CURRENT PROGRESS AND CHALLENGES IN THE DEVELOPMENT OF A HEPATITIS C VIRUS VACCINE

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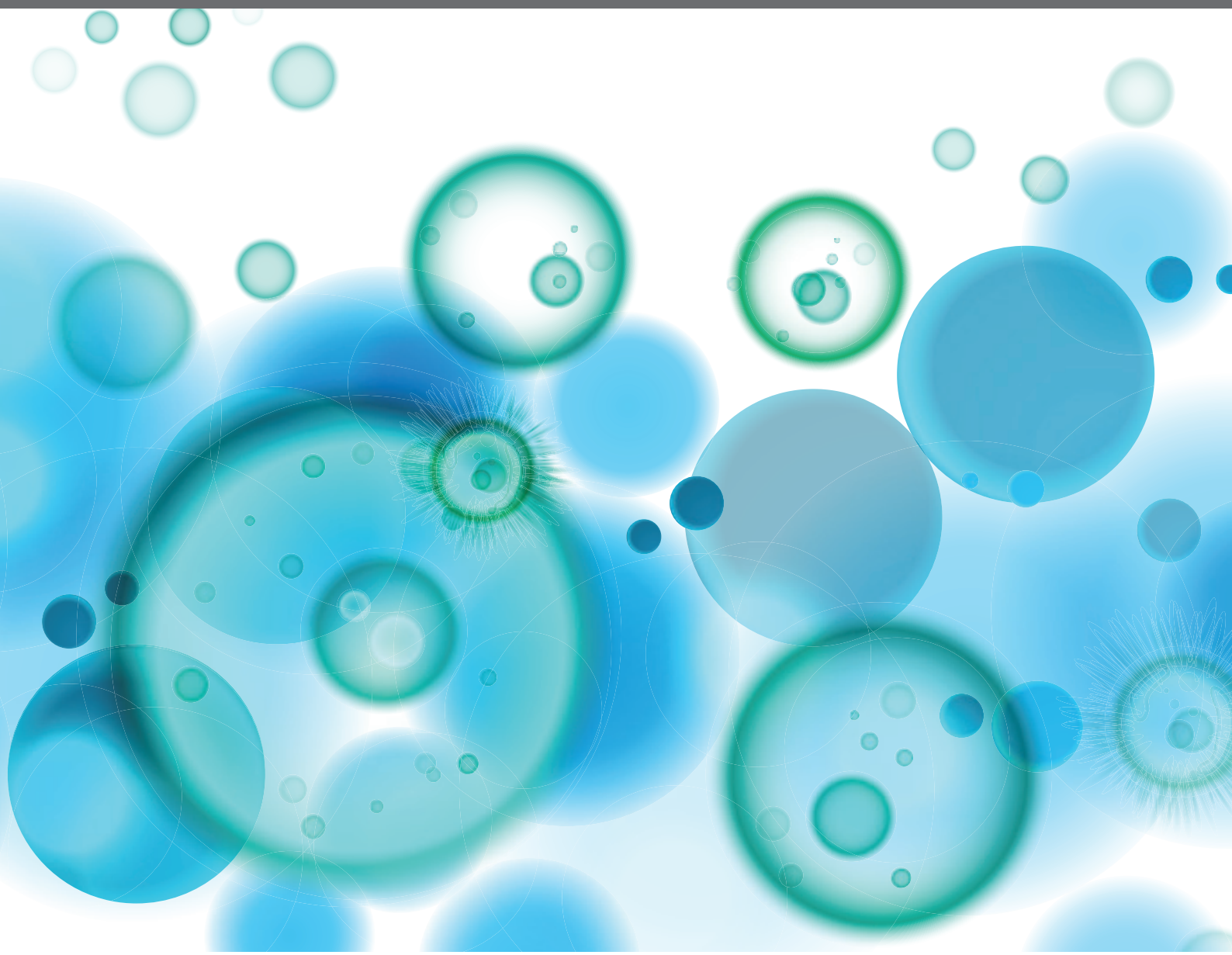

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CURRENT PROGRESS AND CHALLENGES IN THE DEVELOPMENT OF A HEPATITIS C VIRUS VACCINE

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More than 70 million people worldwide are infected with hepatitis C virus, a major cause of liver cirrhosis, liver failure and hepatocellular carcinoma world-wide. In the last decade, this cancer has emerged as the second leading cause of cancer death and the global burden is increasing by two million new infections per year, mainly due to injection drug use. An effective vaccine will be the most effective means to contain the spread of this virus worldwide. The articles in this Research Topic describe the progress that has been made towards a preventive vaccine and the challenges that still need to be overcome to ultimately achieve this goal.

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EDITORIAL
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Editorial: Current Progress and Challenges in the Development of a B Cell Based Hepatitis C Virus Vaccine

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Editorial on the Research Topic

Current Progress and Challenges in the Development of a B Cell Based Hepatitis C Virus Vaccine

More than 70 million people worldwide are infected with hepatitis C virus (HCV), a major cause of liver cirrhosis, liver failure and hepatocellular carcinoma (HCC) world-wide. In the last decade, HCC has emerged as the second leading cause of cancer death. The World Health Organization estimates an increase in the global burden by two million new infections per year and mainly due to injection drug use (IDU). Infection is increasing in young adults in the U.S. because of IDU (1). For patients, the development of highly efficient HCV-specific direct acting antivirals (DAAs) has markedly improved treatment and disease outcome. However, the high costs of DAA limit their access to patients with low income or limited health insurance and in countries with limited resources (2). Indeed these challenges are reasons why overall access to DAA has been estimated to be <10% of the HCV-infected patients on a global level (3). Moreover, the absent access of the majority of patients translates into very limited effect on the global disease burden such as HCV-induced HCC (4). In addition, health care workers with occupational risk for blood-borne pathogens and injection drug users (IDUs) will remain at risk for repeated exposure to HCV, even after successful treatment. This is dramatically illustrated by the growing number of HCV infections in the opioid epidemic (5). Recent clinical evidence suggests that treatment-induced cure in patients in advanced fibrosis does not eliminate the risk of HCC [for review see (6)]. These challenges strongly suggest that DAAs will neither be sufficient to eradicate the disease on a global level nor in distinct patient populations such as IDUs. Taken together, there is a significant need for an effective preventive HCV vaccine to be developed. The articles in this research topic describe the progress that has been made toward a preventive vaccine and the challenges that still need to be overcome to ultimately achieve this goal.

A first step in a "rational vaccine design" approach for HCV is to identify relevant mechanisms of immune correlates of protection. Naglaa Shoukry from the University of Montreal summarizes the challenges to vaccine development and the efforts required to overcome them. Multiple lines of evidence suggest that CD4+ and CD8+ T cell responses are needed to control acute infection but are insufficient for preventing long-term persistence. At the same time, cumulative evidence supports the importance of virus neutralizing antibodies to protect against HCV infection and to facilitate clearance. Most anti-HCV antibodies are directed mainly against the E2 glycoprotein and some to E1 and E1E2. Both envelope proteins are required for viral entry. The function of E2 has been studied in great detail, however much less is known about its "partner in crime," envelope glycoprotein E1 and the interactions of E1 and E2. While Tong et al. from the Institut Pasteur of

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Shanghai describe the function of E1 and aspects of its structure and function that are important for HCV vaccine design, Yost et al. from the University of New Jersey and from the NIH present the complexities of the flexible E2 and E1E2 heterodimer glycoproteins and how the flexibility of disordered regions on the glycoproteins might affect vaccine development. In that regard, computer models of the HCV glycoproteins (E1 and E2) that describe the interplay of E1 and E2 and potential interactions of E1/E2 with host HCV receptors could inform about new approaches to rational vaccine design (reviewed by Guest and Pierce from the University of Maryland and by Kinchen and Bailey from the John Hopkins University School of Medicine).

A significant challenge for a B cell based HCV vaccine is defining conserved epitopes that are capable of eliciting protective antibodies unassociated with viral escape. Keck et al. from Stanford University give a comprehensive overview of the immunogenicity of E2 and summarize epitopes that could be targeted for rational vaccine design. The hypervariable region 1 in the HCV E2 glycoprotein, HVR1, is an immunodominant region associated with neutralization and viral escape as reviewed by Prentoe and Bukh from the University of Copenhagen. Substantial efforts have shown that the majority of antibodies with broad neutralizing activities to diverse HCV isolates recognize conformational epitopes in the HCV E2 glycoprotein, as reviewed by Tzarum et al. from The Scripps Research Institute. Of particular importance is the region of the CD81 binding domain. Ströh et al. from Hanover Medical School focus on the flexibility of this neutralization defining region and discuss the impact for vaccine design. Only some of these conserved epitopes are not associated with viral escape. Cowton et al. from the University of Glasgow identified viral epitopes that were conserved among all strains, giving a promising perspective toward the development of a broadly effective HCV vaccine. An important determinant for viral escape are N-linked glycans. Lavie et al. from the University of Lille highlight the role of N-linked glycans for HCV neutralization and viral escape and give an outlook how modifications of specific glycosylation sites could improve the immunogenicity of vaccine candidates. New strategies will also have keep in mind the close association of HCV with components of the lipid metabolism. In that regard, Wrensch et al. from the University of Strasbourg discuss the interactions of the HCV particle with apolipoproteins and discuss their impact on HCV vaccine design. Vaccine development also requires standardized and sensitive methods two assess the efficacy of vaccine lead candidates. A specific challenge, the development of appropriate test system, that reflects the whole variety of HCV envelope variants is addressed by Kinchen and Bailey from the Johns Hopkins University School of Medicine who stress the need to use extensive well-characterized HCVcc or HCVpp to define neutralization potency and breadth of B cell responses. Furthermore, *in vivo* testing is a key requirement for vaccine development. Burm et al. from Ghent University review existing animal systems for HCV vaccine research and discuss the suitability of liver xenograft models as well as HCV homologs to test vaccine candidates and to assess humoral and cellular immune responses.

In summary, the articles published within this research topic not only give a highly comprehensive overview of the challenges of viral immune evasion required to address for vaccine design, but also informs on the current stage of HCV vaccine research highlighting perspectives and opportunities for the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Hepatitis C Vaccines, Antibodies, and T Cells

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The development of vaccines that protect against persistent hepatitis C virus (HCV) infection remain a public health priority. The broad use of highly effective direct-acting antivirals (DAAs) is unlikely to achieve HCV elimination without vaccines that can limit viral transmission. Two vaccines targeting either the antibody or the T cell response are currently in preclinical or clinical trials. Next-generation vaccines will likely involve a combination of these two strategies. This review summarizes the state of knowledge about the immune protective role of HCV-specific antibodies and T cells and the current vaccine strategies. In addition, it discusses the potential efficacy of vaccination in DAA-cured individuals. Finally, it summarizes the challenges to vaccine development and the collaborative efforts required to overcome them.

Keywords: hepatitis C, vaccines, antibodies, T cells, reinfection

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INTRODUCTION

Direct-acting antivirals (DAAs) against hepatitis C virus (HCV) infection can achieve complete cure in >95% of cases (1, 2) and have been suggested to have the potential to eliminate an infection that affects more than 71 million individuals worldwide (3). It was suggested that with such effective treatments, a vaccine against HCV may no longer be needed (4). However, this argument may be overly optimistic. HCV infection can remain asymptomatic for years during which many infections go undiagnosed. It is estimated that only 5% of HCV cases worldwide are diagnosed (5, 6). Many current and new HCV infections occur in developing countries and among marginalized populations like people who inject drugs (PWIDs), incarcerated individuals, and men who have sex with men (7). These individuals are mostly disengaged from medical care with limited access to HCV screening and treatment. In the meantime, they continue to infect others and contribute to the ongoing epidemic. Indeed, the current opioid epidemic in North America has been associated with increased incidence of HCV (8). Medical procedures remain the major cause of new HCV infections in developing countries with high prevalence of HCV posing another risk factor within the general population and travelers to these areas (9). Finally, DAA treatment does not protect against reinfection further underscoring the need for an effective vaccine (10).

The World Health Organization (WHO) has set elimination targets for 2030 to reduce the rate of new HCV infections by 90% (11). Despite numerous success stories for implementation of national hepatitis C strategies with increased screening, diagnosis, and treatment, notably in places like the Republic of Georgia and Egypt (12–15), this will not be enough to curb HCV transmission on the long-term. Successful vaccination strategies at the population level have been the only reliable method to limit transmission of different viral infections by providing herd immunity (16), especially among vulnerable populations and in low-resource settings. The ongoing effort to eliminate HCV should include two arms: screening and treatment, and enhanced prevention *via* vaccination and harm reduction measures.

The quest to develop a vaccine against HCV has been active, since the discovery of the virus in 1989 but it has been a challenging endeavor due to the high variability of the virus and the lack of small animal models for preclinical testing. Strategies have aimed at either producing broadly neutralizing antibodies (bNAbs) that would neutralize the infectivity of the virus or generating potent virus-specific CD4 and CD8 T cells that can eliminate infected hepatocytes. Various adjuvants, vectors, and vaccination regimens have been tested over the years. At present, two vaccines have made it into human preclinical and clinical trials. The first is a recombinant form of the virus envelope glycoproteins gpE1 and gpE2 aimed at inducing neutralizing antibodies and CD4 helper T cells (17, 18). The second is a vector-based vaccine encoding nonstructural (NS) proteins of the virus (NS3-NS5) using chimpanzee adenovirus priming and modified vaccinia Ankara (MVA) boost. This vaccine regimen was shown to induce high frequencies of virus-specific polyfunctional CD4 and CD8 T cells in healthy volunteers (19) and is currently in phase 2 clinical trials in PWIDs (NCT01436357). Results of this clinical trial are pending and will inform the field about the most appropriate future direction to follow.

This article reviews what we know about the role of antibodies versus T cells in mediating protective immunity against HCV and the pros and cons of targeting each approach in vaccine development. It also discusses the current challenges to HCV vaccine research and suggested collaborative efforts to overcome them.

CORRELATES OF IMMUNE PROTECTION DURING ACUTE HCV

Approximately 25% of individuals acutely infected with HCV are able to eliminate the virus spontaneously while the rest develop persistent infection and chronic liver disease, including fibrosis, cirrhosis, and hepatocellular carcinoma (20). The successful development of a vaccine against HCV is essentially informed by correlates of protective immunity induced during acute resolving infection. Multiple studies in humans and chimpanzees have clearly demonstrated the kinetic association of spontaneous viral clearance with induction of a broad, sustained HCV-specific CD4 and CD8 T cells [reviewed in Ref. (21)]. These T cell responses were also polyfunctional, producing multiple cytokines and effector functions (22). As the infection is cleared, virus-specific T cells develop a memory T cell phenotype and upregulate cell surface expression of the IL-7 receptor CD127 (22–25). The majority of HCV-infected individuals do generate a relatively broad CD4 and CD8 T cell response early on after infection that may afford partial control of viremia. Nevertheless, the abrupt loss of CD4 helper T cell responses, compromises CD8 T cell functionality and facilitates emergence of viral escape mutations in targeted CD8 T cell epitopes (26–28). As CD4 T cell functions are lost, the frequency of virus-specific T cells is reduced and the response becomes limited in breadth and/or functionality [reviewed in Ref. (21)]. Altogether, these dysfunctions result in virus rebound and persistent viremia. As the virus persists, CD8 T cells recognizing intact epitopes (i.e., epitopes that have not mutated) become exhausted and express exhaustion markers like programmed death 1 (PD1), T-cell immunoglobulin and mucin domain-containing-3 (Tim-3), cytotoxic T-lymphocyte protein 4 (CTLA4), 2B4, CD160, KLRG1, T-cell immunoreceptor with Ig and ITIM domains (TIGIT), and CD39 (21, 29). In addition, they upregulate expression of the transcription factor eomesodermin (Eomes), and in some cases the T cell factor 1 (TCF1) while downregulating expression of the T-box transcription factor (T-bet) (30, 31). T cell exhaustion leads to progressive loss of effector functions resulting in reduced polyfunctionality, cytotoxicity, and loss of proliferative capacity [reviewed in Ref. (21)]. As the infection persists, the frequencies of HCV-specific CD8 and CD4 T cells detectable in peripheral blood are dramatically reduced (32, 33). During chronic infection, HCV-specific CD8 T cells are more readily detectable in the liver albeit with an exhausted phenotype (34, 35). Interestingly, CD8 T cells targeting epitopes that have mutated remain functional and detectable in peripheral blood and acquire a CD127+ memory T cell phenotype, similar to memory T cells generated following spontaneous resolution (36).

Early studies of antibodies (Abs) against the HCV glycoproteins gpE1 and gpE2 have suggested that these responses are delayed during acute infection and are not associated with control of viremia (37). Similarly, the early use of viral pseudoparticles suggested that the development of neutralizing Abs (NAbs) is also delayed (38, 39). Yet, changes within the hypervariable region 1 (HVR1) of the gpE2 protein were detected in individuals developing chronic infection and were temporally correlated with Ab seroconversion suggesting immune selection pressure (40, 41). Furthermore, preincubation of virus inoculum with immune serum or anti-HVR1 Abs resulted in reduced infectivity in chimpanzees (42, 43). Passive immunization prolonged the incubation period in infected chimpanzees (44) and provided sterilizing immunity (45) or reduced viral loads in mouse models of HCV infection (46). Studies using multiple viral pseudoparticles representative of the variability of the virus gpE1/gpE2 region demonstrated a correlation between the generation of NAbs and spontaneous resolution (47, 48). The isolation of bNAbs from chronically infected and spontaneously resolved individuals and the capacity of these antibodies to block infectivity in mouse models of HCV infection further underscored the important protective effect of the antibody response (49–51).

EVIDENCE OF PROTECTIVE IMMUNITY AGAINST HCV UPON RE-EXPOSURE AND REINFECTION

Spontaneous clearance of HCV infection in chimpanzees and humans generates long-lived memory T cells that can theoretically protect against reinfection (52, 53). Long-term follow-up of a cohort of German women who were accidently infected with HCV *via* a contaminated blood product demonstrated that spontaneous resolution of acute HCV generated long-lived memory T cells that can be detected up to 20 years post resolution while antibody responses waned with time (52). This observation suggested that T cell responses may provide more durable protective immunity than antibodies. Data from cohorts of PWIDs have reported reduced rates of reinfection and/or chronicity among

individuals with prior immunity to HCV as compared to HCV naïve controls with the same risk exposure (54). Studies in humans and chimpanzees have demonstrated that subsequent HCV exposures in individuals with resolved infection result in lower viral loads and shorter viremia as compared to primary infection in the same individuals (53, 55–60). HCV rechallenge was associated with rapid anamnestic immune response and associated with a blunted secondary infection or faster viral clearance (53, 55–57, 59). Experimental depletion of CD8 T cells from chimpanzees who had resolved primary HCV infection followed by a homologous rechallenge resulted in prolonged infection that resolved only upon recovery of virus-specific CD8 T cells (53). In a complementary experiment where CD4 T cells were depleted, the animals were never able to clear HCV reinfection and accumulated escape mutations in epitopes targeted by the CD8 response leading to viral breakthrough and persistent infection (61). These two studies underscored the important protective role of both CD4 and CD8 memory T cells in preventing HCV persistence.

Protection against viral persistence upon reinfection in PWIDs was associated with an increase in the magnitude and breadth of HCV-specific T cell responses, and polyfunctional memory T cells that can produce more than one cytokine or effector function (57, 59). Analysis of the T cell repertoire demonstrated that CD8 T cells expanding upon reinfection were derived from the memory T-cell repertoire with almost no contribution of *de novo* T cell responses. Furthermore, the T cell repertoire became more focused upon reinfection with selection of T cells of the highest functional avidity (62).

Protection from viral persistence upon reinfection was also associated with generation of cross-reactive NAbs (57). These findings re-emphasized the important role of NAbs in mediating protective immunity and as a key component of a successful vaccine against HCV.

Protective immunity upon re-exposure was not absolute as some chimpanzees and humans could still develop chronic infection despite having strong immune responses upon heterologous rechallenge or infection with variant viruses that are not recognized by the pre-existing memory T cells (59, 63). These observations underscore the importance of inducing broad memory immune responses that target multiple epitopes or variant viruses.

In conclusion, reinfection in humans and chimpanzees confirmed the importance of both T cell responses and antibodies in long-term protective immunity. Furthermore, they underscored the importance of inducing a broadly reactive T cell and antibody response to counteract the variability in the viral quasispecies in real-world exposures. The recent development of better tools to examine the humoral response against HCV like pseudoparticles that are representative of the diverse viral populations (48), isolation of broadly neutralizing antibodies (49, 50), resolution of the crystal structure of the virus E2 glycoprotein (64, 65), understanding the interactions between NAbs and gpE2 (66, 67), and development of gpE2 tetramers that allow direct visualization, characterization, and isolation of HCV-specific B cells (68) will have an important impact on our understanding of the protective role of NAbs against HCV and the design of better vaccines.

STERILIZING IMMUNITY VS PREVENTION OF PERSISTENCE

The ideal goal of most vaccines is to provide sterilizing immunity that will protect against any infection upon exposure to the pathogen. This can only be achieved by induction of strong NAb responses that would neutralize infectivity. This approach has been very effective when targeting conserved viral surface proteins as is the case in vaccines against hepatitis A, B, and yellow fever. In the context of HCV, sterilizing immunity was not observed in chimpanzee rechallenge or human reinfection studies. Vaccination strategies aiming at inducing high titer bNAbs may indeed achieve sterilizing immunity but this will take time. Hence, it is likely that the first-generation vaccines will focus on achieving milder or blunted infections with lower viral loads and shorter periods of viremia and enhanced rate of viral clearance thus preventing viral persistence and protecting against chronic liver disease.

CURRENT HCV VACCINE STRATEGIES

Two main vaccine strategies are currently moving forward with human trials targeting either the cellular or humoral immune response (**Table 1**). The first vaccine is aimed at priming HCV-specific CD4 and CD8 T cells, using an adenovirus-based vector approach and focusing on the virus NS (NS3-NS4A-NS4B-NS5A-NS5B) proteins. The first proof-of-concept for this vaccine was demonstrated in the chimpanzee model of HCV infection. Adenoviral vectors serotype 6 (Ad6) and 24 (Ad24) carrying genes coding for the HCV NS proteins (genotype 1b) were used

TABLE 1 | Current hepatitis C virus vaccine development strategies.

Main Target	Stage	Immunogen	Vaccine regimen	Induced immune response	Potential improvements
T cells	Phase 2	NS3–NS5	Chimpanzee adenovirus 3 priming + modified vaccinia Ankara boost	• Polyfunctional CD4 and CD8 T cells • No antibodies (Abs)	• More potent vectors (e.g., CMV) • Invariant chain combination (enhanced Ag presentation) • Combination with recombinant proteins • Combination with immune check point blockade (for direct-acting antiviral-treated subjects)
Antibodies	Phase 1	gpE1/gpE2	Recombinant gpE1/gpE2 + adjuvant (MF59C.1)	• Some CD4 T cells • Broadly neutralizing antibodies	• Better adjuvants • Better CD8 T cell response inducers • Combination with nonstructural proteins

as prime followed by a plasmid DNA boost. Chimpanzees were then challenged with a heterologous virus (H77 and genotype 1a). This regimen led to priming of cross-reactive HCV-specific CD8 T cells in blood and liver that expanded upon rechallenge and led to suppression of acute viremia and lower viral loads in 4/5 vaccinated chimpanzees as compared to unvaccinated controls where 2/5 chimpanzees developed chronic infection (69). Subsequent testing in healthy human volunteers using human Ad6 priming and chimpanzee adenovirus 3 (ChAd3) boost, primed broad, polyfunctional, and cross-reactive HCV-specific CD4 and CD8 T cells that were sustained for at least a year after boosting with the ChAd3 and exhibited the phenotypic and functional characteristics of long-lived central and effector memory T cells (70, 71). The next-generation of this vaccine involved a heterologous prime-boost vaccination strategy based on ChAd3 priming then boosting with an MVA vector. This latest regimen was tested in healthy human volunteers and demonstrated optimal priming and boosting with the generation of high frequencies of polyfunctional, broad HCV-specific memory CD4 and CD8 T cells (19). This regimen is currently in phase 2 clinical trials as a prophylactic vaccine in high-risk PWIDs (NCT01436357). Results from this trial will provide the first proof of efficacy of this vaccine in real-life exposure to HCV.

The second vaccine is based on recombinant HCV gpE1/gpE2. This vaccine was one of the earliest vaccines tested in chimpanzees. Recombinant genotype 1a gpE1/gpE2 vaccination demonstrated effective immunogenicity and protective immunity against homologous or heterologous HCV rechallenge and even sterilizing immunity in some animals (72, 73). Preclinical evaluation of gpE1/gpE2 adjuvanted with MF59C.1 (an oil-in-water emulsion) in human volunteers induced NAbs as well as proliferative CD4 T cell responses against gpE1/gpE2 (17, 74). This NAb response was cross-reactive and targeted multiple epitopes (18, 75).

These two vaccines targeting either T cell responses or antibodies have demonstrated considerable immunogenicity in healthy volunteers and chimpanzees but whether they will provide protection during real-life exposures remains to be determined. Pending results of ongoing clinical trials will inform the future strategies of vaccine development. Next-generation vaccines against HCV will likely combine both T cell and antibody-based approaches into one single vaccine. The use of other strategies that may enhance immunogenicity like cytomegalovirus-based vectors (76) or fusion of the encoded antigen to major histocompatibility complex class II-associated invariant chain (Ii) (77) that have been reported to enhance CD8 T cell responses can be considered. The development of novel adjuvants and/or strategies that would tailor the T cell and antibody repertoires including the optimal vaccine/boost regimens and schedules are currently active areas of investigation.

VACCINATION IN HCV-CURED INDIVIDUALS

HCV reinfection following DAA-mediated viral clearance remains a problem among individuals with high-risk behaviors like PWIDs (10, 78). Hence, they are one of the main groups in need of effective vaccination strategies that will either provide sterilizing immunity or protection against viral persistence upon reinfection. DAA cure of chronic HCV can normalize the majority of innate immune responses following viral clearance (79). However, data from the few studies that examined reconstitution of HCV-specific T cell responses post DAA cure have suggested only partial restoration of virus-specific immunity. Rapid restoration of the *in vitro* proliferative capacity of HCV-specific CD8 T cells and a slight reduction in the *ex vivo* expression of PD1 on HCV-specific CD8 T cells were reported (80, 81). $TCF1^+CD127^+PD1^+$ HCV-specific CD8 T cells expressing both exhaustion and memory markers were described in chronically infected subjects and maintained during and after treatment (82). However, these "memory-like" CD8 T cells were different as compared to conventional memory T cells as they expressed higher levels of Eomes and TCF1 and produced lower levels of IFN-γ and TNF-α upon antigenic stimulation (82). Effect of DAA treatment on restoration of HCV-specific CD4 T cells, the hallmark of protective immunity, is unknown. Whether such partial restoration of HCV-specific immunity will protect against reinfection has not been tested in humans but a preliminary study in one chimpanzee treated with DAA reported that an intrahepatic HCV-specific CD8 T cell response was maintained at 2 years following cure but was narrowly focused and failed to prevent persistence upon re-challenge (83).

Given that the HCV-specific immune response had already failed once and is likely to be incompletely restored in DAA-cured individuals, it is not clear if they will respond to vaccination and whether they will be able to generate *de novo* T cell and/or Ab responses that can mediate protective immunity. A combined DAA therapy and immunization strategy with genetic vaccines encoding the NS proteins in chimpanzees chronically infected with HCV primed multifunctional T cell responses against non-conserved MHC class I epitopes (84). However, this response failed to contain the infection with the emergence of DAA resistance mutations (84). Vaccination with the NS genes using the ChAd3 prime/MVA, that has demonstrated high immunogenicity in healthy volunteers, did not efficiently reconstitute HCV-specific T-cell immunity in HCV chronically infected patients (85). Vaccine-induced HCV-specific CD8 T-cell responses were induced in 8/12 patients but CD4 T-cell responses were rarely induced. This was true even in patients with low viral loads suppressed with interferon/ribavirin therapy. Furthermore, the overall magnitude of HCV-specific T-cells was much lower than that observed in vaccinated healthy volunteers and the HCV-specific cells exhibited a partially functional phenotype. *In vitro* expansion studies demonstrated that these specificities were derived from pre-existing low-level memory T cell populations that could be expanded by vaccination. Nevertheless, new T cell responses were induced when there was a sequence mismatch between the autologous virus and the vaccine immunogen (85). These preliminary studies suggest that it may be possible to prime new immune responses in chronic and HCV-cured individuals but it is not yet clear if they will be enough to mediate protective immunity upon re-exposure. As suggested elsewhere, it is possible that the vaccination requirements or threshold of priming a protective immune response will be different in this population

(86). Additional strategies that may enhance immunogenicity like the use of novel adjuvants, vectors, or combination with immune checkpoint inhibitors may be interesting avenues to follow. Inclusion of DAA-cured individuals in upcoming vaccine trials will be necessary to evaluate immunogenicity in that special population.

CHALLENGES OF VACCINE DEVELOPMENT AGAINST HCV

Several challenges remain before achieving a vaccine that can be administered to the general population. Overcoming them will require collaborative efforts from the HCV community. These challenges include.

Virus Variability

There are 7 HCV genotypes and 67 subtypes (87). Furthermore, the virus circulates as quasispecies. Vaccination strategies targeting cross-reactive antibodies and T cell responses as well as conserved epitopes are likely to lead to better results. Data so far, suggest that the two HCV vaccines currently in progress can produce such responses, but additional methods to enhance their immunogenicity and broaden the response are needed to provide better vaccine coverage. It is also important to explore additional antigen design approaches that may overcome variability of HCV through the use of consensus sequences, ancestral, or mosaic sequences.

Immunological Challenges

The heterogeneity in the viral populations is compounded by the diversity of the vaccine-targeted human population. Specifically, for T cell-based vaccines, it will be important to design antigens that can be presented by multiple MHC alleles. It will also be critical for vaccination regimens to overcome the intrinsic host factors associated with several of the HCV at-risk populations that may influence the immune response, including: ethnicity, age, liver disease stage, opioid usage, and HIV co-infection. Designing prime/boost strategies and vaccination schedules that would promote expansion of the ideal T cell response (eg., polyfunctional, rapid proliferation, etc.) as well as bNAbs should be considered. Furthermore, as discussed above, tailoring specific vaccination regimens that would broaden the immune response in HCV-cured individuals may be essential.

Despite the progress in our understanding of correlates of protective immunity against HCV, several basic questions remain and require additional research to decipher them. First, although we know a lot about what constitute a good immune response against HCV, we do not understand why only ~25% of infected individuals are able to generate such a response. Second, CD4 T cell dysfunction is definitely key to developing persistent viremia but mechanisms of failure/exhaustion of these CD4 T cells remain unknown. Similarly, the molecular mechanisms underlying CD8 T cell exhaustion and dysfunction remain poorly understood. Third, the function of B cells during acute and chronic HCV, the interaction between T and B cells and how this impacts development of bNAbs are understudied. Future research to answer these questions should inform the vaccine development efforts.

Lack of Preclinical Small Animal Models

Humans and chimpanzees are the only two species that are susceptible to HCV infection. The efficacy of the two vaccines currently in clinical trials was demonstrated first in challenge studies in chimpanzees. With the moratorium on chimpanzee research, use of this model is no longer feasible (88). Furthermore, most mouse models that recapitulate the HCV life cycle are generated on immune-deficient backgrounds thus rendering them of limited use for preclinical vaccine testing but helpful in validating the *in vivo* neutralization capacity of antibodies (89). Novel mouse models using a hepacivirus isolated from the Norway rats of New York city and termed the Norway rat hepacivirus (NrHV) or rodent hepacivirus-nr-1 can recapitulate some but not all aspects of the immune response to a hepacivirus like HCV (90, 91). Specifically, depletion of CD4 T cells is required to achieve persistent viremia in this model (90). Nevertheless, it can still provide important clues about the correlates of immune protection and can be very useful in preclinical testing of novel adjuvants and vectors as well as vaccination regimen. Interestingly, NrHV infection in its original host, the rat, mimics to a great extent HCV infection in humans including the propensity to persist (92). From that perspective, the rat model of NrHV infection may be the more appropriate model to study immunity and vaccination, although the availability of rat-specific immune reagents is limited. Additional research aimed at establishing a more straight forward immune competent small animal model is still needed.

Cohorts for Clinical Trials

One of the difficulties in clinical trials is recruitment of high risk study subjects. Given that the main risk group for HCV infection is PWIDs who also suffer from multiple social, psychological, and marginalization issues, working with this group is challenging (93). Collaborative efforts with organized cohorts around the world are ongoing (94) and should be maximized in preparation for expanding the current clinical trials or new ones. With the current availability of highly effective DAAs, it is tempting to propose clinical trials of vaccines in healthy volunteers followed by challenge and close monitoring where DAA treatment can be administered at the first sign of viral persistence. This is not a novel approach and has been used in trials for malaria vaccines (95). Evidently, the ethical implications are not trivial and will have to be considered carefully.

Standardized Reagents, Reagent Repositories, and Immunological Methods to Assess Vaccine Efficacy

The advancement of our knowledge of protective immunity requires the availability of standardized reagents for testing as well as immune monitoring protocols. Peptides and a number of other reagents are available through the Biodefense and Emerging Infections Research Resources Repository (BEI Resources). Efforts to establish a repository for HCV pseudoparticles were discussed at the 24th International Symposium on HCV and Related Virus. Additional efforts to standardize immune monitoring among different trials should be considered.

Funding

Hepatitis C virus research is far from over. As discussed elsewhere (96), the road to HCV elimination requires additional investment in basic research on HCV to understand the molecular mechanisms of immune control of the virus and to develop effective vaccines. In addition, large-scale clinical trials and infrastructure support for production of large-scale vaccine lots under GMP conditions are costly. Such funding will have to be secured from collaborative initiatives by governments and funding agencies across the world, the WHO, and academic-industrial partnerships.

CONCLUDING REMARKS

Tremendous progress in the development of vaccines against HCV has occurred in recent years. The next-generation of HCV vaccines will have to target both antibodies and T cells for effective protective immunity. Better understanding of correlates of protection from viral persistence in real-life exposure settings and in DAA-cured individuals will be necessary for the design of new clinical trials. Similarly, better understanding of the humoral immune response and factors that may enhance the generation of bNAbs are warranted. Development of novel adjuvants, vectors, and vaccine prime/boost regimens that broaden the specificity and enhance the immunogenicity of vaccines are needed. Collaborative efforts for the establishment of cohorts and conducting vaccine clinical trials are essential.

AUTHOR CONTRIBUTIONS

NHS reviewed the literature and wrote the manuscript.

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Role of Hepatitis C Virus Envelope Glycoprotein E1 in Virus Entry and Assembly

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Hepatitis C virus (HCV) glycoproteins E1 and E2 form a heterodimer to constitute viral envelope proteins, which play an essential role in virus entry. E1 does not directly interact with host receptors, and its functions in viral entry are exerted mostly through its interaction with E2 that directly binds the receptors. HCV enters the host cell *via* receptor-mediated endocytosis during which the fusion of viral and host endosomal membranes occurs to release viral genome to cytoplasm. A putative fusion peptide in E1 has been proposed to participate in membrane fusion, but its exact role and underlying molecular mechanisms remain to be deciphered. Recently solved crystal structures of the E2 ectodomains and N-terminal of E1 fail to reveal a classical fusion-like structure in HCV envelope glycoproteins. In addition, accumulating evidence suggests that E1 also plays an important role in virus assembly. In this mini-review, we summarize current knowledge on HCV E1 including its structure and biological functions in virus entry, fusion, and assembly, which may provide clues for developing HCV vaccines and more effective antivirals.

Keywords: hepatitis C virus, envelope protein, E1, virus entry, virus assembly, fusion

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INTRODUCTION

Hepatitis C virus (HCV) is a major human pathogen that currently infects about 170 million people worldwide. Although recent introduction of highly effective direct-acting antiviral agents has greatly improved hepatitis C treatment outcome, no prophylactic HCV vaccine is available, rendering it difficult to eradicate HCV infections globally (1, 2). HCV is an enveloped, positive-strand RNA virus belonging to the family of *Flaviviridae*. The HCV RNA genome is 9.6-kb in length and encodes a single polyprotein that is co- or post-translationally cleaved into three structural proteins (core, E1, and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (1). The envelope glycoproteins E1 and E2 form a stable heterodimer that mediates virus entry and morphogenesis. HCV virions are associated with host low-density lipoproteins or very-low-density lipoproteins, which play important roles in virus entry, egress, and evasion of the host immune response (3). HCV entry and morphogenesis are highly coordinated processes, which involve all viral structural and non-structural proteins as well as a panel of host factors (4–6). Here, we aim to

Abbreviations: HCV, hepatitis C virus; HCVpp, HCV pseudoparticle; HCVcc, cell-culture derived HCV; CLDN1, claudin-1; nE1, N-terminal domain of E1; pFP, putative fusion peptide; C, cysteine; Asn, asparagine; TMD, transmembrane domain.

summarize current knowledge of HCV E1 including its structure and biological functions in virus entry and morphogenesis.

STRUCTURE OF E1

Domains/Motifs Organization

E1 envelope glycoprotein (192 amino acids) is much smaller than E2 (approximately 365 amino acids depending on the genotypes) but both are type I transmembrane protein with the N-terminal ectodomain residing in the endoplasmic reticulum (ER) lumen and the C-terminus anchoring on the ER membrane. The length of HCV E1 and E2 is similar to that of pestivirus E1 and E2, but many other flaviviruses only encode a single envelope glycoprotein E of 500 amino acids. Bioinformatics analysis of E1 sequences across all genotypes reveals a conserved protein domain organization, including N-terminal domain (NTD, 192–239), putative fusion peptide (pFP, 272–285), conserved region (CR, 302–329), and C-terminal transmembrane domain (TMD, 350–381) (**Figure 1**). NTD contains four conserved cysteines that form intramolecular, and possibly intermolecular, disulfide bonds. In addition, majority of E1 glycosylation sites and identified E1 epitopes reside in this domain, suggesting NTD is likely exposed on the protein surface. The exact roles of NTD remain elusive, although it was shown that a motif (aa 219–221) in NTD may have a cross talk with TMD to determine the complex formation with E2 (7). TMD, also serving as the signal peptide for E2, dictates membrane-bound topology of E1 and is essential for forming a heterodimer with E2. pFP is highly conserved and has been proposed to participate in fusion of viral envelope and host cell membrane during HCV entry (5, 8). CR is highly conserved among all genotypes/subtypes, but its function is poorly defined.

Glycosylation

Both E1 and E2 are heavily glycosylated, and N-linked oligosaccharides are added to asparagine (Asn) within the context sequon Asn-X-Ser/Thr (16). E1 of major genotypes possesses four conserved potential N-linked glycosylation sites (196, 209, 234, and 305) (17), while the fifth glycosylation site at N250 is only specific to genotypes 1b and 6 (18). E1 is not efficiently glycosylated if expressed alone, and requires the co-expression of E2 protein for the full-extent glycosylation (16).

E1 glycosylation contributes to correct protein folding and its biological functions. An early study showed that mutation of N196 or N305 impairs the E1/E2 heterodimerization while mutation of N209 or N234 has little effect (17). A later study based on HCV pseudoparticles (HCVpps) confirmed that glycosylation at N196 or N305 is critical for E1 folding and its incorporation into HCVpp, whereas N209 may modulate the virus entry (19). Using cell-culture derived HCV (HCVcc) system, it was shown that N196 is the most critical among the four E1 glycosylation sites for the HCVcc infectivity (20). In addition, E1 glycosylation pattern may regulate the formation of disulfide bond in E1. For example, disulfide bond involving C306 will likely be prevented by glycosylation at N305 due to spatial restriction (17). Interestingly, removal of this glycosylation increases the immunogenicity of soluble E1 (21). Another study also found that removal of the glycan at N209 improves immunogenicity of the E1/E2 heterodimer (22).

