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Review

The role of neutralizing antibodies in hepatitis C virus infection

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Hepatitis C virus (HCV) is a blood-borne virus estimated to infect around 170 million people worldwide and is, therefore, a major disease burden. In some individuals the virus is spontaneously cleared during the acute phase of infection, whilst in others a persistent infection ensues. Of those persistently infected, severe liver diseases such as cirrhosis and primary liver cancer may develop, although many individuals remain asymptomatic. A range of factors shape the course of HCV infection, not least host genetic polymorphisms and host immunity. A number of studies have shown that neutralizing antibodies (nAb) arise during HCV infection, but that these antibodies differ in their breadth and mechanism of neutralization. Recent studies, using both mAbs and polyclonal sera, have provided an insight into neutralizing determinants and the likely protective role of antibodies during infection. This understanding has helped to shape our knowledge of the overall structure of the HCV envelope glycoproteins – the natural target for nAb. Most nAb identified to date target receptor-binding sites within the envelope glycoprotein E2. However, there is some evidence that other viral epitopes may be targets for antibody neutralization, suggesting the need to broaden the search for neutralization epitopes beyond E2. This review provides a comprehensive overview of our current understanding of the role played by nAb in HCV infection and disease outcome and explores the limitations in the study systems currently used. In addition, we briefly discuss the potential therapeutic benefits of nAb and efforts to develop nAb-based therapies.

Introduction

Hepatitis C virus (HCV) infects approximately 170 million people worldwide (Ascione *et al.*, 2007) and as many as 3 million individuals are newly infected each year (Anonymous, 1999). Currently, there is no available vaccine to prevent HCV infection. In 20–30 % of infections the virus is cleared spontaneously (Santantonio *et al.*, 2008); however, in the majority of patients the virus persists. The mechanism by which some individuals spontaneously resolve infection, while others become chronically infected is not clearly understood. Chronic HCV infection can lead to cirrhosis of the liver and, in some cases, hepatocellular carcinoma (HCC), which ultimately requires a liver transplantation. HCV infection is the predominant indication for liver transplantation and no treatments are currently available to prevent reinfection of a grafted liver. The current standard therapy for chronic infection – pegylated alpha interferon (IFN-α) and ribavirin – is only effective in 40–60 % of cases and response to therapy varies between different viral genotypes. Two recently developed protease inhibitors, boceprevir and telaprevir, given in combination with IFN-α and ribavirin, improve sustained virological response (SVR) rates to 75 % in genotype 1-infected patients (Kwo *et al.*, 2010; McHutchison *et al.*, 2010). This development in the treatment of hepatitis C is encouraging; however, HCV still represents a major disease burden. Recent developments in the model systems used to study virus–receptor and virus–antibody interactions have led to advances in our understanding of the nature of the anti-HCV antibody response and demonstrate the complexity of the virus–host relationship. However, there are still large gaps in our knowledge and understanding of the interplay between HCV and neutralizing antibodies (nAb).

A supplementary figure is available with the online version of this paper.

Virus targets of the antibody response

Antibodies generated during acute infection may be targeted against epitopes within structural and non-structural viral proteins; however, the majority of nAb have been mapped to the envelope glycoproteins E1 and E2 (Johansson *et al.*, 2007; Kato *et al.*, 1993; Keck *et al.*, 2008b; Meunier *et al.*, 2008; Owsianka *et al.*, 2005; Perotti *et al.*, 2008; Shimizu *et al.*, 1996). E1 and E2 are produced by cellular signal peptidase cleavage from the viral polyprotein

(Op De Beeck & Dubuisson, 2003). The C-terminal transmembrane domains have been proposed to function in heterodimerization of the glycoproteins and contain endoplasmic reticulum (ER)-retention signals that are thought to anchor the glycoproteins within lipid membranes (Op De Beeck *et al.*, 2001). The N-terminal ectodomain of E2 possesses the entry determinants for infection of the host cell (Burlone & Budkowska, 2009). A 3D model of the structure of E2 has recently been proposed (Krey *et al.*, 2010). This model assigns a typical class II fusion protein structure to E2, akin to the fusion proteins of members of the alpha- and flaviviruses, consisting of three distinct domains. The structure of E1 is less well defined, although it has been proposed to function as the fusion determinant, triggering fusion of the viral and cellular membranes during entry (Lavillette *et al.*, 2007). However, in other viruses belonging to the family *Flaviviridae*, the proteins analogous to E1 chaperone the fusion protein to ensure correct folding; this is reflected in the model by Krey *et al.* (2010) where the putative fusion peptide is located in domain II of the E2 protein. E1 and E2 form non-covalent heterodimers (Dubuisson *et al.*, 1994) and these were considered to be the mature functional forms on the surface of the virus. However, more recent studies of E1 and E2 present in cell culture infectious HCV particles indicated the presence of larger covalent complexes stabilized by disulphide bridges (Vieyres *et al.*, 2010); it remains to be fully determined which represents the functional form. Despite conserved function between clinical isolates, the E2 glycoprotein tolerates great genetic diversity (Fig. 1, Supplementary Fig. S1, available in JGV Online). The ectodomain contains three highly variable regions. Hypervariable region (HVR) 1 is a 26–28 aa region located at the N terminus of E2 that plays an important role in entry, antibody binding and disease outcome (Bartosch *et al.*, 2003c; Farci *et al.*, 2000; Vieyres *et al.*, 2011). Two additional hypervariable regions, HVR2 and the intergenotypic variable region (igVR) are thought to be involved in E1E2 heterodimerization and virus infectivity (Albecka *et al.*, 2011; McCaffrey *et al.*, 2011). Two additional hypervariable regions have been identified within the subtype 3a E2 glycoprotein. These regions, designated HVR495 and HVR575, are located downstream of HVR1 at aa 495–501 and 575–578, respectively. Both regions are under positive selection during acute (Humphreys *et al.*, 2009) and chronic infection (Brown *et al.*, 2007). Whether or not substitution in these additional variable regions is being driven by antibody responses, and the clinical relevance of this variability, is unknown.

Early evidence of nAb in HCV infection

The earliest studies of the antibody response to HCV were carried out in chimpanzees. Such studies showed that serum from HCV-infected individuals can neutralize virus infectivity *in vitro* and subsequently protect chimpanzees against challenge with HCV (Farci *et al.*, 1994). Similarly, hyperimmune sera raised against peptides homologous to HVR1 also neutralized HCV infectivity *in vitro* (Shimizu *et al.*, 1996) and *in vivo* (Farci *et al.*, 1996). In a separate study, chimpanzees immunized with the envelope glycoproteins E1E2 generated a strong antibody response that partially protected against experimental challenge with autologous HCV (Choo *et al.*, 1994). Evidence that antibodies could protect from natural infection in humans arose from a retrospective study of a cohort of patients receiving polyclonal immunoglobulins against hepatitis B virus surface antigen (HBIG). Patients who received HBIG prior to the introduction of routine screening for HCV infection were less likely to develop HCV than those who received HBIG screened for HCV. Anti-HCV antibodies were detected in HCV-negative patients who had undergone HBIG treatment, indicating a passive transfer of anti-HCV antibodies within HBIG to the recipient (Feray *et al.*, 1998). Furthermore, individuals with hypogammaglobulinaemia (Bjøro *et al.*, 1994) who became infected with HCV experienced a very rapid disease progression, highlighting the potential importance of antibodies in controlling chronic infection. It has however been reported that some hypogammaglobulinaemic patients are able to spontaneously resolve acute HCV infection (Razvi *et al.*, 2001), indicating the importance of cell-mediated immunity in resolved infection.

Model systems to study nAb response to HCV

Chimpanzees are the only species other than humans permissive for HCV infection. Due to ethical and financial constraints, however, they are not an ideal system in which to study the virus and these *in vivo* studies have largely been replaced by *in vitro* systems.

Early *in vitro* methods to study virus–antibody and virus–cell interactions relied upon soluble, recombinant, truncated or full-length versions of E2 expressed in mammalian cells. This system enabled the identification of the first cellular receptors for HCV, namely CD81 (Pileri *et al.*, 1998). Based on this discovery, many early studies to isolate nAb utilized the neutralization of binding (NOB) assay that relies upon E2 binding to Molt-4 cells expressing CD81 (Flint *et al.*, 1999; Rosa *et al.*, 1996). However, as will be discussed later, this system possessed several shortcomings and was superseded by the development of virus-like particles (VLPs), generated in insect cells (Baumert *et al.*, 1998). These VLPs showed structural similarities to the virions isolated from HCV-infected humans and chimpanzees (Baumert *et al.*, 1998) and therefore more accurately represented the native conformation of the envelope glycoproteins. Indeed, comparison of the neutralizing activity of a panel of mAbs to VLPs and soluble E2 ($E2_{661}$) showed a marked difference in their ability to bind the different forms of E2 (Clayton *et al.*, 2002) and to block E2–CD81 interactions (Owsianka *et al.*, 2001). These studies highlight the importance of using E2 representative of the native glycoprotein found on the surface of the viral particle. However, these systems have since been replaced by the HCV pseudoparticle (HCVpp) and HCV cell culture (HCVcc) assays.

