had been engineered to express lower levels of receptor. Huh 7 cells were genetically modified by a colleague to knock down SRBI. Briefly; one allele of the SRBI gene was knocked out using adeno-associated viral vectors, as described in Kohli, 2003. These cells, referred to here as B6 cells, were thereafter used in this study to assess the SRBI dependency of patient derived E1E2 clones, utilising the HCV pseudo particle system, by comparing the infectivity results in these SRBI knock down B6 cells to wild type Huh 7 cells, which natively express SRBI. To assess the levels of SRBI on B6 Huh 7 and wild type Huh 7 cells respectively, SRBI staining and flow cytometry analysis was performed as described previously. Additionally, staining was carried out to ensure that the SRBI knock down did not have an abnormal effect on the other putative Hepatitis C virus receptors such as CD81, claudin and occludin. Moreover, the experiment was performed to confirm any potential difference in HCV pseudo particle infectivity in the following experiments would solely be caused by the variation of SRBI levels on the target cells. Figures 4.5 demonstrate the results of the controls included in the experiments and the results of the B6 Huh 7 SRBI staining which showed a MFI of 18.6 while the parental cells showed a MFI of 36.5 revealing a reduction of SRBI expression by nearly 50% compared to the SRBI expression of the wild type Huh 7 cells. Also, the subsequent experiment

shows that the knock down of SRBI did not impair the expression of CD81, claudin or occludin as the staining of these receptors showed similar MFI values between the parental Huh 7 cells and B6 SRBI knock down Huh 7 cells (Figure 4.6).

A) Controls

MFI Neg =3.7
MFI 2nd FITC=7.3

B) SRBI expression

MFI Huh7wt =36.5
MFI B6 = 18.3
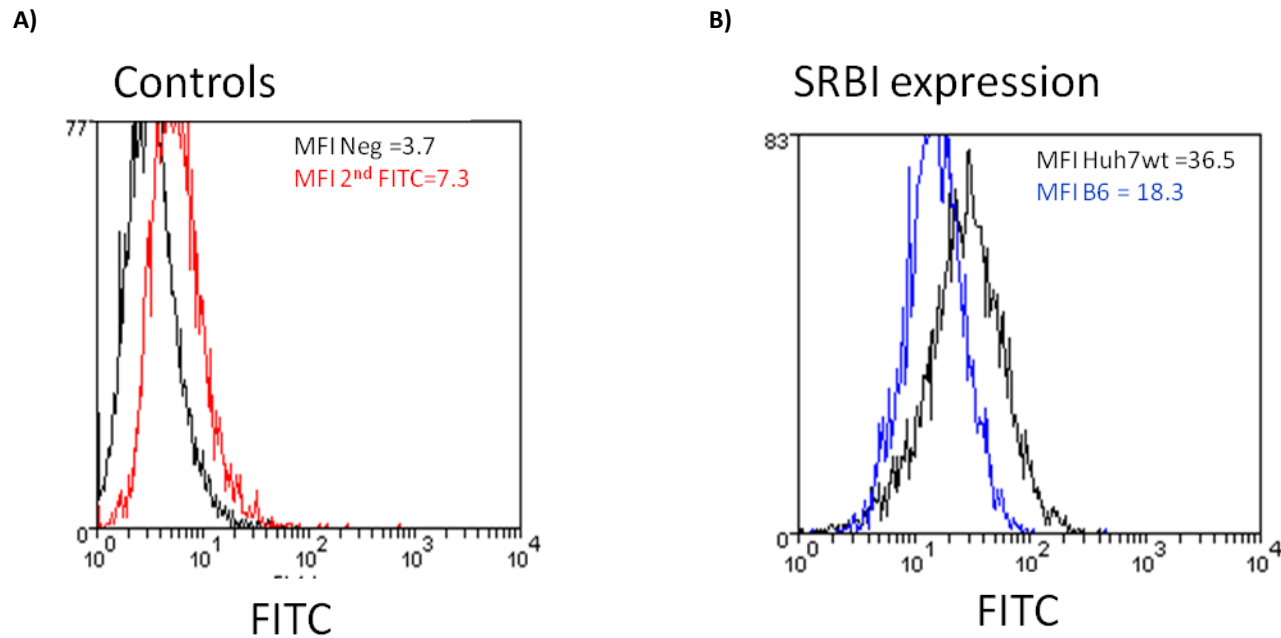
Figure 4.5: cytometry results of intracellular SRBI single stain of wild type Huh 7 cells and SRBI knock down Huh 7 B6 cells.
Flow Wild type Huh 7 cells and SRBI knock down Huh 7 B6 cells were stained with intracellular SRBI antibody to determine the SRBI level expressed on respective cell type. A) illustrates non-stained wild type Huh 7 cells and wild type cells solely stained with FITC- conjugated antibody toa ssess any potential background signal (red). B) demonstrates wild type Huh 7 cells showing MFI value of 36.5 for SRBI expression compared to MFI values of 18.3 for B6 cells (blue) which display over 50% reduction in SRBI expression.

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A) Controls — MFI Neg =3.7, MFI 2nd pCy5 = 4.1 (pCy5)

B) CD81 expression — MFI wt= 97, MFI B6 = 93 (pCy5)

C) Claudin expression — MFI wt = 7.7, MFI B6 = 6.1 (pCy5)