Disulfide Bonds

Eight cysteine residues (C207, C226, C229, C238, C272, C281, C304, and C306) are highly conserved across all HCV genotypes. Although extensive analyses have been performed to decipher the possible disulfide bond matches among these cysteines, these efforts only yielded limited and conflicting information thus far. The solved partial E1 crystal structure revealed an intramolecular disulfide bond between C229 and C238 as well as an intermolecular disulfide bond between C207 and C226 (23). However, this finding is contrasted by another structure modeling study suggesting C226 remains in a free thiol state (24). Instead, this study proposed three different intramolecular disulfide bonds C207–C306, C229–C304, and C238–C281 (24). Moreover, possible disulfide bonds between E1 and E2 have also been proposed based on the proximity of cysteines in the predicted E1/E2 structures, such as C272 (E1) and C452 (E2), C304 (E1), and C486 (E2) (24, 25). These conflicting reports not only reflect the technical difficulty to determine the existence of disulfide bonds but also may reflect the complexity in dynamic changes of disulfide bond formation in E1 during the processes of HCV entry and morphogenesis. Indeed, virion-associated E1 and E2 envelope glycoproteins formed large covalent complexes stabilized by disulfide bridges, whereas the intracellular forms of these proteins assembled as noncovalent heterodimers (26). In addition, C226–C229 form a classical CxxC motif, a key feature of protein disulfide isomerase, which may mediate the isomerization of disulfide bonds in E1 during virus entry (24, 27), as reported in Env fusion proteins from retroviruses (28, 29). More experiments are needed to validate this hypothesis, as reports using thiol-reactive agents indicated that HCV entry is weakly dependent on its redox status, in contrast to other enveloped viruses (30). Mutagenesis of the eight conserved cysteines in E1 indicated that, unlike cysteine mutations in E2 that drastically disrupt virus infectivity (31), the cysteine mutations in E1 have much less effect on virus infectivity (27). Interestingly, the E1 cysteine mutant viruses hardly survive from the freeze-thaw treatment that normally does not harm wild-type HCVcc, suggesting that the disulfide bonds in E1 are more flexible but critical for maintaining stability of infectious virions (27).

Crystal Structure

The NMR structures of partial E1 domains are available, including the region 314–342 (structure 2KNU) (32) that overlaps with the CR domain, and the region 350–369 (structure 1EMZ) (33) that resides in the TMD. The crystal structure of N-terminal domain of E1 (192–270, nE1) was recently solved (23). The nE1 monomer structure contains an N-terminal β-hairpin, a 16 amino acid α-helix in the middle and a three-strand antiparallel β-sheet in the C-terminal. Six nE1 monomers form an asymmetric unit, stabilized by a series of intra- and intermolecular disulfide bonds. Given that the nE1 crystals were prepared at a low pH condition, the covalently linked nE1 dimer may represent the post-attachment conformation of E1 formed in an acidic endosomal compartment. Interestingly, the six-stranded

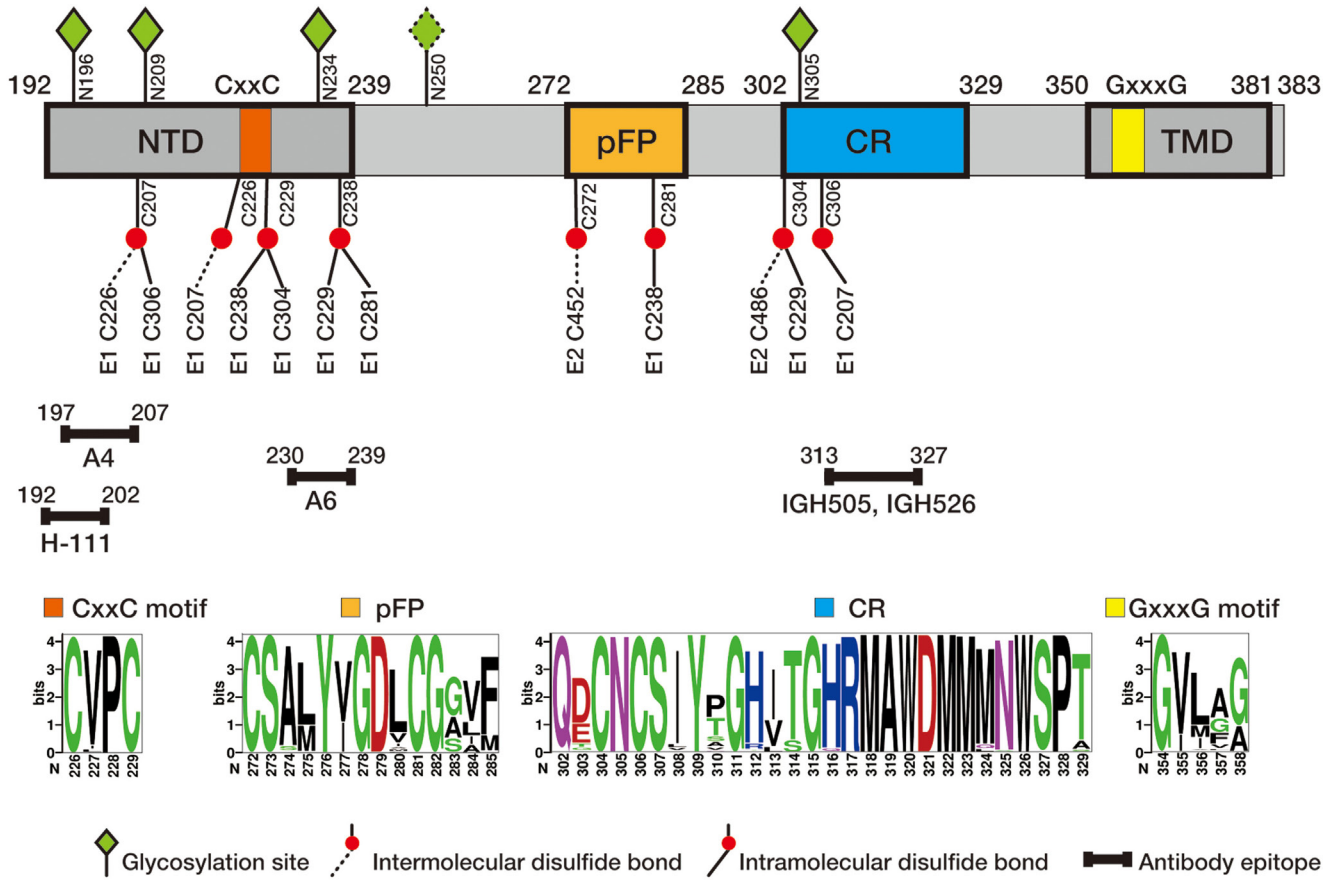

FIGURE 1 | Schematic diagram of hepatitis C virus (HCV) E1 envelope protein. E1 contains N-terminal domain (NTD, 192–239), putative fusion peptide (pFP, 272–285), conserved region (CR, 302–329), and C-terminal transmembrane domain (TMD, 350–381). CxxC motif in NTD and GxxxG motif in TMD are marked in orange and yellow, respectively. Four N-glycosylation sites (N196, N209, N234, and N305) conserved in all genotypes and one genotype 1b/6-specific site (N250) are labeled in green diamond, and conserved cysteine residues are labeled in red ball (intra- and intermolecular disulfide bounds in solid and dash line, respectively). Antibody epitopes, recognized by antibodies H-111 (9), A4 (10), A6 (11), IGH505, and IGH526 (12, 13), are marked with line segments. Of them H-111, IGH505, and IGH526 have been shown to neutralizing viral infection. The numbers correspond to the position of amino acid residues in the HCV polyprotein, with the first residue of E1 starting at 192. Sequence logos were generated based on 184 E1 sequences of genotype 1–6 from the Los Alamos hepatitis C sequence database (14) using WebLogo (15).

β-sheet structure formed by the nE1 homodimers appears similar to that of phosphatidylcholine transfer protein (23), raising a possibility that this domain may be one of the structural elements of HCV envelope proteins to mediate the lipoprotein association during HCV morphogenesis. Unfortunately, this solved nE1 structure gave little structural insight into membrane fusion as the majority of pFP was missing in the structure. The authenticity of this truncated E1 structure still needs to be validated experimentally, and future efforts should be focused on analysis of the full-length E1 ectodomain structure and E1 structure in complex with E2.

E1 Oligomer and E1E2 Heterodimer

Oligomeric status of the global HCV envelope protein complex may fluctuate during the HCV replication cycle. Using HCVcc system, it was shown that trimeric E1 can be detected at the surface of virions by SDS-PAGE under reducing and mild thermal denaturation conditions (34). The formation of trimeric E1 requires the co-expression of E2, and the C-terminal TMDs on the both E1 and E2 appear sufficient to trigger the E1 trimerization. The highly conserved GxxxG motif (Gly354 and Gly358) located in the N-terminal of E1 TMD is critical for the formation of the E1 trimer (34). Interestingly, unlike the trimeric E1, E2 remains a monomer in SDS-PAGE under the same mild thermal denaturation condition. A working model proposes that the TMDs of three E1 monomers form a trimer in the center and simultaneously interact with the TMD of peripheral E2 to form a heterodimer (24, 34). It is unclear whether the conformation of trimeric E1E2 heterodimers is unique to the mature viral particles since this thermal-instable E1 trimer can be also detected in the lysate of infected cells (34).

Expression of E1 and E2 alone can lead to formation of a noncovalent heterodimer, which is retained in the ER inside the cell (35, 36). E1/E2 heterodimerization is critically dependent on interaction between their TMDs which consist of a single α-helix (33, 35, 37).

Truncation or mutation in this α-helix abolishes heterodimerization (38). The result of alanine scanning assay demonstrated that the TMDs consist of charged residues in their centers that act as ER retention signals and are directly involved in heterodimerization (39, 40). Mutagenesis studies show that the residues G354, G358, and Lys 370 in N-terminal of E1 TMD are essential for heterodimerization (33, 41).

THE ROLE OF E1 IN ATTACHMENT AND BINDING DURING VIRUS ENTRY

Hepatitis C virus envelope glycoproteins bind to specific proteins at the surface of hepatocytes to initiate the entry process. This process involves a surprisingly large number of host receptors/co-receptors/factors, and also confers the major determinant of viral tropism (4). These host receptors/co-receptors/factors have been well summarized by recent reviews (2, 4–6). Of them, scavenger receptor BI (SR-BI), cluster of differentiation 81 (CD81), and two tight junction proteins claudin-1 (CLDN1) and occludin1 (OCLN) play the most critical role in HCV entry, and thus are regarded as the real viral receptors/co-receptors. A recent single viral particle imaging analysis on the polarized cell culture revealed a sequential engagement of these receptors/co-receptors during HCV entry which involves the translocation of HCV from the initial contact site on the basolateral membrane to the tight junction (42).

E2 is the major HCV envelope protein that directly interacts with the receptors/co-receptors. The physical interactions between E2 and CD81, SR-BI have been biochemically demonstrated, sometime even in the absence of E1 (43, 44). It is long believed that the role of E1 in this process is mainly to assist E2 by maintaining a functional E2 conformation required for the receptor binding. Indeed, it was showed that the E1E2 complex can interact with CLDN1 whereas E2 alone cannot (45). Consistently, two independent studies showed that mutations in E1 can shift the usage of HCV entry factor from CLDN1 to CLDN6 (46, 47), highlighting the importance of E1 in interaction with CLDN1 during HCV entry process. Furthermore, a critical cross talk between E1 and E2 was identified to modulate E1E2 binding to HCV entry receptors SR-BI and CD81 (45). Interestingly, recent studies suggested that the role of E1 in virus attachment and binding appears more than just assisting E2. A study showed that E1, but not E2, binds ApoE and ApoB, the apolipoproteins that are decorated on HCV virions and are crucial for HCV entry through low-density lipoprotein receptor (48). However, this observation was contradicted by a later study showing that E2 instead of E1 interacts with ApoE (49). Another study showed that E1 directly binds CD36 to facilitate HCV attachment (50).

THE ROLE OF E1 IN MEMBRANE FUSION

Endocytosis takes place upon the engagement of HCV envelope proteins with the receptors. It is well believed that the acidic environment in endosome activates the conformational changes of the envelope proteins and triggers the fusion of viral lipid envelope and endosomal membrane, leading to release of HCV RNA genome to cytoplasm (51). Despite extensive research, the molecular mechanism underlying the membrane fusion during HCV entry remains obscure. For a long time, the key unanswered questions were which viral protein(s) serve the fusion function and how the E1E2 heterodimer changes the conformation to induce membrane fusion.

The E glycoprotein of flaviviruses, a well-characterized prototype of class II fusion protein, consists of three distinct domains (DI, DII, and DIII), containing a fusion peptide buried at the dimer interface at neutral pH (8) and carrying both binding and membrane fusion properties (6). HCV E2 was initially considered as the viral fusion protein because of its size as well as its major role in receptor binding. However, the recent solved HCV E2 core structure exhibits a compact and Ig-like pattern (52, 53), which is different from any class II viral fusion protein-like structures shared by other flaviviruses (8), ruling out the possibility that E2 alone serves as fusion protein. This was further supported by the resolution of E2 structure of BVDV-1, a pestivirus member of the Flaviviridae family (8, 54).

It is now believed that E1 of BVDV and HCV serves as the fusion protein. E1 contains a conserved hydrophobic sequence (CSALYVGDLC, residues 272–281), which has been proposed to be a pFP (55). A number of studies demonstrate that this domain is indeed involved in the HCV fusion process (56–61). In addition, E1 can form a trimer, a typical structure of all fusion proteins. However, E1 seems too small to have a known class II or class III fold that could connect cellular and viral membranes after the fusion peptide insertion. Therefore, HCV E1 may define a new class of membrane fusogen. Interestingly, E1 of Rubella virus in the *Togaviridae* family does not possess the structural features of a classic class II fusion protein, while E1 of alphaviruses in the same *Togaviridae* family harbors a typical class II fusion protein (62). We speculate that constraints on flaviviruses or alphaviruses imposed by alternating life cycles between vertebrates and arthropods may result in more conservative evolution of their fusion proteins than for hepacivirus and rubivirus that infect human only. In the absence of this constraint, the structure of HCV or Rubella virus E1 may have evolved a number of specific features, placing it apart from classical class II fusion proteins of known structure.

Rather than being mediated by a single glycoprotein, HCV fusion appears to be mediated by complex intra- and intermolecular E1E2 dialogs that shape structural and conformational rearrangements of the heterodimer complex, similar to rubivirus and alphavirus (63). Consequently, the characterization of interplays between E1 and E2 is critical to decipher the HCV fusion (45). By combining computational analysis and wet-lab data, it was suggested that E1 co-evolves with the Back Layer domain (BL) of E2, and this genetic association is critical for membrane fusion (64). A soluble BL-derived polypeptide inhibits fusogenic rearrangements and HCV infection, suggesting E1 and E2 BL/Stem regions govern HCV fusion in a concerted manner (64).

THE ROLE OF E1 IN HCV MORPHOGENESIS

Compared to virus entry, much less studies have been conducted to address how E1 contributes to HCV morphogenesis. It is believed that the formation of E1E2 heterodimer is a prerequisite

for assembly of HCV virion. Any mutations that interfere with the dimerization of E1 and E2 would have a severe impact on HCV morphogenesis. For example, the mutations in the GxxxG motif located in TMD of E1 can disrupt the trimerization of E1 and formation of the E1E2 heterodimer, which further prevents the assembly of appropriate tertiary and quaternary structures (25, 34). In addition, E1 and E2 in virions are linked covalently by disulfide bridges (26), suggesting that HCV envelope proteins undergo conformational changes involving disulfide bond modification during virus assembly process.

E1 or the E1E2 complex can interact with NS2 (65, 66), the master viral protein that interacts with multiple viral structural and non-structural proteins to coordinate HCV assembly. This raises a possibility that E1 directly contributes to HCV morphogenesis in a way that may not involve E2. A mutation D263A in E1 abolishes the viral infectivity and leads to secretion of viral particles devoid of genomic RNA (47). Because the direct contact of E1 and viral genome is unlikely, it is tempting to speculate that E1 may regulate the assembly of infectious virions through its interaction with other viral proteins such as NS2.

We recently developed a trans-complementation-based HCV reverse genetics model in which the coding sequence for E1 or E1E2 is deleted from the HCV genome and is provided *in trans* (57, 67). This system allows to perform the mutagenesis study to explore the functional role of individual domains/motifs in the envelope proteins without potential unwanted cis-effects to virus infection by the introduced mutations in the viral RNA genome. By using this system, we found that the pFP in E1 plays an important role in virus morphogenesis in addition to its well-known contributions to HCV entry (57). The deletion of pFP has no effect on the E1E2 heterodimerization, but completely abolishes the production and release of infectious virions. Alanine scanning analysis identified several point mutations within pFP that specifically affect virus morphogenesis rather than virus fusion (57). These results suggest that the pFP of E1 plays a dual role in virus entry and morphogenesis. More systematical studies are needed to reveal the exact underlying molecular mechanisms and also to investigate the contribution of other domains.

CONCLUSION AND PERSPECTIVES

Hepatitis C virus entry and assembly are complicated process that involves numerous viral proteins and host factors, including E1 and E2. As the conformations of E1 and E2 are interdependent, the functional analysis of each of these two envelope proteins should be always put in the context of the heterodimer. For example, the Ig-fold β-sandwich structure of E2 ectodomain displays similarities with domain III class II fusion proteins (53), suggesting that E2 may serve as a chaperone protein for E1 folding to assist its function in virus fusion. Thus, if we regard the E1/E2 complex as an integrated functional protein, their functions may become easier to understand and characterize.

Compared to E2, E1 is less immunogenic. It is probable that most E1 domains are hidden in the E1E2 heterodimer. However, during the heterodimer conformational changes in virus entry process, some E1 domains must be unmasked to finalize the fusion process. Therefore, the characterization of these dynamically exposed E1 domains, such as structural resolution of the E1E2 complexes in their pre- and post-fusion states, should be the keys to fully understand the roles of E1 in HCV life cycle and to accelerate development of HCV vaccines.

AUTHOR CONTRIBUTIONS

YT, JZ, and DL drafted the manuscript; QL performed bioinformatics analysis.

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REVIEW
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Hepatitis C Virus Envelope Glycoproteins: A Balancing Act of Order and Disorder

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Chronic hepatitis C virus infection often leads to liver cirrhosis and primary liver cancer. In 2015, an estimated 71 million people were living with chronic HCV. Although infection rates have decreased in many parts of the world over the last several decades, incidence of HCV infection doubled between 2010 and 2014 in the United States mainly due to increases in intravenous drug use. The approval of direct acting antiviral treatments is a necessary component in the elimination of HCV, but inherent barriers to treatment (e.g., cost, lack of access to healthcare, adherence to treatment, resistance, etc.) prevent dramatic improvements in infection rates. An effective HCV vaccine would significantly slow the spread of the disease. Difficulties in the development of an HCV culture model system and expression of properly folded- and natively modified-HCV envelope glycoproteins E1 and E2 have hindered vaccine development efforts. The recent structural and biophysical studies of these proteins have demonstrated that the binding sites for the cellular receptor CD-81 and neutralizing antibodies are highly flexible in nature, which complicate vaccine design. Furthermore, the interactions between E1 and E2 throughout HCV infection is poorly understood, and structural flexibility may play a role in shielding antigenic epitopes during infection. Here we discuss the structural complexities of HCV E1 and E2.

Keywords: hepatitis C virus, envelope glycoprotein, vaccine design, HCV, E2, E1

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INTRODUCTION

Hepatitis C virus (HCV) presents with mild symptoms; as an acute illness that resolves within weeks; or a lifelong, chronic infection that can lead to cirrhosis, liver cancer, and, if left untreated, death. End-stage, liver disease caused by chronic HCV infection is the leading cause of liver transplantation in the United States, Europe, and Japan (1–3). According to the World Health Organization, there were 1.75 million new HCV infections and 71 million people living with chronic HCV infection worldwide in 2015. Intravenous drug use and unsafe healthcare practices are responsible for a majority of new infections, contributing heavily to the doubling of HCV incidence in the United States between 2010 and 2014 (4, 5).

Despite FDA approval of several direct acting antiviral (DAA) treatments for HCV with very high success rates (>90%) for all genotypes, many at-risk groups are still spreading infection faster than they are being cured (5–8). Chronic HCV prevalence is about 1% of the total world population, but is much higher in many areas where healthcare is not widely accessible. Mongolia,

Uzbekistan, Egypt, and Gabon, for example, have HCV prevalence ranging from 4 to 7% (5) and specific populations in the Nile Delta and Upper Egypt can have infection rates as high as 28%, varying heavily based on socioeconomic status (9). The poorest and least educated in Egypt have the highest HCV infection rates and simply do not have the means to receive treatment. Furthermore, intravenous drug use accounts for about 23% of new HCV infections (10). Populations of intravenous drug users worldwide must overcome several barriers to treatment such as high cost, access to healthcare, compliance, and fear of being discovered as a drug user (8). After a successful course of treatment however, if the patient continues engaging in risky behaviors, they are still at risk to be re-infected. These factors prevent a dramatic improvement in HCV infection rates worldwide. Therefore, it seems unlikely that DAAs alone will eliminate HCV infection without an effective vaccine.

HCV is an enveloped virus containing a positive-sense, single stranded RNA genome. The lipid envelope, derived from the host membrane, is embedded with two type I transmembrane proteins, envelope glycoproteins E1 and E2, which form a heterodimer (11). HCV particles are uniquely associated with lipids and apolipoproteins, which play a role in proper formation and function of secreted virions (12–19). These associations give viral particles an overall low buoyant density (16). The E1/E2 heterodimer is responsible for viral entry from recognition of host cell receptors to membrane fusion. Initial host-virus attachment interactions are through glycosaminoglycans and low-density lipoprotein receptor (20). Several receptors have a necessary role in entry such as claudin-1, occludin, CD81, and scavenger receptor class B type 1, mainly through interaction with E2, although the role of E1 is not fully understood (21–24). E1 and E2 are on the surface of the virion, available for host immune recognition, and are ideal for studies in immunogenicity ultimately leading to vaccine design; however, the conformation of the E1/E2 heterodimer and its interactions have not been well characterized throughout the various stages of virus assembly, host cell attachment, and membrane fusion. High quality, fully glycosylated and disulfide-linked envelope glycoproteins have proven to be difficult to produce in large quantities for biophysical study until recently.

ENVELOPE GLYCOPROTEIN E1

The exact role(s) of E1 during entry, egress, and immune escape is not fully understood (21–24). It has an N-terminal ectodomain of approximately 160 amino acids and exists as a trimer on the surface of cell culture-produced HCV particles, driven by interactions in the E1 C-terminal transmembrane region (25) (**Figure 1**). E1 may aid in recognition of hepatocytes through interactions with apolipoproteins, particularly ApoE, which further interacts with cell surface heparin sulfate during early attachment (26, 27). Structural data of N-terminal 79 amino acids of HCV E1 (nE1) was determined by X-ray crystallography (28). This structure showed a covalently linked, domain-swapped

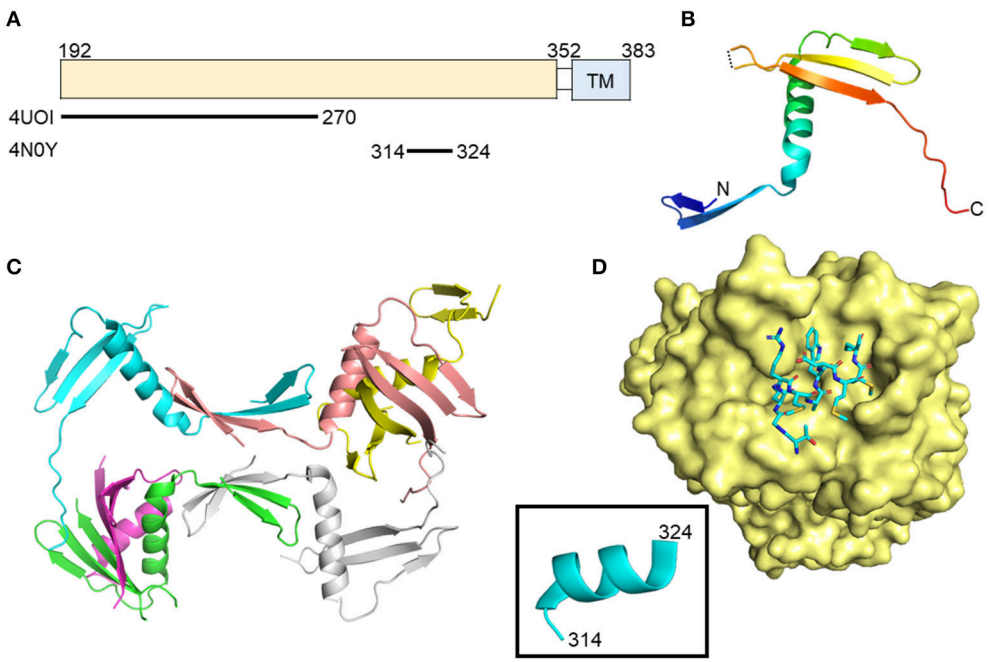

FIGURE 1 | Crystal structures of the N-terminal domain of E1 and an E1 peptide in complex with antibody. **(A)** Linear diagram of E1 glycoprotein. The crystallized E1 constructs and PDB IDs are shown below the diagram. **(B)** N-terminal domain structure of E1 monomer (PDB ID: 4UOI). **(C)** The six molecules of the asymmetric unit of the E1 N-terminal domain. **(D)** Structure of E1 peptide (aa314–324) in complex with antibody IGH526 (PDB ID: 4N0Y). The surface of antibody IGH526 is colored yellow, with the E1 peptide colored according to atom type (light blue, red, orange, and dark blue for carbon, oxygen, sulfur and nitrogen, respectively). The E1 peptide is further shown as ribbon structure in the box.

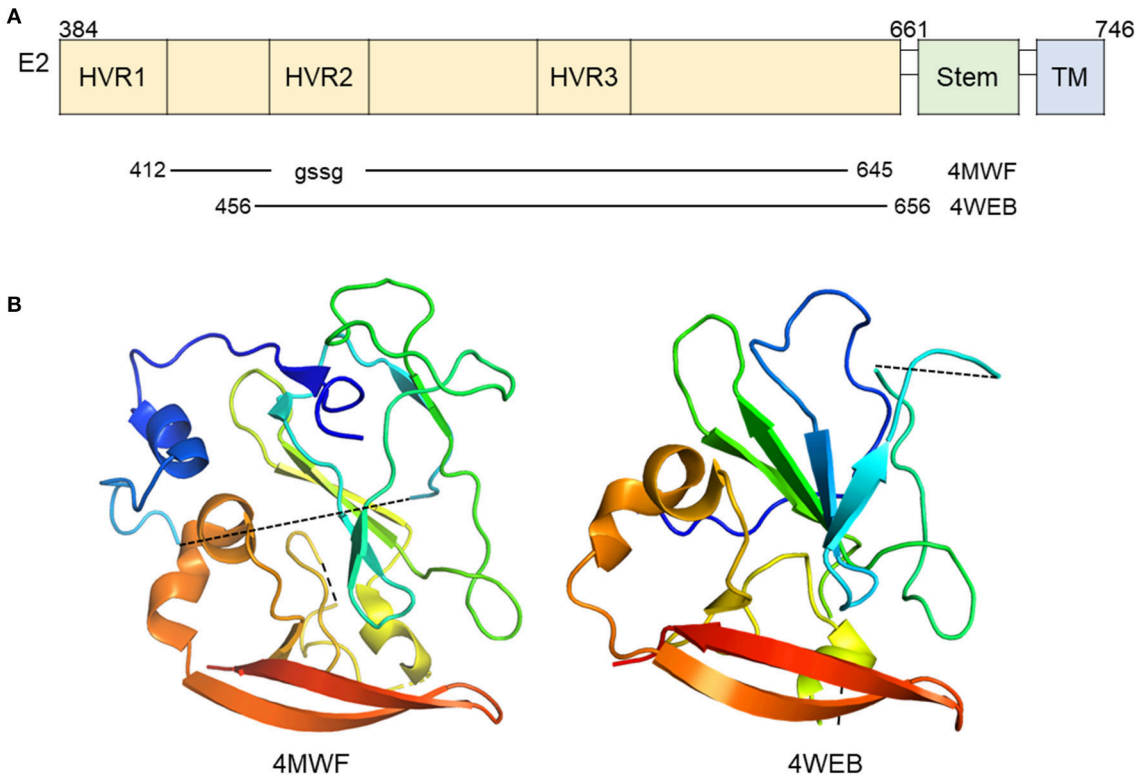

FIGURE 2 | Crystal structure of E2 in complex with antibody. **(A)** Linear diagram of E2 glycoprotein. The crystallized E2 core constructs and PDB IDs are shown below the diagram. **(B)** Ribbon representation of E2 (PDB ID: 4MWF and 4WEB).

homodimer with nE1 forming 16 amino-acid α-helix flanked by a β-hairpin N-terminally and a three-stranded antiparallel β-sheet C-terminally (**Figures 1A–C**). The N-terminus of E1 does not resemble a class II fusion protein as hypothesized, or any other fusion protein conformation, despite having a fusion peptide-like domain (29); however, the published structure may be in a post-fusion conformation as crystals were obtained at a low pH. The cross-neutralizing, anti-E1 antibody IGH526 was shown to bind to an α-helical epitope (residues 314–324) predicted to be highly flexible in molecular dynamics simulations (**Figure 1D**) (30). This is the first E1 antigenic epitope structure described, and may assist in future vaccine design.

ENVELOPE GLYCOPROTEIN E2

The functions of E2 have been more extensively studied relative to E1. E2 is responsible for mediating entry through interactions with several cellular receptors as mentioned above and is highly immunogenic (31–35). Two groups have published the structure of the core domain of E2 bound to Fabs (PDB ID: 4MWF and 4WEB) by X-ray crystallography (36, 37). The two studies employed a similar strategy with varying E2 expression constructs and antibodies for co-crystallization (**Figure 2A**). The 4MWF co-crystal was formed with E2 ectodomain (eE2) from HCV genotype 1a and a neutralizing, human Fab, AR3C

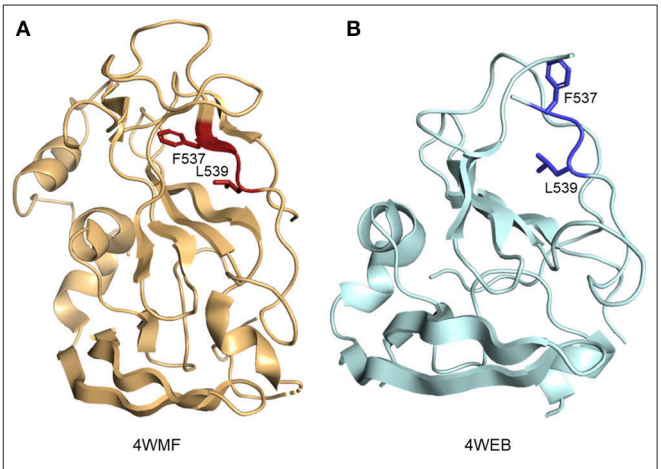

FIGURE 3 | Ribbon representation of E2 hydrophobic residue positions in CD81 binding loop. **(A)** E2 residue F537 and L539 (side chains are shown as red sticks) in the presence of a stabilizing Fab fragment (not depicted) (PDB ID: 4MWF). **(B)** Solvent exposed positions of residue F537 and L539 (side chains are shown as blue sticks) (PDB ID: 4WEB).

that recognizing an N-terminal epitope in E2 and blocks E2-CD81 interaction. The eE2 in this structure does not contain hypervariable region 1 (HVR-1) and replaced HVR- 2 with a Gly-Ser-Ser-Gly linker. The 4WEB co-crystal was formed

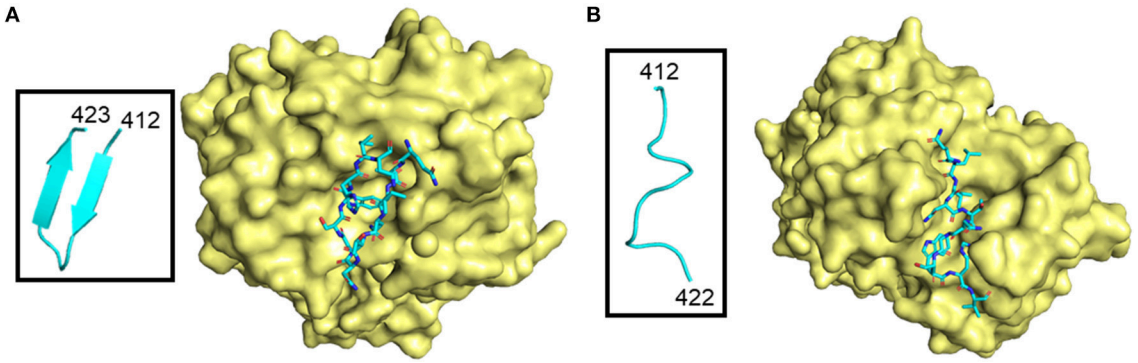

FIGURE 4 | Different conformations of E2 412–423 peptide in complex with antibodies. The antibody surface is colored yellow, and the peptide is shown as sticks colored according to atom type (light blue, red, orange, and dark blue for carbon, oxygen, sulfur and nitrogen, respectively). E2 peptides are further highlighted in boxes and shown as light blue ribbon structures. **(A)** β-hairpin peptide in complex with HCV1 antibody (PDB ID: 4DGY). **(B)** "Open" conformation peptide in complex with neutralizing antibody 3/11 (PDB ID: 4WHY).

with eE2 from HCV genotype 2a, lacking the first 72 amino acids, and non-neutralizing Fab, 2A12, which binds a linear epitope at the C-terminus of eE2. Overall, both structures reveal a monomeric E2 with a globular nature (**Figure 2B**), unlike the class II fusion proteins that E2 was predicted to be similar to, and does not undergo major oligomeric or structural rearrangement upon exposure to low pH (37). Structural stability of the overall fold of the protein is provided by an extensive hydrophobic core and disulfide bonding. Follow-up alanine scanning studies mapped critically important E2 residues for neutralizing antibody recognition to core E2 stability elements and are in agreement with the published structures (38).

The ordered portions of E2 are primarily arranged in β-sheets stabilized by disulfide bonds and hydrophobic interactions; however, a majority of eE2 (62% of it in the case of 4MWF) is in flexible loops or completely unstructured (36). Hydrogen-deuterium exchange and limited proteolysis experiments implicate the first 72 amino acids of eE2 containing HVR1 and region between HVR1 and HVR2 as highly flexible (37). In the 4MWF structure, the AR3C antibody binds this strand and provides stabilization for crystal formation (36). X-ray diffraction data for HVR2 could not be obtained (37). Therefore, in the absence of a stabilizing antibody, that leaves approximately the first 100 amino acids of eE2, containing several glycosylation sites, flexible and solvent exposed. This region is involved in epitope shielding, SR-BI binding, CD81 binding, and neutralizing antibody recognition (31–33, 39–45).

CD-81 BINDING SITE AND NEUTRALIZING ANTIBODIES

Residues of E2 which form the CD81 binding site are found in clusters between aa412–446 and aa519–535 (termed the CD81 binding loop) of HCV genotype 1a strain H77 (36, 46, 47). Distant CD81 binding clusters are brought together by the overall fold of the protein. The two published eE2 structures, when compared, highlight the flexible nature of not only the CD81 binding loop, but the central immunoglobulin-like fold itself. In 4WEB, the CD81 binding loop is disordered, allowing hydrophobic residues to be solvent exposed. In the 4MWF structure, the CD81 binding loop is stabilized by a Fab fragment, bringing order to previously unstructured β-strand E and allowing residues such as F537 and L539 to be flipped into the hydrophobic core of the protein (**Figure 3**). In 2017, Vasiliauskaite et al. expanded on this observation by demonstrating that the hydrophobic residue positions and secondary structure in the CD81 binding loop of E2 were dependent on interactions with different neutralizing antibodies in both HCV pseudoparticles and cell culture-derived HCV particles (48). Given that the current evidence focus on binding to antibodies, the secondary structure of E2 bound to receptor CD81 may further reveal undescribed conformations.

Many neutralizing antibody epitopes overlap CD81 binding residues of E2. For example, antibodies 3/11 and HCV1, as well as others, bind aa412–423, but recognize this flexible stretch of amino acids differently (32, 41, 49–52). The region adopts at least two different: an extended or "open" conformation or a β-hairpin (**Figure 4**) (50). Furthermore, the HCV1 antibody can bind from multiple angles, as visualized by electron microscopy, demonstrating not only local flexibility, but the long-reaching flexibility of the loop (53). Although the aa412–423 epitope is quite tempting for use in vaccine studies because of the cross-neutralizing antibody potential to functionally important residues, very few chronically infected HCV patients (<2.5%) produce a specific antibody response likely due to flexibility, and shielding by HVRs and glycans (45, 54–56). Recent studies seek to improve presentation of candidate epitopes and promote antigen recognition by the immune system using an engineered, cyclic immunogen. Initial data shows a designed E2 cyclic immunogen produced a strong antibody response in mice, whose serum was then used to successfully neutralize HCV infection in culture experiments (57). Further research will determine whether engineered derivations of this epitope will be useful in the pursuit of a viable HCV vaccine.

HCV VIRION-ASSOCIATED E1 AND E2

Structural information of the HCV virion is lacking, in sharp contrast to the closely related flaviviruses and alphaviruses. Obtaining a high-resolution, three dimensional structure of the HCV virion is difficult due to the low level of virus production in cell culture systems and the inherent heterogeneity of the particles owing to its association with apolipoproteins. Cryo-electron microscopy and tomography of HCV virions show spherical particles of highly heterogeneous size (40–100 nm in diameter). The particles displayed no obvious symmetry, no evidence of continuous membrane bilayer, and are covered by electron-dense material; although, the inherent low resolution of the electron micrographs may mask certain features (16). These findings perhaps call into question the hypothesis that HCV adopts a classical, icosahedral scaffold in which its two envelope glycoproteins anchor to the host cell-derived, double-layer lipid envelope. The lack of symmetry and membrane bilayer highlights the unique nature of the HCV virion relative to the other flaviviruses.

Higher-order aggregates of E1 and E2 on secreted virus particles appear to be covalently bonded, whereas non-covalently associated E1/E2 has been detected in the ER (58–60). At the moment, composition and structural information are unavailable for these higher order aggregates; however, one may glean insight from available structural information on E1 and E2 (28, 36, 37). The asymmetric unit of the nE1 structure contains six monomers stabilized by a series of intramolecular and intermolecular disulfide bonds (**Figure 1C**). It is possible that some or all of the intermolecular disulfides in the nE1 structure may be relevant to the higher order structures seen on the virion. The two eE2 structures are highly similar, with an RMSD of less than 0.8 Å for similar carbon-alpha positions with most of the differences located in loop regions. Interestingly, there are discrepancies in the disulfide bonding pattern in these regions. The overall fold of E2 core is unlikely to change in the virion, owing to its extensive hydrophobic core and three disulfide bonds formed between secondary structure elements.

The current structures available for E1 and E2 may reflect the immature forms of the proteins after initial synthesis and during virion assembly. Our hypothesis is that the folds found in these structures would be present on the virion, with the higher order aggregates formed via disulfide bonding through cysteines found in loop regions or within the core domains. During virion assembly and maturation, these core domains fold and higher order structures begin to form within the heterodimer or through interactions with other factors. The environment of the ER and Golgi apparatus during egress is oxidizing and compatible with disulfide bond formation and reshuffling, permitting the formation of the disulfide-linked aggregates. This maturation may contribute to the acid-resistance of extracellular HCV virions and have implications for the mechanisms of entry. Indeed, cell surface-bound HCV needs to be incubated for prolonged periods at 37°C for low-pH-mediated entry to occur (61). This suggests that post-binding events are required to prime the HCV envelope proteins for fusion.