HCVpp are generated by displaying HCV envelope glycoproteins on the surface of retroviral or lentiviral core particles (Bartosch *et al.*, 2003b). This has facilitated studies of viral attachment and entry into target cells and led to the identification of neutralizing sera and antibodies (Bartosch *et al.*, 2003a; Flint *et al.*, 2004; Yu *et al.*, 2004). One advantage of this system is the ability to generate HCVpp displaying E1E2 isolated from a variety of sources (Fafi-Kremer *et al.*, 2010; Tarr *et al.*, 2011). Although useful for studying entry and neutralization, this model system does have its drawbacks. Not all patient-derived E1E2 form functional pseudoparticles (Dowd *et al.*, 2009; Flint *et al.*, 2004; Lavillette *et al.*, 2005), and the reasons for this are unknown. Also, the cell-type used to generate infectious pseudoparticles (human embryonic kidney cells) lacks lipid metabolism machinery (Burlone & Budkowska, 2009); therefore, HCVpp are not associated with any host lipoproteins, unlike serum-derived viral particles (see below). This limitation may greatly affect the interaction of HCVpp with nAb.

HCVcc is based on replication of the genotype 2a stain, JFH-1, in human hepatocarcinoma cells. This is the only strain of HCV so far found to replicate in cell culture without accumulating adaptive mutations. The virus particles produced are infectious allowing studies of the complete virus life cycle (Kato *et al.*, 2001; Wakita *et al.*, 2005). This assay system has been modified to study the properties of genetically diverse viruses by the development of chimeric infectious clones encoding the structural proteins (core, E1, E2 and p7) and non-structural protein 2 (NS2) of all major genotypes (1–7). However, these chimeras often show poor replication kinetics and also acquire cell-culture adaptive mutations (Gottwein *et al.*, 2009; Pietschmann *et al.*, 2006). There is emerging evidence that at least some culture-adaptive mutations render isolates more sensitive to neutralization (Dhillon *et al.*, 2010; Grove *et al.*, 2008). This phenomenon has been widely documented for other viruses, including human immunodeficiency virus (HIV) (Pugach *et al.*, 2004) and respiratory syncytial virus (Marsh *et al.*, 2007). Therefore, an HCV-based single-cycle infection system, particularly one that could be easily supplemented with E1E2 cloned directly *ex vivo*, would provide a more robust method for studying antibody neutralization. *Trans*-complementation of HCV replicons with constructs expressing the HCV structural genes has been shown to result in the production of infectious particles containing a packaged replicon genome, which can then be used to infect permissive cells (Adair *et al.*, 2009). However, the relatively low virus titres produced limit the general applicability of this system.

A small animal model capable of supporting the complete replicative cycle is potentially a better system in which to study systemic virus–host interactions. However, current animal models are far from perfect. The uPA-SCID mouse model uses immunosuppressed chimeric mice transplanted with human hepatocytes, which renders them susceptible to HCV infection (Lindenbach *et al.*, 2006; Mercer *et al.*, 2001). Generation of the chimeric livers is technically difficult and the mice are immunodeficient, preventing any study of the host-adaptive immune response. They have, however, been used to study virus neutralization by passively transferred antiviral antibodies (Law *et al.*, 2008; Vanwolleghem *et al.*, 2008) and anti-receptor antibodies (Meuleman *et al.*, 2008). More recently, a transgenic immunocompetent mouse, expressing the essential HCV entry factors, has been developed. Whilst incapable of supporting significant HCV replication, it supports HCV entry and has been used to demonstrate entry inhibition by virus- and receptor-specific antibodies (Dorner *et al.*, 2011).

The nature of epitopes recognized by nAb

To inform vaccine design and to understand the process of neutralization, it is important to define the nature and location of neutralizing epitopes. Epitopes can be categorized as being either linear or conformational. A number of linear HCV epitopes have been mapped using peptide scanning, a technique where reactivity to a panel of overlapping peptides corresponding to the protein of interest is determined (Clayton *et al.*, 2002; Owsianka *et al.*, 2001). In addition, iterative enrichment of random peptide display libraries can be used to identify peptides capable of binding to mAbs and serum (Tarr *et al.*, 2006; Zhang *et al.*, 2007, 2009). The technique utilizes a suitable host, phage or bacterium, which is genetically modified to display 6–40mer peptides of a random nature, which can be either linear or constrained in a disulphide loop. Multiple rounds of biopanning then enrich peptides able to bind specifically to the target molecule. Alignments of the resulting peptide sequences identify likely antibody contact residues within the linear peptide sequence. Identifying residues constituting conformational epitopes is more challenging. A common approach is to probe panels of glycoproteins possessing single amino acid substitutions (most often using alanine replacement) with the antibody of interest. Marked loss of reactivity indicates that the substituted residue is involved in binding (Roben *et al.*, 1994). However, introduction of substitutions, particularly in structurally important regions of the protein, can have an adverse effect on the overall conformation of the protein. It is important to ensure that the global structure of a mutated protein is maintained, by ensuring that the binding of control, non-competing, conformation-dependent antibodies is unaltered. Using this mutant panel approach, we have successfully mapped the epitopes recognized by a large number of conformation-sensitive and -insensitive antibodies (Johansson *et al.*, 2007; Law *et al.*, 2008; Owsianka *et al.*, 2008; Perotti *et al.*, 2008; Tarr *et al.*, 2006). However, such a directional mutagenesis approach is labour-intensive and requires prior knowledge of the possible contact residues. More recently, a number of novel mAbs were mapped using yeast surface-expressed random- and deletion-mutant E2 libraries (Sabo *et al.*, 2011). Such an approach enables a large number of different residues to be interrogated simultaneously. However, the overall fold of the yeast-expressed proteins was not verified, and this will need to be performed before this approach can be adopted widely. Finally, studies

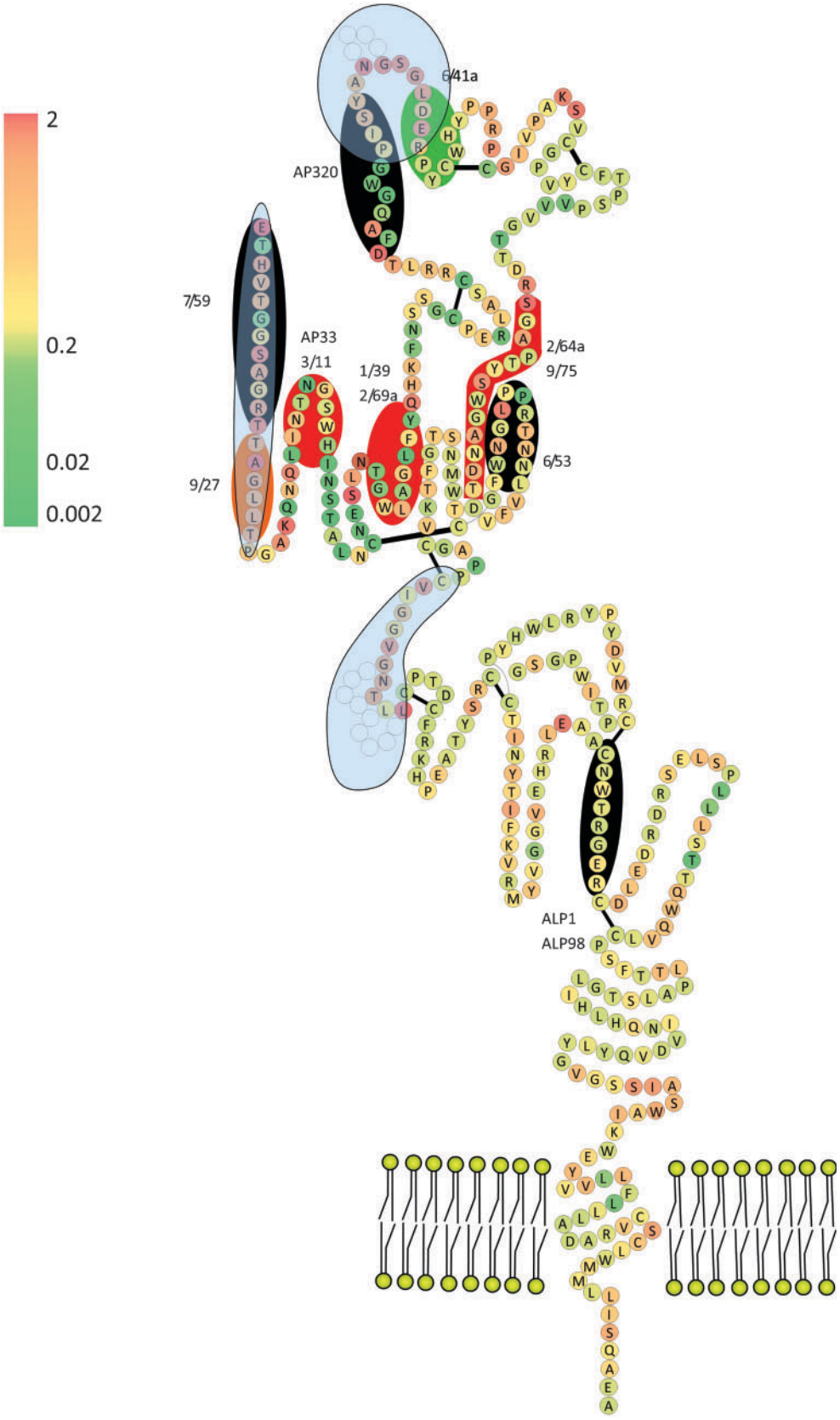
2
0.2
0.02
0.002
6/41a
AP320
7/59
AP33
3/11
1/39
2/69a
2/64a
9/75
6/53
9/27
ALP1
ALP98