D) Occludin expression — MFI wt =5.1, MFI B6 = 5.2 (pCy5)
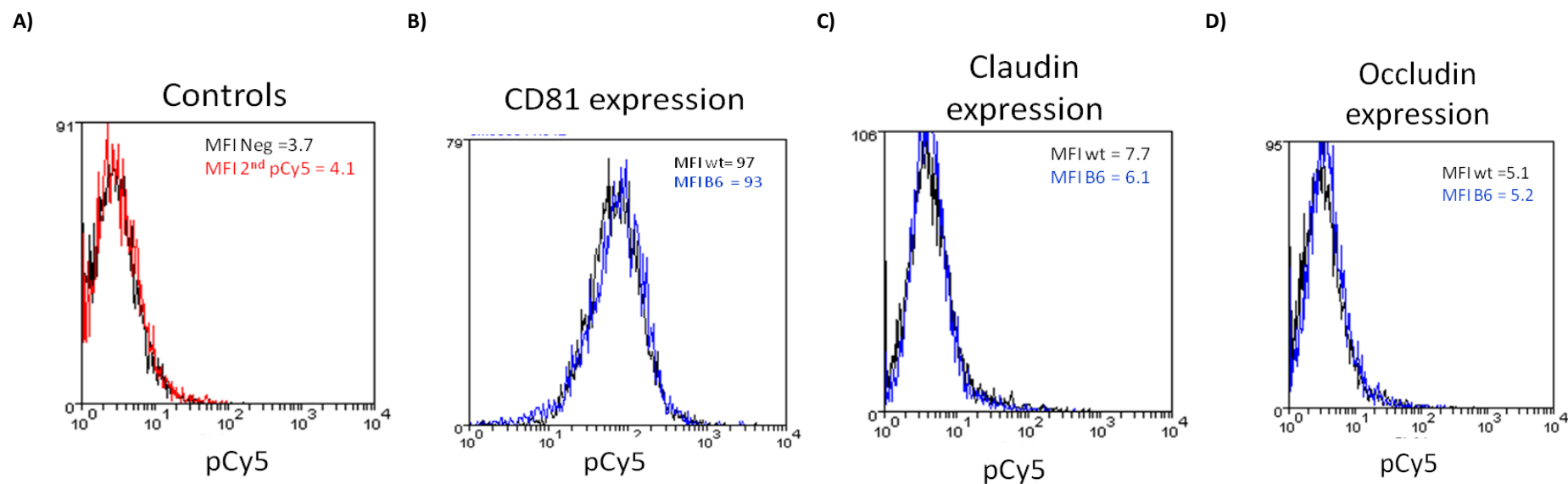
 Figure 4.6: Flow cytometry results of wild type Huh 7 and SRBI knock down Huh 7 B6 cells stain of HCV putative receptors CD81, Claudin and Occludin respectively. In order to assess whether the SRBI knock down had any abnormal effect on the total expression of the other putative HCV receptors on Huh 7 cells, flow cytometry staining of CD81, claudin and occludin was performed. A) shows the histograms of negatively stained cells and background control secondary antibody pCy5. B) to C) CD81, claudin and occluding were stained with surface targeting anti-CD81, anti-claudin antibodies with pCy5 –conjugated secondary antibody. The Mean Fluoroscence Intensity (MFI) values are displayed next to each graph. SRBI down regulation did not have impair the other HCV receptors as the MFI values are comparable between parental Huh 7 cells and SRBI knock down   cells.

4.3.5 HCV pseudo particle infectivity of wild type Huh 7 cells and SRBI knockdown (B6) Huh 7 cells

To assess whether the functionality of patient-derived E1E2 in the Hepatitis C virus pseudo particle assay relies on high levels of accessible SRBI at the point of infection, HEK 293T cells were utilised to produce HCV pseudo particles of nine E1E2 clones; H77 (control strain), 1A20.8, 1B5.23, 1B12.16, 2A1.2, 2A2.4, 2B1.1, 2B2.8 and 3A.1.28. These clones, which represent E1E2 isolates of genotypes 1, 2 and 3, have all previously proven to infect Huh 7 cells with varying degrees of infectivity (Lavillette, Tarr et al. 2005; Tarr, Urbanowicz et al. 2011). To verify that the cells which were used to generate the HCV pseudo particles expressed E1E2 proteins, the cells were lysed and included in an SDS-PAGE and Western blot experiment. All the clones used in the HCV pseudo particle assay produced E1E2 (Figure 4.7).
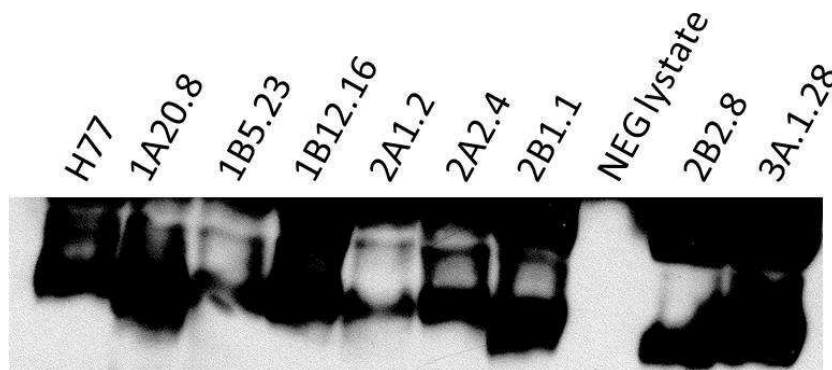
Figure 4.7: Western blot film of the E1E2 clones expressed by the HEK 293 cells which generated the correspondent HCV pseudo particles.
To verify that the HEK 293 cells were able to produce E1E2, the cells were lysed before the lysates were run on a 9% polyacrylamid gel and transferred onto a nitrocellulose membrane. The membrane was then probed with AP33 in a dilution of 1:200, diluted in PBS-Tween and subsequently with polyclonal rabbit anti-mouse horseradish peroxidise at a dilution of 1:1000. The membrane was treated with ECL-reagents before exposed onto a film. The film was developed prior to analysis. Figure 4.5 shows all clones expressing E1E2 protein.

The generated HCV pseudo particles were used to infect wild type Huh 7 cells as well as a SRBI knockdown Huh 7 B6 cell type in this experiment. The infectivity conferred by each E1E2 clone in the B6 cells was expressed as a percentage of that observed in the wild type Huh7 cells.

The results of the HCV pseudo particle assay showed that H77 and 2A2.4 infectivity of Huh 7 B6 was reduced by 45% compared to infectivity of wild type Huh 7 as Figure 4.8 shows. Moreover, 1A20.8, 2B1.1 and 3A.1.28 showed the greatest reduction in B6 infectivity of all the functional clones with 27%, 31% and 18% infectivity compared to wild type Huh 7 infectivity results, indicating a greater SRBI dependency for HCVpp entry for these clones. By contrast, E1E2 clones 1B5.23, 1B12.16, 2A1.2 and 2B2.8 all showed less dependency on SRBI expression, as they all achieved at least 50% infectivity in the B6 cells compared to the wild type Huh 7 cells Figures 4.8 and 4.9.