CONCLUSION

Targeting a conserved epitope with known functional relevance is absolutely essential for production of a broadly neutralizing antibody. Structure-based vaccine design and innovative thinking with regard to stabilization of epitopes will be necessary to forward HCV vaccine efforts. Many of the vaccine studies in the past decade have been done with recombinant HCV E2 or E1E2; however, a majority of the human antibody responses were against E2 HVR1 and ultimately unsuccessful due to the high mutation rate in the region (62). The highly disordered and flexible nature of HCV E2 is a complicating factor to intelligent vaccine design. Not only is local flexibility seen between the same epitope partnered with different antibodies, but large portions of E2 are disordered and variably-sequenced between genotypes (i.e., the HVRs). The described structures of E1 and E2 are only representative of their respective serotypes and may or may not be representative of the many variable HCV isolates that exist. Within the HCV patient population, many circulating isolates are highly resistant to known broadly neutralizing antibodies, and many mutations that allow for resistance to neutralizing antibody recognition have been described (63, 64). Furthermore, inherently flexible, long-chain glycans are responsible for shielding targeted neutralizing antibody binding sites.

Available DAA treatments for HCV are undoubtedly necessary for infected patients; however, taking into account the rate at which high-risk groups are being infected with HCV, a vaccine is an imperative for preventative treatment. In order to achieve this goal, researchers must overcome the problem of HCV which uses an almost shapeshifting mechanism to evade immune detection: shrouding itself with a coat of apolipoproteins, flexibility, and hyper variability. HCV E2 has evolved to maintain a balance between the order of disulfide bonds and hydrophobic interactions necessary to form the overall protein fold, and the flexible chaos which allows the virus to replicate while evading the host immune response.

AUTHOR CONTRIBUTIONS

SY wrote the paper. YW and JM generated the figures. SY, YW, and JM participated in the critical review and revision of the paper.

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REVIEW
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Computational Modeling of Hepatitis C Virus Envelope Glycoprotein Structure and Recognition

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Hepatitis C virus (HCV) is a major global health concern, and though therapeutic options have improved, no vaccine is available despite decades of research. As HCV can rapidly mutate to evade the immune response, an effective HCV vaccine must rely on identification and characterization of sites critical for broad immune protection and viral neutralization. This knowledge depends on structural and mechanistic insights of the E1 and E2 envelope glycoproteins, which assemble as a heterodimer on the surface of the virion, engage coreceptors during host cell entry, and are the primary targets of antibodies. Due to the challenges in determining experimental structures, structural information on E1 and E2 and their interaction is relatively limited, providing opportunities to model the structures, interactions, and dynamics of these proteins. This review highlights efforts to model the E2 glycoprotein structure, the assembly of the functional E1E2 heterodimer, the structure and binding of human coreceptors, and recognition by key neutralizing antibodies. We also discuss a comparison of recently described models of full E1E2 heterodimer structures, a simulation of the dynamics of key epitope sites, and modeling glycosylation. These modeling efforts provide useful mechanistic hypotheses for further experimental studies of HCV envelope assembly, recognition, and viral fitness, and underscore the benefit of combining experimental and computational modeling approaches to reveal new insights. Additionally, computational design approaches have produced promising candidates for epitope-based vaccine immunogens that specifically target key epitopes, providing a possible avenue to optimize HCV vaccines versus using native glycoproteins. Advancing knowledge of HCV envelope structure and immune recognition is highly applicable toward the development of an effective vaccine for HCV and can provide lessons and insights relevant to modeling and characterizing other viruses.

Keywords: hepatitis C virus, vaccines, modeling, design, E1E2, glycoproteins, antibodies

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INTRODUCTION

Hepatitis C virus (HCV) is estimated to have infected over 70 million globally, with millions of new cases every year (1). Chronic HCV infection can lead to cirrhosis and hepatocellular carcinoma (HCC) and deaths due to HCV are rising worldwide (1). In the United States, the yearly rate of deaths resulting from HCV infection has surpassed that of human immunodeficiency virus (HIV) and other infectious diseases (2). Direct-acting antivirals (DAA) for treatment of HCV infection have high cure rates, but face major issues: limited patient accessibility due to high costs of treatment

(3), little to no awareness of infection in most HCV-positive individuals (4), and neither prevention of reinfection (5) nor elimination of HCC risk (6) in cleared HCV patients following DAA treatments. Thus, there is an ongoing major need for an effective prophylactic vaccine for HCV in order to greatly reduce global disease burden (4, 7).

A major barrier to vaccine and targeted therapeutic efforts is the high sequence variability of HCV, as exemplified by its seven confirmed genotypes, which are subdivided into 86 confirmed subtypes as of June 2017 (8) that can differ by greater than 15% in sequence (9). Furthermore, HCV rapidly mutates to form quasispecies within infected individuals, permitting active escape from neutralizing antibodies; this mechanism was clearly demonstrated in a clinical trial of monoclonal antibody HCV therapy followed by deep sequencing of HCV in patients (10, 11). Effective targeting of this diverse virus would be greatly facilitated by a detailed understanding of the molecular determinants of viral fitness, assembly, and function (12).

The envelope glycoproteins E1 and E2 are targets of anti-HCV antibodies (13), and have been used in numerous B cell vaccine development efforts (14–18) and several clinical trials (19, 20) [reviewed by Fauvelle et al. (21)]. Epitope mapping and other characterization efforts have classified E2 antibody epitopes into five antigenic domains (A–E) (22), a nomenclature that will be used in this review. Alternative definitions such as antigenic regions (antigenic regions 1–3) (23) and epitopes I–III (24) have been used to identify these regions on the E2 surface, in addition to epitopes on E1E2 (antigenic regions 4–5) (25). Despite advances from numerous epitope mapping studies, the overall structure of these glycoproteins and the structural basis of neutralizing antibody engagement of many key epitopes have yet to be determined experimentally. Some structures representing portions of these proteins have been determined to date, spanning a conserved "core" region of E2, portions of E1, and multiple mAb-bound E1 and E2 peptides (**Figure 1**; **Table 1**). In contrast, other highly variable viruses, such as HIV and influenza, have likewise been longstanding targets of vaccine design efforts, and the assembly of their envelope glycoproteins, hemagglutinin (HA), and Env have been determined at high resolution (26, 27). Additionally, there are many HA and Env neutralizing antibodies structurally characterized in complex with their epitopes (28–30), providing insights that enabled a number of successful structure-based vaccine design efforts (31–34). Given the relatively limited availability of HCV structural data, as well as the challenges for experimental structure determination presented by innate flexibility (22, 35, 36) and

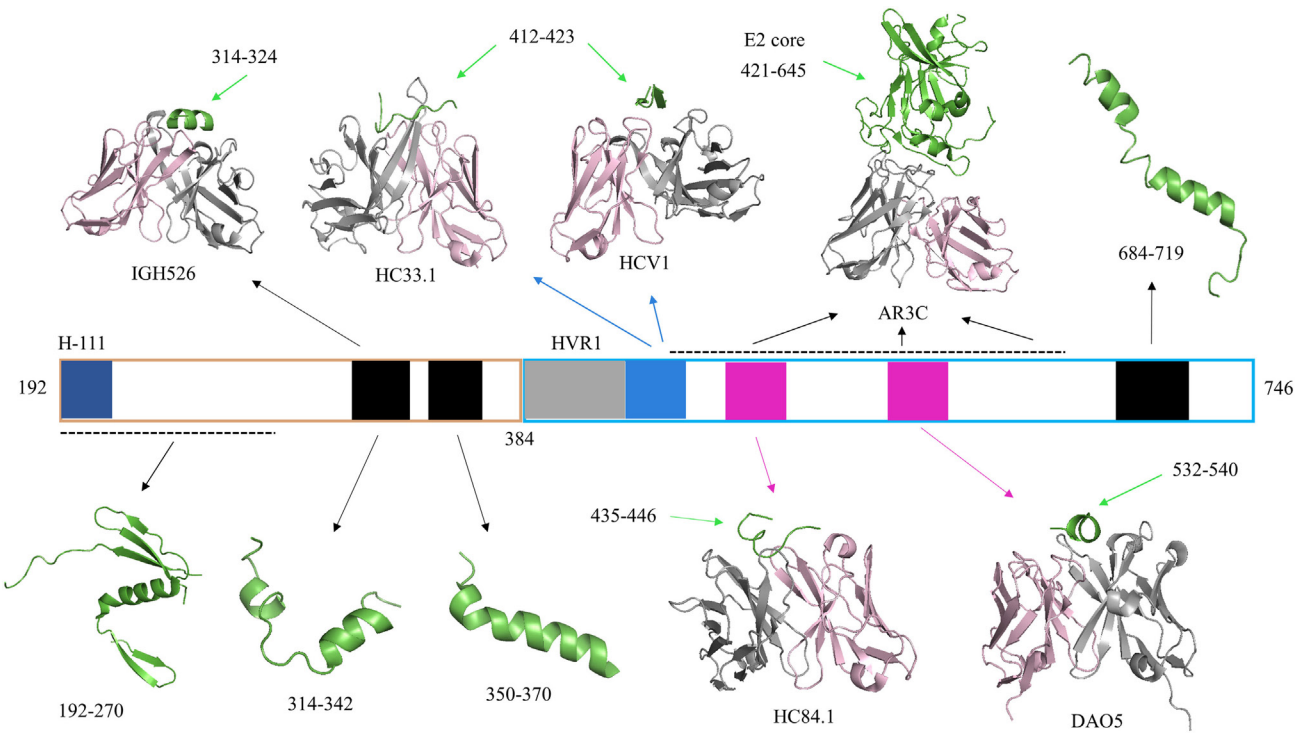

FIGURE 1 | Representative crystallographic and NMR structures of E1E2 regions. Hepatitis C virus (HCV) peptides and proteins in all structures are colored green. Antibody heavy chains are colored gray and light chains are colored light pink. PDB codes of representative structures are 4UOI (192–270), 4N0Y (314–324/IGH526), 2KNU (314–342), 1EMZ (350–370), 4XVJ (412–423/HC33.1), 4DGY (412–423/HCV1), 4MWF (421–645/AR3C), 4JZN (435–446/HC84.1), 5NPJ (532–540/DAO5), and 2KZQ (684–719). Residue ranges of the E1E2 sequence corresponding to specific sites are highlighted by colored bars for reference: H-111 epitope at N-terminus of E1 (aa 192–202, dark blue), E2 hypervariable region 1 (aa 384–410, gray), Domain E (aa 412–423, blue), Domain D/AR3 (aa 434–446, magenta), and Domain B/AR3 (aa 529–535, magenta). Other regions of E1 and E2 with corresponding structures are shown in black bars, and black dashed lines represent gaps in a crystal structure.

TABLE 1 | Experimentally determined structures of E1, E2, and monoclonal antibodies.

Structure code[a]	Hepatitis C virus (HCV) glycoprotein[b]	Residue range[c]	Antibody	Reference
X-ray crystallography				
4UOI	E1	192–270	–	(38)
4N0Y	E1	314–324	IGH526	(39)
4GAG	E2	411–424	AP33	(40)
4GAJ	E2	412–423	AP33	(40)
4GAY	E2	Unbound mAb	AP33	(40)
4DGY, 4DGV	E2	412–423	HCV1	(41)
4G6A	E2	412–423	AP33	(42)
4HS6	E2	412–423	MRCT10.362	(43)
4HS8	E2	412–423	hu5B3.v3	(43)
4WHT, 4WHY	E2	412–423	3/11	(35)
4XVJ	E2	412–423	HC33.1	(44)
5FGB	E2	417–421	HC33.4	(45)
5FGC	E2	415–423	HC33.8	(45)
5EOC	E2	412–422[d]	C2	(16)
5KZP	E2	412–423[d]	HCV1	(17)
5VXR	E2	412–423	MAb24	(46)
4MWF	E2	421–645[e]	AR3C	(47)
4WEB	E2	486–645	2A12	(48)
4Q0X	E2	434–442	mAb#12	(49)
4HZL	E2	430–442	mAb#8	(50)
4JZN	E2	435–446	HC84.1	(51)
4JZO	E2	436–446	HC84.27	(51)
5ERW	E2	438–446	HC84.26	–[f]
5ESA	E2	Unbound mAb	HC84.26	–[f]
4Z0X	E2	435–446	HC84.26.5D	(52)
5NPH, 5NPI, 5NPJ	E2	532–540	DAO5	(53)
3U6R	E2	Unbound mAb	1:7	(54)
4JVP	E2	Unbound nanobody	D03	(55)
Nuclear magnetic resonance				
1EMZ	E1	350–370	–	(56)
2KNU	E1	314–342	–	(57)
2KZQ	E2	684–719	–	(58)
Electron microscopy[g]				
5759	E2	384–717	AR3A	(47)
5760	E2	384–717	AR3A, AR2A	(47)
5761	E2	384–717	AR2A, CD81	(47)
8338, 8339, 8340	E2	412–645	AR1B, AR2A, HCV1	(36)

[a]Protein Data Bank (59) or EMDataBank (60) codes shown. Multiple codes are shown in cases with multiple entries reported from same study containing the same residue range and binding partner(s), corresponding to different crystallographic symmetry forms, electron microscopy reconstructions, or HCV isolate sequences.

[b]In the case of unbound antibody, glycoprotein target of antibody is given for reference.

[c]Residue numbering based on H77 isolate. For crystallographic structures, range reflects resolved residues present in coordinates.

[d]Cyclic epitope-based designs are present in these structures.

[e]This E2 core construct included engineered deletions of residues.

[f]The coordinates for these X-ray structures have been released in the PDB (59) but have no publications associated with them.

[g]These negative stain electron microscopy structures have resolutions of 16–30 Å, thus provide approximate envelopes for fitting high-resolution crystallographic or modeled structures.

high glycosylation (37) of HCV glycoproteins, there is a major opportunity to bridge gaps in knowledge of current structural and mapping data through computational structural modeling, enabling a comprehensive view of glycoprotein structure, recognition, and dynamics.

This review provides an overview of efforts to model HCV envelope structure and recognition, which have collectively yielded many valuable insights into this virus. These efforts include initial work to model the E2 structure, recent modeling of the full-length E1E2 heterodimer, and modeling focused on other aspects of HCV, such as the dynamics of epitopes and recognition of antibodies or coreceptors; a subset of these studies is summarized in **Table 2**. Models and hypotheses from these studies can be used to inform future experimental and computational modeling efforts, as well as structure-based design of effective vaccines.

MODELS OF THE E2 STRUCTURE

Prior to experimentally determined structures of the E2 glycoprotein, computational models were developed to predict its tertiary and quaternary assembly. These efforts used structures of flavivirus and alphavirus class II fusion proteins as modeling templates (61, 62). A crystal structure of the E2 glycoprotein of tick-borne encephalitis virus (PDB code 1SVB) (68) served as the main template for the first of these modeling studies, which was reported over 15 years ago (61). The authors predicted that E2 assembles into an elongated monomer and also described putative E2 homodimerization and a possible site of interaction with E1. Further analysis of this model found that the binding regions predicted for CD81 and multiple E2 mAbs were exposed epitopes on the modeled E2 surface. A more recent E2 modeling study was largely based on the structure of the Semliki Forest virus E1 glycoprotein (PDB code 2ALA) (69), with particular emphasis on shared secondary structure elements, and incorporated nine experimentally determined E2 disulfide bonds as modeling constraints (62). The resulting model included three predicted domains for E2, with domain I (the first in order of amino acid sequence) corresponding to a β-sandwich positioned between the other two domains and forming a tightly packed CD81-binding site that roughly corresponds to antigenic domains B, D, and E. As noted by the authors of the latter modeling study (62), these two E2 models are divergent in several regards, including their predicted disulfide bonds, predicted E2 oligomerization and degree of coverage of the E2 glycoprotein. Subsequent X-ray crystallographic determination of two E2 core crystal structures revealed features distinct from structurally characterized class II fusion proteins (70, 71), including more compactness than the classical three domain organization of class II fusion proteins, despite retaining its immunoglobulin β-sandwich domain (47). Overall differences in architecture presented a likely impediment to template-based modeling, notwithstanding potentially accurate prediction of certain features and secondary structure elements. Regardless, these E2 modeling studies were important first steps in characterizing HCV glycoproteins, providing useful testable hypotheses in the absence of an experimentally determined E2 structure.

MODELS OF E1E2 ASSEMBLY

Currently, no experimentally determined structure is available for the E1E2 complex, which has led to two recent studies that have presented structural models of this assembly (64, 65). For clarity, they will be referred to as E1E2-C and E1E2-F, after their

TABLE 2 | Representative modeling studies of hepatitis C virus envelope glycoproteins and receptors.

Target	Model	Methods[a]	Year	Reference
E2	Structure	Homology-based modeling	2000	(61)
E2	Structure	Homology-based modeling, disulfide mapping	2010	(62)
E2-CD81	Complex structure	Restraints-guided docking	2013	(47)
E2	Front layer dynamics	Molecular dynamics simulation	2016	(36)
E1E2 transmembrane	E1 trimerization, E1E2 heterohexamer	Docking with restraints	2015	(63)
E1E2	Structure	Evolutionary constraints-based structure prediction, homology-based modeling, experimental mapping residue constraints	2017	(64)
E1E2	Structure, high order assembly	Homology-based modeling, *ab initio* structure prediction, experimental mapping residue constraints, docking	2017	(65)
SR-BI	Structure	Homology-based modeling	2013	(66)
CD81-Claudin	Structure	Homology-based modeling, docking	2012	(67)

[a]*Summary of modeling methods used.*

respective first authors. A third E1E2 model has been proposed, but does not contain a complete heterodimer and, therefore, will not be discussed in detail (72). The E1E2-F and E1E2-C models were generated using distinct methodologies. The E1E2-C model was generated through mapping antibody epitopes with shotgun mutagenesis (73), residue contact prediction with evolutionary coupling analysis (74) supplemented by known contacts of the E2 core crystal structure (47), as well as β-sheet pairing predictions using the bbcontacts algorithm (75). The final E1E2-C model of the heterodimer was generated using the CNS suite (76) and a distance geometry simulated annealing protocol. The E1E2-F model was likewise generated using a detailed computational pipeline, while also ensuring that the model corroborated previous experimental findings. Prediction of the E1 structure combined a partial crystal structure of E1 (38) with structural homolog phosphatidylcholine transfer protein (PDB code: 1LN2) (77) in the Molecular Operating Environment program (78). E2 was modeled in the Robetta server (http://robetta.bakerlab.org/), which added missing loops and termini to the E2 core crystal structure. Following *ab initio* prediction and molecular dynamics (MD) simulations of E1 and E2 transmembrane regions (TMs), RosettaDock (79) was used to dock the E1 and E2 models to predict their heterodimeric assembly, followed by symmetric docking of the E1E2 model to form heterohexameric E1E2 models (trimers of E1E2).

Comparison of the E1E2-C and E1E2-F models reveals some similarities, but also major distinctions between them (**Figure 2**). Unsurprisingly, the E2 core region is mostly conserved between the two models, as both E1E2-C and E1E2-F incorporated residue contacts from existing E2 core structures. This conservation includes the overall arrangement of antigenic domains B, D, and E. However, the quaternary structure of the two models display striking differences, with a dramatic change of E1 orientation relative to E2. One notable difference is an inter-chain disulfide bond at C272–C452, which is proposed by E1E2-C on the basis of their antibody epitope mapping data, but is not present in E1E2-F. Additionally, E2 residues 546–547, which are associated with antigenic domain C as well as E1E2 mAb binding based on global epitope mapping studies (80, 81), are located at the predicted interface with E1 in E1E2-F but not E1E2-C. This site has been associated with E1E2 assembly in a recent screening effort, which found that a peptide from JFH-1 (aa 546–560 based on H77 numbering) inhibited HCV entry and bound E1E2 (82). Finally, there are differences in model coverage of E1 and E2 (E1E2-F represents the full glycoprotein sequences), as well as the conformations and orientations of the flexible region at the N-terminus of E2 (HVR1 and antigenic domain E). These models offer intriguing possible modes of E1E2 heterodimerization, providing an avenue to potentially design stabilized vaccines in the absence of an experimentally determined structure, and future studies can confirm (e.g., through structure-guided mutagenesis of predicted interface residues) or refine these models.

RECENT MODELING STUDIES OF E1 AND E2

Other studies have used existing crystal structures to explore conformational flexibility and assembly, capturing the dynamic properties of E2. Flexibility of the CD81-binding site (CD81bs) has been examined in a recent study using MD simulations, hydrogen–deuterium exchange (HDX), and calorimetry (36). The MD simulations suggested that the helical region near residue 434 displays a pronounced tendency to "drift" away from the E2 core, which is supported by crystallographic studies of multiple antibodies bound to the corresponding epitope of the peptide (49). Mobility of these regions has also been examined using an E2 core crystal structure plus modeled domain E, finding a broad range of conformations that occasionally resembled those observed in X-ray structures of the antibody-domain E complex (83).

Studies focused on modeling E1E2 TM domains have provided insights into determinants of E1E2 heterodimerization and assembly. Following descriptions of SDS-resistant E1E2 TM heterodimers and E1 trimers, a trimeric model of E1 TM domains was generated (63). This model was partially based on an experimentally determined structure of the monomeric E1 TM (PDB code: 1EMZ) (56) and also included constraints to enforce putative inter-helical interactions between G_{354}xxxG_{358} residues, a motif essential for E1 TM assembly and conserved in other helix–helix interactions (84). Critical charged and polar residues were exposed in the trimeric model, allowing E1 trimers to form key interactions with E2 such as the putative K370–D728 salt bridge, which was also observed in a separate study that performed MD simulations of the E1E2 TM heterodimer (85, 86). These studies

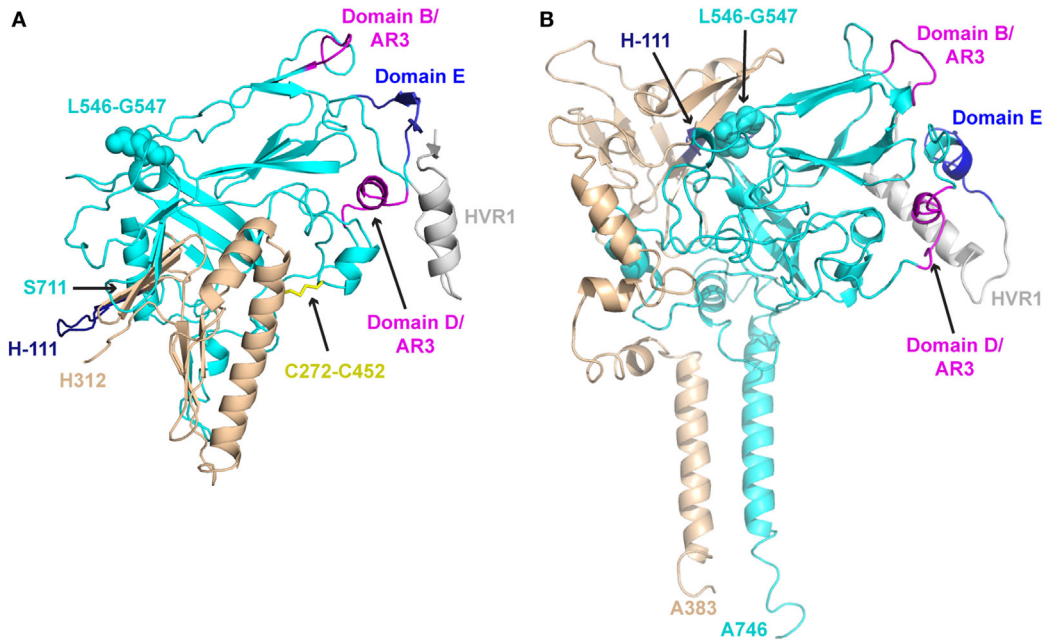

FIGURE 2 | Structural models of E1E2 heterodimeric assembly. **(A)** E1E2 model from Castelli et al. (E1E2-C) (64) in comparison with **(B)** E1E2 model from Freedman et al. (E1E2-F) (65), oriented in the same frame of reference based on E2 core regions. E1 and E2 glycoproteins are shown as tan and cyan cartoons, respectively, while key epitopes are colored and labeled, as in **Figure 1**: H-111 epitope at N-terminus of E1 ("H-111," aa 192–202, dark blue), E2 hypervariable region 1 (HVR1, aa 384–410, gray), Domain E (aa 412–423, blue), Domain D/AR3 (aa 434–446, magenta), Domain B/AR3 (aa 529–535, magenta). Additionally, selected features of modeled E1E2 are highlighted: the predicted E1–E2 disulfide bond of E1E2-C (C272–C452), shown as yellow sticks, and E2 residues L546–G547, predicted to interact with E1 in E1E2-F model, are shown in spacefill on both models. C-terminal residues of E1 and E2 are also labeled for both models (H312, S711 for E1E2-C, A383, A746 for E1E2-F).

and others (87, 88) have used modeling on this small yet critical region to gain a clearer picture of E1E2 association.

In combination with experimental mutagenesis data, modeling has been used to explore how residue substitutions affect glycoprotein stability and structural integrity. Using the program Rosetta, *in silico* alanine mutagenesis of all E2 residues available in one of the E2 core crystal structures predicted changes in protein stability for each mutant (80). Alanine mutants with greatest predicted destabilizing effects on E2 corresponded to those with experimentally measured loss of binding for 14 conformationally sensitive HCV mAbs during global alanine scanning mutagenesis of E2. In the same study, alanine scanning data from each mAb was analyzed by hierarchical clustering to form groups of residues that delineated energetically linked regions on the E2 surface and core. These studies highlight how the incorporation of experimental mutagenesis data and other techniques (e.g., HDX) with modeling methods can reveal key aspects of glycoprotein flexibility and structural determinants.

MODELING ANTIBODY RECOGNITION

Modeling conserved epitopes of HCV glycoproteins has been valuable for elucidating the structural basis of broadly neutralizing antibody (bnAb) recognition. Crystal structures for the domain E peptide (E2 residues 412–423) bound to HCV1 (41), HC33.1 (44), 3/11 (35), and AP33 (40, 42) established different conformations of the same conserved epitope. Understanding the structural basis of these variable conformations was critical for determining why rare domain E mutations evaded neutralization by some of these antibodies, but not all (43, 89). Computational alanine scanning of antigenic domain E bound to HC33.1 predicted a decrease in antibody affinity when key binding residues were mutated, but no change in affinity when a "glycan shift" viral escape mutation was modeled (44). The program GlyProt (90) was used to model E2 glycosylation in the HCV1 and HC33.1 complexes, showing that glycosylated N415 in domain E would be sterically unfavorable for binding by HCV1, which like AP33 engages the β hairpin form of the epitope, but it would be permitted at the exposed N415 residue in the extended conformation bound by HC33.1 (44). Additional modeling of domain E structures in the same study used the PEP-FOLD server (91) to generate *ab initio* peptide models that largely matched a β-hairpin conformation, suggesting that this folding pattern is preferred for domain E in the absence of antibody engagement and that this conformation can be disrupted by several domain E mAbs (35, 44). Computational mutagenesis and modeling not only helped to delineate domain E antibody recognition, but also domain D recognition by an affinity-matured antibody (52). These techniques can be used to build on structural knowledge of other antibody epitopes to E1, E2, or the E1E2 heterodimer, especially if similar crystal structures of antibody–antigen complexes provide informative comparisons.

MODELING RECEPTOR STRUCTURE AND RECOGNITION

Although many E1E2 modeling efforts have focused on antibody–antigen interactions or heterodimerization, some studies have examined the structures of host entry receptors and their interactions. The tetraspanin CD81 (92), scavenger receptor class B type I (SR-BI) (93), and tight junction proteins claudin-1 (CLDN1) (94) and occludin (OCLN) (95) represent the minimal set of HCV coreceptors and together are sufficient for HCV entry (96). Determinants of E1E2, glycoprotein–receptor, and receptor–receptor interactions are shown in **Figure 3**, summarizing current knowledge through high resolution or homologous protein structures that may inform prospective modeling studies. CD81 and SR-BI bind directly to E2 (92, 93) and CLDN1 associates with CD81 to permit HCV entry (97), but the basis of OCLN viral engagement is unknown. CD81 has been characterized the most among these receptors, due to its critical role in HCV entry, infection, and cell-to-cell transmission (98). Kong et al. modeled the CD81–E2 interface using restraints-guided docking using restraints-guided docking (47) with the HADDOCK modeling program (99), which incorporated mutagenesis data into structure prediction. The model was corroborated by a negative stain electron microscopy structure containing E2 and CD81 large extracellular loop (LEL) reported in the same study; the interface contained the CD81-LEL C and D helices, which are implicated in E2 binding (100). To validate this model experimentally, the authors generated E2 mutants based on their docking model that disrupted CD81 binding. A subsequent study (101) concentrated on the interface between CD81-LEL and antigenic domain D, using PEP-FOLD (91) to model the peptide and the AutoDock Vina program (102) for docking to a CD81-LEL crystal structure. CD81 MD have also explored CD81-LEL flexibility, and several crystal structures found pH-dependent conformational changes in these loops (103). The CD81-E2 interface could soon be resolved in greater detail through additional modeling or experimental studies, given that new CD81 crystal structures are available (103, 104) and that CD81 binding determinants on E1E2 have recently been fully delineated through global alanine scanning (81).

Although fewer modeling studies have focused on other HCV receptors, these provide important insights into the structure and recognition of these molecules. SR-BI does not have a reported X-ray structure, making its interactions with E2 relatively challenging to model with protein docking methods. However, the crystal structure of the closely related LIMP-2 (PDB code: 4F7B) led to a homology model of SR-BI, which was then used to elucidate the structural basis of its role in cholesterol uptake (66). Related scavenger receptor CD36 also has a crystal structure available (PDB code: 5LGD) (110), and was recently proposed as an additional coreceptor that binds E1 (111). Several studies have examined the structural determinants of the CLDN1–CD81 interface (97, 112, 113). *In silico* mutagenesis of this interface revealed key binding residues (67), and MD simulations of CLDN1 point mutations showed disruptions of receptor structure thought to diminish HCV entry (114). There is no reported X-ray crystal structure of CLDN1, but several claudin family members have solved structures (115, 116).

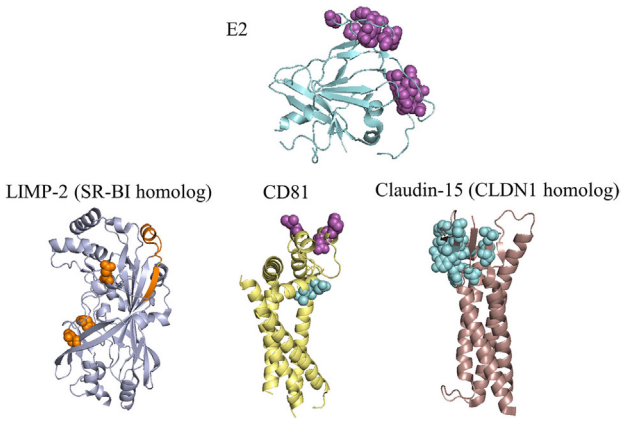

FIGURE 3 | Residues of E2 and coreceptors that influence hepatitis C virus (HCV) entry and infection. E2 and three receptors are depicted with the most complete crystal structure available, or with a crystal structure of a homologous receptor. Purple spacefill residues on E2 showed <20% binding to CD81 when substituted to alanine (81, 105); residues with the same color and representation on CD81 showed reduced or eliminated binding to soluble E2 during random mutagenesis (100). Orange spacefill residues on LIMP-2 showed reduced binding to soluble E2 when mutated to a non-synonymous coding variant or the corresponding residue for mouse SR-BI (106, 107). Binding determinants of E2 to SR-BI are present in HVR1 (108), and are not present on the E2 crystal structure. Cyan spacefill residues on CD81 showed reduced association with CLDN1 when mutated to alanine (67), while cyan spacefill residues on CLDN1 showed either reduced binding to CD81 or decreased entry of HCVpp in alanine substitutions (67, 97, 109). PDB codes used are: 4MWF (E2), 5TCX (CD81), 4F7B (LIMP-2, representing SR-BI), and 4P79 (mouse claudin-15, representing claudin-1). SR-BI and CLDN1 have only moderate sequence identities to their structurally characterized homologs (LIMP-2 has 34% identity with SR-BI, mouse claudin-15 has 35% identity with human CLDN1), thus structures of these receptors may differ from the homologs shown.

DISCUSSION

Given the numerous unknown aspects of the structural basis of HCV envelope glycoprotein assembly, as well as uncertainties regarding antibody and receptor recognition, there is a unique opportunity to leverage modern computational modeling and design algorithms to provide insights and testable mechanistic hypotheses for this system. Based on the challenges inherent in modeling this unique and dynamic viral envelope, future studies can utilize iterative experimental, and modeling approaches, where data-driven modeling is validated through experiments suggested by a model or sets of models. This paradigm has been utilized in previous studies to select and confirm models of antibody–antigen complexes (117, 118), as well as a modeled coiled coil assembly (119).

One additional area of recent interest has been the use of computational structure-based methods to design optimized protein and epitope-based immunogens for vaccines to better engage and elicit neutralizing antibodies, also known as "reverse vaccinology" (120). As seen for modeling, recent work has shown that iterative computational and experimental approaches are quite effective for vaccine design (121). Some have noted that HCV is a promising

potential target for structure-based vaccine design (122), and early efforts have shown promise (15–17). Such work includes the design of scaffolded constructs based on the β hairpin form of antigenic domain E and an epitope from E1 (aa 314–324), and the display of these designs on protein nanoparticles, which showed maintained binding to the epitope-specific antibodies HCV1 and IGH526. In another study, a cyclic peptide design based on antigenic domain E, stabilized with a disulfide bond, was found to be immunogenic in mice; the X-ray structure of an induced murine antibody in complex with this design was determined (16), but no neutralizing antibodies were detected. A more recent vaccine design study reported two other cyclic antigenic domain E peptide designs, as well as a design of E2 with two copies of the antigenic domain E epitope based on structural similarity of a site on the E2 back layer to the β hairpin domain E structure (17). These designs elicited neutralizing antibodies in mice, but varied in H77 neutralization potency and showed limited response to the two non-H77 isolates tested (17). Follow-up studies as well as additional novel designs are needed to demonstrate the potential of rational vaccine design approaches for this virus. Furthermore, though cellular immunology is outside the scope of this review, fine mapping and molecular characterization of T cell epitopes may provide useful information to optimize vaccine constructs that will enhance or focus cellular immune responses, possibly in the context of a B-cell-based vaccine. The recently described structure of a T cell receptor engaging an immunodominant epitope from the HCV NS3 protein (123) is a compelling example for such a strategy.

The increasing application of powerful computational structural modeling techniques has led to a number of insights into HCV and its envelope glycoproteins. With the rapidly growing amount of data, including epitope mapping, structural characterization, and immune repertoire sequencing (124), there will be many opportunities to utilize these methods, to contribute further to the understanding of HCV immunogens, and to design an HCV vaccine. Centralized and up-to-date databases, resources, and standards for those focused on HCV research should facilitate these efforts. Effective resources may be analogous to a database developed for HIV bnAbs (125) or an existing database on HCV sequences and immunology (126). These resources will in turn permit the development of improved algorithms, more accurate models, and additional collaborative efforts focused on elucidating the native assembly and key features of the HCV envelope and eradicating HCV through an effective vaccine.

AUTHOR CONTRIBUTIONS

Both authors wrote and edited this work, and approved it for publication.

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Mapping Determinants of Virus Neutralization and Viral Escape for Rational Design of a Hepatitis C Virus Vaccine

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Hepatitis C virus (HCV) continues to spread worldwide with an annual increase of 1.75 million new infections. The number of HCV cases in the U.S. is now greater than the number of HIV cases and is increasing in young adults because of the opioid epidemic sweeping the country. HCV-related liver disease is the leading indication of liver transplantation. An effective vaccine is of paramount importance to control and prevent HCV infection. While this vaccine will need to induce both cellular and humoral immunity, this review is focused on the required antibody responses. For highly variable viruses, such as HCV, isolation and characterization of monoclonal antibodies mediating broad virus neutralization are an important guide for vaccine design. The viral envelope glycoproteins, E1 and E2, are the main targets of these antibodies. Epitopes on the E2 protein have been studied more extensively than epitopes on E1, due to higher antibody targeting that reflects these epitopes having higher degrees of immunogenicity. E2 epitopes are overall organized in discrete clusters of overlapping epitopes that ranged from high conservation to high variability. Other epitopes on E1 and E1E2 also are targets of neutralizing antibodies. Taken together, these regions are important for vaccine design. Another element in vaccine design is based on information on how the virus escapes from broadly neutralizing antibodies. Escape mutations can occur within the epitopes that are involved in antibody binding and in regions that are not involved in their epitopes, but nonetheless reduce the efficiency of neutralizing antibodies. An understanding on the specificities of a protective B cell response, the molecular locations of these epitopes on E1, E2, and E1E2, and the mechanisms, which enable the virus to negatively modulate neutralizing antibody responses to these regions will provide the necessary guidance for vaccine design.

Keywords: hepatitis C virus, vaccine design, epitopes, virus neutralization, antigenic domains, human monoclonal antibodies

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INTRODUCTION

Estimation of worldwide prevalence of hepatitis C virus (HCV) infections ranged widely from 71 to 185 million people (1, 2) and roughly 400,000 will die annually from HCV associated liver failure and hepatocellular carcinoma (2). An estimated three million people are living with HCV infection in the United States, and there is an annual infection rate of 34,000 new infections (3). A contributing

factor is the consequence of an opioid epidemic that shows no signs of slowing down and is unfortunately associated with increased injection drug use as a major mode to consume illicit opioids. When needles are shared by injection drug users, the risk of becoming infected with HCV increases. Of young adults (≤30 years) living in non-urban areas of states in the central Appalachia area infected with HCV, 73% had a history of injection drug use (4). Data from 2006 to 2012 in some states from these areas showed an astonishing 364% increase in infection amongst young adults. This was primarily in non-minority communities, with non-urban areas increasing from approximately 1.3–4.3 cases per 100,000 population while urban areas increased from 0.3 to 1.5 cases per 100,000 during this 6-year period (4). Because of this alarming rate of HCV infection and its potential lethality, the need for the development of a preventive vaccine is evident. It should be noted that effective direct acting antiviral drugs are available to treat HCV infections. However, susceptibility to reinfection after cure (5) and their prohibitive cost will limit their utility to contain this epidemic.

Vaccination is a powerful and proven method for infection prevention against many pathogens and a vaccine that is effective, accessible, and affordable is needed to control the further spread of HCV. This has been a difficult task because this virus is able to rapidly mutate and escape from protective immune responses. An understanding of what elements of a vaccine are needed and what challenges there are to guide vaccine design are discussed in this review. The focus is on the identification and functional characterization of conserved epitopes that elicit broadly neutralizing antibodies. We will review different immunogenic regions in the virus envelope E2 and in the covalently linked E1 and E2 heterodimer glycoproteins, and the challenges posed by regions of sequence diversity that contribute to viral escape from protective immunity. Collectively, the information gained will form the basis of rational structure-guided design of B cell epitopes in a reverse vaccinology approach to be included in the development of a preventative HCV vaccine (6–8).