Fig. 1. Representation of the tertiary structure of the H77c E2 protein, adapted from the model proposed by Krey *et al.* (2010), showing the natural amino acid diversity. A Shannon entropy plot was performed for each amino acid position in the mature E2 protein, using a panel of 1833 HCV sequences from the GenBank database. Sequences were aligned using the CLUSTAL_X algorithm, with manual adjustment. Amino acid diversity is indicated by colour – dark red denotes extreme variability (an entropy value of 2), while dark green denotes extreme conservation (an entropy value of 0.002). White circles indicate length polymorphisms in other HCV isolates not observed in the H77 sequence. HVRs are highlighted by blue shaded ovals. As previously demonstrated, HVRs 1 and 2, as well as the IgVR display the greatest sequence diversity. However, this analysis highlights the extreme variability observed at specific single amino acids distributed throughout the E2 protein. Linear epitopes recognized by previously described antibodies are highlighted by the following coloured ovals. Black denotes non-nAb, orange denotes nAb that inhibit the interaction with SR-B1, green denotes antibodies that inhibit interaction with CD81 and red denotes antibodies that neutralize entry of HCVpp or HCVcc by interfering with virus–receptor interactions. The presence of highly variable amino acids in the putative epitopes of broadly nAb may indicate evolution of these regions under the selective pressure of the host antibody response to infection.

of antibody escape, particularly *in vitro*, can provide insight into the location of key epitopes. Passaging virus in the presence of nAb and characterizing the emergence of escape mutations has been carried out for HIV (Mo *et al.*, 1997), foot-and-mouth disease virus (Crowther *et al.*, 1993), hepatitis A virus (Ping & Lemon, 1992), influenza A virus (Kaverin *et al.*, 2002) and, more recently, HCV (Gal-Tanamy *et al.*, 2008; Keck *et al.*, 2008b, 2011).

Potential nAb epitopes: HVR1

Early chimpanzee studies demonstrated that nAb have the potential to protect against HCV infection or to reduce the severity of the disease. They identified the HVR1 region of E2 as a major target for nAb. HVR1 possesses multiple linear epitopes between aa 384 and 410. The HVR1 region plays an important role in antibody recognition and disease outcome, and is necessary for binding to scavenger receptor class B type I (SR-BI), a lipoprotein receptor molecule involved in HCV entry (Fig. 2) (Bartosch *et al.*, 2003c; Scarselli *et al.*, 2002). Antibodies targeting HVR1 have been identified *in vivo* (Kato *et al.*, 1993, 1994; Weiner *et al.*, 1992); however, they tend to be highly strain specific (Bartosch *et al.*, 2003a; Shimizu *et al.*, 1996; Vieyres *et al.*, 2011). It has also been suggested that high concentrations of HVR1-specific nAbs are required for effective neutralization (Bartosch *et al.*, 2003a). A number of HVR1-specific mAbs have been generated and characterized (Table 1, Figs 1 and 3, and Supplementary Fig. S1). The rat mAb 9/27 inhibits SR-BI binding to E2, neutralizing infectivity of retroviral pseudotypes bearing HCV E1E2 glycoproteins derived from a genotype 1a virus (Bartosch *et al.*, 2003a; Hsu *et al.*, 2003). It also possesses the ability to neutralize cell-to-cell transfer of HCV (Brimacombe *et al.*, 2011). The epitope recognized by this mAb has been localized to the C-terminal portion of HVR1 (aa 396–407) (Hsu *et al.*, 2003). Other HVR1-specific antibodies (the mouse mAb AP213 and the polyclonal sera R1020 and R140) that show neutralizing activity against HCVpp and HCVcc also map to epitopes within the C-terminal portion of HVR1 (Vieyres *et al.*, 2011). In contrast, the non-neutralizing mAbs 7/59, 6/16 and 6/82a bind to the N-terminal portion of HVR1 (aa 384–395) (Hsu *et al.*, 2003). Thus, it appears that there are two immunogenic regions within HVR1, with the C-terminal portion containing the neutralization determinants.

In addition to mediating the direct binding of E2 and SR-BI, HVR1 is also necessary for interaction between SR-BI and HDL, which has been shown to augment entry (Bartosch *et al.*, 2005; Voisset *et al.*, 2005). This same interaction inhibits neutralization of HCVpp (Bartosch *et al.*, 2005) and HCVcc (Dreux *et al.*, 2006) by anti-HCV antibodies. However, dependency and function of HVR1 in infectivity might vary between different genotypes (Prentoe *et al.*, 2011). Recent studies have also shown that HVR1 is able to mask nAb epitopes within E2, as HVR1 deletion mutants are much more susceptible to neutralization by a panel of human mAbs and patient sera targeting the CD81-binding site within the E2 protein (Bankwitz *et al.*, 2010; Prentoe *et al.*, 2011). This is probably due to masking of the CD81-binding site. Therefore, HVR1 may function to protect viral entry determinants within E2 from neutralization during the early stages of entry (Bankwitz *et al.*, 2010). These myriad roles played by HVR1, and their somewhat contradictory nature, has led some to suggest that HVR1 functions as an immunological decoy, stimulating a strong antibody response towards HVR1 that does not result in viral clearance, but instead drives the selection of antibody-escape mutants (Ray *et al.*, 1999).

Potential nAb epitopes: interactions with CD81

The lack of broadly nAb targeting HVR1 led to the search for other, well-conserved antibody targets. CD81 was the first host factor identified as critical for virus entry and quickly became the focus of the search for nAb. Its role in virus entry is reviewed by Burlone & Budkowska (2009) and is outlined in Fig. 2. CD81 is a member of the tetraspanin family of transmembrane proteins found on most cell types. It was first identified as a receptor due to its ability to bind a recombinant soluble ectodomain of the E2 protein (sE2), a reaction that occurs via the CD81 large extracellular loop (LEL) (Pileri *et al.*, 1998). Both anti-CD81 mAb and soluble CD81 LEL are able to inhibit the entry of HCVpp and HCVcc into hepatoma cells (Bartosch *et al.*, 2003b; Wakita *et al.*, 2005).