4.3.6 The E1E2 clones do not present any correlation between SRBI dependency for HCVpp entry and sensitivity to the neutralising antibodies AP33 and 1:7

To investigate whether the clones which display higher SRBI dependency in the HCV pseudo particle infectivity assay also would show resistance to antibody neutralisation (and vice versa), a neutralisation assay was performed with the monoclonal antibodies AP33 and 1:7. AP33 and 1:7 were added to the supernatant containing the pseudo particles, in five-fold dilution series at the point of wild type Huh 7 infection (Figures 4.10 to 4.13). Therefore, to ascertain whether there was a direct association between SRB1 dependency and neutralisation sensitivity, the IC50 for each isolate and antibody were

plotted against the percentage infectivity observed in the B6 cells (Figure 4.14). Differences in sensitivity to neutralisation were observed as H77 exhibited IC50s of 0.08 µg/ml of AP33 and 0.08 µg/ml of 1:7 while 2A2.4 showed IC50 concentrations at 0.7 µg/ml and 0.2 µg/ml of each mAb, respectively. The data obtained from the correlation graphs also showed that 1A20.8 required approximately 1 µg/ml of both AP33 and 1:7 to inhibit 50% of HCVpp entry. 2B1.1 displayed sensitivity to both mAbs as IC50 concentrations was 0.08 µg/ml of AP33 and 0.3 µg/ml of 1:7, while 3A.1.28 showed IC50 concentrations of approximately 1 µg/ml for both AP33 and 1:7. 1B12.16 displayed the highest IC50 concentrations of both AP33 and 1:7 at 1.3 µg/ml and 1.5 µg/ml, respectively. Fifty percent of 2B2.8 HCVpp infectivity was inhibited at a concentration of 1 µg/ml of AP33 and 1.5 µg/ml of 1:7, while 1B5.23 required 1 µg/ml of AP33 for 50% neutralisation in Huh 7 cells. As 50% inhibition was not observed for 1B5.23 in the 1:7 neutralisation assay, it was, therefore, not included on the graph.

A correlation between SRBI dependency and sensitivity to neutralising mAbs was not observed (Figure 4.14) from the collected data obtained in this work. P values of 0.67 and 0.99 together with a very low coefficient of determination values (0 and 0.022) suggest that there is no co-linearity present in the data.
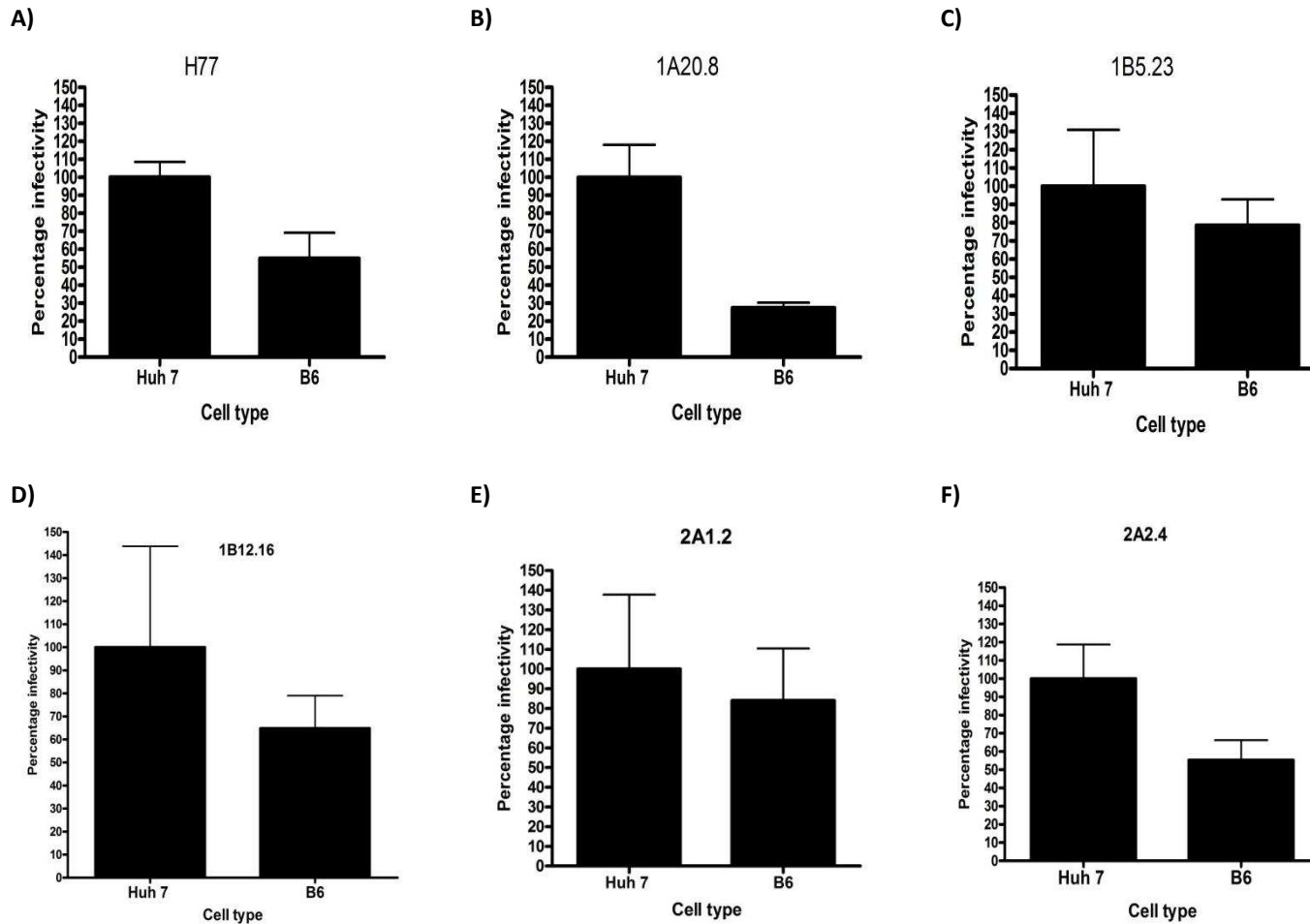
Figure 4.8: HCV E1E2 pseudo particle infectivity of wild type Huh 7 cells and Huh 7 B6 cells. HCV E1E2 clones derived from infected patients were used to generate HCV pseudo particles. These pseudo particles were used to infect wild type Huh 7 cells as well as Huh 7 B6 cells to determine whether some of the clones rely on SRBI for entry in the HCV pseudo particle assay. The clones correspond to the virus genotypes 1, 2 and 3. The infectivity data of wild type Huh 7 was normalised to 100% for every clone. A) H77 (reference strain) showed 55% Huh 7 B6 cell infectivity compared to wild type Huh 7 infectivity, B) 1A20.8 displayed 27% Huh 7 B6 infectivity compared to wild type Huh 7 infectivity and C) 1B5.23 showed 79% Huh 7 B6 infectivity compared to wild type Huh 7 infectivity. D) displays the infectivity data of 1B12.16 which reveals 65% Huh 7 B6 infectivity compared to the wild type Huh 7, E) 2A1.2 demonstrates 84% Huh 7 B6 infectivity compared to wild type Huh 7 cell infectivity. F) 2A2.4 shows 55% infectivity compared to the wild type Huh 7 infectivity results.