TRAITS OF AN EFFECTIVE HCV VACCINE

While there is some debate whether B cell versus T cell responses are necessary for an effective HCV vaccine (9), a brief review of these responses during acute infection suggests that both arms of immunity will be required. During acute HCV infection, 20% of infected individuals will clear infection spontaneously while 80% develop a chronic infection. Spontaneous viral clearance has been associated with robust CD4+ and CD8+ responses that are maintained for several years after the virus has cleared. Losing the robust CD4+ response results in disease progression, hence the importance of the T cell response (10). Neutralizing antibodies elicited during acute infection also appear to contribute to spontaneous clearance. Early and strong neutralizing antibody responses were closely associated with HCV clearance (11, 12). Once viral clearance is achieved, neutralizing antibody levels either decrease or disappear. Those with absent or low serum neutralizing antibody levels during early infection and, therefore, with a delayed neutralizing antibody response pattern tend to develop chronic infections. Knowing that strong and early cellular and humoral immune responses to acute HCV infection is critical for spontaneous clearance, the implication is that both arms of a protective immune response are required to be induced in an effective vaccine design.

Experiences obtained from HIV vaccination studies indicate that antibody-dependent cytotoxicity (ADCC) could be an important determinant of protection (13). While it has been shown that certain subsets of natural killer (NK) cells are associated with disease progression and treatment outcome in chronic HCV patients (14), very little is known about the role of these effector cells during the acute phase of HCV infection. Both, NK cells and the highly abundant Kupffer cells are able to mediate ADCC. Recent publications suggest that ADCC mediated by non-neutralizing antibodies might be impaired in chronic HCV patients potentially due to increased cleavage of CD16 by host cell proteases (15, 16). However, the role of ADCC during acute HCV is only poorly studied. It remains to be determined whether ADCC contributes to viral clearance and might serve as an important determinant for antiviral protection. A key issue is the lack of clear evidence of infected cells expressing surface HCV-encoded antigens.

BROADLY NEUTRALIZING ANTIBODIES TO HCV E2 TARGET EPITOPES CONTAINING VARIABLE AND CONSERVED REGIONS

Broadly neutralizing monoclonal antibodies (MAbs) are an important guide for vaccine design for HCV and other highly variable viruses. These MAbs are directed mainly at epitopes in the E2 glycoprotein (17). The majority of human MAbs (HMAbs) isolated from infected individuals are to conformational epitopes in E2 and can be grouped in distinct clusters of overlapping epitopes. Two commonly employed nomenclatures are clusters designated as antigenic domains (18) or antigenic regions (AR) (19, 20). While there is substantial overlap between these two sets of overlapping epitope clusters, there are differences. The antigenic domains, A–E (**Table 1**) and AR1-3 (**Table 2**) are restricted

TABLE 1 | Immunogenic clusters as defined by antigenic domains.

Antigenic domain	Epitope location and key elements
Hypervariable region 1	384–410, mainly isolate-specific linear epitopes mediating neutralization with some interacting with SR-B1
E	412–423, mainly linear epitopes mediating broad neutralization
D	420–428, 441–443, 616, conformational epitopes on surface layer mediating broad neutralization with a residue 616 located in the back layer
B	431–439, 529–535, conformational epitopes mediating broad neutralization located on the surface layer
C	Conformational epitopes mediating broad neutralization located in part at residues 544–549 that is in the central beta sandwich (aa 492–566)
A	581–584, 627–633 conformational epitopes located on the back layer and mediating non-neutralizing antibodies

TABLE 2 | Immunogenic clusters as defined by antigenic regions.

Antigenic region	Epitope location and key elements
1	E2 non-neutralizing face involving residues 495, 519, 544, 545, 547, 548, 549, and 632
2	E2 back layer region involving residues 625 and 628
3	E2 neutralizing face involving residues 427–443, 529–530. Residues 459, 499, 503, 558, and 616 influence folding of front layer, and residues 424, 425, 517, 518, 520, 523, 535, 536 influence folding of CD81-binding loop
4	E1E2 interface with specific residue 698
5	E1E2 interface with specific residue 639 and 665

to epitopes on E2. Two other ARs, AR4 and AR5, are clusters of epitopes requiring key residues on both E1 and E2 (**Table 2**). Generally, neutralizing MAbs, both human and mouse, are to epitopes on the exposed surface of E2 (**Figure 1**). The neutralizing HMAbs directed at epitopes within antigenic domains B, D, and E include key residues on E2 (residues W420, Y443, and W529 in **Figure 1**) that are also involved in virus binding to the HCV tetraspanin co-receptor, CD81, and thus mediate virus neutralization by blocking virus interaction with this required receptor for virus entry (21, 22). These epitopes are mostly conserved, which explains the wide breadth of virus neutralization of their associated antibodies. Antigenic domain B is also highly immunogenic and antibodies to this region are commonly found in the sera of HCV-infected individuals (21). Epitopes within domains B and D do overlap with shared contact residues in the 441–443 region [but antigenic domain D epitopes do not have residues 529–535 (**Table 1**)] and form a supersite of conformational epitopes on the exposed surface of the E2 core structure that contributes to CD81 binding (23, 24). Residues within antigenic domain B participating in virus binding to CD81 include 529, 530 and 535 (21). For antigenic domain D epitopes, the 441–443 region is involved in CD81 interaction (25). In the AR system, both antigenic domain B and D are included in AR3 (**Figure 1**; **Table 2**). Another highly conserved region is antigenic domain E located just downstream of hypervariable region 1 (HVR1) at a E2 segment encompassing residues 412–423 that also is responsible for broadly neutralizing antibodies (**Table 1**) (22, 26, 27). Antibodies to domain E are directed at mainly linear epitopes that include residue 420, which has been shown to be needed in virus binding to CD81 (28). A key residue that affects a broadly neutralizing HMAb, HC33.4 to domain E, is located at 408, within HVR1 (21, 22). Taken together, the ability to elicit antigenic domain B, D, and E antibodies will be important for vaccine design as these antibodies will prevent virus entry. Non-neutralizing antibodies are to overlapping epitopes on the back layer of E2 (**Figure 2**) and mainly in antigenic domain A (**Table 1**) and AR1 (**Table 2**). A shared residue for all domain A epitopes is 632. Interestingly, antigenic domain C, which mediates broad neutralization, is located in part at residues 544–549 in the central beta sandwich (residues 492–566), a region that is flanked by the front and back layers of the E2 core structure. Although some antigenic domain C residues are shared with AR1, domain C epitopes are exposed on the virus surface to allow access for antibody binding that leads to virus neutralization (**Tables 1** and **2**; **Figure 2**). This is further supported by the observation of overlap between the epitopes of CBH-7, a neutralizing antibody to domain C, and AR5A, a broadly neutralizing HMAb to E1E2 (19, 20). Global E2 and E1E2 antibody epitope mapping studies from Foung and Law lab groups have been described (29, 30).

Hepatitis C virus has multiple variable regions in E2 that aid the virus in evading from protective immunity. They include the HVR1 located at the N-terminus encompassing residues 384–410, hypervariable region 2, residues 460–485 and the intergenotypic variable region, residues 570–580. The HVR1 downmodulates protective immunity by at least three mechanisms. Although antibodies to this region are neutralizing, rapid mutation occurs leading to escape variants without compromised viral fitness that stay one step ahead of the antibody response (32). Another mechanism is shown with infectious recombinant cell cultured virions, HCVcc, with and without HVR1. Broadly neutralizing HMAbs neutralized HCVcc without HVR1 with greater potencies than against wild-type HCVcc (33, 34). Thus, it can be argued that a HVR1-deleted vaccine antigen could help boost broadly neutralizing antibody responses. *In vitro* studies with infectious HCV variants also help to define the region of HVR1 that is important for virus interaction with another HCV co-receptor, scavenger receptor class B type 1, SR-B1, and the region responsible for rapidly escaping variants to neutralizing antibodies (35).

Less is known about the immunogenic regions on E1 and E1E2. While a few HMAbs to E1 have been described (36, 37), two have been shown to exhibit broad virus neutralization (36). Two regions, AR4 and AR5, require contact residues on both E1 and E2, and some of these antibodies mediate broad virus neutralization (20). HMAbs to AR4 and AR5 do not mediate neutralization by blocking virus binding to CD81 but are postulated to inhibit conformational changes to the E1E2 heterodimer during viral entry. Because E1 has a relatively high degree of conservation within different genotypes and subtypes, elicited antibodies to E1 tend to exhibit broad neutralization. For these reasons, it is probable that vaccine constructs composed of both E2 and E1 in the form of heterodimers will be superior than vaccine constructs composed of E2.

VIRAL ESCAPE IN THE DEFINED REGIONS OF THE EPITOPE

The elusive virus is able to escape from the immune containment, in part due to its high rate of mutation and driven by presence of neutralizing antibodies. These mutations can occur either in areas directly targeted by neutralizing antibodies, or not directly targeted. CBH-2 is a neutralizing HMAb to an epitope within the antigenic domain B cluster. As discussed earlier, the epitope for CBH-2 has residues that participate in virus binding to CD81. However, there are at least two regions in E2 that are required to form the CBH-2 epitope. A conserved region located at 529–535 and a region at 425–431 with some variability. A single amino acid substitution at 431 results in viral escape from CBH-2 and a variant without compromised viral fitness (38). In contrast, another domain B antibody, HC-11 also requires these two regions to

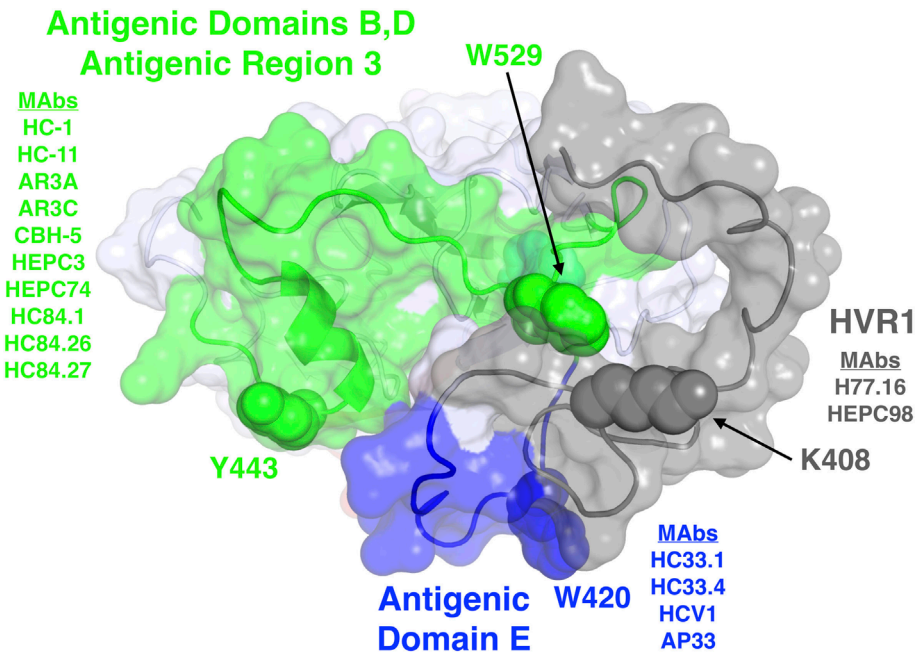

FIGURE 1 | Structure and mapped epitope sites on the front layer, or neutralizing face, of E2. Structure shown is the E2 core crystal structure (23), with N-terminal residues corresponding to hypervariable region 1 (HVR1) and antigenic domain E (residues 384–423), which are mostly absent or unresolved from the E2 core crystal structure, modeled using the FloppyTail protocol in Rosetta (31) and shown for reference. Epitope sites are colored according to antigenic domains/regions or HVR1, with shared key epitope residues shown as spacefill and labeled. The remainder of E2 is shown as light gray. Representative monoclonal antibodies with binding residues mapped to each domain or HVR1 are also shown.

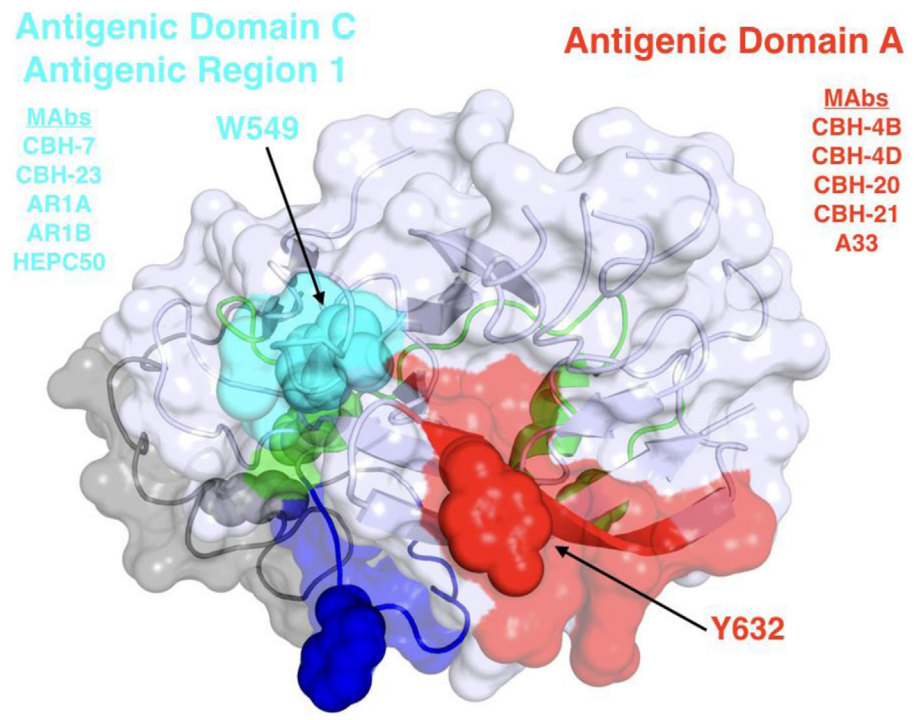

FIGURE 2 | Structure and mapped epitope sites on the back layer and proximal β sandwich residues of E2. The structure corresponds to a distal face of the E2 core structure shown in **Figure 1**, with antigenic domains/regions labeled and representative monoclonal antibodies listed.

form the targeted epitope but the derivative escape variant with a mutation at 438 has reduced fitness. A third domain B HMAb, HC-1, is not associated with viral escape and its epitope has not been defined beyond the conserved region 529–535 (39).

Another region of great interest for vaccine development is the E2 segment 412–423 that is highly conserved. The importance of this region was first recognized by studies with a murine MAb, AP33 that recognizes mainly a linear epitope at 412–423 (40). However, a mutation at N417 that leads to a glycan shift to N415 will result in a robust variant able to escape neutralization by AP33 (41). A similar mechanism of escape has been documented with an HMAb designated as HCV1 (42). A different HMAb, HC33.1, to this region, interestingly, has a completely different neutralization profile. The variant with a N417 glycan shift is more sensitive to be neutralized than wild-type virus HC33.1 (41, 43). Structural studies revealed that this conserved E2 region, designated as antigenic domain E is highly flexible (43, 44). The implication for vaccine design is to stabilize domain E in a conformation that mirrors the HC33.1-bound epitope and not AP33 nor HCV1.

VIRAL ESCAPE MUTATIONS OUTSIDE OF THE DEFINED EPITOPES

Mutations that occur outside of epitope and receptor binding sites can also result in structural changes that will lead to escape from broadly neutralizing antibodies (45). These mutations lead to reduced efficiency of broadly neutralizing antibodies targeting different ARs, although viral fitness and efficiency of CD81 interaction are also reduced. Another example is studies with HC33.4 and AR4A, two neutralizing HMAbs to epitopes without any overlap and with different mechanisms of virus neutralization. The key binding region of HC33.4 is to the amino terminus of E2, 412–423, while for AR4A, it is the carboxy terminus of E2 along with residues on E1 to form its epitope (20, 22). HC33.4 is able to neutralize HCV by blocking interaction with CD81 and AR4A mediates neutralization by presumably the inhibition of E1E2 conformational change associated with viral entry. Unexpectedly, both antibodies have similar neutralization profiles against a large panel of genotype 1 isolates and both antibodies poorly neutralize variants with mutations at 403, a residue not known to be involved in the non-overlapping epitopes of these two HMAbs (46). Thus, extra-epitopic mutations need to be considered as well when discussing mutations that lead to viral escape. Escape mutations are especially important for HCV infected individuals receiving a liver transplant. Once the patient receives a new liver, reinfection of the liver by HCV is common as the virus is still able to avoid the immune system. Indeed, several of the escape variants isolated from patients undergoing liver transplantation were found to be located outside the defined epitopes. Many of these variants, selected post-liver transplantation, contained mutations within the CD81 binding domains II and III (47).

VIRAL ESCAPE MUTATIONS BY ALTERED RECEPTOR DEPENDENCY

Accumulating evidence suggests that HCV can escape from broadly neutralizing antibodies that target envelope receptor-binding sites by altering the dependency on viral cell entry factors CD81 and SR-B1. For HCV, this has been first demonstrated for a cell culture adapted JFH1 variant that was characterized by an increased affinity for CD81: a mutation of E2 G451 to R resulted in increased E2 binding affinity to CD81 and at the same time reduced the dependency on the entry factor SR-BI. The mutant virus showed highly increased sensitivity to neutralizing anti-envelope antibodies (48). A similar effect has been observed for L403F and L438V that modulate resistance to the HMAbs HC33.4 and AR4A by altering E2 binding to SR-B1, as discussed above (46). Furthermore, engineered mutations of the E2 protein revealed nine different polymorphisms that were associated with reduced SR-B1 dependency and increased resistance against a panel of neutralizing antibodies (49). This indicates that escape *via* changes in SR-B1 binding efficiency is a relevant motif for HCV escape variants. A different mechanism has been observed for E2 residues 447, 458, and 478 that have been identified in clinically relevant transplant escape variants as important determinants for susceptibility to neutralizing antibodies (50). Mutations F447L, S458G, and R478C conferred resistance against conformational HMAbs CBH-2, CBH-5, CBH-23, and HC1 by altering the interaction of E2 with cell surface CD81. Furthermore, increased entry efficiency might also contribute to viral escape of these clinically relevant patient variants. Thus, changes in receptor usage by the HCV envelope protein is an important mechanism for viral escape that needs to be addressed in prospective vaccine studies or immunotherapeutic approaches using anti-E2 antibodies.

To address this challenge, antibodies targeting different HCV entry factors have been proposed for immunoprevention (51–56). More specifically, antibodies targeting SR-B1, CLDN1, and CD81 have demonstrated broad antiviral effects against HCV infections and have been suggested for the prevention of liver graft infection complementary to treatment with direct-acting antivirals (57). Single treatment with antibodies or small molecule inhibitors targeting viral receptors can potentially result in escape mutations as it has been reported for the SR-BI antagonist ITX-5061 (58) or during a clinical trial of an anti-CD4 monoclonal antibody targeting HIV-1 infection (59). Due to the reduced rate of transmission and the increased barrier for escape mutations, targeting of multiple entry host factors likely prevent viral escape due to changes in receptor tropism. Interestingly, when neutralizing antibodies that inhibit virus interaction against different co-receptors are tested simultaneously (as discussed more extensively in the next section), there can be antagonism or synergy in their combined effect (60, 61). Taken together, simultaneous targeting of multiple factors can lead to synergistic inhibition of infection (62), further strengthening this approach.

ATTRIBUTES OF HVR1 THAT SHOULD BE CONSIDERED IN VACCINE DESIGN

There are functions of HVR1 (aa 384–410) located at the N-terminus end of E2 that argue against and for its retention in vaccine design. It is highly immunodominant and virtually all HCV infected individuals will have serum antibodies to HVR1. The high rate of mutations in this region helps the virus escape

protective antibody responses to this region by first serving as an immunological decoy (63). Second, it physically shields the more conserved antigenic domain B and D regions in E2 (33, 64). HVR1 partially blocks broadly neutralizing domain B and D antibodies from binding to their respective epitopes; removal of HVR1 leads to a defective virus that is more susceptible to these antibodies. Third, we recently proposed a new mechanism in which HVR1 adversely affect the function of broadly neutralizing antibodies (65). When some antigenic domain E HMAbs, e.g., HC33.4, were more extensively mapped, a residue located in HVR1 at 408 was found to affect HC33.4 binding to E2. This raised the possibility that when an anti-HVR1 antibody is bound to E2, it can interfere with the binding of HC33.4 and other domain E HMAbs. This was found to be the case as postulated with H77.16, a murine neutralizing MAb to HVR1 (60). Surprisingly, the binding of H77.16 also inhibited the binding and neutralization of antigenic domain B and D HMAbs. Additional studies showed that this interference is by steric hindrance and collectively supported the hypothesis that an anti-HVR1 response can interfere with more protective neutralizing antibody responses. Based on these observations, vaccine design should be based on a HVR1-deleted E1E2 immunogen to increase the production of broadly neutralizing antibody responses.

However, HVR1 also facilitates viral entry cells by interacting with SR-B1 and thus allowing attachment and eventual entry of the host cell *via* CD81. Although the majority of anti-HVR1 antibodies are isolate-specific, some antibodies to this region have broad neutralization profiles. J6.36, H77.39, and H77.16 are murine MAbs that target regions within HVR1 (**Figure 1**) (60). H77.39 inhibited binding of HCV to CD81 and SR-B1. While both J6.36 and H77.16 block E2 attachment to SR-B1, J6.26 also reduces E2 binding to CD81 (60). Binding studies revealed that H77.39 and H77.16 are reactive to all six major HCV genotypes. Therefore, antibodies to HVR1 could play a key role in vaccine design for their abilities to have a wide breadth of neutralization and for their ability to block SR-B1 attachment. This perspective has been supported by a recent study that shows a similar antibody to H77.16, HMAb HEPC98 (**Figure 1**), synergistically neutralize HCVcc when combined with a HMAb, HEPC74 that maps to an antigenic domain B region (61). Their observation contrasts with the observation that H77.16 when combined with other antigenic domain B or D antibodies led to antagonism (65). These contrasting results are likely the result of greater spatial separation between E2-bound HEPC98/HEPC74 relative to E2-bound H77.16/HC-11.

CONCLUSION

This review outlines the functional organization of antigenic domains and epitopes within each domain that are associated with escape or are more invariant and essential for viral fitness, receptor binding, and viral entry. The findings raise the possibility of antagonistic relationship between immunogenic decoys, e.g., HVR1, which elicit antibodies associated with escape and may be responsible for antibody-mediated interference in the protective antibody response. Collectively, these studies begin to create a high-resolution map of conserved neutralizing epitopes not associated with viral escape and how other antigenic domains serve as diversions of the immune response and are able to elicit antibodies that negatively modulate neutralizing antibodies. Having this information, a future direction is to employ the knowledge in vaccine design. One approach is to stabilize flexible regions on E2 known to encode broadly neutralizing epitopes to be more favorable to antibodies that are not associated with rapid escape. For example, antibodies to the E2 region comprising residues 412–423 have broad neutralizing activities. However, an adaptive mutation in this linear epitope, Asn417Ser, is associated with a glycosylation shift from Asn417 to Asn415 that enables HCV to escape neutralization by MAbs such as HCV1 and AP33. By contrast, the HMAb HC33.1 can neutralize virus bearing the Asn417Ser adaptive mutation. Structural studies showed that $E2_{412–423}$ when bound by this antibody has a distinct structure than either AP33 or HCV1 (44). The results highlight the structural flexibility of the $E2_{412–423}$ epitope, which may serve as an evasion mechanism to reduce antigenicity. It is probable that other E2 regions with similar structural flexibility impede the induction of neutralizing antibodies by a similar mechanism.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Hypervariable Region 1 in Envelope Protein 2 of Hepatitis C Virus: A Linchpin in Neutralizing Antibody Evasion and Viral Entry

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Chronic hepatitis C virus (HCV) infection is the cause of about 400,000 annual liver disease-related deaths. The global spread of this important human pathogen can potentially be prevented through the development of a vaccine, but this challenge has proven difficult, and much remains unknown about the multitude of mechanisms by which this heterogeneous RNA virus evades inactivation by neutralizing antibodies (NAbs). The N-terminal motif of envelope protein 2 (E2), termed hypervariable region 1 (HVR1), changes rapidly in immunoglobulin-competent patients due to antibody-driven antigenic drift. HVR1 contains NAb epitopes and is directly involved in protecting diverse antibody-specific epitopes on E1, E2, and E1/E2 through incompletely understood mechanisms. The ability of HVR1 to protect HCV from NAbs appears linked with modulation of HCV entry co-receptor interactions. Thus, removal of HVR1 increases interaction with CD81, while altering interaction with scavenger receptor class B, type I (SR-BI) in a complex fashion, and decreasing interaction with low-density lipoprotein receptor. Despite intensive efforts this modulation of receptor interactions by HVR1 remains incompletely understood. SR-BI has received the most attention and it appears that HVR1 is involved in a multimodal HCV/SR-BI interaction involving high-density-lipoprotein associated ApoCI, which may prime the virus for later entry events by exposing conserved NAb epitopes, like those in the CD81 binding site. To fully elucidate the multifunctional role of HVR1 in HCV entry and NAb evasion, improved E1/E2 models and comparative studies with other NAb evasion strategies are needed. Derived knowledge may be instrumental in the development of a prophylactic HCV vaccine.

Keywords: hepatitis C virus (HCV), hypervariable region 1 (HVR1), viral entry, vaccine design, neutralization

INTRODUCTION

It is estimated that at least 2 million people become infected with hepatitis C virus (HCV) every year (1). The majority of these individuals will develop chronic infections adding to the more than 71 million chronically infected people worldwide, who are consequently at increased risk of developing liver diseases, such as cirrhosis and hepatocellular carcinoma (1, 2). HCV-related mortality is estimated at 400,000 people every year, and although direct-acting antiviral therapies with cure-rates >95% are now available, treatment is often not accessible for multiple reasons,

including frequent occult infection and high cost (3, 4). Thus, the development of a prophylactic vaccine is required to control HCV worldwide, but this challenge has proven difficult owing in part to the complex measures HCV employs to avoid the host immune responses (5).

HCV is an enveloped, positive-stranded RNA virus of the *Hepacivirus* genus in the *Flaviviridae* family (6, 7). The genome is ∼9.6 Kilobases and encodes 10 functional viral proteins from a single polyprotein. Virus structural proteins form part of the virus particle with the Core protein assembling into the viral capsid that protects the HCV genome, and envelope proteins 1 and 2 (E1 and E2) imbedded in the viral envelope as the heterodimeric glycoprotein complex, E1/E2 (8, 9). *In vitro* systems for studying the role of E1/E2 in HCV entry and neutralization have been developed. Cell culture infectious HCV (HCVcc) can be produced in cell lines of hepatic origin and yields particles that share many similarities with *ex vivo* derived HCV (10–12). HCVcc recombinants encoding at least the structural proteins Core, E1 and E2 of a given HCV isolate, but depending on the unique replication capabilities of the JFH1 isolate (13), typically do not require cell culture adaptive envelope mutations (14–19), thus making these HCVcc recombinants particularly useful in studies of entry and neutralization. Such recombinants, including marker viruses, have been developed for major genotypes 1–7 (2, 20, 21).

Another model, used primarily for the study of HCV entry and neutralization, is HCV pseudo-particles (HCVpp), in which lentiviral or retroviral particles harbor authentic HCV envelope proteins (22–24). However, these particles are produced in non-hepatic 293T cells and therefore lack lipoprotein-association, potentially introducing additional bias in the *in vivo* relevance of obtained results. For example, many studies have shown that HCV particles associate with apolipoproteins, mainly ApoE, ApoCI, ApoAI, and debatably, ApoB (25–30). This is likely explained by the fact that HCV hijacks the very-low-density lipoprotein (VLDL) production machinery of the infected hepatocyte for virion production (30). In fact, HCV particles from patients and HCVcc systems display low density in gradients due to similarities with VLDL, whereas this is not the case with HCVpp (31–34). A study found that ApoE decreased accessibility of E2 neutralization epitopes (35). In addition, both ApoE and ApoCI appear to facilitate rapid virus entry, which promotes neutralizing antibody (NAb) resistance by decreasing time spent in the extracellular environment (36–38).

Initial attachment of HCV to the target hepatocyte has been shown to depend on virion-associated ApoE interacting with cell-surface expressed syndecan-1, syndecan-2 and T cell immunoglobulin and mucin domain-containing protein 1 (39–41). Following attachment, the HCV particle interacts with important entry co-receptors, such as scavenger receptor class B, type I (SR-BI), and CD81 (13, 14, 18, 23, 42–45). In addition, HCV relies on additional co-receptors, such as low-density lipoprotein receptor (LDLr) (46–48) and the late-stage entry receptors claudin-I and occludin (49, 50). Most recently, cellular factors that modulate HCV co-receptor localization and possibly prime the cell for infection have also been described (51–55). While it has been reported that LDLr may facilitate non-infectious uptake of HCV (48), it seems clear that the receptor must play an important role in infectious uptake, as recently confirmed for a number of HCV co-receptors, including LDLr (56). In addition, one study found redundancy in HCV entry dependency for SR-BI and LDLr, suggesting some overlap in function (57). As will be reviewed in the following sections evidence is mounting that the early entry co-receptors LDLr, and particularly SR-BI, are involved in HCV antibody evasion, possibly in an interplay with CD81 (45, 58–61).

Patient studies have found that an early induction of HCV-specific NAbs is correlated with resolving HCV infection (62–65). However, the virus employs mechanisms to avoid NAbs. The high mutation rate of HCV, due to the error-prone polymerase NS5B, permits continuous escape from NAb responses (66, 67). On a global scale, this heterogeneity has resulted in the emergence of six epidemiologically important genotypes and numerous clinically relevant subtypes (2, 6, 7). This has important implications for treatment and vaccine development, but this topic is outside the scope of this review. HCV also avoids NAbs by the capacity for cell-to-cell spread (68) and association with apolipoproteins as mentioned above (35–38, 69). Finally, HCV NAb sensitivity is intrinsically modulated by incompletely understood properties of E1/E2, such as envelope polymorphisms (70–73), N-linked glycans (the glycan shield) (74–77) and hypervariable region 1 (HVR1) at the N-terminus of E2 (58, 78, 79) (**Figure 1A**).

The study of the role of HVR1 in the HCV life cycle is a great example of how methodological breakthroughs advance and refine scientific questions. The development of HCVpp and HCVcc models (13, 14, 22–24), as well as the advent of novel tools, such as comprehensive panels of monoclonal antibodies with non-overlapping E1/E2 epitopes (80–91), have facilitated an increasing number of studies that improve understanding of the role of HVR1 in important aspects of the HCV life cycle, particularly immune evasion and viral entry.

CHARACTERIZATION OF HVR1 IN PATIENT STUDIES

Shortly after the discovery of HCV, sequencing efforts identified the N-terminus of E2 as a hotspot of sequence variation, and it was termed HVR1 (92–94). The length of HVR1 was initially debated, but has since been agreed to be 27 amino acids long (amino acids 384–410 in the H77 reference strain), except for some subtypes of genotype 6 in which it appears to typically be 26 amino acids. In patients, HVR1 begins accumulating substitutions in the acute phase of infection (95, 96) and continues evolving during chronic infection (94, 97–99). The reason for this has been the subject of debate. One study, finding no evidence of positive selection and no correlation between evolutionary rate and HVR1-specific antibody responses in patients, suggested that random drift might be the cause for HVR1 variation (100). However, many studies did observe strong positive selection of HCV, particularly in HVR1 (101–103) and a large body of data now supports that HVR1 variation is due to antibody-driven immune selection.

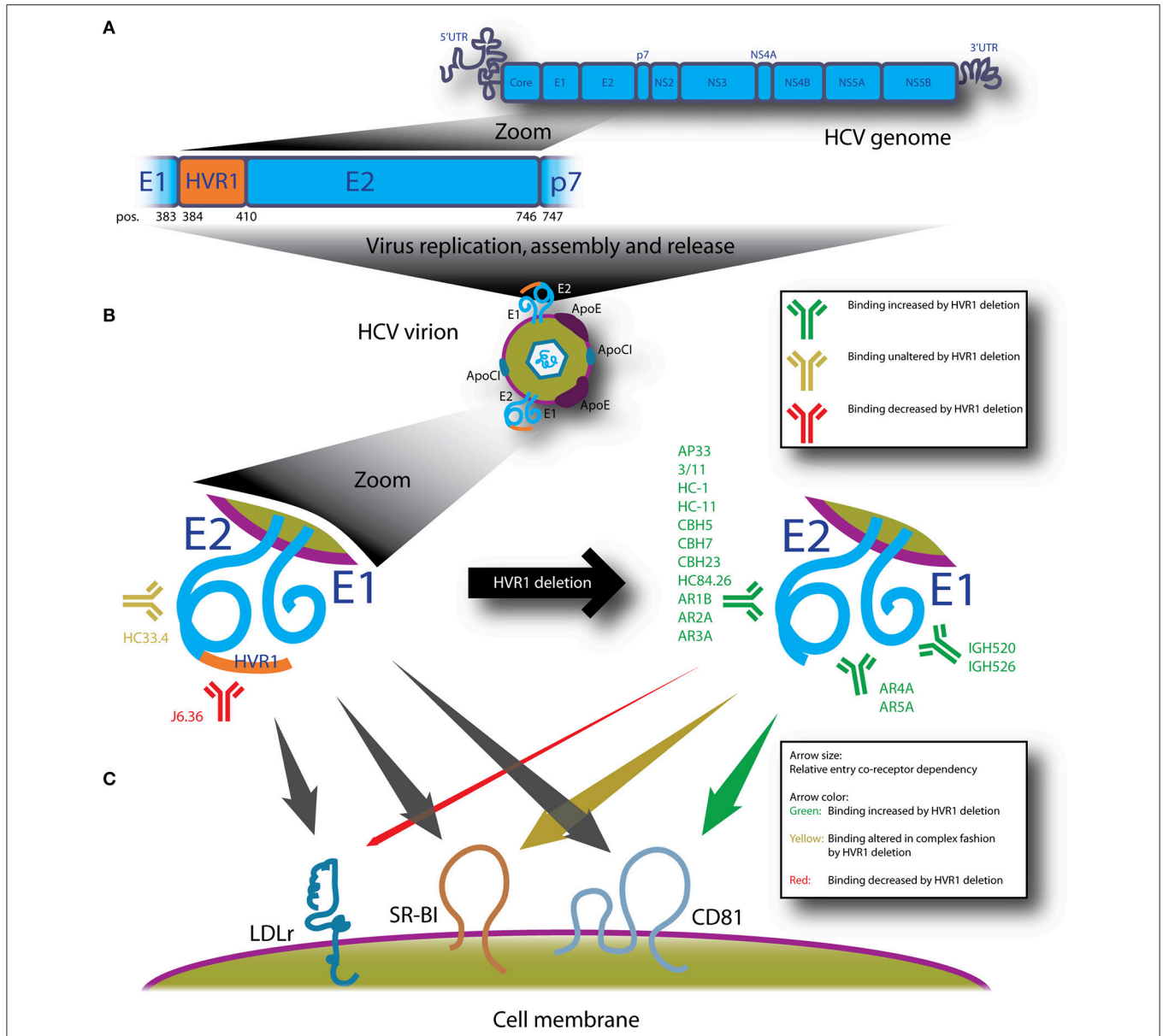

FIGURE 1 | HVR1 of HCV is located at the N-terminus of E2 and protects the virus from diverse neutralizing antibodies and modulates entry interactions with LDLr, SR-BI and CD81. **(A)** Depicts HCV genome organization with a zoom of E2 showing that HVR1 corresponds to the 27 N-terminal amino acids of E2 (H77 reference sequence; amino acid position 384–410). **(B)** Replication of the HCV genome in a permissive cell leads to assembly and release of HCV virions with the E1/E2 complex embedded in the viral envelope. For HVR1-deleted HCV, sensitivity to NAbs is dramatically altered as compiled from multiple studies referenced in the text of this review. Monoclonal NAbs shown are part of comprehensive panels mentioned in the introduction and their specificities are: E1 (IGH520 and IGH526), E2; HVR1 (J6.36), E2; antigenic domain B (CBH5, HC-1 and HC-11), E2; antigenic domain C (CBH7 and CBH23), E2; antigenic domain D (HC84.26), E2; antigenic domain E/epitope I (AP33, 3/11, HC33.4), E2; antigenic region 1 (AR1B), E2; antigenic region 2 (AR2A), E2; antigenic region 3 (AR3A), E1/E2; antigenic region 4 (AR4A) and E1/E2; antigenic region 5 (AR5A). **(C)** HVR1-deleted HCV interacts differently with entry co-receptors LDLr, SR-BI and CD81, both in terms of dependency for entry (size of arrows) and how readily binding to the receptors occurs (color of arrow). Data is compiled from multiple studies cited in the text on the effects of deleting HVR1 from HCVcc, HCVpp or expressed forms of E2 or E1/E2.

Firstly, antibodies against HVR1 are commonly detectable in chronically infected patients (37, 104–110) and the early induction of such antibodies is associated with acute self-limited infection (111). Interestingly, an early reduction in HVR1 sequence diversity is associated with acute self-limited infection (112), suggesting that a rapid anti-HVR1 response curtails virus proliferation before the virus is able to adequately establish a virus population in the host from which to adapt (e.g., diversify the HVR1 sequence). Secondly, although HVR1 variants are, at least to some extent, able to co-exist with

the antibodies that recognize them (98, 106, 110, 113), emerging HVR1 variants have been found to have decreased reactivity with autologous patient serum antibodies, indicating that these variants represent escape (98, 106, 110, 113, 114). In addition, with the advent of the HCVpp entry model, HVR1 variants emerging in patients have been shown to be directly responsible for decreased *in vitro* neutralization with homologous serum (64). Finally, HVR1 variation is decreased or non-existent in HCV-infected patients with various types of immunoglobulin deficiencies (115–119).

The neutralization epitopes in HVR1, responsible for this antibody-driven hypervariability, seem to commonly reside in the C-terminus of the region (98, 106, 120, 121). Interestingly, despite the extremely high sequence diversity of HVR1, significant cross-reactivity of patient antibodies between HVR1 variants has been reported (104, 106–108). This may be because HVR1 contains highly conserved positions, such as conserved hydrophobic and positively charged residues, indicating functional constraints on HVR1 evolution (122).

CHARACTERIZATION OF HVR1 IN STUDIES OF EXPERIMENTALLY INFECTED CHIMPANZEES

Chimpanzees represent the first infection model of HCV and it has been used extensively to study HCV pathogenesis (123–125), including the role of HVR1. Incubation of hyper-immune serum raised against HVR1 peptide with a well-characterized homologous HCV chimpanzee inoculum prevented acute HCV infection in chimpanzees in one out of two cases (126), thus identifying HVR1 as the first HCV neutralization epitope. Interestingly, a minor variant of the inoculum had a different, serum-resistant, HVR1 sequence and this variant became dominant in the non-protected animal. It is therefore not surprising that anti-HVR1 antibodies in chimpanzees have been associated with HVR1 sequence variation (127), although HVR1 apparently does accumulate sequence changes more slowly in HCV-infected chimpanzees than it does in humans (128). This is likely due to subtle differences in HCV infection of chimpanzees compared with the human infection (129, 130), most notably the lower, and typically late, anti-HCV antibody response in chimpanzees (131).

Interestingly, it was possible to infect chimpanzees by intra-hepatic injection of HCV RNA with the HVR1 coding sequence deleted (132), resulting in acute infections, which in one case became an attenuated chronic infection. It was since shown that the animals had not raised NAbs and, in fact, that the chimpanzee that cleared acute infection with HVR1-deleted HCV could be chronically infected with the homologous virus following re-challenge (133). These studies confirm that NAbs are not critical for preventing chronic infection in chimpanzees and that HVR1 is not essential for HCV infectivity and persistence *in vivo*.