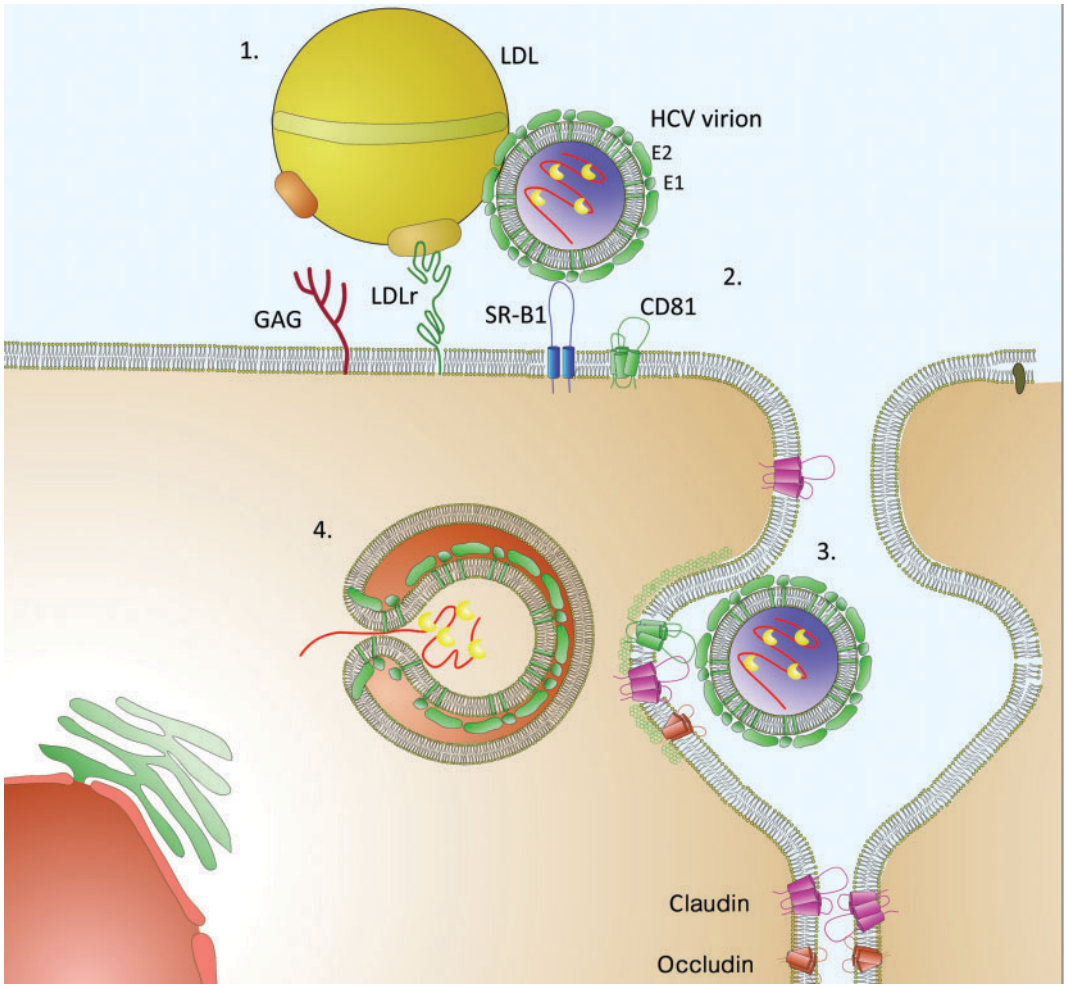

Fig. 2. Stages in the HCV entry pathway targeted by antibody-mediated neutralization. HCV entry is a multi-step process requiring both localization and entry receptors. Binding and internalization into susceptible cells results in release of viral RNA that translocates to replication sites on the ER. 1. HCV particles circulate as LVPs in the vascular system. These LVPs consist of lipoproteins (yellow) in complex with HCV particles possessing E1 and E2 glycoproteins (green) on the surface. Current models suggest that the virus and lipoprotein form a single particle, but this has not been confirmed. Following localization to the surface of hepatocytes, initial association of LVP with the cell surface occurs by interaction with glycosaminoglycans (GAGs) and the low density lipoprotein receptor (LDLr). 2. Specific interactions with SR-B1 and CD81 are mediated by the E1 and E2 glycoproteins. 3. Following engagement with SR-B1 and CD81, viral particles are translocated to regions of the membrane possessing tight junction proteins occludin and claudins-1, -6 or -9. Binding to these receptors forms a co-receptor complex that results in clathrin-mediated endocytosis of the viral particle. 4. The endocytic vesicle containing the virus particle translocates through the cytoplasm, where maturation into a low pH late endosome results in conformational changes in the envelope glycoproteins that mediate fusion of the viral envelope and endosome membrane. This allows the viral nucleocapsid to enter the cytoplasm.

Early studies showed that anti-HCV serum from chimpanzees and humans was able to block E2 binding to target cells due to the presence of NOB antibodies (Ishii *et al.*, 1998; Rosa *et al.*, 1996). The identification of CD81 as a cellular receptor by Pileri *et al.* (1998) confirmed that NOB antibodies are able to inhibit HCV binding to CD81.

Antibody competition studies provided the first insight into regions of E2 involved in CD81 binding. These suggested that CD81-binding sites exist within E2 regions aa 412–423 (Owsianka *et al.*, 2001), aa 432–447 (Clayton *et al.*, 2002), aa 480–493 (Flint *et al.*, 1999), aa 528–535 (Owsianka *et al.*, 2001) and aa 544–551 (Flint *et al.*, 1999). Subsequent mutagenesis studies confirmed the importance of most of these regions by showing that the specific residues critical for E2 binding to CD81 include W^{420}, Y^{527}, W^{529}, G^{530} and D^{535} (Owsianka *et al.*, 2006) and the ^{436}GWLAGLFY^{443} motif (Drummer *et al.*, 2006). However,

there was no evidence for the involvement of conserved residues within region aa 480–493 (Owsianka *et al.*, 2006). These somewhat conflicting data demonstrate the importance of the choice of E2 used in mapping studies. The initial suggestion that aa 480–493 function as a CD81-binding site was based on studies with a soluble, truncated form of E2 (Flint *et al.*, 1999), whereas subsequent studies used E2 expressed in the context of VLPs (Owsianka *et al.*, 2001) or E1E2 (Owsianka *et al.*, 2006). Therefore, the antigenic exposure of epitopes in different forms of E2 can greatly affect our understanding of E2–receptor and E2–antibody interactions.

There is a high degree of conservation of the residues W^{420}, Y^{527}, W^{529}, G^{530} and D^{535} and the ^{436}GWLAGLFY^{443} across different genotypes. Analysis of human and murine mAbs recognizing both linear and conformational epitopes, demonstrated that the most broadly nAb are targeted to the CD81-binding site (Johansson *et al.*, 2007; Law *et al.*, 2008; Owsianka *et al.*, 2005, 2008; Perotti *et al.*, 2008) (Table 1 and Fig. 3). The region immediately downstream of HVR1 is recognized by the broadly neutralizing mouse mAb AP33 (Owsianka *et al.*, 2005). Key residues within E2 that are essential for AP33 binding, identified by analysis of alanine replacement glycoproteins and enrichment of random peptide display libraries, are L^{413}, N^{415}, G^{418} and W^{420} (Tarr *et al.*, 2006). Whilst its epitope is predominantly linear in nature, maximal binding is dependent on the overall local conformation of the E2 protein. The neutralizing rat mAb 3/11 recognizes an epitope overlapping that of the mAb AP33 (Tarr *et al.*, 2006) (Fig. 1 and Supplementary Fig. S1). However, <5 % of individuals with resolved or chronic infection harbour antibodies targeting this region of the E2 protein (Tarr *et al.*, 2007), suggesting that this region is less immunogenic in humans than in rodents, or that the presentation of this region is different in experimental immunogens compared with the native viral particle.

The majority of human mAbs targeting the CD81-binding site recognize conformational epitopes. These mAbs exhibit either a cross-reactive broadly neutralizing or an intermediate neutralizing phenotype (Table 1 and Fig. 3). Our mapping studies showed that all the broadly neutralizing conformation-sensitive antibodies targeted the highly conserved E2 residues W^{529}, G^{530} and D^{535} (Johansson *et al.*, 2007; Keck *et al.*, 2008b; Law *et al.*, 2008; Owsianka *et al.*, 2006; Tarr *et al.*, 2006). In addition to these residues, the human Fab fragment e137 also required T^{416} and W^{420} (Perotti *et al.*, 2008). Thus, based on their epitopes, the broadly neutralizing CD81-binding site antibodies fall into three groups: those that recognize predominantly linear epitopes located between E2 residues 412 and 423 (e.g. AP33 and 3/11); those that recognize conformational epitopes where key contact residues are located between residues 529 and 535 (e.g. 1:7, A8, AR3A and CBH2); and those whose epitopes span these two important CD81-binding regions (e.g. e137).