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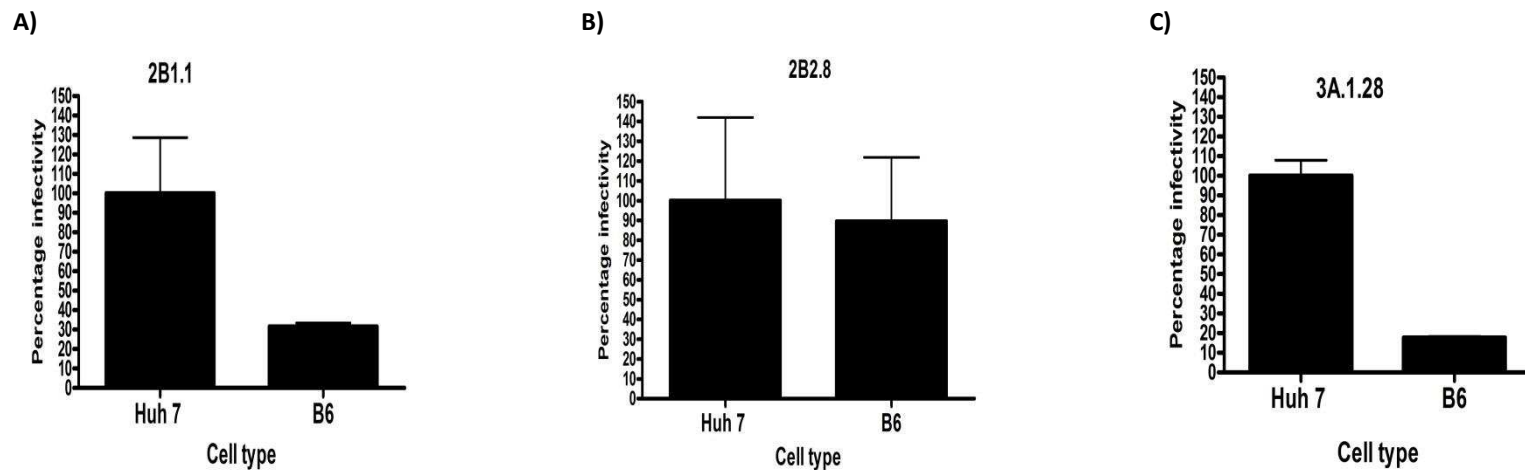
Figure 4.9: HCV E1E2 pseudo particle infectivity of wild type Huh 7 cells and Huh 7 B6 cells HCV E1E2 clones derived from infected patients were used to generate HCV pseudo particles.

These pseudo particles were used to infect wild type Huh 7 cells as well as Huh 7 B6 cells to determine whether some of the clones rely on SRBI for entry in the HCV pseudo particle assay. The clones corresponds to the virus genotypes 1, 2 and 3. The infectivity data of wild type Huh 7 was normalised to 100% for every clone. A) 2B1.1 showed 32% Huh 7 B6 cells compared to wild type Huh 7 infectivity, B) 2B2.8 displayed 90% Huh 7 B6 infectivity compared to wild type Huh 7 infectivity and C) displays the infectivity data of 3A.1.28 which reveals 18% Huh 7 B6 infectivity compared to the wild type Huh 7.