HVR1 PROTECTS HCV FROM NEUTRALIZING ANTIBODIES

It was initially discovered that E2 expressed on the surface of cells did not appear to lose proper folding upon deletion of HVR1 (134). Subsequently it was shown that chimpanzees could be acutely and chronically infected with HCV by intrahepatic injection with HVR1-deleted HCV RNA transcripts, although infection was attenuated (132). With the advent of the HCVpp model of HCV entry it became possible to perform detailed studies of viral entry and neutralization (22, 23), but the deletion of HVR1 in the HCVpp model decreased infectivity 10 to 100-fold, making it challenging to study (44, 60). However, HVR1-deleted HCVpp was found to have increased susceptibility to NAbs targeting cross-subtype conserved epitopes (78), suggesting a role of HVR1 in NAb protection.

These studies were complemented with the advent of the HCVcc model (13, 14). The removal of HVR1 from HCVcc harboring E1/E2 from multiple isolates, including genotype 1–3, 5, and 6, had very different effects on culture viability (79). Some recombinant viruses were only slightly attenuated, whereas the fitness of others depended on one or two adaptive envelope substitutions, and the genotype 4a recombinant was non-viable (58, 79). Interestingly, while the H77(1a) envelope substitutions identified in the HCVcc model rescued infectivity of the HVR1-deleted H77 HCVpp, the opposite was true for HVR1-deleted S52(3a) HCVpp, in which the HCVcc adaptive envelope substitution decreased HCVpp infectivity even further (60). However, in all cases the resultant HVR1-deleted HCVcc displayed dramatically increased sensitivity to HCV NAbs and patient sera (58, 79). This phenomenon was initially believed to mainly involve epitopes that overlapped with the CD81 binding site of E2 (58), but it was recently shown that HVR1 protects a much wider variety of epitopes, such as antigenic regions 1–5 (AR1-5; on E2 and E1/E2), antigenic domains B-E (on E2) and even E1 epitopes (135) (**Figure 1B**). An exception to the broad increase in sensitivity is that viruses with and without HVR1 were similarly sensitive to the antigenic domain E antibody, HC33.4, and it has been suggested that this might indicate that HVR1 does not protect certain epitopes within antigenic domain E (136). However, it should be noted that HC33.4 has a secondary contact residue at position 408 within HVR1 (137), which could explain why HVR1-deleted viruses were not more sensitive to this antibody. The breadth in epitopes protected by HVR1 makes it less likely that direct steric epitope shielding alone accounts for the observed differences in NAb sensitivity of HCV with and without HVR1, but more studies are needed to address this in detail. Importantly, the ability of HVR1 to protect HCV from NAbs was recently confirmed *in vivo* by infusing HCV-permissive human liver chimeric mice with antibodies from a chronically infected patient prior to challenge using mouse pools of HCV with and without HVR1 (11).

The broad NAb-sensitizing effect of removing HVR1 has enabled the use of HVR1-deleted viruses to study virus escape in culture using lower doses of NAb than would otherwise have been needed (138). Although resistance substitutions identified in this manner for NAb AR5A were relevant for HCVcc retaining

HVR1 (138), clear differences were observed in similar studies for NAb AR4A, which also appeared to have a higher barrier to resistance (139). It was recently found that HVR1-mediated NAb protection could be increased even further through the binding of HVR1-specific antibodies, possibly by increased steric occlusion mediated by HVR1-bound antibody (137). The HVR1-mediated NAb protection may function in concert with other highly variable region in E2 (140, 141), but how this interplay functions is largely unknown.

A related mechanism by which HVR1 has been proposed to protect HCV from NAbs is in serving as a decoy epitope, diverting the humoral immune system away from more conserved epitopes. This is supported by the correlation between persistence and higher non-synonymous to synonymous substitution rates in HVR1 (142), thus indicating that HVR1-directed immune responses can help the virus persist. The observed positive selection of HVR1 (101–103), combined with studies of HVR1 variants in immune-complexed HCV further supports this hypothesis (143, 144). In addition, the appearance of HVR1-specific antibodies in patient sera was associated with emergence of immune-complexes of particles carrying that specific HVR1 sequence, leading to a large reduction of that viral population within the patient (144). The idea that HVR1 contains immuno-dominant antibody epitopes with a high propensity for accumulating fitness-permissive escape substitutions fits well with the idea that HVR1 also protects other NAb epitopes on E1/E2. It could be hypothesized that immuno-dominance would be a possible consequence of the aforementioned epitope protection.

IN VITRO STUDIES OF THE ROLE OF HVR1 IN HCV ENTRY

SR-BI was identified as a possible HCV co-receptor by its ability to interact with soluble E2 (43). It was also found that HVR1-deleted soluble E2 protein lost most of the ability to interact with this receptor (43), although the interaction could be restored by the introduction of HVR1-deletion adaptive envelope substitutions previously identified *in vivo* (43, 132). These findings suggested that HVR1 modulates SR-BI interaction, but may not be directly interacting with SR-BI. The fact that an antibody against HVR1 blocked soluble E2 binding with SR-BI (43) is not proof of an HVR1/SR-BI interaction as the antibody could be sterically interfering with the SR-BI/E2 interaction without binding directly to the SR-BI binding site, much like the binding of antibody to an epitope tag on E2 neutralized tagged HCV (145). It was subsequently shown that HVR1-deleted soluble E2 more effectively bound CD81 (146). The advent of the HCVpp model confirmed CD81 (23, 44), and SR-BI (44) as co-receptors of HCV entry and facilitated in depth studies of their role in this process.

It was discovered that the human serum component, high density lipoprotein (HDL), enhanced HCVpp infectivity and this phenomenon was confirmed in multiple ways to be both HVR1 and SR-BI dependent (78, 147). In addition, HDL appeared to decrease NAb sensitivity of HCVpp (78, 148), possibly by increased speed of viral entry, thus minimizing the window during which neutralization could occur (149). These findings were corroborated in HCVcc studies (45, 148, 149). In parallel with these studies it was found that the HDL component, ApoCI, was sufficient to induce HCV infection enhancement (37). Interestingly, it appeared that ApoCI was transferred from HDL to HCV in an HVR1 and SR-BI dependent fashion, linked with the native lipid transfer function of the receptor (38). HDL does not interact directly with HCV in the absence of SR-BI (147, 149), but free ApoCI is able to do so, thus bypassing SR-BI (38). In fact, low doses of free ApoCI confer enhancement, while high ApoCI doses destabilize the virus, potentially through modulating virion fusogenicity (38).

SR-BI/HCV interaction was confirmed with HCV particles derived from human serum (150). However, this interaction did not depend on E2, but rather VLDL-like properties of these particles (150), most likely virion-associated ApoE. The fact that the interaction with SR-BI was energy-dependent and that suramin (a compound that reduces ApoE/receptor interaction) could not decrease the HCV/SR-BI association suggested that SR-BI might serve a role in endocytosis (150). However, the results could also be explained by secondary E2/SR-BI interactions, which might not be inhibited by suramin. HCVcc, which unlike HCVpp, is associated with apolipoproteins like ApoE, was used to address this possibility (151). It was found that the lipid-transfer function of SR-BI was critical for infection, but particles with densities above 1.1 g/ml depended on SR-BI specifically for cell attachment (151). While both these phenomena were independent of E2/SR-BI interaction, a third interaction involving a complex HVR1/E2/SR-BI/HDL interplay to enhance infectivity of HCV was also described (151), which is in line with findings from studies of HCVpp and HCVcc outlined above.

In addition, ApoE was found to be associated with HCV both with and without HVR1, but may serve different roles in the interaction with SR-BI (59, 60). While the nature of these differences remains unclear it is tempting to speculate that the high density HVR1-deleted particles interact with SR-BI through ApoE, as shown to be the case for high-density HCV retaining HVR1 (151). The fact that temporal blocking of CD81 and SR-BI yield similar HCV entry inhibition profiles may suggest that these HCV/receptor interactions are closely linked in time (45, 61), further stressing the possibility that SR-BI interactions lead to exposure of the CD81 binding site and downstream entry events.

It was found that the removal of HVR1 greatly increases accessibility of the CD81 binding site on E2 (58, 59). While HVR1 did not appear to modulate late-stage HCV entry co-receptor dependency for claudin-I and occludin, it did appear to influence the ability of HCV to interact with SR-BI (59). However, another study found that HVR1-deletion adaptive envelope mutations were responsible for altered SR-BI dependency as opposed to the deletion of HVR1 itself (60). Non-HVR1 E2 determinants of SR-BI binding would also be better in line with the fact that HVR1-deleted soluble E2 binding to SR-BI could be rescued by envelope mutations (43).

The part of HVR1 involved in modulating these processes, including the ability of HVR1 to protect HCV from NAbs,

was since narrowed down to polyprotein positions 400–408 in the HCVpp model (121) and found to include conserved basic residues in HVR1, such as R408. However, in the HCVcc model it was also found that changing the N-terminal position 385 of HVR1 broadly influenced NAb sensitivity (152). It seems clear that intrinsic properties of the HVR1 sequence helps determine the level of HVR1-mediated NAb protection (120), but to what degree this depends on E1/E2 properties outside of HVR1 remains to be determined. Interestingly, many of the effects of removing or mutating HVR1 can be reproduced by the introduction of point mutations outside of HVR1 (153–155), suggesting the existence of non-HVR1 determinants.

HVR1 has also been proposed to interact with glycosaminoglycans in the HCVpp model, thus suggesting a role in attachment (156). However, the HCVpp model is typically deficient in ApoE, which is now believed to be the primary mediator of HCV attachment (39, 40), suggesting the results may not be as relevant for native HCV. Finally, HVR1-deleted HCV was shown to have decreased LDLr entry dependency (59, 60). In addition, HVR1-deleted HCVcc particles lost most of the ability to interact with soluble LDLr, suggesting a role of HVR1 in the interaction (60). Thus, HVR1 modulates the interaction of HCV with no less than three entry co-receptors (**Figure 1C**). Not surprisingly, several open questions remain, both with regards to receptor usage and NAb protection.

FUTURE PERSPECTIVES FOR DEFINING THE ROLE OF HVR1

HVR1 apparently modulates interactions with no less than three HCV entry co-receptors, which may explain the functional constraints on HVR1 evolution. In addition, the inherent high variability of HVR1 permits it to serve as a rapidly changing decoy epitope, while directly protecting the virus from NAbs targeting a wide array of both conserved and less conserved E1/E2 epitopes. Not surprisingly, the deletion of HVR1 from soluble E2 protein fails to fully recapitulate these effects, which severely impairs reliability of molecular interaction studies and modeling. The structural flexibility of HVR1 has so far hindered crystallography studies of E2 protein retaining HVR1 (157, 158) and consequently we know very little about how this important region interacts with the remaining part of E2. Being able to produce and study a recombinantly expressed, native (i.e., as it sits in the virus membrane) E1/E2 heterodimer is urgently needed to further elucidate the contentious multi-functionality of HVR1 at a molecular level. The lack of native recombinant E1/E2 is also likely why the obvious interest in using HVR1-deleted vaccine candidates, in which conserved epitopes should be more exposed and consequently more immunogenic, has yielded conflicting results (159, 160). It is likely also evidence for the fact that HVR1 multi-functionality is dependent on the E1/E2 context on the virion. However, little is known about how much of the effect of HVR1 on NAb sensitivity and receptor dependency is intrinsic to the HVR1 sequence and how much depends on the E1/E2 context. In addition, the interplay between E1/E2 NAb protection caused by polymorphisms, N-linked glycans and HVR1 is virtually unknown. Such studies should offer a novel way to insights on how HVR1 serves its many functions, including the capacity to protect such a wide array of NAb epitopes.

The research on the role of HVR1 in the HCV viral lifecycle and host responses remains highly relevant, but despite great advances in our understanding of this unique genome region for HCV, particularly during the past 15 years, many questions remain. Providing answers to the role of HVR1 may prove critical in designing a successful HCV vaccine and stemming this global epidemic.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Neutralizing Face of Hepatitis C Virus E2 Envelope Glycoprotein

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The high genetic variability of hepatitis C virus, together with the high level of glycosylation on the viral envelope proteins shielding potential neutralizing epitopes, pose a difficult challenge for vaccine development. An effective hepatitis C virus (HCV) vaccine must target conserved epitopes and the HCV E2 glycoprotein is the main target for such neutralizing antibodies (NAbs). Recent structural investigations highlight the presence of a highly conserved and accessible surface on E2 that is devoid of N-linked glycans and known as the E2 neutralizing face. This face is defined as a hydrophobic surface comprising the front layer (FL) and the CD81 binding loop (CD81bl) that overlap with the CD81 receptor binding site on E2. The neutralizing face consists of highly conserved residues for recognition by cross-NAbs, yet it appears to be high conformationally flexible, thereby presenting a moving target for NAbs. Three main overlapping neutralizing sites have been identified in the neutralizing face: antigenic site 412 (AS412), antigenic site 434 (AS434), and antigenic region 3 (AR3). Here, we review the structural analyses of these neutralizing sites, either as recombinant E2 or epitope-derived linear peptides in complex with bNAbs, to understand the functional and preferred conformations for neutralization, and for viral escape. Collectively, these studies provide a foundation and molecular templates to facilitate structure-based approaches for HCV vaccine development.

Keywords: hepatitis C virus, neutralizing antibodies, crystal structure, neutralizing face, vaccine design

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Hepatitis C is a worldwide epidemic that can cause liver failure and hepatocellular carcinoma. Hepatitis C virus (HCV) infects 1–2% of the world population with estimated 1.5–2 million new infections each year (1–4). Direct-acting antivirals have now been developed to treat patients with persistent HCV infection, yet the reports of increasing number of new HCV infections highlight the urgency in developing an effective HCV vaccine for global control of HCV infection (5).

Hepatitis C virus is an enveloped, positive-strand, RNA virus classified within the *Hepacivirus* genus, one of the four genera of the *Flaviviridae* virus family. The HCV particles consist of a nucleocapsid containing the viral genome surrounded by an endoplasmic reticulum-derived membrane crowned by the E1–E2 envelope proteins (6). It was suggested that the HCV particle is a hybrid lipoviral particle (7) that incorporates a thick shell of host-derived apolipoproteins coating the viral surface (8) and may reduce virus sensitivity to neutralizing antibodies (NAbs) (9, 10). This unique coating of the HCV virion is structurally distinct from other members of the *Flaviviridae* family. The E1 and E2 are type I transmembrane glycoproteins with C-terminal transmembrane domains that form a heterodimer on the viral envelope to enable viral entry into the host cells (11). Of note, it has been shown in mammalian cell expression systems that E1 and E2 form noncovalent heterodimers (12, 13), whereas in the cell culture HCV system, the virion-associated E1–E2 complex can be linked

covalently by disulfide bonds (14). It is unclear that which form represents the functional E1E2 heterodimer, or whether they could represent different maturation stages of E1E2.

Hepatitis C virus entry is a complex and multistep process that involves interactions of the viral particles with cell surface glycosaminoglycans and many host factors, with the tetraspanin CD81, scavenger receptor class B member 1 (SR-B1), claudin-1, and occludin considered to be the essential set of entry factors (15–18). E2 may serve as the receptor binding protein of HCV and directly interacts with the CD81 and the SR-B1 [for review see Feneant et al. (19)]. In contrast, the role of E1 is poorly understood and appears to help modulate the E2-receptor interactions and fusion with the host cell membrane (20–22).

E2 is the main target of NAbs and it has been suggested that the major mechanism for HCV neutralization is blockage of interaction between E2 and its receptor CD81 (23). Several broadly NAbs (bNAbs) have been isolated from infected patients or immunized animals. The majority of these bNAbs target three overlapping/adjacent neutralizing sites (as defined by antibody competition) and block E2 binding to the CD81 receptor [for review see Ref. (23, 24)]. These epitopes include antigenic site 412–423 (AS412, antigenic domain E, or epitope I), antigenic site 434–446 [AS434, part of E2 front layer (FL), antigenic domain D, or epitope II], and antigenic region 3 (AR3). When the first E2 structure was determined, these neutralizing sites were found to cluster on an exposed surface devoid of glycans on E2, known as the neutralizing face (25). Here, we summarize recent knowledge on the E2 neutralizing face, based on structures of the E2 core domain and peptide–bNAb complexes corresponding to different E2 epitopes.

STRUCTURAL STUDIES OF E2 ENVELOPE GLYCOPROTEIN

Structural studies of the HCV envelope glycoproteins are essential for a better understanding of the viral entry mechanism as well as for vaccine and drug design. Yet, since overexpression of the HCV envelope glycoproteins often results in misfolded or aggregated proteins, structural studies have been technically challenging. To date, there are no available high-resolution structures of the E1E2 heterodimer, the entire E1, or the entire E2. Moreover, since the E2 transmembrane region is required for folding of E1 (13), only the ectodomain of the E2 can be expressed as a folded and soluble protein (26–28) and, therefore, is more amenable for structural studies.

E2 CORE DOMAIN STRUCTURES

The E2 glycoprotein (amino acid 384–746 in the H77 prototypic strain) is heavily modified post translationally by up to 11 N-linked glycans (29) and 9 strictly conserved disulfide bonds. E2 possesses three variable regions (VRs), hypervariable region 1 (HVR1), and VRs 2 and 3 (VR2 and VR3, **Figure 1A**), that comprise ~25% of the E2 sequence and contribute to the high genetic diversity of HCV. The VRs and N-linked glycans increase the inherent heterogeneity of E2, which in turn influence the accessibility of antibody epitopes. The E2 ectodomain is a highly stable protein with a melting temperature (T_m) of ~85°C (30). Yet, two independent hydrogen–deuterium exchange (HDX) mass spectrometry experiments indicate high flexibility of the E2 protein, mostly in the VRs, the FL, and CD81 binding loop (CD81bl) (30, 31) that further hinder structural studies of E2.

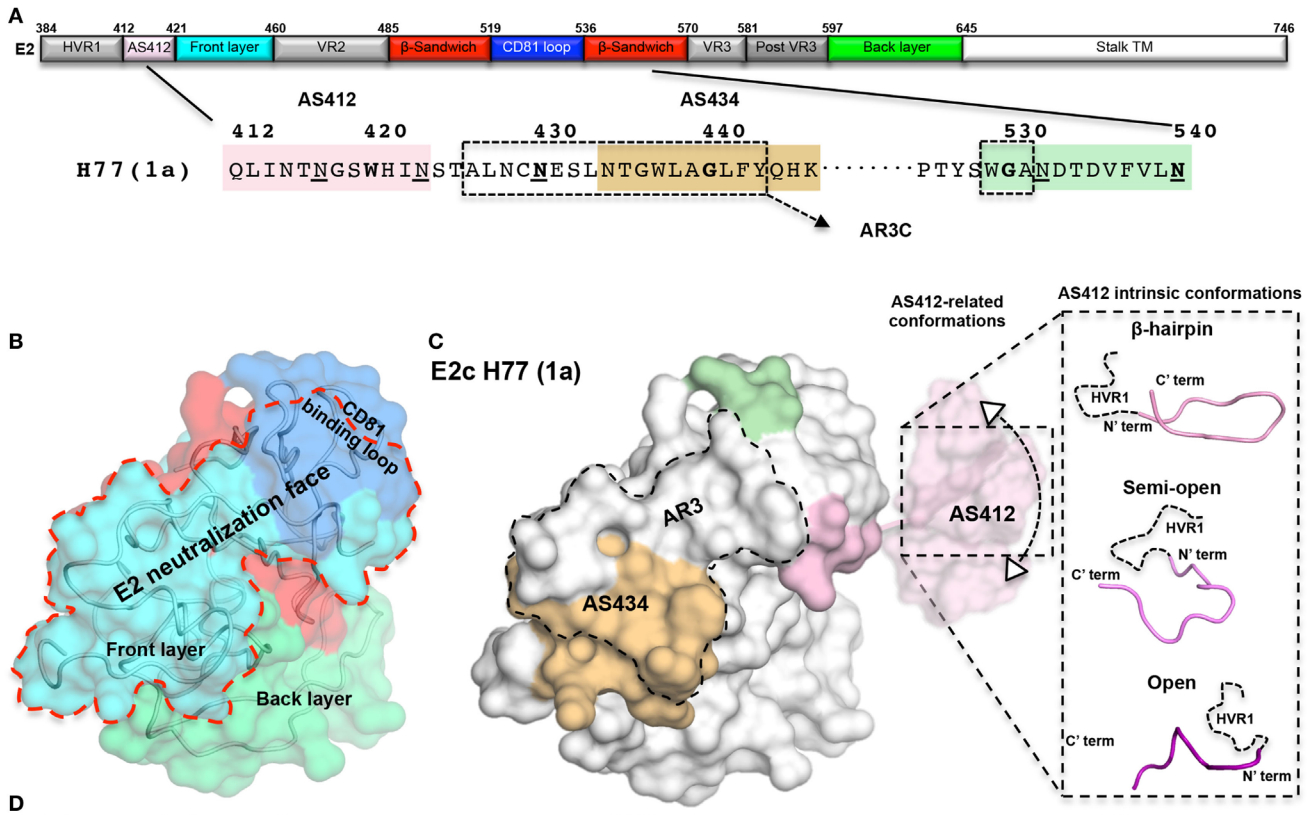
To determine the structure of E2, the E2 ectodomain was engineered by removal of the E2 flexible regions in two independent studies (25, 31). In both cases, a bound mAb facilitated crystallization of E2 (**Figures 1B,D**). The first structure of the prototypic strain H77 isolate (genotype 1a) in complex with bNAb AR3C (see below), consists of E2 residues 412–645 with an internal truncation of VR2 and removal of the N448 and N576 glycosylation sites (E2c) (25). The second structure, of the J6 isolate (genotype 2a) in complex with non-neutralizing mAb 2A12 that binds to the back layer (BL), consists of E2 residues 456–656 (456–652 based on H77 isolate numbering) (31). Overall, both structures share a similar fold but with significant conformational variation around the VR3 region (564–612) and some differences in their disulfide bonds (32). The E2 core domain adopts a globular structure with a new protein fold consisting of a central immunoglobulin (Ig) β-sandwich fold that is stabilized by conserved disulfide bonds and flanked by a FL and a BL (N- and C-terminally). FL is mostly a β-strand with a short helix that packs against the central β-sandwich and BL consisting of antiparallel β-sheets and short helices (**Figures 1A,B**). Both E2 structures indicate that more than 60% of the residues are disordered or in loops, despite the Ig β-sandwich scaffold being highly stabilized by disulfide bonds that can accommodate conformational flexibility of VRs and FL (30).

THE E2 NEUTRALIZING FACE

Based on the H77 E2c structure and epitope mapping experiments, four structural surface regions, or faces, are defined: glycan face, occluded face, non-neutralizing face, and neutralizing face (25). Of note, FL and CD81bl are not modeled in the J6 E2 structure (31). The neutralizing face is a predominantly hydrophobic surface that overlaps most of FL (421–459) and CD81bl (519–535, **Figures 1A,B**) (25) and consists of highly conserved residues. The neutralizing face is accessible on the viral surface and is immunogenic both in infection and in immunization (23, 33). Negative-stain electron microscopy (EM) of the E2 ectodomain in complex with bNAb AR3C suggested that, although surrounded by N-glycosylation sites, the neutralization face is not obstructed by glycans (excluding the AS412 region, see below) or VRs. Moreover, the neutralization face can be recognized by NAbs with different angles of approach to E2 (30). Intriguingly, it was recently suggested that non-neutralizing mAbs that target HVR1 (34) could shield the neutralizing face and protect HCV from binding of NAbs.

E2 ANTIGENIC REGION 3

The AR3 is a cluster of discontinuous epitopes formed by E2 FL and CD81bl (**Figure 1A**) that was originally defined by a panel of human antibodies isolated from a chronically infected HCV patient (35, 36). The AR3 is a target for bNAbs AR3A, AR3B, AR3C, and AR3D that exhibit cross-genotype neutralization



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	Epitope	mAbs Name	Antigen	PDB ID		References
	AS412	HCV1	H77 (1a) pep412-423 in β hairpin conformation	4DGV/Y	Human bNAb	Kong et al., 2012b (65)
		AP33	H77 (1a) pep412-423 in β hairpin conformation	4G6A 4GAG/J	Mouse bNAb	Kong et al., 2012a (64) Potter et al., 2012 (66)
		MRCT10.v362	H77 (1a) pep412-423 in β hairpin conformation	4HS6	Humanized and affinity-matured bNAb (from AP33)	Pantua et al., 2013 (55)
		Hu5B3.V3	H77 (1a) pep412-423 in β hairpin conformation	4HS8	Humanized and affinity-matured bNAb (from muB53)	Pantua et al., 2013 (55)
		3/11	Glasgow (1a) pep412-423 in open conformation	4WHY/T	Rat bNAb	Meola et al., 2015 (68)
		HC33.1	H77 (1a) pep412-423 in semi-open conformation	4XVJ	Human bNAb	Li et al., 2015 (69)
		HC33.4	H77 (1a) pep406-425 in semi-open conformation	5FGB	Human bNAb	Keck et al., 2016a (34)
		HC33.8	H77 (1a) pep406-425 in semi-open conformation	5FGC	Human bNAb	Keck et al., 2016a (34)
		MAb24	H77 (1a) pep412-423 in β hairpin conformation	5VXR	Mouse bNAb	Gu et al., 2018 (67)
*	**AR3**	**AR3C**	**H77 (1a) E2c**	**4MWF**	**E2 core from genotype 1 with bNAb AR3C**	**Kong et al., 2013 (25)**
	AS434	HC84.1	H77 (1a) pep434-446	4JZN	Human bNAb	Krey et al., 2013 (78)
		HC84.27	H77 (1a) pep434-446	4JZO	Human bNAb	Krey et al., 2013 (78)
		HC84.26	H77 (1a) pep434-446	5ERW	Human bNAb	Keck et al., 2016b (79)
		HC26 AM	H77 (1a) pep435-446	4Z0X	Affinity-matured human bNAb	Keck et al., 2016b (79)
		8	H77 (1a) pep430-446	4HZL	Murine neutralizing mAb (genotype 1a only)	Deng et al., 2013 (80)
		12	H77 (1a) pep421-446	4Q0X	Murine non neutralizing mAb	Deng et al., 2014 (81)
	a.a. 529-540	DAO5	J4 (1b) and JFH-1 (2a) pep529-540	5NPH/I/J	Non-neutralizing mAb	Vasiliauskaite et al., 2017 (82)
*	**Back layer**	**2A12**	**J6 (2a) E2c**	**4WEB**	**E2 core from genotype 2 with non-neutralizing mAb 2A12**	**Khan et al., 2014 (31)**
	cycAS412	C2	Cyc pep412-422	5EOC	Cyclic peptide derived from the AS412 epitope	Sandomenico et al., 2016 (74)
		HCV1-C1	Cyc pep412-423	5KZP	Cyclic peptide derived from the AS412 epitope	Pierce et al., 2017 (75)

FIGURE 1 | The neutralizing face of the hepatitis C virus E2 glycoprotein. **(A)** Schematic representations of E2 regions (a.a. 384–746, based on the prototypic isolate H77 numbering system) colored by structural components with variable regions in gray, AS412 region in pink, front layer (FL) in cyan, β-sandwich in red, CD81 binding loop (CD81bl) in blue, back layer (BL) in green, and the stalk and transmembrane region (TM) in white. The a.a. sequence of the neutralization face (a.a. 412–446 and 525–535) and the epitope of mAb DAO5 is shown below. The AS412, antigenic region 3, and AS434 neutralization epitopes are marked in pink, dashed rectangle, and wheat. The epitope of the non-neutralizing mAb DAO5 is marked in green. The N-linked glycosylation sites surrounding the neutralizing face (N417, N423, N430, N532, and N540) are underlined. **(B)** Surface representation of the E2c structure (25) (PDB entry 4MWF) with the structural components colored as in **(A)**. The neutralizing face is marked by a red dashed line. **(C)** The E2 neutralizing epitopes on the E2c structure. For the AS412 epitopes, a.a. 412–420 are modeled onto E2c based on the AP33 bNAb-AS412 crystal structure (PDB entry 4G6A). The conformational flexibility of AS412 related to the E2 (30) is schematically shown. The three known AS412 conformations (β-hairpin, semi-open, and open) for neutralization are shown on the right. **(D)** Summary of the E2 crystal structures. The two E2 core domain-mAb structures are marked by stars.

by blocking E1E2 binding to CD81. The AR3 mAbs have been demonstrated to protect against HCV in passive antibody transfer experiments in both the human hepatocyte-chimeric mouse model and the genetically humanized mouse model (35, 36). The AR3 mAbs share a similar genetic background with their heavy chain (HC) encoded by the germline gene family V_H1–69 (36), which is known to be germline gene precursors for the generation of bNAbs against HCV (37–39), influenza (40–43),

and HIV (44). This group of mAbs interacts with conserved hydrophobic residues in their antigens *via* hydrophobic residues at the tip of their complementarity-determining region 2 loops. Recently, two independent studies reported the isolation of bNAbs, from patients spontaneously cleared HCV, also target AR3 and are encoded by V_H1*–69* genes (45, 46).

Alanine scanning mutagenesis experiments together with the structural analysis of H77 E2c–AR3C complex mapped the AR3 epitopes to the E2 FL (426–443) and the tip of the CD81bl (529–531) (25, 35, 47), overlapping with the majority of E2 neutralizing face (**Figures 1B,C**). AR3 comprises mostly highly conserved residues across the HCV genotypes (25) although variability has been observed in several binding residues (e.g., E431, L433, and F442).

The structure of the E2c in complex with the AR3C bNAb indicates a well-defined secondary structure of AR3, where E2 FL consists of β-strands and an α-helix (436–443) that packs against the β-sandwich region and BL. However, this defined conformation is probably induced or stabilized by binding of the AR3C mAb. When unbounded, AR3 on recombinant E2 is highly flexible as shown by HDX mass spectrometry and molecular dynamics simulations (30). Such flexibility may explain the poor quality of NAb responses to the E2 neutralizing face in immunization studies using recombinant E2.

E2 ANTIGENIC SITE 412–423 (AS412)

AS412 is a highly conserved linear antigenic site that overlaps with the N-terminal region of E2 neutralizing face and contains residues that are critical to CD81 binding [e.g., W420 (48)]. AS412 (412–423) is located between the C-terminus of HVR1 and the N-terminus of FL and contains the first two N-glycosylation sites (N417 and N423) of E2 (**Figure 1A**). AS412 is the target for some of the most characterized cross-genotype NAbs, isolated from both infected donors and E2-immunized animals (49–56) (**Figure 1D**). Moreover, bNAbs against AS412 show passive protection in animal models (chimpanzee and humanized mice) inoculated with HCV (57, 58) as well as delaying HCV recurrence post-transplant in clinical trials [for HCV1 bNAb (59, 60)]. However, natural elicitation of such bNAbs in infection is rare and is detected only in 2–15% of the patients (54, 61, 62). In animal immunization experiments, only low levels of NAbs against AS412 have been elicited (23, 63).

Although AS412 is present in the H77 E2c construct, only its C-terminus (421–423, **Figures 1A,C**) could be modeled in the E2c–AR3C complex structure, suggesting high flexibility of this region. The flexibility or conformational heterogenity of AS412 relative to E2 was validated by a recent EM study on the H77 E2c-HCV1 bNAbs complex, which revealed a 10–22° variation in the angle that the HCV1 Fab fragment approaches E2 (30) (**Figure 1C**, left). A second level of flexibility, likely reflecting the intrinsic conformational variability of the region, was observed in crystal structures of linear peptides corresponding to AS412 in complex with different NAbs. Three main conformations have been reported for AS412 in these antibody complexes (**Figure 1C**, right). The most common and the first to be determined is the β-hairpin conformation, as observed with HCV1, AP33, MRCT10.v362, hu5B3.v3 bNAbs, and MAb24 (55, 64–67) (**Figures 1C,D**). An extended or "open" conformation was observed in the complex with rat mAb 3/11 (68) and a semi-open conformation in complexes with mAbs HC33.1, HC33.4, and HC33.8 (**Figures 1C,D**) (34, 69). Despite these differences in the AS412 conformations, alanine scanning mutagenesis and structural analysis indicate that L413, G418, and W420 are critical for binding of AS412 bNAbs [beside 3/11, see below (70)].

The β-Hairpin Conformation

The first conformation of AS412 to be determined and the most common is the β-hairpin conformation, stabilized by a number of internal backbone hydrogen bonds, with a β-turn at residues 416–419. In complexes of HCV1, AP33, MRCT10.v362, hu5B3.v3 bNAbs, and MAb24, the β-hairpin conformation is highly similar with slight changes in the β-turn type [type IV hairpin turn in the hu5B3.v3 complex, while type I for all of the others (55, 64–67)]. The hydrophobic face of the hairpin is recognized by a binding pocket composed of the antibody heavy and light chains, whereas the N417 and N423 glycosylation sites project from the opposite side of the peptide and are solvent exposed (65), indicating that AS412 is likely not closely packed against E2. Superposition of the AS412 C-terminus (421–423) of AP33 and HCV1 on the E2c structure results in steric clashes between the FL and the epitope-bound mAb, supporting the notion that AS412 is flexible on E2. Escape of HCV from neutralization by bNAbs targeting AS412 has been reported in several studies (55, 57, 59, 67, 71, 72), including the N415D/K and N417S/T mutations. The N417S/T mutations can result in a glycosylation shift from N417 to N415. Structural analysis of these AS412 complexes provides an explanation for the viral escape mechanism. The side chain of N415 is buried in the antibody binding pocket and, therefore, mutation of N415 or the glycosylation shift to N415-glycan would create steric clashes in the antibody binding pocket and interfere with antibody binding.

The Semi-Open Conformation

The semi-open conformation was observed in the complex structures with human bNAbs HC33.1, HC33.4, and HC33.8 (34, 69). In this conformation, beside residues 414 and 415 that form an antiparallel β-sheet with the long HC CDR3, the antigen adopts an extended conformation that is stabilized by one internal backbone hydrogen bond (69). Residues 416–419 adopt a β-turn conformation as in the original β-hairpin structures (69). The neutralization potency of the HC33 bNAbs is not impaired by the N417S/T mutation and the glycosylation shift to N415 because the side chain of N415 (as well as N417 and N423) is solvent exposed in the antibody–peptide complex structure (54, 69, 73). Modeling of N-linked glycans on N415 indicates potential interactions with the HC33 HC (69) that may explain the higher neutralization potency of HC33.1 against glycan-shifted virus (73). These properties indicate that the semi-open conformation would be a useful template for structure-based vaccine design.

The Open Conformation

The extended open conformation of the AS412 was observed in its crystal structure with rat NAb 3/11 (68). This conformation

is stabilized by internal backbone hydrogen bonds, similarly to the β-hairpin conformation, but through creation of a different interaction network. The side chains of N415, W420, and H421 are critical for the binding of 3/11 (68, 70). AS412 is immersed in a deep cavity formed by both the HC and LC of 3/11 with only the side chains of N417 and S419 exposed to solvent, providing a structural explanation for viral escape by point mutations N415Y and G418D and the glycosylation shift mutation N417S (68, 71, 72).

Cyclic AS412

So far two groups have reported structure-based design of AS412 as cyclic immunogens (cycAS412) (74, 75) (**Figure 1D**). In the first study, cycAS412 did not elicit NAbs in immunized mice (74). Structural studies of one of these mAbs, C2, in complex with cycAS412, revealed that cycAS412 retains a β-hairpin conformation but binding to the C2 mAb is mediated by the opposite face of the epitope. Consequently, the structure suggests that cycAS412 failed to mimic the AS412 conformation required for binding of bNAbs leading to the lack of neutralization capability. In the second study, another cyclic AS412 peptide, C1, was studied (75). Mice immunized with this cyclic peptide conjugated to a protein carrier produced better binding and NAb responses than the equivalent linear peptide, albeit neutralization was restricted to the virus from which the peptide was derived. In the same study, AS412 was also grafted onto a hairpin at E2c BL region. The addition of a second copy of AS412 to E2c was not detrimental to the engineered protein (T2) and the antibody response elicited appeared to be similar to the standard, soluble, C-terminally truncated, E2 ectodomain (384–661). It is yet to be determined why the antibody response was still restricted to the autologous virus despite NAbs to AS412 being elicited.

E2 ANTIGENIC SITE 434–446 (AS434)

AS434 is a short hydrophobic 1.5 turn α-helix (helix α-1, 437–442) encircled by a N- and C-terminal extended regions spanning FL residues 434–446. Several NAbs that target AS434 have been isolated from chronically infected HCV donors (39) and from immunized mice (76) (**Figure 1**). Beside NAbs, AS434 can also elicit non-neutralizing mAbs that were proposed to interfere with NAbs that target AS412 (39, 76, 77). AS434 is highly conserved among HCV genotypes (excluding residues 434 and 444) and escape mutants have not been observed *in vitro* (39), indicating that it is a good target for structure-based vaccine design.

AS434 has been structurally defined by six crystal structures of mAbs in complex with the corresponding linear peptides. Four of them are human bNAbs (HC84.1, HC84.27, HC84.26, and HC84.26AM) (78, 79) and the other two are the weakly and non-neutralizing murine mAbs (12 and 8) (80, 81) (**Figure 1D**). Although some variations are found in the conformation of the N- and C-terminal regions, residues 437–442 of the different peptides adopt an α-helical conformation that is similar to that observed in E2c FL (25). Notwithstanding, the biological activities of the mAbs vary greatly because of the way they approach their epitopes. When superposed on the E2c crystal structure, the human bNAbs bind to AS434 using an angle of approach that is similar to bNAb AR3C with only minor structural clashes with the E2 protein. In contrast, superposition of the murine mAbs onto the E2c structure results in structural clashes with the central β-sandwich scaffold. These clashes suggest that the murine mAbs bind an opposite face of the epitope (almost 180° rotation of the helix) and, therefore, would require some conformational rearrangement of AS434 upon antibody binding. The transition between the two modes of binding is possibly supported by the high flexibility of E2 FL as indicated by HDX experiments (30).

In the structures of the HC84 human bNAbs, the C-terminal but not the N-terminal loop of the AS434 was modeled. Yet, the interactions are dominated by hydrophobic interactions between the side chains of the α-helical residues L441 and F442 and the antibody CDRH2 hydrophobic tip (39, 78). Similar to the AR3 bNAbs, the HCs of HC84 bNAbs also originate from the V_H1-*69* family genes. In contrast, in the murine mAb 8 structure, only the N-terminal loop was modeled with a different mode of binding to AS434: hydrophilic interaction with the side chains of E431 and N434 and hydrophobic interaction with the side chains of W437 and L438 that are essential for antibody binding. This different mode of binding requires conformational rearrangement of AS434 on E2 to expose the side chains of W437 and L438 buried in the E2c structure.

With the goal of using bNAbs for HCV immunotherapy, a recent study (79) applied yeast display to affinity mature the human HC84.26 bNAb. The affinity-matured mAb, HC84.26AM, showed improved affinity and neutralization against diverse HCV isolates and the capability to protect humanized mice against challenge with infectious human serum. Structural study of HC84.26AM in complex with the AS434 peptide showed that the conformation of the epitope is similar to that with the wild-type mAb, where mutations in the light chain improved the biological activity of the antibody.

THE CD81 BINDING LOOP

CD81bl, spanning residues 519–535, connects β-strands 5 and 6 of the E2 β-sandwich scaffold and contains critical residues for CD81 receptor binding, including Y527, W529, G530, and D535 (48). Although fully modeled as a loop in the H77 E2c structure, the CD81bl is highly flexible when unbound as indicated from HDX experiments (30) and disordered in the J6 E2 core structure (31). Intriguingly, the alanine scanning mutagenesis indicates that many residues, despite being distal from the CD81 binding surface, can severely suppress E2 binding to CD81 when mutated (47, 48). These results suggest that the overall conformation of CD81bl is important in positioning W529, G530, and A531 toward E2 FL so as to form the receptor binding site and neutralizing face of E2.