A number of antibodies recognizing the CD81-binding site that exhibit intermediate or restricted neutralization breadth have also been described. These include antibodies targeting the region ^{436}GWLAGLFY^{443} (7/16b and 11/20), although there is conflicting data about the ability of 7/16b to compete for CD81 binding (Flint *et al.*, 1999) and the region ^{524}APTYSWGA^{531} of E2 (2/64a). However, mAb 9/75, whose minimal epitope is also located between residues 528 and 535, was unable to neutralize HCVpp at a concentration of 50 μg ml^{-1} (Hsu *et al.*, 2003). Thus, subtle differences in the specificity of antibodies targeting CD81-binding sites might influence their neutralizing potency. In addition, accessibility of the antibody-binding site will also influence neutralizing potency. Early studies comparing the accessibility of epitopes on various forms of E2 protein, including E1E2 heterodimers, soluble truncated E2 ectodomain and E2 in the context of insect expressed VLPs, demonstrated differential exposure of epitopes in the various presentations of E2 (Clayton *et al.*, 2002). Finally, it is worth highlighting the apparent differences between the human and the murine antibody response. The most potent and broadly neutralizing murine antibodies target linear epitopes within residues 412–423, whereas similar human antibodies are rare. By contrast, the most broadly neutralizing human antibodies are conformation sensitive and target epitopes centred on the key CD81-binding residues W^{529}, G^{530} and D^{535}, whereas murine antibodies targeting this region (e.g. mAb H35 and H48) are highly restricted (Owsianka *et al.*, 2006).

Are there other targets for nAb?

The majority of antibodies identified to date target receptor-binding epitopes within E2. This can, in part, be attributed to the use of assays, such as mammalian-expressed sE2 (Pileri *et al.*, 1998) and NOB assays (Rosa *et al.*, 1996), which are biased towards E2. It is important to note that the NOB assays that first identified nAb targeting CD81 were carried out on Molt-4 cells, a lymphoma cell line that expresses only CD81 but none of the other HCV entry receptors. Therefore, the presence of antibodies inhibiting interactions between the virus and other receptors may have been overlooked. Indeed, nAb which do not target the E2–CD81 interaction have been found (Hsu *et al.*, 2003) (Table 1). Subsequent studies comparing Molt-4 cells and Huh7 human hepatoma cells showed that NOB antibodies inhibit the binding of E2 to Molt-4 cells more readily than to Huh7 cells (Heo *et al.*, 2004). Additionally, in early studies the NOB assay served as an initial screen for 'neutralizing' potential of mAbs, promoting the selection of anti-E2 antibodies targeting this site for further study. This has introduced an inherent bias towards reports of nAb that disrupt the interaction between E2 and CD81. Multiple cell surface receptors are involved in HCV entry to target cells; however, they do not all interact directly with E2 (Fig. 2). In addition to CD81, SR-BI and the tight junction protein occludin are both thought to interact with E2 directly. Whilst HVR1 is thought to be important for SR-BI interaction, and mAbs targeting linear

Table 1. mAbs targeting the envelope glycoproteins of HCV

All amino acid numbering is based on the H77 reference strain.

mAb	Epitope (aa)	Epitope class	Interaction targeted	Source of antibody	Reference(s)
H-111	192–211	Linear	Unknown	Human	Keck *et al.* (2004b)
IGH505	312–327 (E1)	Linear	Unknown	Human	Meunier *et al.* (2008)
IGH526	312–327 (E1)	Linear	Unknown	Human	Meunier *et al.* (2008)
9/27	396–407 (HVR1)	Linear	SR-BI	Rat	Hsu *et al.* (2003)
7/59	384–391 (HVR1)	Linear	SR-BI	Rat	Hsu *et al.* (2003)
H77.39	415 and 417	Linear	CD81/SR-B1	Mouse	Sabo *et al.* (2011)
3/11	412–423	Linear	CD81	Rat	Flint *et al.* (1999)
HCV1	412–423	Linear	CD81	Human*	Broering *et al.* (2009)
95-2	412–423	Linear	CD81	Human*	Broering *et al.* (2009)
AP33	412–423	Linear	CD81	Mouse	Owsianka *et al.* (2005); Tarr *et al.* (2006)
2/69a	436–443	Linear	Unknown	Rat	Flint *et al.* (1999); Hsu *et al.* (2003)
1/39	432–443	Linear	CD81?	Rat	Flint *et al.* (1999)
AP320	464–471	Linear	Unknown	Mouse	Clayton *et al.* (2002)
6/41a	480–492	Linear	Unknown	Rat	Flint *et al.* (1999)
11/20	436–447	Linear	CD81	Rat	Hsu *et al.* (2003)
2/64a	524–531	Linear	CD81	Rat	Hsu *et al.* (2003)
H53	540–550	Conformation dependent	Unknown	Mouse	Cocquerel *et al.* (1998); Owsianka *et al.* (2006)
H35	523 and 530	Conformation dependent	CD81	Mouse	Cocquerel *et al.* (1998); Owsianka *et al.* (2006)
H48	530	Conformation dependent	Cd81	Mouse	Cocquerel *et al.* (1998); Owsianka *et al.* (2006)
Fab e137	416, 420, 529, 530 and 535	Conformation dependent	CD81	Human	Perotti *et al.* (2008)
Fab e20	529, 530 and 535	Conformation dependent	CD81	Human	Mancini *et al.* (2009)
AR3A	424, 436–447, 523, 530,535, 538 and 540	Conformation dependent	CD81	Human	Law *et al.* (2008)
AR3B	424, 436–447, 530, 535 and 540	Conformation dependent	CD81	Human	Law *et al.* (2008)
AR3C	424, 530, 535, 538 and 540	Conformation dependent	CD81	Human	Law *et al.* (2008)
AR3D	424, 436–447, 530 and 535	Conformation dependent	CD81	Human	Law *et al.* (2008)
1 : 7	523, 529, 530 and 535	Conformation dependent	CD81	Human	Allander *et al.* (2000); Johansson *et al.* (2007)
A8	523, 529, 530 and 535	Conformation dependent	CD81	Human	Allander *et al.* (2000); Johansson *et al.* (2007)
L1	Unknown	Conformation dependent	Unknown	Human	Allander *et al.* (2000); Johansson *et al.* (2007)
CBH4B	Unknown	Conformation dependent	Unknown	Human	Hadlock *et al.* (2000); Keck *et al.* (2004a)
CBH4D	Unknown	Conformation dependent	Unknown	Human	Hadlock *et al.* (2000); Keck *et al.* (2004a)
CBH4G	Unknown	Conformation dependent	Unknown	Human	Hadlock *et al.* (2000); Keck *et al.* (2004a)

Table 1. cont.

mAb	Epitope (aa)	Epitope class	Interaction targeted	Source of antibody	Reference(s)
CBH5	523, 525, 530, 535 and 540	Conformation dependent	CD81	Human	Hadlock *et al.* (2000); Owsianka *et al.* (2008)
CBH7	540 and 549	Conformation dependent	CD81	Human	Hadlock *et al.* (2000); Owsianka *et al.* (2008)
ALP98	644–651	Linear	Unknown	Mouse	Clayton *et al.* (2002); Owsianka *et al.* (2001)
ALP1	647–658	Linear	Unknown	Mouse	Clayton *et al.* (2002); Owsianka *et al.* (2001)

*Denotes antibodies generated in transgenic mice containing human antibody genes.

epitopes within HVR1 block E2 interaction, efficient binding is E2 conformation-dependent (Scarselli *et al.*, 2002). Recently, a number of mAbs have been described which appear to interfere with both CD81 and SR-BI binding. Whilst most of these have limited breadth, mAb H77.39, whose epitope overlaps that recognized by mAb AP33, was shown to inhibit E2 binding to both CD81 and SR-BI (Sabo *et al.*, 2011).

Occludin co-precipitates with E2 (Benedicto *et al.*, 2008; Liu *et al.*, 2009); however, no virus-specific antibodies that can neutralize this interaction have been identified. To date, direct interaction between E2 and claudin-1 has not been demonstrated (Evans *et al.*, 2007; Krieger *et al.*, 2010). Experimentally derived anti-claudin-1 antibodies have been shown to neutralize HCV infectivity by inhibiting the interaction between CD81 and claudin-1, which is necessary for the formation of the E2-CD81-claudin-1 co-receptor complex (Fofana *et al.*, 2010; Krieger *et al.*, 2010). Although these antibodies do not target the virus particle itself, targeting host factors may represent an alternative approach to preventing HCV entry. Indeed a number of anti-receptor antibodies targeting CD81 (Meuleman *et al.*, 2008; Molina *et al.*, 2008) and SR-BI (Catanese *et al.*, 2010) have been shown to block viral entry.