Figure 4.10: HCV pseudo particle neutralisation assay with AP33 and 1:7.
In order to investigate whether a relationship between SRBI dependency in HCVpp infectivity and a corresponding resistance to neutralising antibodies exists, the generated HCV pseudo particles were combined with the monoclonal antibodies AP33 and 1:7 in five fold titrations prior to Huh 7 cell infection. As a positive control, the HCV pseudo particles were added to the cells in the absence of neutralising antibodies and PBS acted as a negative control in the assay. A) and B) show H77 pseudo particle infectivity in the presence of AP33 and 1:7, respectively. H77 proves to present sensitivity to the neutralising antibodies. C) and D) display the neutralising data of 2A2.4 that shows intermediate sensitivity to the monoclonal antibodies. The mAb concentration of which 50% HCVpp infectivity is inhibited (IC50) is shown next to each graph.
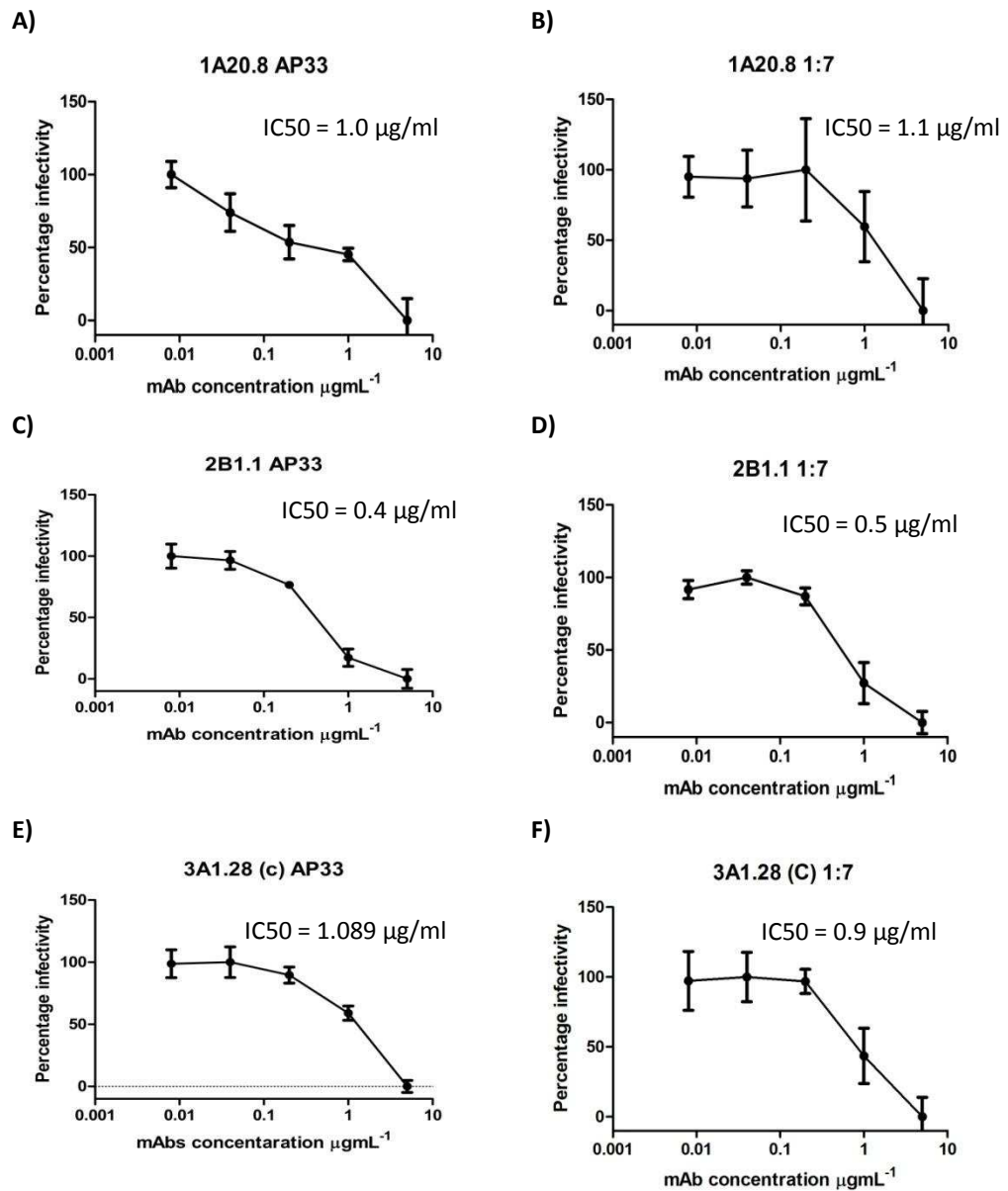
Figure 4.11: HCV pseudo particle neutralisation assay with AP33 and 1:7.
In order to investigate whether a relationship between SRBI dependency in HCVpp infectivity and a corresponding resistance to neutralising antibodies exists, the generated HCV pseudo particles were combined with the monoclonal antibodies AP33 and 1:7 in five fold titrations prior to Huh 7 cell infection. A) to B) show 1A20.8 pseudo particle infectivity in the presence of AP33 and 1:7, respectively. C) to D) display the neutralising data of AP33 and 1:7 to 2B1.1and E) to F) illustrates the neutralisation data of AP33 and 1:7 to 3A1.28. The mAb concentration of which 50% of HCVpp infectivity is inhibited (IC50) is shown next to each graph.
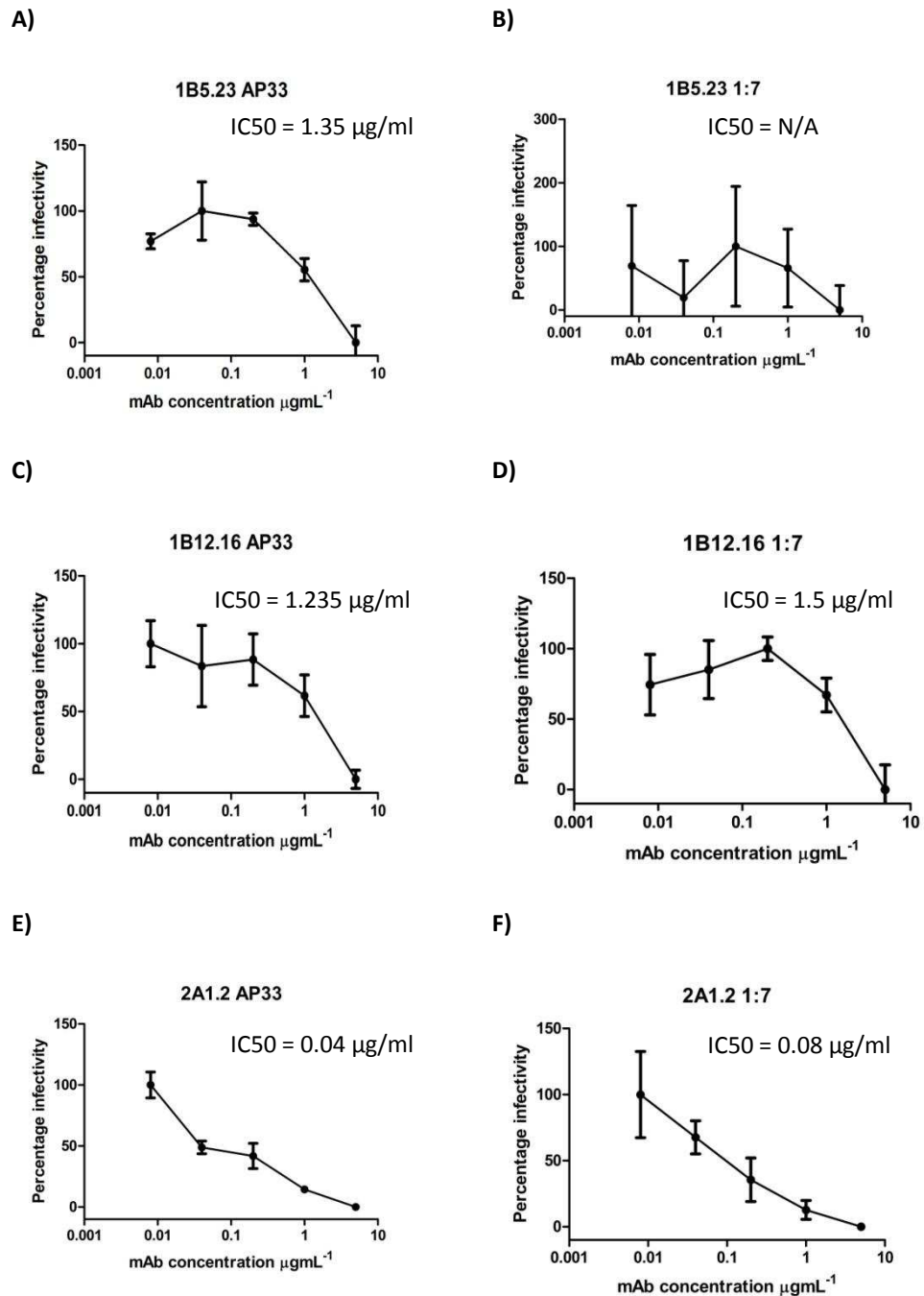
Figure 4.12: HCV pseudo particle neutralisation assay with AP33 and 1:7.