The mAb DAO5 is a non-neutralizing mAb that can compete with CD81 binding to soluble E2 (82), and targets the C-terminus of CD81bl and β-strand 6 of the β-sandwich scaffold region (residues 529–540). Crystal structures of mAb DAO5 in complex with peptides derived from the J4 and JFHI isolates (82) indicate that the peptide adopts a one-turn α-helical conformation with the side chains of F537 and L539 buried in the antibody binding interface. In the E2c–AR3C structure, this region adopts a β-strand conformation with F537 and L539 side chains pointing toward

the core of the β-sandwich. Since DAO5 is non-neutralizing, it is possible that it recognizes misfolded E2 presented in the antigens used in immunization.

CONCLUSION

The mechanisms used by HCV, as well as by HIV and influenza virus, to evade the humoral immunity include high genetic variability, glycan shielding of immune epitopes, and conformational flexibility near the neutralizing sites on the viral envelope proteins (83). An effective vaccine for HCV must address these challenges, *inter alia*, by targeting conserved neutralizing epitopes to improve the immune response. Despite the challenges in structural studies of HCV envelope glycoproteins, the recent E2 structures (E2c and linear epitopes) have contributed to the identification of the E2 neutralizing face. Structural characterization of HCV antigen–antibody complexes has improved our understanding of how the immune system recognizes HCV to achieve broad neutralization. These studies provided the field with useful molecular templates to enable structure-based design of candidate vaccine antigens.

AUTHOR CONTRIBUTIONS

NT, IAW, and ML prepared and wrote the review manuscript.

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Conformational Flexibility in the CD81-Binding Site of the Hepatitis C Virus Glycoprotein E2

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Numerous antibodies have been described that potently neutralize a broad range of hepatitis C virus (HCV) isolates and the majority of these antibodies target the binding site for the cellular receptor CD81 within the major HCV glycoprotein E2. A detailed understanding of the major antigenic determinants is crucial for the design of an efficient vaccine that elicits high levels of such antibodies. In the past 6 years, structural studies have shed additional light on the way the host's humoral immune system recognizes neutralization epitopes within the HCV glycoproteins. One of the most striking findings from these studies is that the same segments of the E2 polypeptide chain induce antibodies targeting distinct antigen conformations. This was demonstrated by several crystal structures of identical polypeptide segments bound to different antibodies, highlighting an unanticipated intrinsic structural flexibility that allows binding of antibodies with distinct paratope shapes following an "induced-fit" mechanism. This unprecedented flexibility extends to the entire binding site for the cellular receptor CD81, underlining the importance of dynamic analyses to understand (1) the interplay between HCV and the humoral immune system and (2) the relevance of this structural flexibility for virus entry. This review summarizes the current understanding how neutralizing antibodies target structurally flexible epitopes. We focus on differences and common features of the reported structures and discuss the implications of the observed structural flexibility for the viral replication cycle, the full scope of the interplay between the virus and the host immune system and—most importantly—informed vaccine design.

Keywords: hepatitis C virus, glycoprotein E2, neutralizing antibodies, conformational flexibility, immunoglobulin-like domain, CD81-binding site, vaccine design

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INTRODUCTION

Approximately 71 million people worldwide are chronically infected with hepatitis C virus (HCV), which is one of the major causes of liver cirrhosis, liver failure, and hepatocellular carcinoma (1). Small-molecule drugs targeting HCV proteins termed direct-acting antivirals achieve cure rates of >95% (2), but high treatment costs, lack of awareness about hepatitis C, the emergence of multi-drug resistant viruses, and the need to protect patients from re-infection indicate that a prophylactic vaccine is still urgently required. In most viral infections, neutralizing antibodies (nAbs) are in the first line of defense of the adaptive immune response. For HCV, rapid induction of nAbs along with a broadly reactive T-cell response correlates with spontaneous clearance during acute infection and several studies highlighted the role of humoral immunity for the control both in the acute and chronic phase of infection (3, 4).

The two glycoproteins E1 and E2 of HCV are the major targets for nAbs. In particular, the receptor-binding glycoprotein E2 contains major antigenic determinants of HCV, mostly overlapping with binding sites for cellular receptors, including scavenger receptor class B type 1 (SR-B1) (5), the low-density lipoprotein receptor (LDLr) (6), and the tetraspanin CD81 (7). In addition to an extensive disulfide bridge network involving 8 and 18 conserved cysteines in E1 and E2, respectively, both proteins are heavily glycosylated in their N-terminal ectodomains (8, 9). Glycans are important for protein folding and affect epitope presentation and/or accessibility (10). The C-terminal transmembrane domains of E1 and E2 are anchored in the lipid envelope and interact to form an E1E2 heterodimer in HCV particles that are associated with lipoproteins and therefore also termed "lipo-viro particles" (11). Moreover, an E1 trimer observed at the surface of cell culture-derived HCV (HCVcc) and pseudoparticles suggested the presence of E1E2 heterodimers assembled as heterohexameric complexes (12, 13). However, due to the lack of structural information, many features of the architecture and glycoprotein arrangement at the surface of infectious HCV particles remain elusive.

E2 contains four hypervariable regions (HVR) termed HVR1 (residues 384–410 in the prototype H77 sequence), HVR2 (residues 460–485) (14, 15), HVR3 (residues 431–466) (16), and the intergenotypic variable region (igVR, residues 570–580) (17). The fact that the HVR1 interacts with SR-B1 and LDLr during virus entry (5, 6) would *per se* render this segment an interesting target for nAbs. Indeed, the first described HCV neutralization epitope is localized in HVR1 (18). However, nAbs targeting the HVR1 tend to be mostly strain specific, making the HVR1 less interesting for vaccine design (19). Although viruses lacking the HVR1 infect chimpanzees (20) they are more susceptible to neutralization by patient sera and other human mAbs (21–24), indicating that the HVR1 masks neutralization epitopes and serves as an "immune decoy," recombinant glycoproteins lacking the HVR1 are not superior vaccine antigens (25). In addition, the binding of poorly neutralizing Abs to HVR1 can block the binding of broadly neutralizing Abs (bnAbs) to adjacent, conserved regions on E2 (26). These observed antagonistic effects suggest that the induction of anti-HVR1 Abs can interfere with a protective humoral response against HCV infection. By contrast, both HVR2 and the igVR seem neither to be direct targets for nAbs nor be directly involved in receptor binding. Nevertheless, similar to HVR1, both regions were found to modulate the accessibility of the CD81-binding site and the presentation of neutralizing epitopes on the E2 ectodomain (17, 27).

NEUTRALIZATION EPITOPES

On the quest to develop a safe and efficient B-cell vaccine, numerous neutralization epitopes within the HCV glycoproteins have been mapped using a variety of approaches. Peptide scanning approaches using overlapping peptide libraries or random peptide display libraries have revealed a number of linear epitopes, but such an approach is not suitable to identify residues that contribute to conformation-sensitive epitopes (27). Another powerful approach is alanine scanning, probing panels of protein variants with distinct amino acid substitutions for binding to the Abs of interest (28–33). However, amino acid substitution frequently results in protein misfolding and thereby in false contact residues in case of conformation-sensitive epitopes—as illustrated for the bnAb AR3C, where the crystal structure revealed different contact residues than expected from previous alanine scanning (34). This pitfall is often alleviated by the use of non-competing conformational Abs to probe overall protein conformation and cross-competition analysis using a panel of well-characterized nAbs. *In vitro* studies of antibody escape can provide or confirm information about key epitopes (35–43). The gold standard to identify neutralization epitopes still remains the structural analysis of the immune complex, however, HCV glycoproteins are difficult to crystallize and only one neutralization epitope has been structurally characterized in complex with the E2 ectodomain to date (34). The combination of peptide and alanine scanning together with Ab cross-competition studies have yielded different nomenclature systems to describe and cluster epitopes on E2 to date such as antigenic domain A–E (44), antigenic region 1–5 (45), and epitope I–III (46). Of note, extensive overlap exists between these three systems of epitope nomenclature (47).

E1 is less immunogenic but two regions targeted by nAbs have been identified: residues 192–202 (in the prototype H77 sequence), which are recognized by the weakly nAb H-111 (48) and residues 313–324, which interact with the cross-reactive nAbs IGH-526 and IGH-505 (49, 50).

E2 STRUCTURE AND CONFORMATIONAL FLEXIBILITY

The two crystal structures of E2 core fragments, one in complex with the non-nAb 2A12 and the other with the bnAb AR3C, show that E2 features a central immunoglobulin (Ig)-like β-sandwich with two adjacent layers, one in front and one at the back (34, 51). Several regions of the protein are found in loop configurations or are disordered suggesting a high flexibility in parts of the structure (34). The igVR forms a disulfide-constrained loop within a flexible region spanning residues 567–596 but HVR1 and HVR2 are not included in the expression construct (51) or are only partially resolved in the electron density (34). Both structures are highly similar in the overall fold but the disulfide bond connectivity differs, suggesting that E2 features an enhanced plasticity compared to other viral glycoproteins, allowing for rather drastic local structural changes without affecting the overall fold of E2 (52). Of note, free thiol groups within the viral glycoproteins are required for virus entry (53), indicating a functional role of the observed plasticity.

To date, no detailed structural information on the CD81–E2 interaction is available but different techniques including alanine scanning mutagenesis, negative stain electron microscopy, nAb competition experiments, and *in silico* docking have been applied to map the CD81-binding site on E2 (34, 54, 55). Critical contact residues include highly conserved residues W^{420}, Y^{527}, W^{529}, G^{530}, D^{535} (54), and the G^{436}WLAGLF motif (55, 56) most of which are located within (1) a conserved N-terminal region (aa412–423), (2) a front layer region (aa428–446), and (3) an adjacent loop named CD81-binding loop (aa518–542). The majority of α-E2

bnAbs identified to date compete with CD81 for binding to E2. Hence, it is not surprising that their epitopes overlap with one or more of these three regions corresponding to three antigenic regions named epitope I, II, and III (46) (**Figure 1**). Interestingly, not all Abs targeting one of these three epitopes neutralize HCV infection, in spite of similar contact residues (57–59). For non-nAbs directed against epitope II interference with neutralization by nAbs targeting epitope I was proposed (60), but also cooperativity effects between nAbs directed against epitope I and nAbs targeting epitope II have been reported (29).

Within the last years a number of crystallographic studies have revealed molecular details of how Abs interact with these three epitopes, illustrating a great structural heterogeneity in particular within the epitope I (26, 41, 61–66), but also epitope II (59, 67–69), and more recently epitope III comprising the CD81-binding loop and parts of the core Ig-like domain (58). Of note, all three segments are largely conserved in sequence across all HCV genotypes and subtypes (**Figure 1**).

In the E2 core–AR3C Fab complex structure, epitope I is mostly disordered but synthetic peptides mimicking this epitope were complexed and crystallized with Fabs from bnAbs isolated from immunized rodents or from HCV-infected individuals. Human nAb HCV1, mouse nAbs AP33 and mAb24, and humanized and affinity-matured nAbs MRCT10.v362 and hu5B3.v3 (derived from AP33 and mu5B3, respectively) bind such epitope I peptides in a very similar β-hairpin conformation (**Figure 1B**) (41, 62, 63, 65, 66). However, the superposition of the linear epitope in complex with AP33 and HCV1 Fab reveal a 22° difference in the binding angle highlighting that both Abs engage the epitope on E2 from different directions (62). nAb paratopes are similar in shape and surface charge but interactions with E2 are realized by different nAb residues resulting in small conformational differences within a conserved β-hairpin conformation. In both cases, residues L^{413}, N^{415}, G^{418}, and W^{420} of E2 are deeply buried in Fab-peptide interface.

By contrast, the same peptide is recognized in an extended conformation in a deep cleft between heavy and light chains of the Fab from the rat nAb 3/11 (**Figure 1B**) (61). E2 residues N^{415}, W^{420}, and H^{421} are especially critical for the 3/11-antigen interaction in line with epitope mapping by alanine scanning mutagenesis (32, 61). A third conformation of epitope I is recognized by a group of human mAbs named HC33 that were isolated from HCV-infected blood donors (**Figure 1B**) (64). In complex with the HC33.1 Fab, residues I^{414} and N^{415} form an anti-parallel β-sheet with strand F of the heavy chain variable region Ig domain and the remaining part of the peptide is recognized in an extended coil conformation. This interaction mode results in a turn (residues T^{416}–S^{419}) superimposing with the turn observed in the β-hairpin conformation in complex with HCV1 and AP33 Fabs. Residues L^{413}, G^{418}, and W^{420} constitute key anchors for the interaction and are deeply buried in the HC33.1-E2 interface (29, 64). An adaptive mutation $N^{417}S$ is associated with a shift of an N-linked glycosylation site from N^{417} to N^{415} that abolishes neutralization by nAbs HCV1, AP33, and mAb24. Residue N^{415} is buried by HCV1, AP33, and mAb24 but it is solvent-exposed in the HC33.1 Fab-peptide complex structure and allowing for glycosylation at N^{415} (29, 41, 64). Mutations $N^{417}S$ and $N^{415}D$ enhance the sensitivity to HC33.1 neutralization but also to neutralization by other human nAbs targeting different epitopes, suggesting that this region has a global impact on the conformation of HCV glycoproteins (41).

In the complex structures of the related HC33.4 and HC33.8 Fabs, a similar extended conformation is observed for E2 aa418–423 and aa415–423, respectively (26) (**Figure 1B**), but N-terminal residues aa412–414 are disordered. Although the HVR1-residue K^{408} was identified by alanine scanning mutagenesis to be part of the HC33.8- and HC33.4- but not of the HC33.1-epitope, no structural evidence for further epitope–paratope interactions beyond epitope I was observed (26). In summary, epitope I adopts at least three distinct conformations and greatly differs in its nAb interactions depending on the individual nAb. However, in all cases, the hydrophobic interaction networks involves W^{420}, which is strictly conserved across HCV genotypes (**Figure 1B**) and serves also as a critical residue for CD81 binding (54). A recent electron microscopy study demonstrated that the HCV1 Fab binds soluble E2 from different angles of approach thereby further highlighting the conformational flexibility in epitope I (70).

At the surface of HCV particles, the epitope is either present in different conformations or readily converts between them (i.e., with a minimal kinetic barrier for conversion) and individual nAbs bind the epitope with their particular conformational selectivity. Indeed, the dose-dependent neutralization of nAbs 3/11 and AP33 suggests that the different conformations are in a dynamic equilibrium and can be converted in either direction (61). Interestingly, *in silico* predictions of the peptide alone propose a β-hairpin similar to the one observed in complex with Fabs from HCV1, AP33, and mAb24 (64). Together with the fact that the β-hairpin was observed in the majority of Fab complex structures, this suggests that the β-hairpin represents a preferred, but extremely unstable conformation on the HCV particle that can be readily converted into different conformations following an "induced-fit" binding mode to the antibody. This is further supported by the reported differences in neutralization potency of nAbs targeting the three described epitope I conformations (39, 71). The observation that nAbs targeting this segment usually have a broad neutralization activity suggests that genotype-specific sequence variations do not dictate the predominant epitope I conformation, although neutralization efficiency may be modulated by these sequence variations (61). The observed structural flexibility could explain the limited immunogenicity to the epitope I observed in HCV-infected patients (72).

Similarly, Fab-peptide structures provided molecular insights into recognition of epitope II. Structural information is available for epitope II in complex with different Abs—potent human nAbs on the one hand and weakly and non-nAbs derived from immunization with synthetic peptides on the other hand. When recognized by potent nAbs HC84.1, HC84.27, and the affinity-maturated nAb HC84.26.5D, an E2 peptide comprising aa434–446 forms a short α-helix spanning residues W^{437}–F^{442} with an extended conformation on the C-terminal side comprising residues 443–446 (68, 69). This short α-helix can also be found in the AR3C Fab–E2 complex structure (34). Two other crystal structures of murine Fabs from the non-nAb #12 and the weakly neutralizing mAb #8 reveal an epitope that is located few amino acids upstream, but also includes the short α-helix (59, 67). Of note, residues W^{437} and

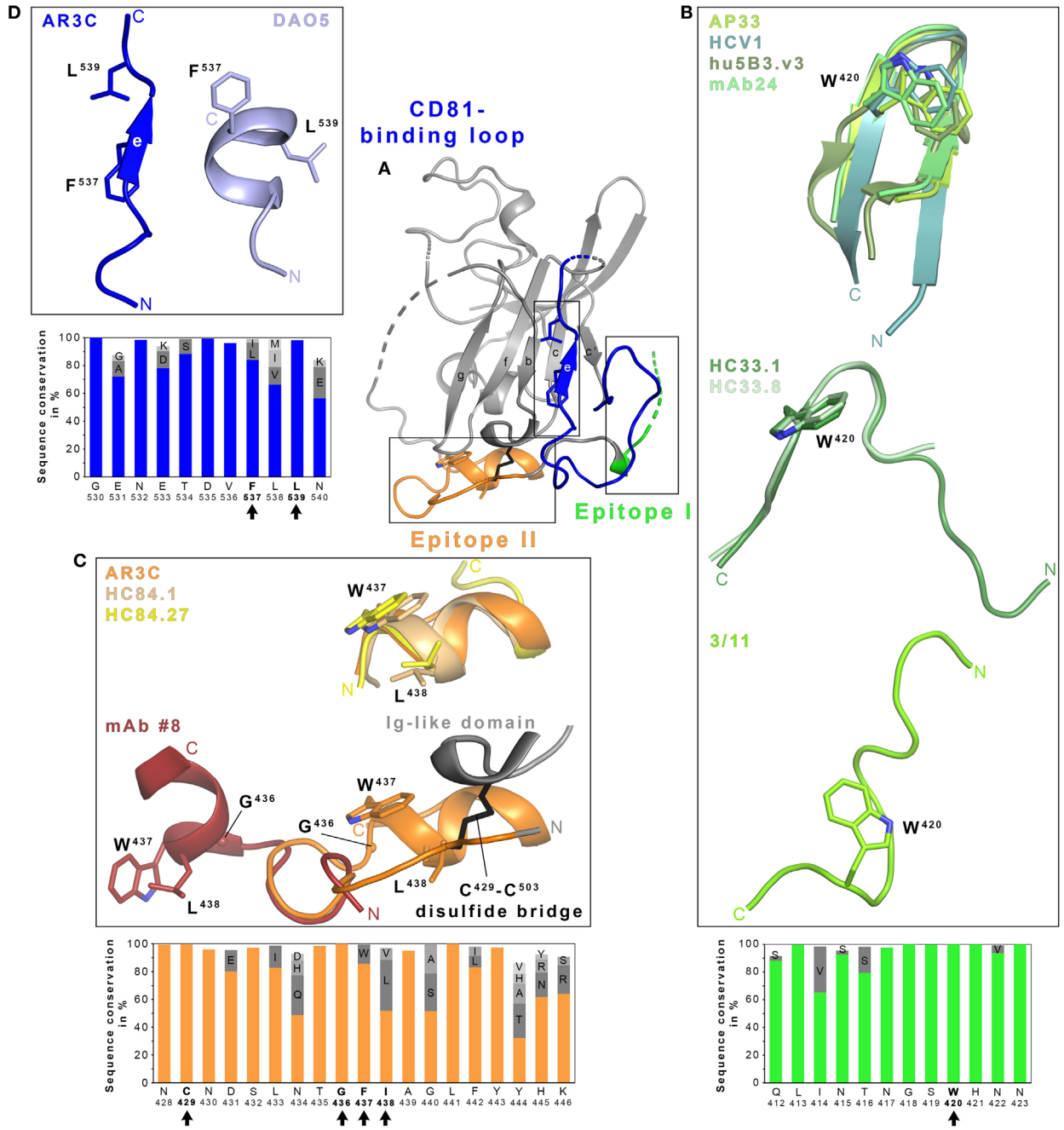

FIGURE 1 | Structural flexibility of the hepatitis C virus (HCV) E2 glycoprotein. **(A)** Cartoon representation of the E2 ectodomain crystallized in complex with AR3C Fab (PDB 4MWF). The composite CD81-binding site consisting of epitope I (aa412–423; green), epitope II (aa428–446; orange), and the CD81-binding loop (aa518–542; blue) is highlighted in color and sidechains of selected residues are displayed as sticks. **(B–D)** Close-up views of the three antigenic sites mentioned above. **(B)** Epitope I is disordered in the context of the E2 structure but a synthetic epitope peptide folds as β-hairpin in complex with neutralizing antibodies (nAbs) AP33 (PDB 4GAJ), HCV1 (PDB 4DGV), hu5B3.v3 (PDB 4HS8), and mAb24 (PDB 5VXR) (upper panel). By contrast, the same peptide adopts two distinct extended conformations in complex with nAbs HC33.1 (PDB 4XVJ) and HC33.8 (PDB 5FGC) (middle panel) or 3/11 (PDB 4WHY; bottom panel), respectively. The peptide in the nAb HC33.4 complex (PDB 5FGB) adopts an amino acid backbone conformation identical to the one in complex with nAb HC33.8 and is not shown for simplicity. **(C)** Superposition of the epitope II peptide structure in complex with HC84.1 and HC84.27 Fabs (PDB 4JZN and 4JZO, respectively) onto the E2 ectodomain structure (upper panel) reveals a conserved 1.5-turn α-helix (aa437–442) with an extended C-terminal segment containing aa443–446. Superposition of the N-terminal loop of epitope II (aa430–434) from the peptide structure in complex with mAb #8 (PDB 4HZL) onto its counterparts in the E2 structure suggests that the short α-helix flips out to expose residues W^{437} and L^{438} for mAb #8 binding (bottom panel). **(D)** Residues 532–540 of the CD81-binding loop were observed in an extended conformation in the context of the E2 ectodomain structure **(A)** and in a helical conformation in the DAO5 Fab–E2 peptide complex structure (PDB 5NPJ) suggesting thereby a putative open and closed conformation of the immunoglobulin-like domain. Amino acid sequence conservation of the respective antigenic site was calculated across the six HCV genotypes for 481 isolate sequences (100 sequences each for genotypes 1, 2, 3, 6, and 70, 9 and 2 sequences for genotypes 4, 5, and 7, respectively) obtained and analyzed from the ViPR database (http://www.viprbrc.org) and is shown below each close-up view. Residues with side chains shown as sticks are highlighted by black arrows.

L^{438} crucial for binding of mAb #12 and #8 are not accessible in the AR3C Fab–E2 complex, suggesting that a conformational change exposing these two residues is required to allow E2 binding. In line with this observation, superposition of the respective peptide structures using the N-terminus, which should be anchored to the Ig-like domain *via* a disulfide bridge (C^{429}–C^{503}), reveals that the C-terminal α-helix has to flip out to allow for mAb #8 binding (**Figure 1C**). This flexibility has been attributed to the strictly conserved G^{436} constituting a hinge between N- and C-terminus of the polypeptide chain, thereby resulting in an open and a closed state of E2 that implies the different presentation of epitope II (59, 70). Potent nAbs HC84.1, HC84.26.5D, and HC84.27 recognize the closed state similar to AR3C, indicating that this represents the preferred state of E2 in the viral particle and the open state targeted by weakly or non-nAbs is less frequently observed on virus particles. However, minor differences in the spatial arrangement of the C-terminal part of epitope II (aa443–446) (69) suggests that additional local structural changes may also occur in the closed conformation (59, 69).

A detailed functional and structural analysis of the non-nAb DAO5 provided a glimpse onto conformational changes in the CD81-binding loop (epitope III) and the adjacent part of the Ig-like domain (58) (**Figure 1D**). In the AR3C–E2 complex structure, the CD81-binding loop is stabilized by the Fab and the side chains of residues F^{537} and L^{539} (located on β-strand E) are buried inside the hydrophobic core of the Ig-like domain resembling a hypothetical closed conformation (34). In the absence of stabilizing Fab interactions, residues 524–535 are disordered and F^{537} is solvent-exposed (51). The crystal structure of non-nAb DAO5 Fab in complex with the E2 peptide aa532–540 reveals a helical conformation in which residues F^{537} and L^{539} are buried in the Fab interface, suggesting that on E2 they need to be solvent exposed to allow for interaction with DAO5 in a putative open conformation. A high sequence conservation within this region suggests that the observed conformational flexibility in the Ig-like domain is an intrinsic feature of E2. Indeed, both conformations are present simultaneously on infectious particles; hence, it is tempting to speculate that the open conformation recognized by non-Ab DAO5 acts as an immunological decoy that distracts the humoral immune system from the relevant CD81-binding conformation (58).

In addition to the static crystal structures, representing snapshots of an apparently highly dynamic protein, solution-based studies such as hydrogen deuterium exchange mass spectrometry (HDXMS) and limited proteolysis help to characterize flexible regions in E2 (51, 70). HDXMS detects the deuterium incorporation into the backbone amides when proteins are exposed to deuterated solvent. The exchange rate depends on the conformational flexibility and accessibility of individual residues to the solvent (73). As expected, HDXMS data confirmed the high structural flexibility in the E2 front layer including the composite CD81-binding site overlapping with epitopes of most bnAbs (70). Moreover, HVR1, HVR2, and igVR are highly flexible and heterogeneous in presented conformations in addition to being hypervariable in sequence (70). Interestingly, despite its unique conformational flexibility E2 has a high thermal stability when compared to proteins from thermophilic organisms or other viral envelope proteins such as HIV-1 env or influenza hemagglutinin presumably also due to its dense disulfide bridge network (70).

CONCLUSION AND FUTURE PERSPECTIVES

The conformational flexibility within HCV E2 extends to the entire composite CD81-binding site, which overlaps most of the conserved neutralization epitopes present in E2. This finding raises the question how such a conformational flexibility emerges during virus evolution? Which functional importance does this flexibility have—or in other words—which selective advantage does this flexibility provide for the virus?

One possible explanation could be that the observed conformations represent different stages during virus entry, where a number of changes in environmental conditions (e.g., receptor binding, endosomal acidification, or a putative conformational change to fuse viral and endosomal membrane) may require different glycoprotein conformations. However, all nAbs mentioned above targeting epitope I block CD81 binding, suggesting that the different epitope I conformations can be adopted upstream of receptor binding. To date, the epitope I conformation in complex with CD81 remains elusive, but a conformationally flexible surface could be required for receptor binding. It is estimated that ~30% of protein–protein interactions include disordered protein regions (74) and the region within the large extracellular loop of CD81 thought to interact with E2 was described to display marked conformational fluctuations (75, 76). Therefore, a conformationally flexible surface on the glycoprotein may be favorable to establish a highly specific receptor interaction *via* an ordered interface following an induced fit binding mechanism. Structural studies on E2 in complex with CD81 will be required to further address this hypothesis.

Another possible explanation could be that the observed conformational flexibility is required for a putative dynamic rearrangement at the virus surface during infection of the host cell, resulting in exposure of the conserved receptor-binding region in E2—similar to the structural dynamics or "virus breathing" described for the related flaviviruses [reviewed in Ref. (77)]. Such an "opening" rearrangement would be in line with the observed time- and temperature-modulated exposure of neutralization epitopes on HCV virions (78).

A third possible explanation could be that this flexibility constitutes a viral mechanism to efficiently evade from nAbs. In general, the stability of peptides has been reported to directly correlate with their capacity to induce a humoral immune response (79), suggesting that conformational flexibility implies a modest immunogenicity. In line with this finding, immunization with a synthetic HCV epitope I peptide did not elicit bnAbs, likely due to its intrinsic structural flexibility (80) and several studies reported that even a cyclic variant of epitope I does not elicit high titers of Abs neutralizing HCV infection (80, 81).

This has important implications for vaccine design, suggesting that— although many subunit vaccine candidates based on HCV glycoproteins are currently under development—an unmodified form of the latter is limited in its capacity to elicit nAbs. Structure-guided stabilization of neutralization epitopes within E2 toward

the conformation targeted by nAbs can potentially improve its immunogenic properties. Alternatively, a recent innovative approach termed epitope-focused vaccine design (82, 83) facilitates the design of epitope-specific immunogens to elicit nAbs where conventional vaccines failed to raise an immune response. For this purpose, a structurally characterized neutralization epitope is grafted onto an unrelated protein scaffold containing a segment with an identical backbone conformation. A successful example of this strategy is the development of an epitope scaffold presenting a single neutralization epitope of the human respiratory syncytial virus F protein and its neutralization potency can potentially be further augmented by the incorporation of further neutralization epitopes (82). Epitope-focused design has also been applied to HCV neutralization epitopes (80, 84) albeit with limited success. However, more recently an anti-idiotypic Ab, which also functions by mimicking a neutralization epitope on an unrelated protein (in this case an antibody), was demonstrated to robustly induce HCVcc-nAbs (85), suggesting that epitope-focused immunogens represent a viable strategy to develop a safe and efficient B cell vaccine and elicit a protective nAb response.

AUTHOR CONTRIBUTIONS

LS, KN, and TK participated in the design and coordination of the manuscript. All authors wrote, read, and approved the final version of the manuscript.

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Predicting the Effectiveness of Hepatitis C Virus Neutralizing Antibodies by Bioinformatic Analysis of Conserved Epitope Residues Using Public Sequence Data

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Hepatitis C virus (HCV) is a global health issue. Although direct-acting antivirals are available to target HCV, there is currently no vaccine. The diversity of the virus is a major obstacle to HCV vaccine development. One approach toward a vaccine is to utilize a strategy to elicit broadly neutralizing antibodies (bNAbs) that target highly-conserved epitopes. The conserved epitopes of bNAbs have been mapped almost exclusively to the E2 glycoprotein. In this study, we have used HCV-GLUE, a bioinformatics resource for HCV sequence data, to investigate the major epitopes targeted by well-characterized bNAbs. Here, we analyze the level of conservation of each epitope by genotype and subtype and consider the most promising bNAbs identified to date for further study as potential vaccine leads. For the most conserved epitopes, we also identify the most prevalent sequence variants in the circulating HCV population. We examine the distribution of E2 sequence data from across the globe and highlight regions with no coverage. Genotype 1 is the most prevalent genotype worldwide, but in many regions, it is not the dominant genotype. We find that the sequence conservation data is very encouraging; several bNAbs have a high level of conservation across all genotypes suggesting that it may be unnecessary to tailor vaccines according to the geographical distribution of genotypes.

Keywords: neutralizing antibodies, hepatitis C virus, vaccine, bioinformatics, HCV-GLUE, sequence conservation

INTRODUCTION

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is a major cause of liver disease worldwide. Recent estimates indicate that HCV infects approximately 71 million people globally (1). Approximately 70% of infected individuals develop a chronic infection that can lead to liver cirrhosis and hepatocellular carcinoma (HCC). Termed a "silent killer," the initial infection is usually asymptomatic and individuals are often unaware that they carry the infection until symptoms develop several decades later. In recent years, a number of effective direct-acting antiviral (DAA) drugs have been developed. However, the silent nature of initial infection makes timely diagnosis and treatment more challenging. The long period of chronic infection may already have caused irreversible liver damage or initiated a chain of events that will ultimately result in HCC even if the virus is successfully cleared by DAA-treatment post-diagnosis (2, 3). Further studies are required to address this question. This and other factors including cost, access to treatment, and reinfection enforces the pressing need for a prophylactic vaccine for HCV.

One of the major barriers to vaccine development for HCV is the sequence diversity of the virus. Currently, there are seven genotypes and 67 subtypes that have at least 33 or 15% nucleotide variation, respectively (4). As a result, an effective vaccine must be capable of protecting against challenge by an extremely diverse viral population. The question is how to design such a vaccine? HCV has two surface glycoproteins E1 and E2 that form a heterodimer. These proteins govern the entry process of the virus. The E2 glycoprotein, which contains the receptor-binding site (RBS) for the cellular receptors CD81 and SR-BI is the most studied (5, 6). E2 contains a number of variable regions; hypervariable region 1 (HVR1) is located at the N-terminus (aa384-427), this region has been shown to be important for interaction with the SR-BI receptor and to play a role in antibody evasion by shielding epitopes and preventing neutralization (7–12). The roles of the other variable regions are less defined, they are hypervariable region 2 (aa461-481) and the intergenotypic variable region (aa570-580) (8, 13). E2 has ~11 N-linked glycosylation sites that form a glycan shield, which has also been shown to be involved in immune evasion (14). An insight into possible targets for HCV vaccine development, i.e., surface-exposed, conserved regions of the HCV glycoproteins can be gleaned from studies into broadly neutralizing antibodies (bNAbs). Viral neutralizing antibodies have been shown to inhibit infection by either blocking interaction with the RBS or by inhibition of the post-entry fusion mechanism (15, 16). By definition, bNAbs do this by targeting highly conserved regions within the viral glycoproteins that are involved in these processes. We generated the HCV bNAb, AP33 in 2001 and demonstrated in 2005, with the development of the HCV pseudoparticle (HCVpp) system that it was able to neutralize particles decorated with diverse HCV E1E2 glycoproteins (17, 18). Since then, there has been significant progress in the isolation and characterization of HCV bNAbs, as reviewed by Ball et al. (19). The majority of HCV bNAbs have been shown to target the E2 glycoprotein particularly the CD81 RBS. Within the literature, several different nomenclatures are used to describe these regions, herein, we will use Epitopes 1–4 (20, 21). The potential of utilizing HCV bNAbs to inform rational vaccine design and the associated challenges has been the topic of recent reviews (22, 23). With the plethora of HCV bNAbs now available, which of these would be the most promising for further analysis and vaccine design? In this study, we have probed a large HCV sequence dataset to determine the level of conservation of each bNAb epitope. Using this data and documented neutralization studies, we conclude that the most promising candidates to date as a starting point for development of a bNAb-based vaccine approach are HC84.20, AR4A, 1:7, A8 and AP33. 95-2, HCV1, and Hu5B3.v3 also have strong potential, but there are insufficient neutralization data available at this time.

MATERIALS AND METHODS

Epitope Identification

For each bNAb, the epitope reported in the literature cited was used. For the majority of bNAbs, this was straightforward; however, for a small group, the data in different publications were conflicting. We have used an eight-residue binding motif for bNAb AR3C that differs from the original epitope identified by alanine-scanning (24). These eight residues were consistent between two subsequent reports; the crystal structure of AR3C bound to core E2 and also in an extensive alanine-scanning study (25, 26). AR3A, AR3B, and AR3D were excluded from our analysis as the alanine-scanning data were conflicting and no structural data were available to corroborate either study. We have also updated the binding motifs of several conformational bNAbs (HC84.20, HC84.24, HC84.26, HC-1, HC-11) reported by the Foung lab to incorporate a later study by Pierce and coworkers that includes a comprehensive E1E2 alanine-scanning study (27). Residues that inhibited binding by at least 80% were selected as critical-binding residues. Crucially, as these antibodies bind conformational epitopes, alanine mutation may alter the overall structure of E1E2; therefore, mutations in regions that affected binding of all conformational antibodies were not included.

Bioinformatic Analysis

The analysis of public HCV sequence data was performed within Genes Linked by Underlying Evolution (GLUE) (28). GLUE is an open source, data-centric bioinformatics environment specialized for the analysis of virus genomic sequence data.

GLUE was used to create a public sequence data resource called HCV-GLUE (28) for the study of HCV genomes. HCV-GLUE provides an interactive web application for public use; the underlying dataset may be also downloaded to a local computer. This dataset currently contains approximately 92,000 HCV sequences derived from the public GenBank database (29) and is updated on a daily basis. Sequences from non-human hosts, <500 bases in length, recombinant, or patent-related are excluded from the set. Within HCV-GLUE, each sequence is assigned a genotype and where possible a subtype according to a maximum likelihood method based on the scheme proposed by Smith et al. (4). Furthermore, each sequence is maintained in alignment to a closely related reference sequence. The GLUE software system provides basic functions for the analysis of amino acid residues across sets of stored sequences. A residue numbering scheme proposed by Kuiken et al. (30) is used within HCV-GLUE.

GLUE allows existing projects such as HCV-GLUE to be extended to address-specific research questions. For the current article, we created an extension, HCV-NABS, which may be downloaded from https://github.com/giffordlabcvr/HCV-NABS. The HCV-NABS extension augments the HCV-GLUE dataset with data relating to 38 neutralizing antibodies and their putative-binding locations. We then also created scripts within the HCV-NABS extension to analyze the frequency of amino acid residue patterns both at individual binding locations and at combinations of binding locations pertaining to each bNAb. Procedures were also added to report the numbers of sequences within each genotype containing a substantial part (90%) of the E2 region of the HCV genome. These data were stratified according to the country of origin, which had been annotated in the GenBank record, if any. We used the HCV-GLUE characterization of sequence genotypes and subtypes to stratify the analysis. The bioinformatics analysis may be reproduced by installing GLUE, HCV-GLUE, and the HCV-NABS extension on any computer.

RESULTS

Identification of the bNAb Epitopes

A group of 38 monoclonal antibodies that has been shown to have broad neutralization activity were selected from the literature. Importantly, the epitopes of this group of antibodies have been characterized by alanine-scanning mutagenesis and/or structural analysis. The bNAbs have been isolated and characterized by many different groups. However, often several antibodies were isolated from the same source as indicated by the nomenclature. The largest such group is the HC84 group; these are all designated as HC84. xx and tend to share overlapping epitopes. The bNAbs used in this study are shown in **Table 1**, together with the region of E1E2 that they target and the specific residues that are critical for antibody binding. It is well documented that most neutralizing antibodies target particular regions of the E2 glycoprotein that are involved in CD81 binding; Epitope 1 (aa412–423), Epitope 2 (aa434–446), Epitope 3 (aa523–535), and Epitope 4 (aa611–617) as shown in **Figure 1** (20, 21). We compared the specific residues bound by all 38 bNAbs and identified 47 E1E2 residues, 27 of which lie within these four epitopes. Certain residues seem to be key target residues as they are recognized by several bNAbs from different sources these include; W420 in Epitope 1 and F442 in Epitope 2 that are targeted by 12 and 11 bNAbs, respectively (**Figure 2**). We have shown that W420 is a critical residue modulating interactions with the cellular receptors CD81 and SR-BI (31). Other residues only form part of the epitope for 1 bNAb, for instance, T416, A439, and D533.

Analysis of the Level of Conservation of bNAb Target Residues

Rather than focusing on the designated epitope regions, we determined the level of conservation of all 47 residues recognized by HCV bNAbs, as a significant number of the antibody-interacting residues are outside these regions (refer to **Table 1** for details). We used the HCV-GLUE to analyze the level of conservation for each genotype (1–7) and 10 subtypes (**Figure 3**; Table S1 in Supplementary Material). For the majority of bNAbs

TABLE 1 | Broadly neutralizing antibodies and their epitopes analyzed in the study.