HCV entry into target cells occurs via clathrin-mediated endocytosis of the viral particle (Blanchard *et al.*, 2006). Subsequent release of the viral genome into the cytosol requires the pH-dependent fusion of viral and cellular membranes. There is evidence to suggest that patient-derived antibodies may be able to inhibit this fusion process and thus block virus infectivity (Haberstroh *et al.*, 2008; Kobayashi *et al.*, 2006). The fusion determinant or determinants within the envelope glycoproteins are still incompletely defined; therefore, it is not possible to identify the epitopes to which these antibodies may bind. A precedent for virus neutralization by targeting the fusion determinant has been set by studies into other viruses. For example, the highly conserved HIV-1 fusion determinant lies within the gp41 envelope glycoprotein and is targeted by broadly nAb (reviewed by Karlsson Hedestam *et al.*, 2008). Antibodies have also been identified that target the fusion determinant within the influenza virus haemagglutinin (HA) and these show broad neutralizing activity against all HA subtypes within a specific group (Sui *et al.*, 2009). Therefore, this presents another promising target for virus neutralization that requires further investigation.

Antibodies targeting epitopes within the envelope glycoprotein E1 have been identified in some patient sera, but these are generally rare (Pestka *et al.*, 2007). The reasons for this are unclear but might be due to technical difficulties in detecting E1 responses, as the protein misfolds unless it is co-translated with E2 (Dubuisson *et al.*, 1994). Alternatively, the presence of E2 might mask E1 epitopes or be immunologically dominant (Garrone *et al.*, 2011). Despite these challenges, there are reports of anti-E1 responses in the literature. For example, mAb H-111 binds the ^{192}YEVRNVSGVYH^{211} region of E1 and is cross-reactive to genotypes 1a, 1b, 2b and 3a. In studies, this mAb partially reduced VLP entry into Molt-4 cells (Keck *et al.*, 2004b). The mAbs IGH505 and IGH526 recognize a linear epitope encompassing aa 313–327 and show broad neutralization of HCVpp bearing E1E2 derived from different genotypes (Meunier *et al.*, 2008). Previous trials of E1 protein vaccine candidates induced E1-specific antibody responses (Garrone *et al.*, 2011; Leroux-Roels *et al.*, 2004; Nevens *et al.*, 2003), but these did not reduce viral load in chronically infected patients (Nevens *et al.*, 2003). A recent study in chimpanzees showed that anti-E1 antibodies induced by immunization with a recombinant form of E1 conferred protection against experimental infection with heterologous HCV (Verstrepen *et al.*, 2011). The authors reported that the protective effect of the E1-based vaccine was better than that induced by an E2-based vaccine (Verstrepen *et al.*, 2011). More recently, VLPs expressing E1E2 and E1 constructs were used in prime–boost experiments in an attempt to elicit nAb. Despite being able to elicit E1-specific responses, these were not neutralizing, and

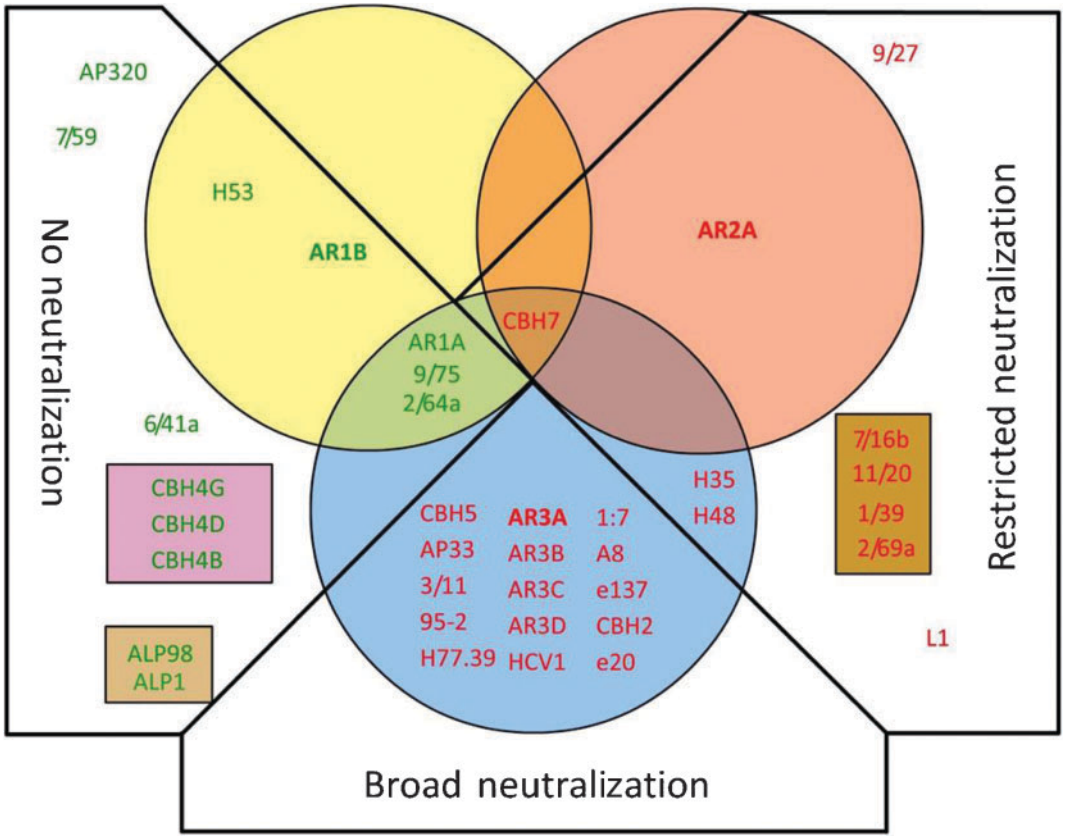

Fig. 3. Organization of the antigenic regions (AR) of the E2 glycoprotein. E2 has previously been classified into three antigenic 'domains' (Keck *et al.*, 2004a) or three AR (Law *et al.*, 2008). This Venn diagram summarizes all previous data on the specificity of mAbs directed to E2, as determined by competition assays and point mutagenesis studies. Antibody names are placed relative to their location on this map, with non-nAb highlighted in green text and nAb in red text. This organization highlights at least six partially overlapping AR (denoted by coloured circles and rectangles), as well as other discrete epitopes recognized by mAbs. Anti-E2 mAbs can be clearly classified as non-neutralizing, broadly neutralizing, or narrowly neutralizing, which generally correlate with the three AR. AR1, 2 and 3 (Law *et al.*, 2008) are highlighted as yellow, red and blue circles, respectively. These AR partially overlap, with the epitope recognized by mAb CBH7 overlapping all three epitope clusters. Many of the reported antibodies map to AR3. Interestingly, all broadly nAb exclusively recognize the discontinuous AR3. AR1 contains epitopes that are exclusively recognized by non-nAb, with part of this region possessing epitopes that overlap with AR3. AR2 is defined by a single antibody, AR2A, which neutralizes a restricted number of HCV isolates. In addition to these three AR, a discrete region is defined by the conformation-sensitive antibodies CBH4B, CBH4D and CBH4G, as well as two conformation-independent regions defined by ALP98 and ALP1, and 7/16b, 11/20, 1/39 and 2/69a. The previously described antigenic 'domains' (Keck *et al.*, 2004a) broadly agree with these AR. Domain 'A' appears to be a separate AR that does not overlap with other epitope clusters. Domain 'B' significantly overlaps AR3, and domain C is a shared epitope that overlaps AR1, AR2 and AR3. Mapping the relative specificities of these mAbs reveals complex immunogenic surfaces of E2 that broadly equate to a non-neutralizing face, a broadly neutralizing face and a restricted neutralizing face to the E2 protein. It remains to be demonstrated if these antibody reactivity data agree with structural analyses of the E2 protein.

neutralization was only observed when animals were boosted with E1E2 (Garrone *et al.*, 2011). Due to the limited understanding of the role and structure of E1 and how it interacts with E2, the mechanism by which these antibodies act remains unknown. It is also important to remember that the HCV fusion determinant has yet to be identified but has been proposed to reside within E1 (Lavillette *et al.*, 2007). If this were the case, E1 may become an important target for antibodies inhibiting the fusion step.

Do nAb influence infection outcome?