In order to investigate whether a relationship between SRBI dependency in HCVpp infectivity and a corresponding resistance to neutralising antibodies exists, the generated HCV pseudo particles were combined with the monoclonal antibodies AP33 and 1:7 in five fold titrations prior to Huh 7 cell infection. A) to B) show 1B5.23 pseudo particle infectivity in the presence of AP33 and 1:7, respectively. C) to D) display the neutralising data of AP33 and 1:7 to 1B12.16 and E) to F) illustrates the neutralisation data of AP33 and 1:7 to2A1.2 The mAb concentration of which 50% of HCVpp infectivity is inhibited (IC50) is shown next to each graph.
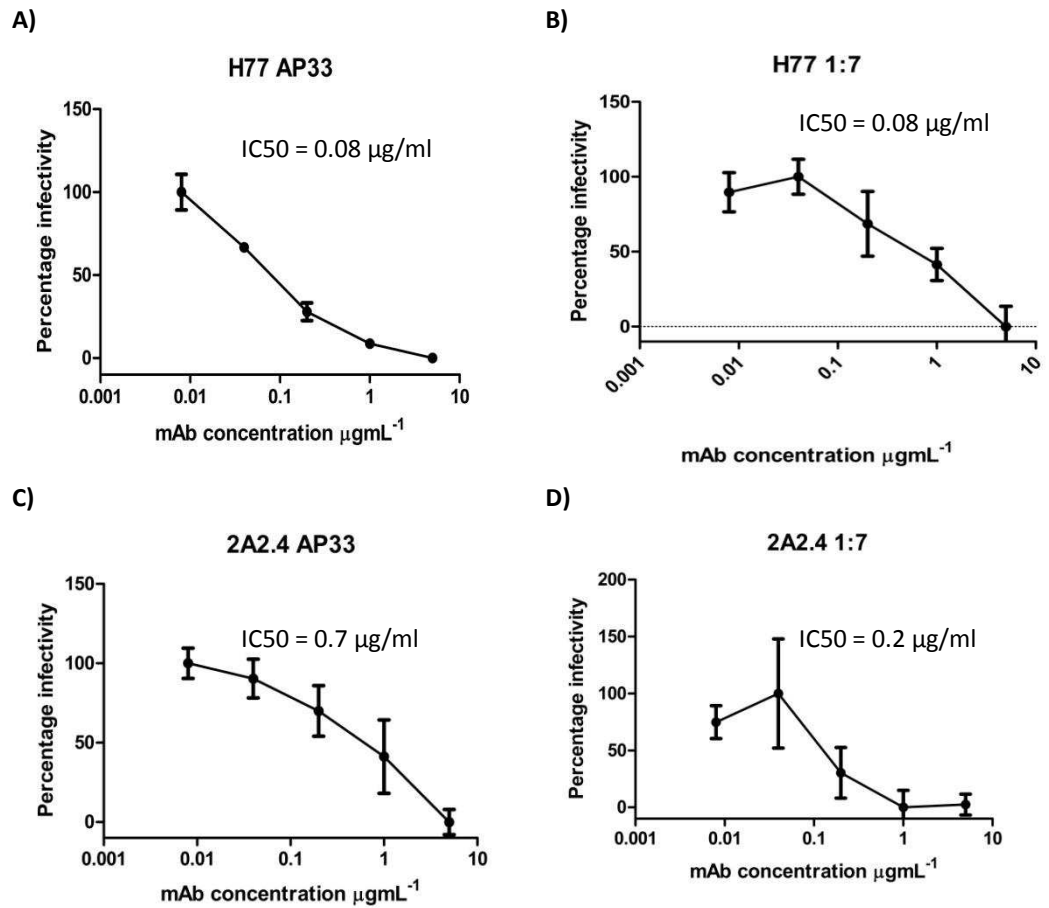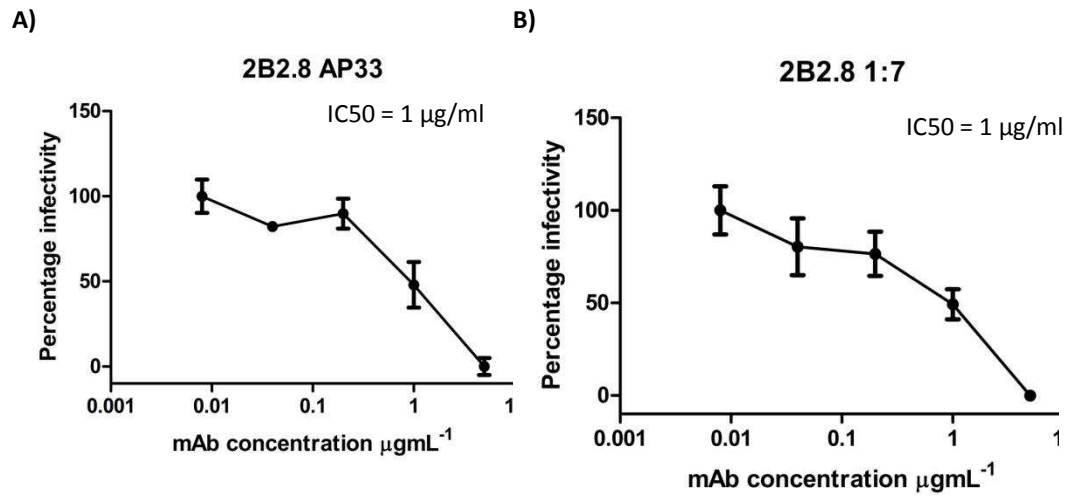
Figure 4.13: HCV pseudo particle neutralisation assay with AP33 and 1:7.
In order to investigate whether a relationship between SRBI dependency in HCVpp infectivity and a corresponding resistance to neutralising antibodies exists, the generated HCV pseudo particles were combined with the monoclonal antibodies AP33 and 1:7 in five fold titrations prior to Huh 7 cell infection. A) to B) show 2B2.8 pseudo particle infectivity in the presence of AP33 and 1:7, respectively. 2B2.8 proves to present intermediate sensitivity to both AP33 and 1:7. The mAb concentration of which 50% of HCVpp infectivity is inhibited (IC50) is shown next to each graph.
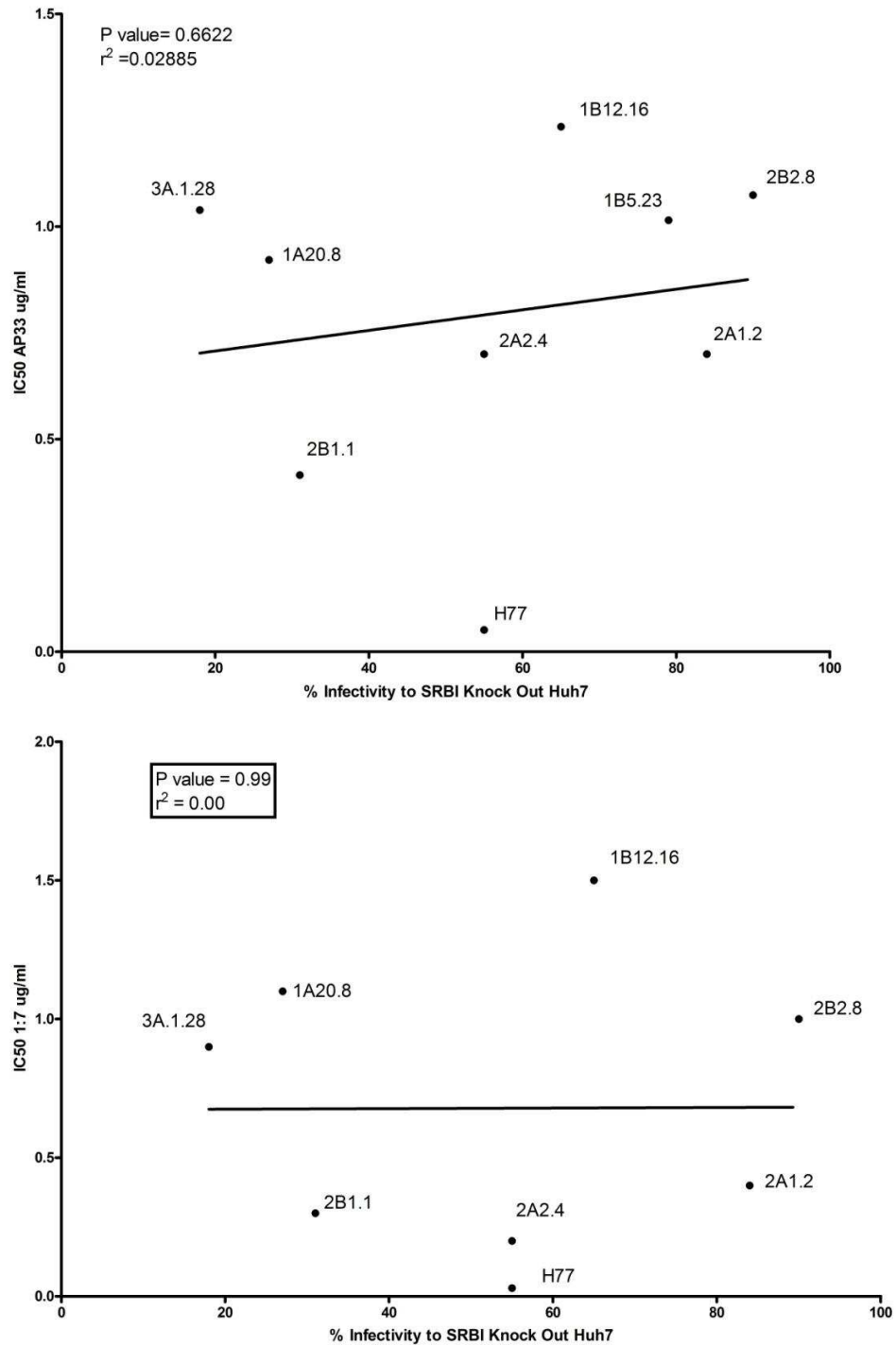
Figure 4.14: Linear regression graph of the neutralising IC50 of AP33 and 1:7of each E1E2 clone and the correspondent Huh 7 B6 infectivity.
To obtain a view of the relationship between the SRBI dependence for HCVpp infectivity and sensitivity to neutralising antibodies, a linear regression curve was created in GraphPad version 5. A) displays the correlation between Huh 7 B6 infectivity and AP33 neutralisation. B) illustrates the correlation between Huh 7 B6 infectivity and 1:7 neutralisation. Fifty percent inhibition was not observed with 1B5.23 in the 1:7 neutralisation assay and was therefore not included in the graph. The results of these graphs show no significant correlation between SRBI dependency and sensitivity to neutralising antibodies.