Name	E2-binding residues	Region targeted	Identification of residues	Reference
AR4A	*Y201, T204, N205, D206, R657, L692, D698*	E1E2	Mutagenesis	(32)
AR5A	*Y201, T204, N205, D206, R639, R657*	E1E2	Mutagenesis	(32)
J6.36	*F403, G406*	Hypervariable region 1 (HVR1)	Mutagenesis	(33)
J6.103	*F403, G406*	HVR1	Mutagenesis	(33)
H77.16	*G406, N410, I411*	HVR1	Mutagenesis	(33)
HC33.4	*K408*, L413, W420	HVR1, Epitope 1	Mutagenesis	(34)
HC33.8	*K408*, L413, G418, W420	HVR1, Epitope 1	Mutagenesis	(34)
HC33.29	*K408*, L413, G418, W420	HVR1, Epitope 1	Mutagenesis	(34)
AP33	L413, N415, G418, W420	Epitope 1	Structure	(17, 35, 36)
Hu5B3.v3	L413, N417, W420, I422	Epitope 1	Structure	(37)
HC33.1	L413, G418, W420	Epitope 1	Structure	(34, 38)
HC33.32	L413, G418, W420	Epitope 1	Mutagenesis	(34)
HCV1	L413, N415, G418, W420	Epitope 1	Structure	(39, 40)
95-2	L413, W420	Epitope 1	Mutagenesis	(39)
H77.39	N415, N417	Epitope 1	Mutagenesis	(33)
3/11	N415, W420, H421	Epitope 1	Structure	(41–43)
Mab24	T416, G418, W420, H421	Epitope 1	Mutagenesis	(44)
HC84.22	W420, *N428*, *C429,* W437, L441, F442, Y443, W616	Epitope 1, 2, and 4	Mutagenesis	(27, 45)
HC84.23	W420, *N428*, *C429,* W437, L441, F442, Y443, W616	Epitope 1, 2, and 4	Mutagenesis	(27, 45)
AR3C	*T425, N428, C429,* L438, L441, F442, Y443,W529	Epitope 2 and 3	Structure	(24–26)
e20	*T425, L427, N428,* W437, F442, W529, G530, D535, W616	Epitope 2 and 3	Mutagenesis	(46–48)
HC-11	*T425, N428, C429,* G436, W437, L438, F442, Y443, D520, G530, D535	Epitope 2 and 3	Mutagenesis	(27, 49)
HC-1	*C429,* W529, G530, D535	Epitope 3	Mutagenesis	(49, 50)
HC84.20	*C429,* L441, Y613, W616	Epitope 2 and 4	Mutagenesis	(27, 34)
HC84.21	*C429,* L441, F442, Y443	Epitope 2	Mutagenesis	(27, 34)
HC84.24	*C429,* F442, Y443	Epitope 2	Mutagenesis	(27, 34)
HC84.25	*C429,* L441, F442, W616	Epitope 2 and 4	Mutagenesis	(27, 34)
HC84.27	*C429,* L441, F442, Y443, K446, W616	Epitope 2 and 4	Structure	(27, 34, 51)
mAb#8	W437, L438	Epitope 2	Structure	(52, 53)
mAb#41	W437, L438	Epitope 2	Peptide mapping	(52)
CBH-2	W437, A439, G530, D535	Epitope 2 and 3	Mutagenesis	(49)
HC84.1	L441, F442	Epitope 2	Structure	(34, 51)
HC84.26	L441, F442	Epitope 2	Mutagenesis	(34)
1:7	G523, T526, Y527, W529, G530, D535	Epitope 3	Mutagenesis	(54)
A8	G523, T526, Y527, W529, G530, D535	Epitope 3	Mutagenesis	(54)
MAb44	G523, P525, *N540, W549*, Y613	Epitope 3 and 4	Mutagenesis	(44)
J6.27	A524, W529	Epitope 3	Mutagenesis	(33)
H77.31	W529, G530, D533	Epitope 3	Mutagenesis	(33)

[a]*Italicized residues are not in Epitopes 1–4. Numbering is according to the H77 polyprotein. Antibodies are listed in numerical order according to the first residue of their epitope.*

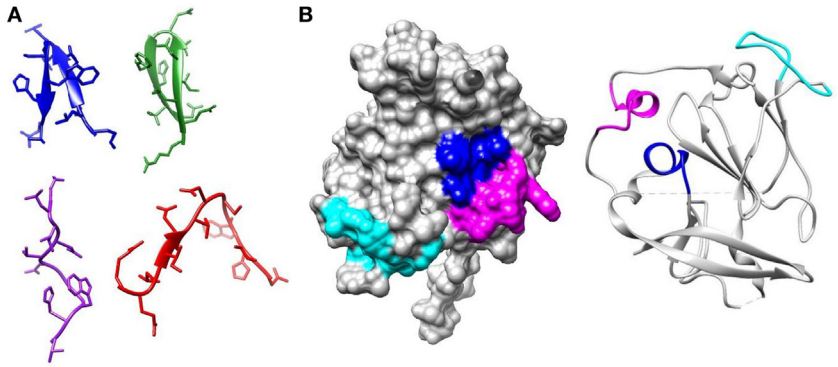

FIGURE 1 | Regions of E2 targeted by broadly neutralizing antibodies. **(A)** Epitope 1 (412–423) is flexible. The structure of this region has been solved bound to several broadly neutralizing antibodies. In AP33 (blue) (PDB 4GAG) and HCV1 (green) (PDB 4DGV), this region forms a β-hairpin structure. In HC33.1 (red) (PDB 4XVJ), it has an intermediate structure between a β-hairpin and a coil and in 3/11 (purple) (PDB 4WHT), it has an extended conformation. **(B)** The core E2 structure (PDB 4MWF) with Epitope 2 (434–446) in magenta, Epitope 3 (525–535) in cyan, and Epitope 4 (611–617) in blue.

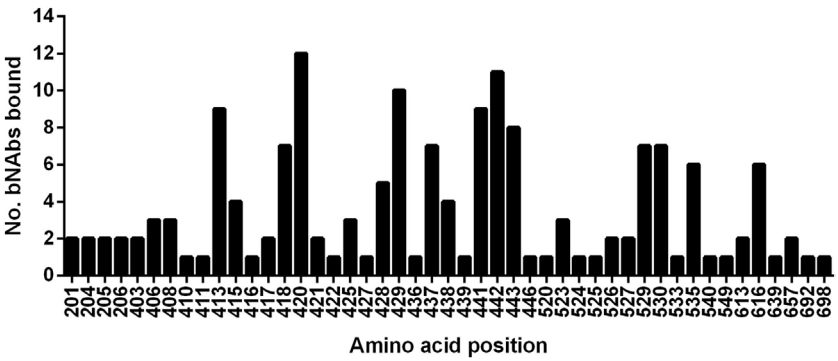

FIGURE 2 | Relative usage of residues bound by broadly neutralizing antibodies. The graph plots the number of broadly neutralizing antibodies in this study that use each amino-acid as part of their epitope.

alanine-scanning mutagenesis in the genotype (gt), 1a H77 strain was used to determine the bNAb antibody-interacting residues. The J6 group of bNAbs is the exception as this was mapped using the gt2a J6 virus strain. Consequently, in our analysis, we used the sequence that was used to map the antibody interaction as the reference sequence. Predictably, the overall level of conservation among these residues, which are bound by bNAbs, was high. Positions 408K, 410N in HVR1 and 411I just downstream were less well conserved. In Epitope 1, threonine at position 416 is substituted by serine in a large proportion of gt2a, gt2c, gt3b, and gt4a. In Epitopes 2 and 3, two residues 437W and 533D were well-conserved for gt1 and gt1a but were found to be preferentially replaced by a similar residue, phenylalanine, and glutamic acid, respectively, in all other genotypes and subtypes. Likewise, at position 438, the leucine residue found in the H77 gt1a sequence was an isoleucine in the majority of sequences. Some variants were very genotype specific. In gt2, position 446K was generally serine, arginine, or asparagine depending on the particular subtype. The majority of gt3 and gt6 sequences have a glutamic acid residue replacing the asparagine at position 540; however, in the subtype gt3b, this is commonly a threonine residue. Both variants are particularly interesting as substitution of N540 removes a potential N-linked glycosylation motif at this position.

The number of amino-acid variants identified (excluding that of the reference sequence), for each position is shown in **Figure 4**. Generally, gt1 has a greater range of variants for each position, although this may be skewed due to the large number of gt1 sequences (>23,000) in the database compared to the other genotypes. Even so, it is clear from the data that certain positions are less tolerant of variation than others, most notably residues within E1 (201–206) and toward the E2 C-terminal end (613, 639, 692, 698). Interestingly, of the other six positions whereby nearly all the genotypes/subtypes have fewer than three variants namely 406, 413, 421, 436, 523, 526, three of these are glycine residues.

Analysis of the Level of Conservation of bNAb Epitopes

To assess how well the complete binding motif of each bNAb was conserved, we determined the residue with the lowest

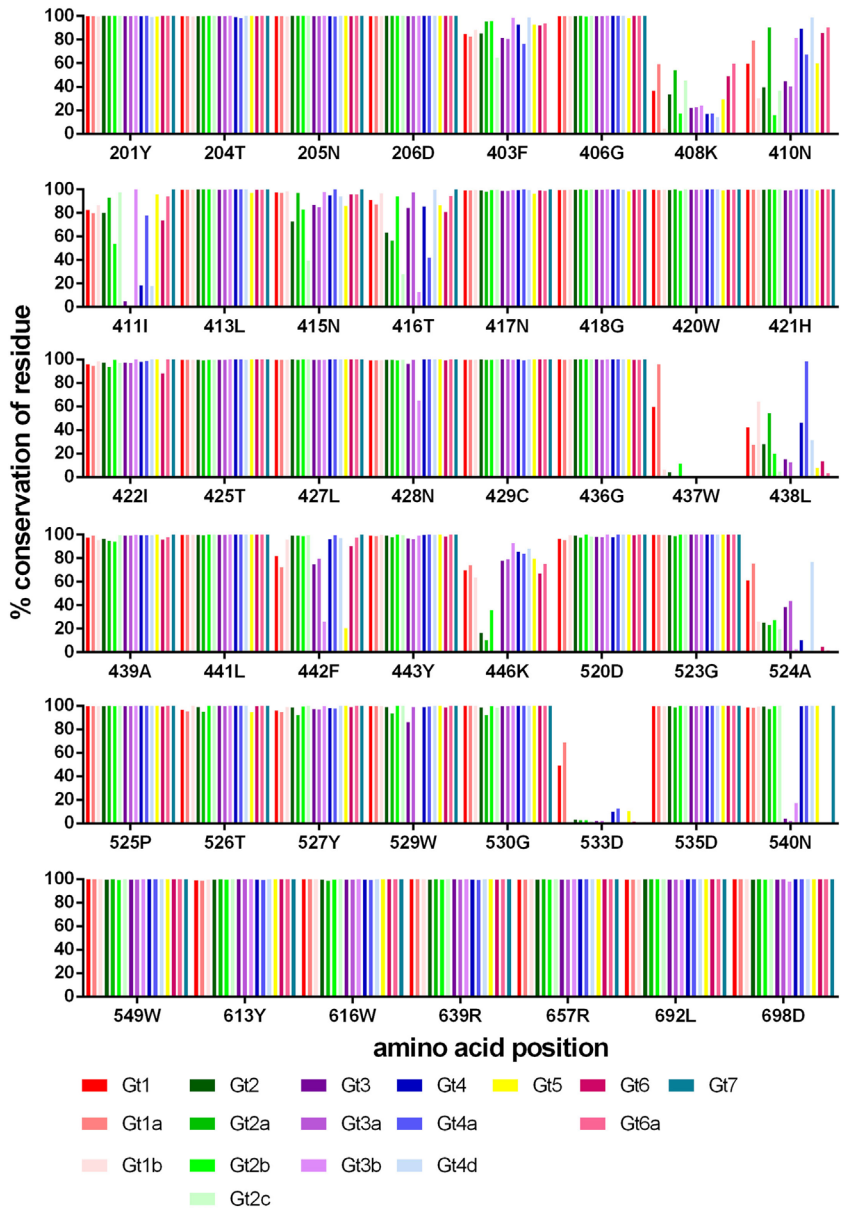

FIGURE 3 | Conservation of bNAb-bound residues. The level of conservation (%) with respect to the reference sequence is shown for each genotype and subtype. Gt1 (red), Gt2 (green), Gt3 (purple), Gt4 (blue), Gt5 (yellow), Gt6 (pink), and Gt7 (teal).

level of conservation relative to the reference sequence for each motif (refer to **Table 2**). There is the possibility that a particular residue may be differentially conserved between genotypes and therefore, this was done for all seven genotypes. These values were combined to give the % conservation for all genotypes for each binding motif, and this figure was used to rank the bNAb panel from the most conserved overall (HC84.20) to the least conserved (mAb#8, mAb#41 and HC-11). The number of genotypes where the binding motif was conserved by at least 90% is also reported in **Table 2**. Epitopes of six antibodies had this level of conservation in all seven genotypes. In contrast, the epitope residues of 15 antibodies did not reach this degree of conservation in the glycoprotein of any genotype. The level of conservation varied with respect to each genotype. The epitopes of gt4 E1E2 had the highest level of conservation, with 22/38 antibodies displaying >90% conservation of their binding motifs. This compares to only 7/38 antibodies in the case of gt3, suggesting that this genotype may be the most difficult to neutralize with this bNAb panel.

The epitopes of antibodies AR4A and AR5A, which bind residues in E1 and toward the C-terminus of E2, were highly conserved. Epitope 1 is also well-conserved, indeed, the majority of bNAbs that target this region are ranked highly overall. The differences in rank, of the Epitope 1-binding bNAbs, can be attributed to the specific residues recognized by each antibody. For

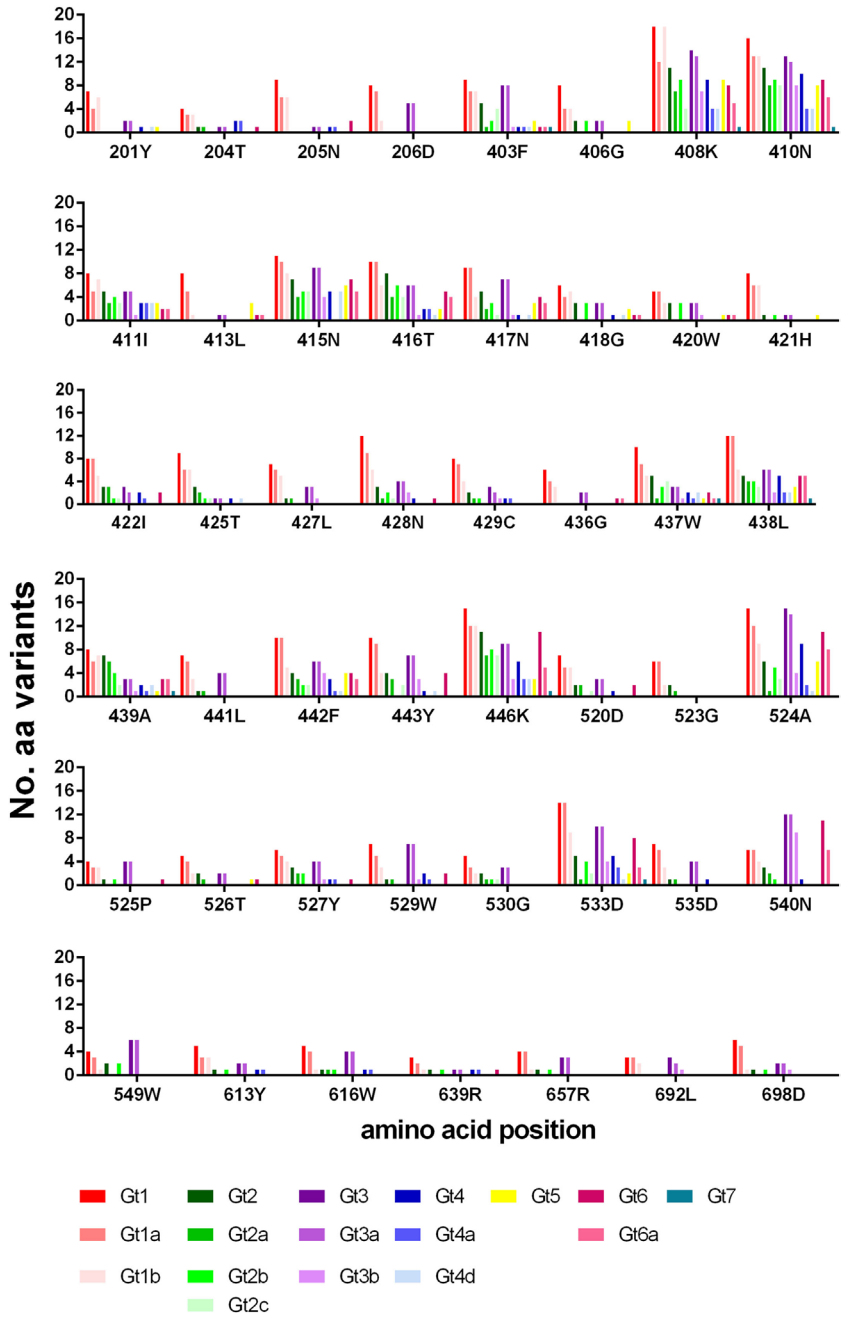

FIGURE 4 | Variance of bNAb bound residues. The number of different amino-acid variants for each residue is shown across the different genotypes and subtypes. Gt1 (red), Gt2 (green), Gt3 (purple), Gt4 (blue), Gt5 (yellow), Gt6 (pink) and Gt7 (teal).

instance, bNAbs AP33, HCV1, H77.39, and 3/11 all bind N415, which has a lower level of conservation in gt2, gt3, and gt5. This reduces the level of conservation of these antibodies compared to HC33.1, 95-2 or Hu5B3.v3, which do not require N415 for binding. Similarly, the Mab24 epitope is not as well conserved due to its requirement for T416. For the HC33 group of antibodies, those that require K408 are ranked lower overall as this residue, which is located in the HVR1, perhaps unsurprisingly, is poorly conserved. However, in contrast, the residues 403 and 406 in HVR1 that are recognized by J6.36 and J6.103 are unexpectedly well-conserved (>90% conservation in gt4, gt5, and gt6).

The bNAbs that bind to Epitope 2 generally perform less well, the exception is HC84.20, which ranked at the top of **Table 2**. Many of the HC84 series of the bNAbs are the highest ranking Epitope 2-interacting antibodies. There is >90% conservation of the binding motif within this region of E2 of gt2, gt4, and gt6, whereas the corresponding residues in the gt1 and gt3 glycoproteins are less conserved (81.85 and 74.83%, respectively). This is even more

TABLE 2 | The lowest conservation (%) for each bNAb epitope binding motif across genotypes.

Name	Gt1% Con	Gt2% Con	Gt3% Con	Gt4% Con	Gt5% Con	Gt6% Con	Gt7% Con	Sum% Con	No. gt >90%	Region targeted	Rank
AR4A	99.79	99.78	99.72	99.15	99.65	99.86	100	697.95	7	E1E2	2
AR5A	99.79	99.78	99.8	99.15	99.65	99.65	100	697.82	7	E1E2	3
J6.36	85.02	85.28	81.48	92.85	92.76	91.93	0	529.32	3	Hypervariable region 1 (HVR1)	21=
J6.103	85.02	85.28	81.48	92.85	92.76	91.93	0	529.32	3	HVR1	21=
H77.16	59.4	39.75	4.8	18.6	2.72	25.16	0	150.43	0	HVR1	29
HC33.4	36.58	33.51	21.96	16.88	29.53	49.3	0	187.76	0	HVR1, Epitope 1	25=
HC33.8	36.58	33.51	21.96	16.88	29.53	49.3	0	187.76	0	HVR1, Epitope 1	25=
HC33.29	36.58	33.51	21.96	16.88	29.53	49.3	0	187.76	0	HVR1, Epitope 1	25=
AP33	97.57	72.57	86.88	94.94	85.82	95.62	100	633.4	4	Epitope 1	11=
Hu5B3.v3	96.05	97.19	97.24	98.05	96.30	88.03	100	672.86	6	Epitope 1	8
HC33.1	99.66	99.56	99.85	99.87	97.06	99.83	100	695.83	7	Epitope 1	5=
HC33.32	99.66	99.56	99.85	99.87	97.06	99.83	100	695.83	7	Epitope 1	5=
HCV1	97.57	72.57	86.88	94.94	85.82	95.62	100	633.4	4	Epitope 1	11=
95-2	99.73	99.56	99.9	100	97.06	99.83	100	696.08	7	Epitope 1	4
H77.39	97.57	72.57	86.88	94.94	85.82	95.62	100	633.4	4	Epitope 1	11=
3/11	97.57	72.57	86.88	94.94	85.82	95.62	100	633.4	4	Epitope 1	11=
Mab24	91.08	63.21	84.21	85.73	86.67	80.96	100	591.86	2	Epitope 1	15
HC84.22	59.59	4.2	0	0.92	0	0.51	0	65.22	0	Epitope 1, 2, and 4	32=
HC84.23	59.59	4.2	0	0.92	0	0.51	0	65.22	0	Epitope 1, 2, and 4	32=
AR3C	42.23	28.13	15.14	46.2	7.69	13.59	0	152.98	0	Epitope 2 and 3	28
e20	59.59	4.2	0	0.92	0	0.51	0	65.22	0	Epitope 2, 3, and 4	32=
HC-11	42.23	4.2	0	0.92	0	0.51	0	47.86	0	Epitope 2 and 3	36=
HC-1	99.78	98.49	86.15	99.15	100	98.62	100	682.19	5	Epitope 3	7
HC84.20	99.81	99.58	99.8	99.51	100	100	100	698.7	4	Epitope 2 and 4	1
HC84.21	81.85	99.12	74.83	96.08	20.51	90.47	100	562.86	4	Epitope 2	16=
HC84.24	81.85	99.12	74.83	96.08	20.51	90.47	100	562.86	4	Epitope 2	16=
HC84.25	81.85	99.12	74.83	96.08	20.51	90.47	100	562.86	4	Epitope 2 and 4	16=
HC84.27	69.80	16.37	74.83	85.60	20.51	66.97	0	334.08	0	Epitope 2 and 4	24
mAb#8	42.23	4.2	0	0.92	0	0.51	0	47.86	0	Epitope 2	36=
mAb#41	42.23	4.2	0	0.92	0	0.51	0	47.86	0	Epitope 2	36=
CBH-2	59.59	4.2	0	0.92	0	0.51	0	65.22	0	Epitope 2 and 3	32=
HC84.1	81.85	99.12	74.83	96.08	20.51	90.47	100	562.86	4	Epitope 2	16=
HC84.26	81.85	99.12	74.83	96.08	20.51	90.47	100	562.86	4	Epitope 2	16=
1:7	95.81	98.49	86.15	97.86	94.74	98.62	100	671.67	6	Epitope 3	9=
A8	95.81	98.49	86.15	97.86	94.74	98.62	100	671.67	6	Epitope 3	9=
Mab44	98.73	99.43	4.03	99.51	100	0.56	100	502.26	5	Epitope 3 and 4	23
J6.27	61.23	25.14	38.41	1.26	0	4.52	0	130.56	0	Epitope 3	30
H77.31	49.09	3.02	2.12	9.83	10.53	1.4	0	75.99	0	Epitope 3	31

Shading denotes >90% conservation. Each antibody has been ranked from the most to the least conserved according to the sum of the lowest level of conservation (%) across all genotypes. An = symbol shows broadly neutralizing antibodies that have equivalent rank.

striking in gt5 where conservation of the binding motif is only 20.51%. These reductions are attributable to the phenylalanine at position 442 of the binding motif; this hydrophobic residue is changed to an aliphatic residue in a significant proportion of sequences. The particular aliphatic residue varies depending on genotype, e.g., leucine (12.38%) in gt1, isoleucine (18.6%) in gt3, and methionine (35.9%) in gt5. This change is likely to affect antibody binding. HC84.20 ranks so highly because F442 is not a critical binding residue for this bNAb. The HC84.22 and HC84.23 binding motifs are poorly conserved, due to their interaction with W437. This residue also forms part of the binding motif for other bNAbs that interact with Epitope 2, CBH-2, mAb#8, mAb#41, and e20. However, in the majority of instances (>95% for all but gt1), it is replaced by the hydrophobic phenylalanine residue: this amino acid has very similar properties; therefore, it is likely that a degree of antibody binding would be retained. Remarkably, the binding motif of the strongly neutralizing bNAb AR3C has a low level of conservation across all genotypes. Further inspection of the sequences shows that this is due to L438, which is replaced by an isoleucine in the majority of sequences. This is a conservative change; therefore, it is probable that antibody interaction may be retained.

Investigation of bNAb Epitopes in HCV Subtypes

The bNAbs with the most conserved binding motifs were further analyzed for the level of conservation at the HCV subtype level. For the analysis, we selected the 10 best represented subtypes in the dataset (1a, 1b, 2a, 2b, 2c, 3a, 3b, 4a, 4d, and 6a). This scrutiny revealed some interesting observations at the subtype level, as shown in **Table 3**. Surprisingly, the conservation of the binding motif of bNAbs HC-1, 1:7 and A8 was 0% in the 3b subtype compared to 99.09% for gt3a indicating that these antibodies may not neutralize gt3b. Further examination of the epitope sequence showed that this is due to replacement of W529 to a phenylalanine in all gt3b sequences ($n = 351$) in the database. This is a relatively conservative change from one hydrophobic residue to another; therefore, it is probable that the bNAbs will still bind gt3b. Likewise, further dissection of the genotypes into subtypes for the Epitope 1-binding bNAbs AP33, HCV1, H77.39 and 3/11 highlighted differences at the subtype level for both gt2 and gt3. This is due to the asparagine residue at position 415. While in gt2a, there is a high level of conservation at this position (97.27%), this drops to 82.94% in gt2b and more substantially to only 39.47% in gt2c. While in gt2b, this drop is due to an increase in the presence of serine at this position, the majority of gt2c sequences (57.68%) were found to have a histidine residue instead. Serine and asparagine are both hydrophilic, neutral residues and therefore this change may not substantially affect antibody binding. Histidine, however, is positively charged and this more dramatic change, found in gt2c, is more likely to abrogate antibody interaction. Similarly, in gt3a, there is a drop in conservation of N415 to 86.88%. This decrease is due to a higher prevalence of the positively charged arginine residue at this position, which would also be predicted to inhibit antibody binding.

Analysis of the Binding Motif Pattern

The conservation analysis reported above treats each position within the antibody motif as a single entity. This approach would not identify changes within the binding motif pattern that modify more than one substitution within the epitope. To investigate this, we analyzed the complete binding motif pattern for the 10 bNAbs with the most conserved epitopes (**Table 4**; Table S2 in Supplementary Material). The most prevalent amino-acid patterns, i.e., found in at least 10 sequences are shown in **Table 4**. These data confirm the high level of conservation of these epitopes across all seven genotypes. In all but one example, the most prevalent sequences only had a single substitution within the binding motif. For bNAbs AR4A and AR5A, the level of epitope conservation is particularly dramatic. From over 9,700 sequences analyzed, there was only a single alternative sequence that was present in at least 10 sequences. This had a single conservative change at E1 204 replacing threonine with serine. The HC84.20 epitope motif was also very highly conserved with a single alternative sequence present in 1.1% of gt1 sequences.

The bNAb-binding motifs in Epitope 1 bound by 95/2 and HC33.1 and HC33.32 were marginally more variable; however, each alternative sequence was still represented at a low frequency (fewer than 50 copies) in the database. There was more sequence variation across the Hu5B3.v3-binding motif in Epitope 1, with 12 different sequences identified. For all the Epitope 1-interacting bNAbs, there was no obvious preference for mutations at specific positions as substitutions were found to occur at all residues in the bNAb epitopes and all but one sequence was reported in at least two genotypes. Similarly, in Epitope 3 where the HC-1, 1:7, and A8 binding motifs share three common residues (W529, G530 and D535), there was no pattern for which residue in the binding motif was altered. Gt3 sequences had the lowest frequency of conservation, due to the substitution of W529 with a phenylalanine residue.

Geographical Distribution

Our analysis is based on the sequence data available in Genbank. To investigate how the available data reflected the global distribution of HCV, we analyzed the country of origin of the deposited sequences. We focused on E2 as this is the principal target of bNAbs; however, the data for both E1 and E2 are shown in

TABLE 3 | The lowest level of conservation (%) for each bNAb epitope-binding motif across subtypes.

Name	Gt1a% Con	Gt1b% Con	Gt2a% Con	Gt2b% Con	Gt2c% Con	Gt3a% Con	Gt3b% Con	Gt4a% Con	Gt4d% Con	Gt6a% Con	Region targeted
HC84.20	98.58	99.73	98.48	99.72	100	99.79	99.75	99.24	100	100	Epitope 2 and 4
AR4A	99.76	99.57	99.8	99.7	100	99.74	98.13	98.13	99.23	100	E1E2
AR5A	99.76	99.57	99.8	99.7	100	99.76	100	98.13	99.23	100	E1E2
95-2	99.66	99.83	100	98.7	100	99.91	99.75	100	100	99.7	Epitope 1
HC33.1	99.55	99.83	100	98.7	100	99.82	99.75	100	99.82	99.7	Epitope 1
HC33.32	99.55	99.83	100	98.7	100	99.82	99.75	100	99.82	99.7	Epitope 1
HC-1	99.78	99.66	92.21	99.72	98.44	99.09	0	99.38	100	100	Epitope 2 and 3
1:7	94.62	98.87	92.21	99.43	98.44	99.09	0	97.5	100	100	Epitope 3
A8	94.62	98.87	92.21	99.43	98.44	99.09	0	97.5	100	100	Epitope 3
Hu5B3.v3	94.46	98.44	93.49	98.7	96.93	96.86	99.51	98.77	99.45	98.81	Epitope 1
AP33	96.98	98.49	97.27	82.94	39.47	84.85	97.8	100	93.91	95.87	Epitope 1
HCV1	96.98	98.49	97.27	82.94	39.47	84.85	97.8	100	93.91	95.87	Epitope 1
H77.39	96.98	98.49	97.27	82.94	39.47	84.85	97.8	100	93.91	95.87	Epitope 1
3/11	96.98	98.49	97.27	82.94	39.47	84.85	97.8	100	93.91	95.87	Epitope 1

Shading denotes > 90% conservation.

TABLE 4 | The most prevalent sequences for the complete bNAb epitope motifs.

bNAb	Sequence	Percentage of sequences (%)							Total no. sequences	Gt range
		Gt1	Gt2	Gt3	Gt4	Gt5	Gt6	Gt7		
HC84.20	C/L/Y/W	98.6	99.2	99.5	99.5	100	100	100	11,379	1–7
	C/L/H/W	1.10	0	0	0	0	0	0	89	1
AR4A	Y/TND/R/L/D	99.3	99.2	99.2	97.5	100	99.6	100	9,704	1–7
	Y/SND/R/L/D	0.11	0	0	1.97	0	0	0	12	1, 4
AR5A	Y/TND/R/R	99.5	99.5	99.4	97.5	100	99.1	100	9,741	1–7
	Y/SND/R/R	0.11	0	0	1.97	0	0	0	12	1, 4
95/2	L/W	99.6	99.6	99.9	100	96.3	99.7	100	29,658	1–7
	L/R	0.15	0.07	0.05	0	0	0.17	0	39	1, 2, 3, 6
	P/W	0.07	0	0.05	0	0	0	0	19	1, 3
	L/[a]	0.05	0.07	0	0	0	0	0	12	1, 2
	L/C	0.04	0	0	0	0.75	0	0	11	1, 5
HC33.1 and HC33.32	L/G/W	99.3	99.3	99.7	99.9	94.8	99.5	100	29,565	1–7
	L/N/W	0.19	0	0.03	0	0	0	0	45	1,3
	L/G/R	0.15	0.07	0.05	0	0	0.17	0	39	1, 2, 3, 6
	L/D/W	0.07	0	0.10	0	0	0	0	21	1, 3
	P/G/W	0.07	0	0.05	0	0	0	0	19	1, 3
	L/S/W	0.05	0.07	0	0	0.75	0.17	0	15	1, 2, 5, 6
	L/G/[a]	0.05	0.07	0	0	0	0	0	12	1, 2
	L/G/C	0.04	0	0	0	0.75	0	0	11	1, 5
HC-1	C/WG/D	99.4	98.1	85.7	98.3	100	98.6	100	13,764	1–7
	C/FG/D	0.04	0	13.4	0.43	0	1.08	0	380	1, 3, 4, 6
	C/WG/G	0.12	0	0	0	0	0	0	12	1
	C/RG/D	0.07	0	0.11	0	0	0	0	10	1, 3
1:7 and A8	G/TY/WG/D	91.9	97.5	82.9	96.6	94.7	97.2	100	12,806	1–7
	G/TF/WG/D	3.64	0	2.6	2.14	0	1.10	0	453	1, 3, 4, 6
	G/TY/FG/D	0.02	0	13.4	0.43	0	1.10	0	377	1, 3, 4, 6
	G/AY/WG/D	3.33	0.19	0	0	0	0	0	341	1, 2
	G/TH/WG/D	0.37	0.19	0.07	0	0	0	0	41	1, 2, 3
	G/TY/WG/G	0.11	0	0	0	0	0	0	11	1
Hu5B3.v3	L/N/W/I	95.0	96.1	96.1	97.7	92.5	87.0	100	27,806	1–7
	L/N/W/L	2.37	0.07	0.03	0.65	0	0.51	0	555	1, 2, 3, 4, 6
	L/N/W/V	1.40	2.59	2.70	1.30	0	11.3	0	537	1, 2, 3, 4, 6
	L/S/W/I	0.40	0.37	0.23	0	0.75	0.34	0	109	1, 2, 3, 5, 6
	L/D/W/I	0.16	0.15	0.05	0	0	0.17	0	42	1, 2, 3, 6
	L/N/R/I	0.13	0.07	0.05	0	0	0.17	0	35	1, 2, 3, 6
	L/Q/W/I	0	0	0.62	0	1.50	0	0	26	3, 5
	L/N/W/T	0.10	0.07	0.05	0	0	0	0	25	1, 2, 3
	P/N/W/I	0.07	0	0.05	0	0	0	0	15	1, 3
	L/R/W/I	0.05	0	0	0	0	0	0	12	1
	L/N/[a]/I	0.05	0.07	0	0	0	0	0	12	1, 2
	L/N/C/I	0.04	0	0	0	0.75	0	0	11	1, 5

[a]*Signifies a stop codon, sequence differences are shown in bold.*

Table S3 in Supplementary Material. The number of sequences that cover at least 90% of E2 for gt1–6 per country is shown in **Figure 5**. Genotype 7 is not shown as there are only two sequences, both of which were isolated in Canada. There are an additional 1,320 sequences representing gt1–6 where no country is specified. Even for gt1, the most prevalent genotype, while the USA is well-represented, large areas of the globe have severely limited or no sequence information for this region of the HCV genome. Indeed, there are no sequences for Central America, the Middle East, most of the African continent, Eastern Europe, and South America. This trend is even more pronounced with regard to the less prevalent genotypes. There are approximately 3,000 more E1 sequences available compared to E2; however, the overall trend with respect to the global distribution is similar (Figure S1 in Supplementary Material).

DISCUSSION

The development of the GLUE software has enabled rapid analysis of large datasets of viral sequences (28). HCV-GLUE is the most advanced project; however, similar data resources for other viruses including HIV and HBV are under development (R. Gifford and J. Singer, personal communication). In this study, we have assessed the level of conservation of the critical residues of HCV glycoproteins that are recognized by a panel of HCV bNAbs using HCV-GLUE. This analysis has identified a group of

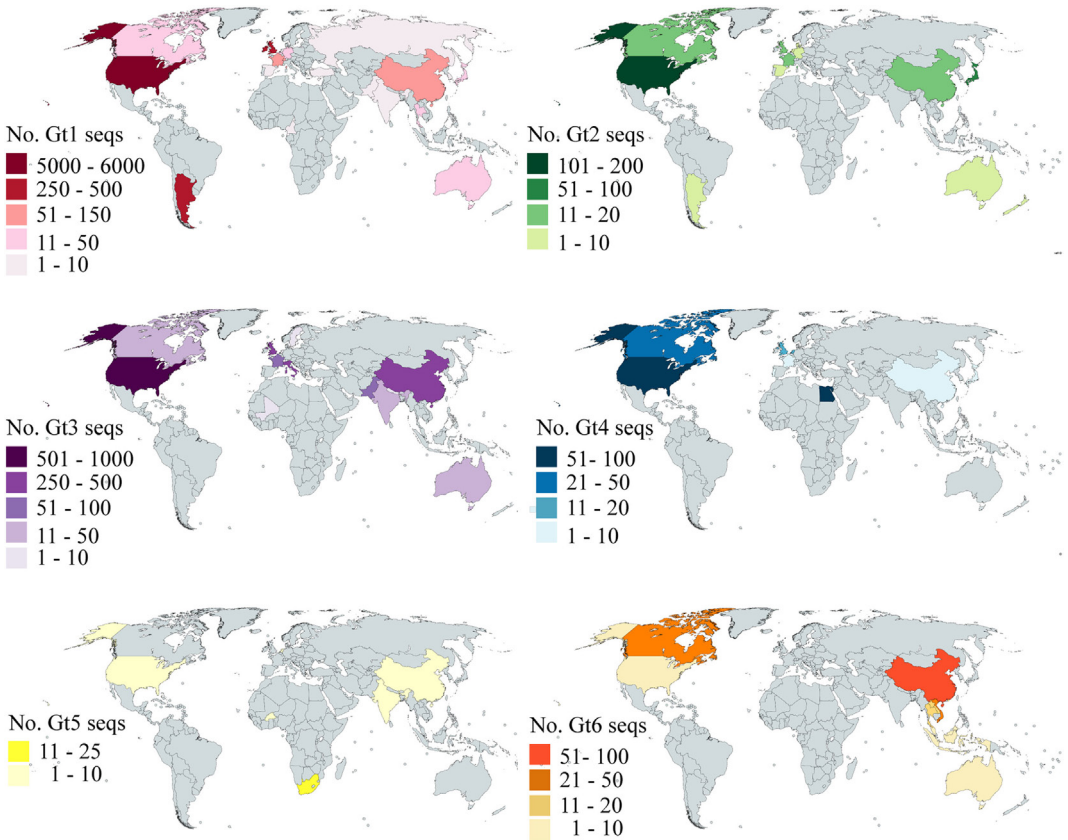

FIGURE 5 | Global distribution of E2 sequence coverage. The maps are color-coded to show the number of E2 sequences (with at least 90% coverage) per country for Gt1 (red), Gt2 (green), Gt3 (purple), Gt4 (blue), Gt5 (yellow), and Gt6 (orange). Countries shaded in gray have no sequences reported.

bNAbs that theoretically, based on epitope conservation, would be the best leads for a vaccine design strategy. However the presence of the epitope sequence is not the only factor dictating the efficacy of an antibody; the affinity of the antibody for its target is also important. We and others have noted that some isolates of E1E2 are more resistant to neutralization either by patient sera or purified bNAbs (12, 55–58). Indeed, two of these studies, which both performed large-scale neutralization studies of >80 E1E2 isolates identified three groups, with those that were either highly sensitive or highly resistant at the extremes and the majority falling somewhere in between. The reason for this is unclear, Urbanowicz and coworkers suggest that multiple mechanisms are likely to be involved as they could not identify any common sequence substitutions that explained the extreme phenotypes (55). In contrast, El-Diwany and coworkers demonstrated that two polymorphisms at positions 403 and 438 modulate neutralization phenotype and suggest that this is *via* alteration of binding to the receptor SR-BI (57). Moreover, in the context of an HCV infection, it has been demonstrated that the glycan shield formed by extensive glycosylations of E1E2 can prevent bNAbs binding to their neutralizing epitopes (14, 59). Studies also indicate that the HVR1 of E2 functions to block access to neutralizing epitopes, this is variable between E1E2 isolates strongly influencing the neutralization phenotype (12). Also relevant in the case of HCV, which is particularly diverse in sequence, is the possibility that a contributing factor to the breadth of binding by bNAbs may be due to the antibody paratope being able to accommodate alternative residues. Therefore, we examined the available neutralization data for the bNAbs with the most conserved epitopes.

By definition, all bNAbs can neutralize more than one viral genotype, although they have been assessed by different groups in different systems with different isolates and methods and thus it is difficult to compare the results directly. With regards to the conservation of the epitope sequence, we identify HC84.20 as the top candidate; it has been assessed for neutralization activity in the HCV cell culture (HCVcc) system and was able to neutralize all E1E2 sequences (gt1–6) except for gt3. AR4A, which ranked second overall performed better in neutralization studies reported by Giang et al. (32). AR4A was able to neutralize the full complement of E1E2 sequences (gt1–6) tested in both the HCVpp and HCVcc systems. The AR4A epitope was conserved in all 24 sequences tested. AR4A was able to neutralize 85.8% of E1E2 isolate in a large-scale neutralization study (57). According to our data, as the AR5A epitope is also conserved in all the sequences tested, it should behave similarly. In practice; however, this bNAb could neutralize gt 2 sequences in the HCVcc system but not in the HCVpp system and could

not neutralize any gt3 sequence tested (32). From the alanine-scanning mutagenesis, AR4A and AR5A have overlapping epitopes; however, our results together with the neutralization data suggest that AR5A binds additional residues that have yet to be identified (32). This is supported by the observation in the original paper that the epitope of AR5A but not AR4A overlaps with the epitope of the bNAb CBH-7 (32). CBH-7 was not included in our analysis as, despite much effort, the epitope has not yet been defined (27, 60, 61).