Due to the asymptomatic nature of acute HCV infection it is difficult to identify, and therefore study, patients in the

early phase of disease. Despite this, several studies of acute HCV infection have been performed and these have shown that development of a broad and potent T-cell response is important for clearance (reviewed by Bowen & Walker, 2005). More recent studies have also indicated that nAb also play a critical role in disease outcome. Rapid induction of nAb early during infection is associated with spontaneous recovery and these antibodies appear to be more cross-neutralizing (Dowd *et al.*, 2009; Pestka *et al.*, 2007). By contrast, clearance in chimpanzees is associated with cellular immunity (Barth *et al.*, 2011; Grakoui *et al.*, 2003; Shoukry *et al.*, 2003) rather than nAb (Logvinoff *et al.*, 2004; Meunier *et al.*, 2005). There are some important differences in the outcome of HCV infection in chimpanzees compared with humans. Chimpanzees tend to suffer a milder disease, show a higher rate of viral clearance and do not appear to develop liver fibrosis or cirrhosis (reviewed by Jo *et al.*, 2011). Therefore, chimpanzees may not be the most reliable indicator of the role played by the humoral immune response in clearance of the virus during acute infection. In contrast to spontaneous resolvers, patients who developed a chronic infection generated no antibodies or a very low titre antibody response in the early phase of the infection (Dowd *et al.*, 2009; Pestka *et al.*, 2007). Assessment of nAb levels several years later demonstrated high antibody titres in chronically infected patients, although these were unable to clear the virus (Pestka *et al.*, 2007).

Intriguingly, limited studies of viral evolution during acute infection have shown that resolved infection is associated with stable HVR1 sequences, whereas persistence is accompanied by noticeable HVR1 sequence change (Farci *et al.*, 2000; Ray *et al.*, 1999). This observation led to the hypothesis that HVR1 is acting as an immunological decoy (Ray *et al.*, 1999) – targeting the antibody response to this region of E2 at the expense of other neutralization epitopes that are functionally or structurally less amenable to change. This hypothesis gained support from a detailed analysis of the nAb response in patient H, over a period of 26 years. This study found that the early antibody response was targeted towards HVR1, which led to HVR1 sequence change. A more broadly nAb response was only observed much later during chronic infection (von Hahn *et al.*, 2007). These findings can be interpreted in one of two ways. In those virus populations able to undergo rapid HVR1 evolution, mutations arise and lead to antibody escape, whilst in those populations where HVR1 evolution is limited, escape does not occur and the virus is cleared (Ray *et al.*, 1999). It is, however, equally plausible that the antibody response in individuals who experienced viral clearance targeted more conserved epitopes outside of HVR1 and, therefore, HVR1 sequence evolution was not observed. The finding that the early antibody response in acute resolvers is more broadly neutralizing supports our alternative hypothesis.

Even in chronically infected individuals, there is evidence that antibodies partially control the virus. Firstly, hypogammaglobulinaemic individuals exhibit a marked rapidity and severity in disease progression (Bjøro *et al.*, 1994). Secondly, B-cell depletion during rituximab therapy has been reported to cause an increase in viral load, which returns to normal after cessation of therapy (Ennishi *et al.*, 2008), presumably as the B-cell population recovered. It is likely that during chronic infection there is an intricate interplay between host and virus such that mutations that lead to immune escape might also reduce viral fitness. This is particularly compelling given that most nAb responses are targeted to functionally important regions.

Viral persistence in the presence of nAb

Early studies using the HCVpp system suggested that broadly nAb were a common feature of long-term chronic HCV infection. Therefore, how does the virus persist in the face of this response? Very often antibodies or sera have only been tested against a limited number of genotypes or subtypes (Bartosch *et al.*, 2003a; Broering *et al.*, 2009) and therefore may not truly be broadly neutralizing. Also, data from our laboratory showed that E1E2 clones differ greatly in their sensitivity to neutralization, and the E1E2 derived from H77c, which has been most frequently used in the HCVpp studies, is easily neutralized by the majority of sera. By contrast, most E1E2 clones derived *ex vivo* are far more resistant to neutralization and truly broadly neutralizing sera are rare (Tarr *et al.*, 2011).

Even so, HCV can still persist in those patients with more broadly neutralizing responses and the virus must utilize mechanisms to evade these. The most widely reported evasion mechanism is mutational escape. HCV contains a positive-sense ssRNA genome that is replicated by a virus-encoded RNA-dependent RNA polymerase. This polymerase lacks proof-reading capabilities which, when coupled with the high replication rate of the virus, results in the generation of a highly diverse population of viral variants (reviewed by Simmonds, 2004). This population is often referred to as a quasispecies, although it is questionable if this assignation is strictly accurate (Holmes, 2010). The diverse virus population will harbour neutralization escape variants that have a selective advantage over neutralization-sensitive variants. Positively selected amino acid sites are located within and around known receptor- and nAb-binding regions (Brown *et al.*, 2005, 2007). Recent work has shown that E1E2 evolution is driven by the nAb response and escape variants soon become the dominant circulating strain (Dowd *et al.*, 2009; Farci *et al.*, 2000; von Hahn *et al.*, 2007). Both von Hahn *et al.* (2007) and Dowd *et al.* (2009) show that sequential serum samples are limited in their ability to neutralize the concurrently circulating viral strains, but are able efficiently to neutralize virus strains from earlier time points. Therefore even within one individual, a single viral envelope sequence may not be sufficient to 'pull-out' all nAb.

The most broadly nAb described to date target the CD81-binding site and HCV has evolved various methods of shielding this region of the E2 protein. E2 contains up to 11 potential *N*-linked glycosylation sites, nine of which are conserved across genotypes (>97 %) (Helle *et al.*, 2007).

Glycans are important for the structure and function of glycoproteins (Goffard *et al.*, 2005) and are critical for HCVpp entry into target cells (Falkowska *et al.*, 2007). Specific glycans are known to mask the CD81-binding site and, therefore, nAb epitopes. Removal of these glycans results in increased binding to CD81 and increased sensitivity to neutralization by patient sera and mAb (Falkowska *et al.*, 2007; Helle *et al.*, 2007, 2010). Changes occur in the frequency and position of glycans on both HIV-1 gp120 and influenza HA glycoproteins, and these 'evolving glycan shields' have been shown to decrease sensitivity to antibody neutralization (Abe *et al.*, 2004; Wei *et al.*, 2003). Whilst there is some variability in the location and number of glycosylation sites across different functional E1E2 clones (Helle *et al.*, 2007), there is little evidence that the glycans undergo significant intra-host evolution (Brown *et al.*, 2007; Helle *et al.*, 2007) and some highly conserved glycans are critical for entry (Falkowska *et al.*, 2007).

Lipid shielding may represent an additional strategy used by HCV to evade the antibody response. In patient sera, HCV exists as a heterogeneous population with a range of buoyant densities. Low density particles, termed lipoviral-particles (LVPs) are the most infectious, and current data suggest that key neutralizing epitopes are less accessible on LVPs. These are associated with very low density lipoproteins (VLDL), such as the apolipoproteins apoB and apoE (reviewed by Burlone & Budkowska, 2009). In addition, several *in vitro* studies have demonstrated that high density lipoprotein (HDL) components of human serum, such as apoCI, can enhance the infectivity of HCVpp and HCVcc via an HVR1-dependent mechanism (Bartosch *et al.*, 2005; Dreux *et al.*, 2007; Meunier *et al.*, 2005). HDL also reduces the sensitivity of HCVpp to antibody neutralization, possibly by accelerating the entry of HCV via SR-BI-mediated lipid uptake (Dreux *et al.*, 2006). ApoCI is able to further enhance infectivity by promoting fusion between viral and cellular membranes (Dreux *et al.*, 2007). Lipids clearly play a crucial role in the infectivity and entry of viral particles as well as the neutralization sensitivity, and further studies are required to fully elucidate this interplay.

More recently, HCV has been found to be capable of direct cell-to-cell transmission, which is largely resistant to antibody neutralization (Timpe *et al.*, 2008; Witteveldt *et al.*, 2009), although the rat mAbs 9/27 (targeting aa 396–407 within HVR1) and 11/20c (targeting the CD81-binding residues aa 412–423 and 436–447) are able to partially inhibit cell-to-cell transmission (Brimacombe *et al.*, 2011). Cell-to-cell transmission requires all four entry receptors: CD81, SR-BI, claudin-1 and occludin, although SR-BI appears particularly important (Brimacombe *et al.*, 2011). Many other enveloped viruses, including herpes simplex virus 1, human T-cell lymphotropic virus and measles virus, utilize direct cell-to-cell transmission in a bid to evade the host immune response (reviewed by Mothes *et al.*, 2010). HIV-1 also employs a number of mechanisms to spread directly from cell to cell. These include promoting the fusion of infected and uninfected cells; hijacking actin-containing structures which physically join infected and uninfected cells; and exploiting the immunological synapse formed between dendritic cells and T-cells (reviewed by Sattentau, 2008). The exact mechanism of HCV cell-to-cell transmission is still unknown, but regardless of the mechanism, direct cell-to-cell transmission represents an ideal method of immune evasion and may explain why nAb do not always clear the virus.