4.4 Discussion

As the details of true HCV replication and entry have been difficult to unravel for many years, the introduction of HCVcc and HCVpp systems enabled HCV researchers to study HCV entry from different perspectives.

In HIV research, studies have been published describing the virus presenting a decreased dependency on CD4 (a HIV receptor that is crucial for entry) in vitro as well as an enhanced sensitivity to neutralising antibodies (Platt, Wehrly et al. 1998; Dumonceaux, Chanel et al. 1999; Hoffman, LaBranche et al. 1999; Bannert, Schenten et al. 2000; Blish, Nguyen et al. 2008).

We aimed to investigate whether this was applicable to the entry process of HCV and whether SRBI would act similarly to CD4. Nine E1E2 clones derived from patients with chronic HCV infection were included in the HCVpp assay. These clones, H77 (control strain), 1A20.8, 1B5.23, 1B12.16, 2A1.2, 2A2.4, 2B1.1, 2B2.8 and 3A.1.28, were representatives of HCV genotypes 1, 2 as well as 3, and were used to infect wild type Huh 7 cells (with natural levels of expressed SRBI on the cell surface) and SRBI knock down Huh 7 cells. To also determine whether there is any correlation between high SRBI dependency and sensitivity to neutralising antibodies, these clones were also included in HCVpp neutralisation assay with E2 targeting mAbs AP33 and 1:7.

The HCV pseudo particle assay had recently been established in the lab at the time of designing this study and although could have been useful to include

this assay in previous studies presented in Chapter 2 and 3, the option was not available.

As SRBI is utilised in the liver for lipid uptake, SRBI expression is understandably high on primary hepatocytes (Regeard 2008). In this work, we found by flow cytometry, that the natural SRBI expression varied slightly between different Huh7 cell populations. However, authors of a study published in 2009 did not observe any variation in SRBI expression in phenotypically distinct Huh 7 cell lines (Sainz, Barreto et al. 2009).

An initial attempt was made to isolate two separate populations of high and low SRBI expressing Huh 7 cells by staining wild type Huh7 cells for SRBI and sorting them using a fluorescence activated cell sorter (FACS). This experiment proved to be unsuccessful as the cells, following FACS sorting, reverted back to a mixed population of high and low SRBI expressers. This observation suggests that SRBI expression might be regulated epigenetically. Moreover, the growth media that was added to the sorted cells contained serum with lipids. Lipids are the natural ligands of SRBI and lipid starvation has been demonstrated to up regulate SRBI expression in human hepatoma cells (Kapadia, Barth et al. 2007). Re-addition of lipids to hepatoma cells caused SRBI expression to drop again, confirming that ligand availability controls the regulation of SRBI. Hence, in our study, once the sorted cells were placed back in media which naturally contained lipids in an optimal cell stimulating environment to maximize the proliferation rate of the cells, this could cause the cells to revert back to mixed populations of varying SRBI-expressing cells.

A second attempt to generate cells with varying amount of SRBI expression was via Lentiviral gene delivery. Although the positive control, the GFP gene proved to be successful, the over-expression of human SRBI was unsuccessful. The fact that the GFP delivery worked successfully shows that the conditions for correct gene delivery were optimal.

The third attempt to over-express SRBI was by lipid starvation. Wild type Huh 7 cells were grown in MAC media containing lipid-deficient serum to examine whether limited availability of lipid would up-regulate the SRBI expression of the Huh 7 cells. However, the Huh 7 cells proved to be quite sensitive to such drastic a change in nutrients and began to display signs of stress. Whilst FACS analysis after 18 days was possible with cells grown in T75 flask, it was however, more difficult with cells grown in a 6-well plate. Huh 7 cells have during this present study been shown to prefer a high density environment. Under such conditions, the cells grow at a higher rate and are less sensitive to minor trauma. This was observed during the duration of this work and could explain the drastic difference in cell health upon lipid starvation.

The expression levels of SRBI have shown to be associated with HCVpp and HCVcc infectivity. (Bartosch, Verney et al. 2005; Lavilette, Tarr et al. 2005; Grove, Huby et al. 2007; Regeard, Trotard et al. 2008). The over-expression of SRBI, by lentiviral transduction, on human hepatoma cell lines has proved to enhance HCVcc J6/JFH1 infectivity by three-fold (Grove, Huby et al. 2007). In association, silencing SRBI expression has resulted in reduction of HCVpp infection (Lavillette, Tarr et al. 2005), re-enforcing that role for SRBI as a crucial entry factor in HCV infectivity.

Lavillete et al conducted a study that included different HCV E1E2 clones representing HCV genotypes 1 to 6 to examine whether different E1E2 clones exhibit various levels of SRBI dependency (Lavillette, Tarr et al. 2005). SRBI expression was down regulated by ten-fold in Huh 7 cells compared to parental cells. From the results of that study, H77 (genotype 1a) exhibited most dependency with a ten-fold reduction in HCVpp infectivity. However, E1E2 clone 2A1.2 showed the least SRBI dependency as the Huh 7 infectivity was only reduced by 30%. In our study, 50% of SRBI down-regulation was achieved on human hepatoma cells and from the nine E1E2 clones included in this study, 3A1.28 showed the highest SRBI dependency with 82% reduction in infectivity observed with the B6 cells compared to the parental Huh 7 cells, while 2B2.8 showed only approximately 10% decrease in infectivity with the B6 cells compared to the parental Huh 7 cells. In the Lavilette study, 3A1.28 and 2B2.8 exhibited over 60% and approximately 40% reduction in infectivity in the SRBI-silenced cells. However, coinciding with Lavilette et al, 2A1.2 showed less than 30% percent reduced infectivity with the SRBI down-regulated cells, indicating a lower dependency on SRBI at entry for this clone. H77 displayed 45% reduced infectivity in the B6 cells compared to wild type Huh 7 in our study. E1E2 clone 2B2.4 was also common between the studies. In our study, 2B2.4 showed approximately 45% reduced infectivity with the B6 cells compared to the wild type huh 7 cells, while it displayed over 60 % reduction in the Lavilette paper. As we achieved 50% SRBI down-regulation, while Lavilette et al achieved a ten-fold decrease in SRBI expression, the discrepancy in HCVpp infectivity could be explained by the difference in achieved SRBI down-regulation between the studies.

All the clones were included in HCVpp neutralisation assay with two monoclonal antibodies targeting HCV E2 glycoprotein. This experiment was performed to assess whether there was a relationship between the SRBI dependency and sensitivity to neutralisation by these mAbs. Regression analysis showed that there was no correlation or statistical significance to confirm an association.

Five of the clones, 1A20.8, 2B1.1, 3A.1.28, H77 and 2A2.4 showed reduced HCVpp infectivity with the SRBI-knockdown cells compared with wild type Huh 7. As this dependency is observed with clones that are representative of different genotypes, this effect is likely to be genotype-independent. Therefore, developing therapeutic antibodies against SRBI that exhibit broad neutralisation abilities across HCV genotypes could be potentially interesting. If these findings strengthen the hypothesis that some HCV isolates naturally could require higher levels of host factors proven important in entry, such as SRBI, for survival and cell invasion, understanding the behaviours and the survival requirements of individual viral genotypes can be useful in the development of tailored therapy in the defence of HCV infection.

Since this study only investigated the properties of nine isolates, to reinforce our findings, more HCV E1E2 clones from different genotypes must be included in the work. However, due to time constraints this study could not be expanded.

To add to this investigation, it would be interesting to examine whether dependencies to the other entry receptors occurs among different HCV E1E2

clones in the HCVpp system. Moreover, since SRBI is naturally a lipid receptor on cells (Scarselli, Ansuini et al. 2002), it would be preferable to include HDLs in the assay and observe potential change in HCVpp infectivity. Also, since wild type Huh7 cells, expressing natural levels of SRBI, and Huh7 SRBI-knockdown cells were used in this work, including a hepatoma cell line over expressing SRBI would increase the physiological relevance of the work. The outcome of those assays would help strengthen or refute the hypothesis questioned in this study.

5 Final conclusion

The lack of an appropriate HCV cell culture model previously impaired the study of HCV entry since the discovery of the virus in 1989 (Choo, Kuo et al. 1989). The recent development of the HCVpp and HCVcc systems have allowed significant progress in this area and confirmed the importance of glycoproteins E1 and E2 in receptor binding. The importance of E1 and E2 as targets of neutralising antibodies has also been revealed.

An essential step in developing immuno-focussed vaccines is identifying the epitopes to which neutralising antibodies are directed. This is intimately linked with identifying regions of the glycoproteins that interact with host cell receptors. HCV possesses at least four entry receptors, highlighting the complex, multi-step entry process that confers tissue tropism to the virus. These receptor interactions are all potential targets for immune intervention. The interplay between binding of HCV glycoproteins to entry receptors and the effect this has on neutralisation sensitivity has been the main focus of this investigation.

Greater understanding of the interaction between the host receptors and the HCV glycoproteins, and how this influences antibody binding to E2, will be useful to designing novel antibody therapies. It is also important to appreciate the individual requirements of different virus isolates, as isolate-specific therapeutics might be required for clearance of HCV infection. The production of neutralising antibodies that are able to compete and interfere with receptor binding events could prove to be beneficial in blocking entry. Understanding

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the different entry properties of the HCV genotypes could be helpful in therapeutic settings; tailoring treatments for the patients might increase the chances of clearing the infection. Finally, as the details of HCV entry have not yet been fully disclosed, the combined results of this body of work will aid the understanding of the mechanisms utilised by HCV in cell invasion and the means to study them.

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