It has also been postulated that non-nAb present within patient sera bind distinct epitopes within E2 and block or inhibit the binding of nAb to neutralization epitopes (Zhang *et al.*, 2007, 2009). This has been proposed as a reason for the failure of polyclonal immunoglobulin preparations to successfully treat HCV (Davis *et al.*, 2005). This does, however, contradict observations made by Féray *et al.* (1998) in which polyclonal immunoglobulins were found to protect against HCV infection. Zhang and colleagues reported that antibodies targeting the region of E2 encompassing aa 434–446 strongly interfere and abrogate the neutralization exhibited by antibodies targeting epitopes located between residues 412 and 423. However, we have shown that murine mAbs and affinity enriched human immunoglobulin fractions targeting epitopes overlapping the aa 434–446 region of E2 exhibit restrictive neutralization of both HCVpp and HCVcc (A. W. Tarr, unpublished data). Previously, some of the mAbs targeting this region of E2 had been shown to neutralize E2–CD81 binding (Clayton *et al.*, 2002; Owsianka *et al.*, 2001) as well as HCVpp supplemented with autologous H77c E1E2 (Hsu *et al.*, 2003).

Insights into the structure of HCV envelope glycoproteins

Studies of murine and human mAbs also provide insight into the structure of the E1E2 glycoproteins. HCV belongs to the family *Flaviviridae* and its genomic organization is characteristic of other members of this group (Lemon *et al.*, 2001). Similarities between the primary amino acid sequence of HCV E2 and the envelope glycoproteins of flavivirus (E) and alphavirus (E1) suggest that E2 is a class II fusion protein (Krey *et al.*, 2010). Several conserved cysteine residues within E2 form disulphide bonds to stabilize the structure (Dubuisson & Rice, 1996). The identification of conformation-dependent antibodies, such as H53 and CBH5 (Table 1) demonstrated that the structure of E2 is highly ordered (Hadlock *et al.*, 2000; Owsianka *et al.*, 2006). Delineation of the disulphide bridged cysteine partners, together with knowledge of the CD81-binding sites, antibody epitopes and location of potential *N*-linked glycosylation sites facilitated the generation of a 3D model of E2 based on the structure of other class II fusion proteins. In this model, E2 is composed of three domains (DI, DII and DIII). HVR1 precedes DI, HVR2 is found within DII, and IgVR links DI and DIII (Krey *et al.*, 2010). In this model, key CD81-binding regions are brought together into DI, with the glycosylation sites of E2 clustering around the CD81-binding surface. A putative highly conserved fusion peptide is located within DII, which may also form a

region of contact with E1 (Krey *et al.*, 2010). The study by Krey *et al.* (2010) used a soluble E2 construct truncated at amino acid residue 715 and therefore does not precisely represent the native E2 glycoprotein found on the surface of viral particles. However, it does provide us with the best indication yet of the structure of E2. A model such as this could help to focus future studies at those regions of E2 that are surface exposed and therefore likely to be a target for antibodies. It could also help to identify highly conserved features or domains of E2 that might elicit broadly nAb and thus aid in the rational design of therapeutic immunogens. Indeed, if the fusion determinants within the envelope glycoproteins are identified, it would greatly facilitate the search for antibodies capable of neutralizing this important step in viral entry.

Potential application of nAb in vaccines or therapeutics

Although our knowledge of HCV has increased dramatically in the two decades since it was first identified, development of an effective vaccine to prevent or treat infection has yet to be achieved. Studies of the immune response suggest that any vaccine would need to target both cellular and humoral arms of the immune system in order to be effective. A strong $CD8^+$ and $CD4^+$ T-cell response targeting multiple epitopes is associated with spontaneous viral clearance (reviewed by Thimme *et al.*, 2008) and both cellular and humoral arms of the immune response drive evolution of HCV sequences leading to the generation of escape mutations (von Hahn *et al.*, 2007). Any vaccine or therapy targeting a single viral epitope would lead to the development of resistant strains. Therefore, the rational design of vaccine or therapeutic immunogens requires an approach similar to that adopted for the treatment of HIV; that is, the simultaneous targeting of multiple viral (and possibly host) targets to prevent the emergence of resistance mutations.

The protective effect of a high titre, broadly nAb response in the acute phase of the disease (Dowd *et al.*, 2009; Pestka *et al.*, 2007) and evidence that spontaneous resolution of primary infection affords some protection against persistent reinfection in IVDUs (Osburn *et al.*, 2010) give strong support to the idea that immunity to HCV can be elicited by immunization. In addition, it is believed that passive immunotherapy may be able to reduce viral titres to undetectable levels. A number of products assessed in clinical trials have attempted to replicate this protective immunity but have met with mixed results. Trials of polyclonal anti-HCV immunoglobulins (Civacir) (Davis *et al.*, 2005) and an anti-E2 mAb (HCV-Ab^{XTL}68) (Schiano *et al.*, 2006) in liver transplant patients resulted in high levels of serum antibodies, which correlated with a reduction in HCV RNA levels. However, sustained reduction of HCV RNA has not been reported and no long-term protection against liver pathology has been demonstrated (Davis *et al.*, 2005; Schiano *et al.*, 2006). Perhaps the most promising vaccine candidate to date is a recombinant form of the envelope glycoproteins E1E2 (Chiron Corp.). It induced a strong cellular and humoral response in chimpanzees, resulting in sterilizing immunity against homologous viral challenge. This vaccine also prevented development of chronic infection following heterologous viral challenge (Houghton & Abrignani, 2005). Preliminary safety and immunogenicity studies of an adjuvanted form of the vaccine (HCV E1E2/MF59.C1) suggest that it is capable of inducing both cellular and humoral immunity against the envelope glycoproteins in healthy humans with few adverse events (Frey *et al.*, 2010). Phase I clinical trials of this vaccine candidate have been completed and are awaiting publication of the results.

This review has focused on the role played by nAb in preventing or limiting HCV infection of target cells and much effort has been invested in this area of research. However, non-nAb may also play a role in disease outcome through the action of Fc-mediated effector functions including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). ADCC and CDC are both involved in the immune response to HIV (Chung *et al.*, 2008), but Fc-mediated effector functions in the context of HCV infection are less well understood. Sera from both the acute and chronic phase of infection can mediate ADCC via binding to E2 expressed at the cell surface (Nattermann *et al.*, 2005), while several E2-specific mAbs are able to induce CDC of E2-expressing cells (Machida *et al.*, 2008). Viral mechanisms to evade the effects of ADCC and CDC have also been reported (Machida *et al.*, 2008; Maillard *et al.*, 2004). Optimizing these non-nAb effector functions may prove critical in the design of effective therapeutic antibodies (Jiang *et al.*, 2011).

Conclusion

Our current understanding of the nAb response raised against HCV suggests that E2 is the major target and that multiple epitopes within E2 may be targeted by both linear- and conformation-dependent antibodies. Predominantly these neutralization epitopes overlap with CD81-binding sites and clearly demonstrate a role in inhibition of entry. However, this interaction is not the only target for nAb. Antibodies are able to inhibit the fusion of viral and cellular membranes, target as-yet-unidentified epitopes within E2, bind E1 and may be able to inhibit the interaction between the virus and other cellular receptors. Claudin-1 and occludin have only recently been identified as entry receptors and as such are less well studied than CD81. Switching the focus to these receptors may lead to the discovery of new nAb epitopes. Defining the fusion determinants within the envelope glycoproteins would facilitate the discovery of antibodies targeting these epitopes. This may be a step closer thanks to the recently described 3D structural model of E2 (Krey *et al.*, 2010). It will be interesting to see whether the proposed fusion domains are targeted by patient-derived antibodies capable of inhibiting fusion. It will also be interesting to see if E1 has a role to play in fusion, as has been suggested. Very few

antibodies to E1 have been identified so far (Keck *et al.*, 2004b; Meunier *et al.*, 2008); however, the exposed nature of this glycoprotein on the surface of the viral particle makes it a natural target for the immune response. If fusion determinants within E1 are identified, it would be an ideal candidate for the design of rational immunogens.

The protective effect of nAb *in vivo* in some individuals gives hope that such antibodies can be used therapeutically. The challenge for researchers will be to find antibodies which target highly conserved epitopes with broad neutralizing capabilities that will be effective against all genotypes of HCV. Identifying antibodies which inhibit many different virus–cell interactions would greatly enhance our repertoire of agents to use in the fight against this virus and we should continue searching for these antibodies.

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