Several of the top-ranking bNAbs bind to Epitope 1; of these, bNAb 95-2 has the most conserved epitope composed of only two residues L413 and W420. This antibody was isolated by Broering and coworkers along with bNAb HCV1 (39). They report that both antibodies were able to neutralize all six HCVpp tested (gt 1–4) suggesting broad neutralization, although HCV1 was marginally less effective. There are no data available for gt 5 or 6. The fact that only two residues (L413 and W420) are required by bNAb 95-2 for binding contributes to the high level of conservation. It should be noted that, in the original study, bNAb HCV1 was also reported to only require the same two residues. Subsequent work by Kong et al. (40) who showed by alanine-scanning mutagenesis, and more importantly, by co-crystallization of HCV1 in complex with Epitope 1 peptide that N415 and G418 were also required (40). To our knowledge, no further work with 95-2 has been reported in the literature and therefore it remains possible that 95-2 may also require additional residues for binding. Other Epitope 1 bNAbs HC33.1, HC33.32, and H77.39 do not neutralize as well as we would predict from our results even though their epitopes are conserved in all the sequences tested (33, 34). Contrary to our predictions, in practice, H77.39 is the most effective, neutralizing gt1, gt2, gt4, and gt5 but not gt3 or gt6. HC33.1 did not efficiently neutralize gt2, gt3, or gt6 and HC33.32 performed marginally less well neutralizing only gt2, gt4, and gt5 (33, 34). These data suggest that other factors are influencing antibody performance. As mentioned above, these may be attributes of the antibody themselves such as affinity and avidity. Alternatively, properties of the E1E2 glycoproteins such as glycosylation or HVR1 structure may be preventing access to the epitope. Hu5B3.v3 could neutralize both gt1 and gt2, but it has not been tested against other genotypes (46).

AP33 and 3/11 that both recognize E2 Epitope 1 residues (L413, N415, G418, W420 for AP33 and N415, W420, H421 for 3/11) have been tested against a wider range of E1E2 sequences although these studies were performed prior to the development of the HCVcc system (18, 41). Tarr et al. compared the performance of both antibodies in parallel (41). AP33 performed significantly better than 3/11, neutralizing 17/18 HCVpp (gt 1–6) compared to 6/18 isolates (gt1, gt2, gt4, gt5, and gt6). This is despite the 3/11 epitope being completely conserved, in contrast to that of AP33. Indeed, the sequence that AP33 did not neutralize contained two mutations in the AP33 epitope N415Q and G418S, which explains the lack of neutralization. Notably, a sequence that was neutralized by AP33 had a change in one of the antibody-interacting residues whereby N415 was replaced by a histidine. This is particularly interesting as nearly 60% of gt2c sequences have a histidine at this position. Therefore, this data suggests that AP33 can still bind and neutralize sequences containing this variant, which would increase the predicted level of AP33 binding to gt2 sequences. It has been shown that a mutation N417S/T results in a glycan shift within Epitope 1 from 417 to 415 that can block binding with neutralizing antibodies, such as AP33, which require N415 (37, 62). The shift is due to a change in position 417 to either serine or threonine that creates an N-linked glycosylation site. Consequently, we checked the frequency of these mutations in the database. We find that these mutations have been detected in the majority of genotypes, with the exception of gt4 and gt7, albeit at low frequency (0.23–0.74%). AP33 performed well in a large-scale neutralization study with a mean IC_{50} value of 0.69 μg/ml (55). Hu5B3.v3 has been shown to neutralize gt1 and gt2 sequences, but there is no data for other genotypes (37).

The Epitope 3-binding antibodies 1:7 and A8 both have been shown to perform well in neutralization studies. They were tested against 10 different E1E2 sequences (gt1–6) in the HCVpp system (54). The epitope was conserved in all the sequences. 1:7 was marginally better than A8 neutralizing HCVpp bearing all 10 sequences by at least 50%. A8 only neutralized 9/10 isolates by >50%; however, this was at a relatively low concentration (15 μg/ml) compared to other studies. 1:7 was included in a large-scale neutralization study and shown to have a mean IC_{50} value of 2.1 μg/ml (55). HC-1, which binds in part to Epitope 3 did not function as well in neutralization tests. Although its epitope is conserved, HC-1 only neutralized 3/8 E1E2 sequences (gt1 and gt 5) tested (63). Similarly to bNabs 1:7 and A8, the highest concentration tested was 20 μg/ml compared to 50 μg/ml for other studies; therefore, this might improve at higher concentrations. From our bioinformatics analysis, the top bNAbs would be HC84.20, AR4A, AR5A, and 95/2; however, if we take into account the available neutralization data we can conclude that HC84.20, AR4A, 1:7, A8, and AP33 are the most promising lead candidates to date. 95-2, HCV1, and Hu5B3.v3 performed well in our analysis but have not been tested as extensively across different genotypes for neutralization. AR5A, HC33.1, HC33.32, HC-1, H77.39, and 3/11 all have highly conserved epitopes; however, they were not as effective in neutralization studies.

One unexpected result from our analysis was the relatively low level of conservation of the AR3C antibody epitope residues, this was principally due to the requirement for L438. This residue is poorly conserved with isoleucine and valine being the main variants. Our data are in stark contrast to the neutralization studies reported for AR3C (24, 64). AR3C neutralized 27/29 E1E2 sequences tested in the HCVpp system (24, 64). Examination of the sequences showed that several isolates had either isoleucine or valine at position 438, thereby providing strong evidence that AR3C can still bind and neutralize these variants. If we adjust for this in our analysis, the level of conservation is significantly improved, indeed, AR3C would be ranked 16th overall.

The analysis of the bNAb epitope motifs shows that there is a strong preference for the bNAb epitope consensus sequence, there are very few variants circulating at significant levels. This makes it feasible to assess whether the major variants remain susceptible to neutralization. Indeed, only 30 variants would be

required to test this for the most conserved bNAbs as the epitopes have several residues in common.

A recent publication by Messina et al. was the first to estimate the relative prevalence of HCV globally (65). Prevalence was estimated in 20 geographical regions and by country, data permitting. Gt1 was the most prevalent in 15/20 geographical regions accounting for 46.2% of cases. Nearly one-third of cases worldwide are due to gt3. The remaining ~25% are due to gt2, gt4, and gt6. Of these, gt2 is the most widespread globally although it does not have the highest prevalence in any region. Gt4 is localized mainly to Africa, indeed, it has the highest frequency in North Africa and the Middle East and Central sub-Saharan Africa. Like gt2, gt6 is not the most prevalent genotype in any region; however, it is significant in East and Southeast Asia. Finally, gt5 accounts for the lowest frequency globally, but is highly localized in southern sub-Saharan Africa. Our data show that the top candidate bNAbs identified here should be effective against the majority of variants. Indeed, the HC84.20 and AR4A epitopes are conserved in >99% of available sequences for each genotype; therefore, a vaccine based on these epitopes would be predicted to be effective against all genotypes. While well-conserved against the most prevalent genotype, gt1, both 1:7 and AP33 epitopes are less well conserved in gt3. However, in the case of 1:7, this was specifically gt3b sequences whereas AP33 epitope residues were more conserved in gt3b than gt3a suggesting that these antibodies may complement each other. In the same way, conservation of the AP33 epitope was reduced in gt2b and gt2c; however, the 1:7 epitope was highly conserved in these subtypes.

LIMITATIONS

There are some caveats to the analysis reported in this study. The first is that our analysis is based on the publicly available sequence data available in Genbank. HCV sequence data in GenBank captures the known genomic diversity of this virus, and this dataset has for example underpinned the genotype and subtype definitions for the virus (4). HCV sequences may be submitted to GenBank as part of research projects with a wide variety of aims. Large sequence sets for a particular kind of research, for example, focusing on specific patient cohort types, may dominate. GenBank does not, therefore, accurately represent the range of HCV genomic diversity that a vaccine strategy would face "in the wild." However, GenBank does provide a reasonable number of sequences within certain major genotypes and subtypes and so it does give a view on which genomic patterns are viable for the virus; which can, therefore, be presumed to exist in the wider epidemic. Another factor is that while the developed world has some coverage of the HCV glycoproteins, significant areas of the globe have little or no sequence data available. As a result, particular genotypes such as gt5 and gt6 are poorly represented, and it is possible that other circulating genotypes/subtypes have yet to be identified. To truly understand the extent to which a bNAb-based vaccine may be effective globally, this data gap should be addressed. The analysis also depends on the availability and quality of the bNAb epitope mapping data. Obviously, bNAbs that have not been finely mapped could not be included in our analysis. Although the number of co-crystallization studies has increased in recent years, the majority of epitope mapping is based on alanine-scanning mutagenesis. While this is an important tool, the results may also identify residues that prevent antibody-interaction due to conformational changes. There are also examples whereby alanine-scanning experiments have not been able to conclusively identify the epitope, for instance, bNab e137 where mutation of 15 different residues prevent antibody binding (46, 47, 66). This study is based on sequence conservation of bNAb epitopes, in practice, other factors including antibody affinity, epitope shielding by both the glycan shield, and HVR1 will all influence the efficacy of each antibody. Furthermore, the epitope residues of many of the bNAbs have been determined by alanine-scanning mutagenesis, which does not unequivocally prove direct contact in the absence of structural information of the antigen/antibody complex. Unfortunately, the neutralization data reported in the literature is also variable for the different bNAbs, the assays have not been standardized and few comparative studies are available.

Despite the limitations, from our study, we can conclude that based on epitope conservation and the available neutralization data that the most promising bNAbs for potential vaccine leads are HC84.20, AR4A, 1:7, A8, and AP33. Our results are encouraging as several bNAb epitopes were highly conserved across all genotypes. This finding supports the notion that a single HCV vaccine could indeed fit all, and that ultimately tailoring vaccines to specific regions may prove to be unnecessary. However, further analysis of how the bNAbs perform in large-scale neutralization trials will be required to conclusively test this. Our analysis of the most prevalent epitope variants in circulation should provide useful information when designing these experiments. Nonetheless to counter escape mutations and provide sterilizing immunity, it is likely to be desirable to develop a multi-target vaccine, this could be a B-cell and T-cell-based combination or a combination of different B-cell targets.

AUTHOR CONTRIBUTIONS

Conceptualization (VC and AP), design and development of HCV-GLUE (RG and JS), generation of data (JS), analysis of data (VC), writing—original draft (VC and JS), writing—review and editing (AP and RG), funding (AP and RG).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01470/full#supplementary-material.

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Glycan Shielding and Modulation of Hepatitis C Virus Neutralizing Antibodies

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Hepatitis C virus (HCV) envelope glycoprotein heterodimer, E1E2, plays an essential role in virus entry and assembly. Furthermore, due to their exposure at the surface of the virion, these proteins are the major targets of anti-HCV neutralizing antibodies. Their ectodomain are heavily glycosylated with up to 5 sites on E1 and up to 11 sites on E2 modified by N-linked glycans. Thus, one-third of the molecular mass of E1E2 heterodimer corresponds to glycans. Despite the high sequence variability of E1 and E2, N-glycosylation sites of these proteins are generally conserved among the seven major HCV genotypes. N-glycans have been shown to be involved in E1E2 folding and modulate different functions of the envelope glycoproteins. Indeed, site-directed mutagenesis studies have shown that specific glycans are needed for virion assembly and infectivity. They can notably affect envelope protein entry functions by modulating their affinity for HCV receptors and their fusion activity. Importantly, glycans have also been shown to play a key role in immune evasion by masking antigenic sites targeted by neutralizing antibodies. It is well known that the high mutational rate of HCV polymerase facilitates the appearance of neutralization resistant mutants, and occurrence of mutations leading to glycan shifting is one of the mechanisms used by this virus to escape host humoral immune response. As a consequence of the importance of the glycan shield for HCV immune evasion, the deletion of N-glycans also leads to an increase in E1E2 immunogenicity and can induce a more potent antibody response against HCV.

Keywords: hepatitis C virus, neutralizing antibodies, glycosylation, humoral immune response, glycoproteins

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INTRODUCTION

With approximately 70 million people chronically infected worldwide, hepatitis C virus (HCV) is a major health burden. In most cases, HCV establishes chronic infection that can lead to the development of cirrhosis and hepatocellular carcinoma. For a long time, standard treatment for HCV infection consisted in a non-specific combination therapy with pegylated interferon and ribavirin, which was relatively toxic and effective in half of treated patients. Advances in *in vitro* and *in vivo*

Abbreviations: HCV, hepatitis C virus; HVR1, hypervariable region 1; HCVpp, HCV pseudoparticles; HCVcc, cell culture-derived HCV; ER, endoplasmic reticulum; SR-BI, scavenger receptor BI; mAb, monoclonal antibody; Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine.

HCV infection systems resulted in a great increase of our understanding of the HCV life cycle. This led to the development of several successful direct acting antivirals that allow for the achievement of high HCV clearance rates (>90%). However, the high cost of these antivirals therapy precludes their accessibility to the large majority of HCV-infected patients (1). In this context, the development of a preventive HCV vaccine would constitute the most cost-effective means to limit HCV spread. Studies have shown that a successful HCV vaccine would induce the production of neutralizing antibodies and a potent HCV-specific T cell response (2). However, a key challenge in HCV vaccine development is to overcome the high diversity of this virus. Several vaccine candidates targeting the envelope glycoproteins have been shown to induce strong humoral and cellular immune response in animal models or clinical trials in humans. However, their efficiency was limited by viral escape from immune response due to the high genetic variability of the virus (2–5). In this context, the design of an efficient vaccine will require a good knowledge of the strategies used by the virus to escape host immune response. One of these strategies is the presence of a glycan shield that protects E2 conserved epitopes from neutralizing antibodies. Here, we present the glycosylation of HCV envelope glycoproteins and we review the different aspects of the modulation of neutralizing antibodies by HCV glycan shield.

GLYCOSYLATION OF HCV ENVELOPE PROTEINS

Distribution of E1 and E2 N-Glycans

E1 and E2 are highly glycosylated with N-glycans representing one-third of the heterodimer mass. N-glycosylation occurs on the asparagine (Asn) residue belonging to aparagine–X–serine/threonine (Asn–X–Thr/Ser) motifs where X denotes any residue but Proline. In most genotypes, E1 contains four conserved glycosylation sites that are located at amino acid position 196 (E1N1), 209 (E1N2), 234 (E1N3), and 305 (E1N4) in genotype 1a H77 strain (**Figure 1**). However, an additional glycosylation site is present at position 250 in genotypes 1b and 6, or at position 299 in genotype 2b (6).

Up to 11 glycosylation sites can be detected in most E2 glycoprotein sequences. Nine of them are conserved across HCV genotypes, and they are located at positions 417 (E2N1), 423 (E2N2), 430 (E2N3), 448 (E2N4), 532 (E2N6), 556 (E2N8), 576 (E2N9), 623 (E2N10), and 645 (E2N11) in the H77 reference strain (**Figure 1**). The two other glycosylation sites are also conserved in most genotypes except in genotype 1b for the site at position 476 (E2N5) and in genotypes 3 and 6 for the site at position 540 (E2N7). Thus, despite high sequence variability in HCV, the majority of N-glycosylation sites are highly conserved, suggesting that glycans play a major role in the HCV life cycle. Importantly, all these sites have been confirmed to be occupied by glycans (7, 8).

In a minority of HCV genomes, additional glycosylation sites can also be observed. For instance, another glycosylation site has been reported to be present in the intragenotypic hypervariable region HVR495 of E2 in a minority of genotype 3a isolates from Pakistani patients (9). An additional glycosylation site has also been shown to appear in hypervariable region 1 (HVR1) after selection of a mutant resistant to monensin treatment in cell culture (10). The appearance of such natural or selected glycans suggests that HCV can adapt to environmental changes by generating novel glycosylation sites.

Type of Glycans Associated With E1 and E2 Glycoproteins

N-linked glycosylation occurs by the transfer en bloc of a Glc3Man9GlcNAc2 oligosaccharide from a lipid intermediate to an Asn residue in the consensus sequence Asn–X–Thr/Ser of a nascent protein. Three major types of N-glycans can be observed on glycoproteins. The first type corresponds to high mannose glycans which are composed of two *N*-acetylglucosamine molecules linked to several mannoses residues; the second type of glycans are complex oligosaccharides which are mainly composed of two *N*-acetylglucosamines, galactose and can contain sialic acid and fucose. Hybrid type glycans constitute the third type of N-glycans. They are composed of *N*-acetylglucosamine, galactose, mannose, and can contain sialic acid. Complex and hybrid glycans are generated during the transit of the protein through the Golgi compartment by addition or removal of sugar residues by specific enzymes. It has been shown that cell-associated E1E2 mainly display high-mannose-type oligosaccharides, which is in agreement with their retention in the endoplasmic reticulum (ER) of infected cells (11). Furthermore, the characterization of E2 glycosylation sites by mass spectrometry confirmed that the majority of these sites are occupied by high mannose glycans on a recombinant form of the glycoprotein (12).

Since they are assembled in the ER, HCV particles have to cross the secretory pathway before being released by infected

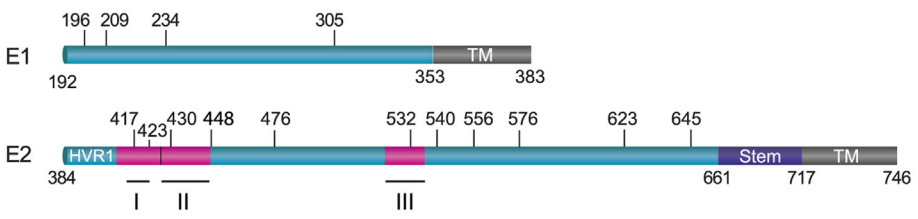

FIGURE 1 | Position of N-linked glycans on hepatitis C virus envelope glycoproteins. E1 and E2 are schematically represented by boxes with their transmembrane domains shown in brown. The glycosylation sites and their position are indicated by vertical bars (on reference strain H77). The localization of three major neutralizing epitopes on E2 (I: 412–423; II: 427–446; III: 523–535) is also shown.

cells. During this process, the glycoproteins associated with the virions are modified by cellular glycosidases and glycosyl transferases. In the absence of a cell culture system for HCV, the first evidence of glycan modifications linked to secretion of viral particles came from the characterization of retroviral pseudotypes harboring HCV envelope glycoproteins [HCV pseudoparticles (HCVpp)] (13, 14). However, later on, different patterns of glycosylation have been observed between cell culture-derived HCV (HCVcc) and HCVpp associated glycoproteins (11). Thus, HCVcc-associated E1E2 heterodimers contain both high-mannose and complex type N-linked glycans, whereas HCVpp associated E1E2 display a majority of complex-type glycans. Complex glycans are hallmarks of protein transit through the Golgi apparatus since they result from the processing of high-mannose-type glycans by Golgi glycosidases and glycosyltransferases (15). The incomplete maturation of HCVcc E1E2 glycans indicates that some glycans are not accessible to Golgi enzymes. By contrast, HCVpp-associated glycans are more efficiently matured. These results are likely due to differences in the assembly process of HCVpp and HCVcc. Indeed, HCVcc assemble in an ER-derived compartment (16), whereas HCVpp assemble in a post-Golgi compartment (17). Therefore, in the HCVpp system, E1E2 transit through the Golgi compartment without any other viral components and are thus fully accessible to Golgi enzymes. In the HCVcc system, E1E2 are already associated with nascent viral particles when they travel through the secretory pathway and might thus be less accessible to Golgi enzymes.

It is worth noting that, in addition to N-linked sugars, O-linked glycans have also been identified on a recombinant form of E2 protein (18). Four of these O-linked carbohydrates were identified in HVR1 and two in the core structure of E2 (Thr473 and Thr518). However, these types of glycans were not found by another group which used a similar approach for their detection (19). Since the two groups used an E2 protein from different genotypes, one cannot exclude genotype differences in terms of O-glycosylation, but no O-linked glycans were reported on the structure of E2 (20, 21). It is also possible that the O-glycans observed by Braütigam and coworkers (18) are present on a misfolded structure of E2.

N-Glycans on E1 and E2 Structures

In 2014, the crystal structure of the N-terminal sequence (residues 192–270) of E1 expressed in the absence of E2 was obtained. This polypeptide contained N-glycosylation sites E1N1 and E1N2, but the E1N3 N-glycosylation site was removed from the sequence by mutagenesis. However, to facilitate the crystallization process, the molecule was produced in the presence of an N-glycosylation inhibitor (22). In this structure, the N-terminus forms a beta-hairpin followed by a domain composed of a 16 amino acid long alpha-helix flanked by a three strands anti-parallel beta-sheet. The oligomeric arrangement displays two types of dimers. In the first type, the two monomers interface is formed by the interaction of the N-terminal beta-hairpin forming an anti-parallel beta-sheet and by hydrogen bonding between Y1 residue and the *N*-acetyl-D-glucosamine of the E1N1 glycosylation site. The second dimer interface corresponds to a six-stranded beta-sheet formed from two sets of three strands from two monomers that is stabilized by two disulfide bridges.

The structure of the central E2 ectodomain was solved by two independent groups in 2013 and 2014 (20, 21). The obtained structures were very similar [residues 412–645 of E2 of E2 from H77 isolate of genotype 1a in Kong et al. (20); residues 456–656 of E2 from J6 isolates of genotype 2a in Khan et al. (21)]. E2 core shows a globular structure. Indeed, it is composed of a central immunoglobulin fold beta domain as found in other viral envelope proteins. This central beta sandwich is flanked by front and back layers consisting of loops, short helices, and beta sheets. Most of the N-glycosylation sites present on E2 could be observed in the crystal structure (H77 strain sequence) obtained by Kong and collaborators (4MWF) (20). Only E2N1, E2N5, E2N4, and E2N9 are absent due to truncations or mutations introduced in the E2 sequence to facilitate crystallization (20). The majority of the N-linked glycans were disordered in the crystal structure. Only glycan N430 could be modeled as Man6GlcNAc2. E2 structure revealed that 7 of the 11 N-linked glycans form an extensive glycan shield that masks E2 neutralizing epitopes (**Figure 2**) (20). Residues E2N7, E2N8, E2N10, and E2N11 were also modeled in the final E2 core structure obtained by Khan and collaborators (21). They were located on the periphery of the core on a highly basic surface.

ROLE OF E1E2 N-GLYCANS IN THE HCV LIFE CYCLE

Role of the N-Glycans of E1 and E2 in the Early Secretory Pathways

One of the major roles of N-linked glycans is their involvement in protein folding (15). Indeed, the presence of large polar saccharides directly influences the local orientation of the protein. In addition, N-glycans indirectly affect protein folding through their interaction with the ER chaperones, calnexin, and calreticulin. Calnexin and calreticulin are lectin-like chaperones, which show an affinity for monoglucosylated N-linked oligosaccharides (15, 25). Calnexin has been shown to interact with HCV envelope glycoproteins (26–28) and has been suggested to be involved in the folding of E1E2 envelope glycoproteins. In the case of HCV, several N-linked glycans of E1 (E1N1 and E1N4) and E2 (E2N7, E2N8, and E2N10) have been shown to play a role in E1 and E2 folding and heterodimerization. The alteration of the folding observed for these mutants was not due to a lack of recognition by the calnexin chaperone (8).

Functions of Virion-Associated Glycans

The extended glycosylation of E1 and E2 suggests that interactions between lectin receptors and virus might play a role during HCV infection. In agreement with this hypothesis, several lectin receptors have been shown to participate in HCV entry (29). Thus, HCV is thought to initially bind the endothelium of the liver *via* the mannose-binding lectins L-SIGN and the dendritic cells *via* DC-SIGN. Both cell surface proteins are believed to function as capture receptors that concentrate the virus before subsequent interaction with the hepatocytes (29). HCV interaction with

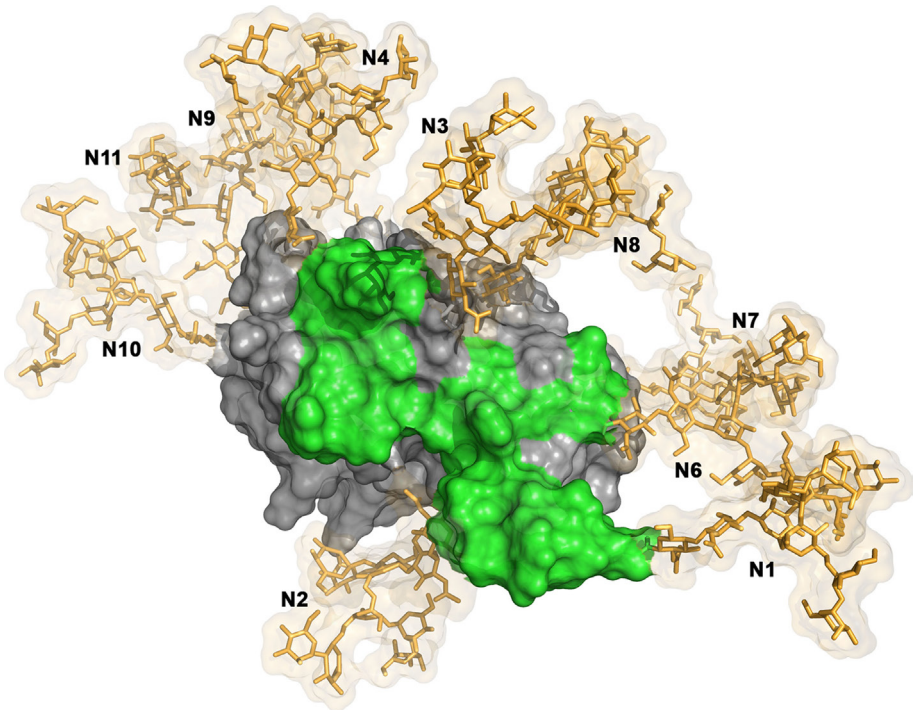

FIGURE 2 | Glycan shield masking E2 neutralizing epitopes. The model of E2 structure is composed by the E2 core structure (PDB ID: 4MWF) (20) and its N-terminal antigenic region 412–423 (PDB ID: 4DGY) (23). The E2 structural model has been built in a similar way than in Fuerst et al. (24). The hypervariable region 1 located at the N-terminus of E2 is not shown. The E2 molecular surface is displayed in gray and its neutralizing epitopes are highlighted in green. High mannose N-glycans (Man9GlcNac2) have been modeled at the 10 N-glycosylation sites available in the E2 structural model using the Glycoprotein Builder tool of the GLYCAM-webserver (http://glycam.org), with an energy minimization step. The glycans (N1, N2, N3, N4, N6, N7, N8, N9, N10, and N11) are shown in sticks representation (in gold) with their transparent molecular surface. The figure was generated with PyMOL (The PyMOL Molecular Graphics System, Version 1.83 Schrödinger, LLC).

hepatocytes is then mainly promoted by scavenger receptor BI (SR-BI), CD81, and the tight-junction proteins claudin-1 and occludin. Interestingly, the type of glycans associated with E1E2 proteins has been shown to influence their binding affinity for lectin receptors as well as for the non-lectin receptors (30).

Site-directed mutagenesis in HCVpp and HCVcc systems enabled to further characterize the functional role of N-glycans associated with HCV envelope proteins (8, 31, 32) (**Table 1**). These studies confirmed that several glycans (E1N1, E2N8, and E2N10) are involved in E1E2 folding and heterodimerization. Glycans can also modulate E1E2 entry functions by affecting the affinity of the envelope proteins for receptors. Indeed, mutation of E2N6 glycosylation site led to an increase in HCVcc infectivity. Moreover, this mutant was more sensitive to infectivity inhibition by a soluble form of the CD81 large extracellular loop (32). In agreement with these data, a soluble form of E2 devoid of E2N6 glycan exhibited a higher affinity for CD81 (31). Altogether, these data suggest that the improved infectivity of E2N6 mutant is due to its increased affinity for CD81. Interestingly, the loss of E2N6 glycosylation site has been observed among naturally occurring HCVcc variants adapted to cell culture (33, 34). It is worth noting that on the 3D structure of E2, the CD81-binding site is surrounded by glycans (20), and the removal of the glycan at position E2N6 likely provides more space for CD81 binding.

By contrast, mutation of the E2N7 glycosylation site led to a strong decrease in HCVcc infectivity without affecting viral particle secretion, suggesting that the glycan present at this position modulates virus entry (32). However, the exact mechanism was not determined. Furthermore, the role of E2N7 glycan in virus entry is likely genotype specific since this glycan site is absent in genotype 3 and 6.

Different Roles for N-Glycans in HCVpp and HCVcc Systems

Noteworthy, in some cases, envelope glycoprotein entry functions were differently affected by glycan loss in HCVcc and HCVpp systems (32). It is the case for the E2N2 or E2N4 mutations that slightly affected HCVcc infectivity but abolished HCVpp infectivity (32). Several studies could also demonstrate differences between the entry functions of envelope proteins in HCVpp and HCVcc systems (35–39). These differences might be due to the distinct assembly processes of HCVcc and HCVpp particles, that lead to different glycan processing and different organization of the proteins at the virion surface (11, 14, 17, 40). Moreover, the association of HCVcc with lipoproteins might also account for the differences in the properties of E1E2 in HCVpp and HCVcc systems (40, 41). Importantly, despite differences in glycosylation

TABLE 1 | Summary of the features of HCV glycosylation mutants [adapted from Helle et al. (92)].

Virus	HCVcc Infectivity[a]	HCVpp infectivity[a,b]	Core release[c]	Sensitivity to neutralization[d]
wt	+++	+++	++	+
Mutant				
E1N1	+/–	++	–	ND (+)
E1N2	++	+	+	ND (+)
E1N3	+++	++	++	ND (+)
E1N4	++	+	+/–	ND (+)
E2N1	+++	++	++	++
E2N2	++	– (–)	++	++[e]
E2N3	+	+++	+	ND (+)
E2N4	++	– (–)	+	++[e]
E2N5	++	++	++	+
E2N6	+++	++	++	++
E2N7	+/–	+++ (+)	+	ND (–)
E2N8	–	–	+/–	ND
E2N9	+++	+++	++	+
E2N10	–	–	–	ND
E2N11	+	+	+/–	++
HVR495	+++	+	ND	++

[a]*Percentage of infectivity relative to the wild type (wt): +++, >90%; ++, between 30 and 90%; +, between 10 and 30%; +/–, between 2 and 10%; –, <2%.*
[b]*Infectivity of HCVpp of genotype 1a and 3a for the HVR495 glycan (9). The results in brackets are obtained for genotype 2a HCVpp.*
[c]*Percentage of core release relative to the wt: ++, >75%; +, between 30 and 75%; +/–, between 12 and 30%; –, <12%.*
[d]*Sensitivity to antibody neutralization: +, similar to the wt; ++, more than fourfold increase in sensitivity to neutralization with most antibodies tested; –, decrease in sensitivity to neutralization. The values in brackets were obtained for genotype 1a HCVpp only.*
[e]*Results obtained with the HCVcc system only.*
ND, not determined; HCV, hepatitis C virus; HCVpp, HCV pseudoparticles; HCVcc, cell culture-derived HCV.

patterns, there is a strong correlation between HCVpp and HCVcc in their sensitivity to antibody neutralization, indicating that the type of glycans associated with HCV envelope proteins might not drastically affect the recognition of neutralizing epitopes (39).

Antiviral Strategies Targeting E1E2 N-Glycans

The importance of N-glycans for the folding of E1E2 envelope proteins, for HCV entry and for the protection of the virus from neutralizing antibodies make them promising targets for antiviral strategies. Accordingly, many studies have shown the antiviral activity against HCV of several carbohydrates binding agent (CBA), such as cyanovirin-N, griffithsin, or scytovirin lectins, as well as pradimicin-A (42–47). Surprisingly, the selection of resistance mutations by propagating HCVcc in the presence of increasing doses of CBA did not lead to the appearance of mutations in the envelope glycoproteins. Resistance was rather conferred by mutations in the core and the non-structural proteins (48), suggesting indirect mechanisms of resistance.

In agreement with the functional role of glycosylation in the HCV life cycle, inhibition of α-glycosidases I and II, that are essential for N-linked glycan processing, impaired HCV production. Indeed, treatment of infected cells with competitive inhibitors of α-glucosidases led to the degradation of E2 and to the consequent inhibition of HCV assembly and secretion (49, 50). Despite the great potential of glycosidase inhibitors as broad-spectrum antiviral drugs, their clinical development has been hampered by their relatively low efficacy. Such a compound like celgosivir showed only a modest antiviral effect in chronically HCV-infected patients in a phase II clinical trial (5% of the tested patients experienced a 10-fold reduction in viremia) (51).

HCV GLYCAN SHIELD AND HOST IMMUNE RESPONSE

Neutralizing Determinants in HCV Envelope Glycoproteins

Neutralizing antibodies inhibit viral infection by binding to viral particles. This leads to the blockade of the interaction with receptors or prevents the envelope glycoproteins conformational changes required for the fusion step. E2 envelope glycoprotein is the main target of the humoral immune response against HCV (52). The importance of neutralizing antibodies to eliminate HCV infection has been shown in a humanized mouse model (53). Several regions of E2 are targeted by neutralizing antibodies. Among them the first 27 amino acids of E2 that correspond to HVR1, a highly variable region of the protein, play important roles in interaction with the HCV co-receptor SR-BI, in viral fitness, and in assembly and release of viral particle (54–57). However, antibodies targeting HVR1 exhibit poor cross-neutralization potency across HCV genotypes due to the high variability of this region (58). HVR1 deletion mutants are more susceptible to neutralization by monoclonal antibodies (mAbs) and patient sera (54, 56). Since CD81-binding site is the main target of neutralizing antibodies, this finding suggests that HVR1 masks this site on E2. Additional lines of evidence gave rise to this hypothesis. Indeed, no interaction could be observed between HCV and CD81 at the cell surface in the absence of SR-BI (59). This led to the proposal that HVR1 shields CD81-binding site and that the interaction of SR-BI with HVR1 allows for the exposure of CD81-binding region on E2 (54, 60, 61).

Interestingly, most antibodies endowed with broad neutralizing activity target conserved conformational epitopes on E2 and inhibit the interaction between E2 and CD81 (52). Neutralizing antibodies targeting the CD81-binding site either recognize linear epitopes located in amino acids 412–423, conformational epitopes with contact residues located between residues 523 and 535 or epitopes spanning these two CD81-binding regions (**Figure 1**). Importantly, the most potent neutralizing murine antibodies target linear epitopes covering residues 412–423, whereas antibodies isolated from HCV-infected patient sera targeting this epitope are rare. By contrast, most human neutralizing antibodies recognize conformational epitopes centered on the CD81-binding residues W529, G530, and D535 (62).

While most neutralizing antibodies target CD81-binding sites, some neutralizing antibodies targeting conserved epitopes overlapping the SR-BI binding site have been described (63). Interestingly, a synergistic neutralization has been observed between the HEPC74 and the HEPC98 antibodies that, respectively, block E2-CD81 and E2-SR-BI binding (64). Moreover, the pairing of these antibodies showed an enhanced neutralizing breadth and

their mechanisms of action were found to be independent. Thus, the reliance of HCV on multiple cellular receptors constitutes a source of vulnerability that could be exploited in the design of an efficient vaccine.

Over the past few years, the interaction between E2 and several different neutralizing antibodies could be precisely mapped by resolution of the crystal structure of E2 peptide-antibody complexes (23, 65–67), providing a molecular framework to better understand HCV neutralization.

Role of Glycosylation in HCV Resistance to Neutralization

The most common mechanism of evasion to antibody neutralization is mutational escape. For its replication, HCV relies on a RNA-dependent RNA polymerase that lacks proofreading capabilities and allows a high replication rate of the virus. These features result in the generation of a high diversity of viral variants that constitute quasispecies (68). The neutralization escape variants contained in the viral population have a selective advantage over sensitive variants and can become the dominant circulating strain (52, 69–71).

Apart from its high genetic heterogeneity, HCV has developed various ways to escape the host immune response. One of them is a protection by a glycan shield that reduces the immunogenicity of the envelope proteins and masks conserved neutralizing epitopes at their surface. Indeed, glycans associated with viral envelope proteins are synthesized by the host cell and are recognized as self-structures. Thus, many viruses that impact human health use glycosylation to evade the host immune response (72).

Characterization of N-glycosylation mutants in the HCVcc system has shown that at least five glycans (E2N1, E2N2, E2N4, E2N6, and E2N11) on E2 reduce the sensitivity of the virus to neutralization (32). Indeed, the absence of one of these glycans leads to an increase in the sensitivity of the virus to neutralization by antibodies purified from HCV positive patients or mAbs. These data further confirm those obtained with the HCVpp system for E2N1, E2N6, and E2N11 mutants (8, 31). However, in this latter system, E2N2 and E2N4 mutations resulted in the production of non-infectious particles. HCV glycans have been shown to mask the neutralizing activity of mAbs targeting conserved epitopes while having no effect on the recognition of HVR1 epitopes (32). Moreover, E2N1, E2N2, E2N4, and E2N6 modulate the inhibition of HCV infectivity by a soluble form of CD81 receptor. Since most broadly neutralizing mAbs target CD81-binding site on E2, these results suggest that this site is the neutralizing antibody target that is protected by N-glycans. In agreement with these data, the modeling of N-linked glycans on E2 core structure confirmed the presence of an extensive glycan shield that masks CD81-binding site (**Figure 2**) (20). Since HVR1 is also thought to hide CD81-binding site, it would be interesting to determine whether glycans and HVR1 shielding effects are additive.

E1 glycosylation had no effect on the sensitivity of HCVpp to neutralization with purified antibodies from HCV positive patient sera (6). However, this result could be due to the fact that the neutralizing immune response against HCV is dominated by anti-E2 antibodies. Thus, it would be interesting to determine the role of E1 glycans in the protection of HCV from neutralization by using anti-E1 neutralizing mAbs. Unfortunately, the availability of such antibodies remains very limited (73, 74).

N-Glycosylation Escape Mutants

Further highlighting the importance of N-linked glycosylation in shielding E2 epitopes from recognition by broadly neutralizing antibodies, Pantua and collaborators (75) observed the appearance of escape mutants from AP33 neutralizing mAb that exhibited a glycan shift. Indeed, *in vitro* resistance selection led to the identification of N417S and N417T HCVcc variants that were resistant to broadly neutralizing antibodies targeting the 412–423 E2 epitope. The two variants presented a glycosylation shift from N417 (E2N1) to N415. N415 residue has been shown to be important for the recognition of the 412–423 epitope by AP33 and HCV1 neutralizing antibodies. Moreover, N415 appeared to be buried in the antibody-peptide interface in the crystal structure of 412–423 epitope in complex with several neutralizing antibodies (66, 67, 75). Consequently, attachment of a glycan at N415 and not at N417 would create a steric bulk that would abrogate AP33 and HCV1 binding. These data led to the conclusion that the glycosylation shift from residue N417 to N415 causes HCV resistance to AP33 and HCV1 neutralizing antibodies (75).

Interestingly, this glycosylation shift could also be observed in the absence of neutralizing antibody selection, thus showing that residue 417 is polymorphic (N, S, or T) (76–78). Furthermore, in a minority of genotype 3a isolates, an additional glycosylation site appears in the intragenotypic hypervariable region HVR495, which has been shown to provide some protection against neutralizing antibodies (9). Therefore, as previously described for HIV, glycosylation shift is another mechanism leading to the appearance of HCV resistance to neutralizing antibodies (79). However, as compared to HIV, this glycosylation shift remains very limited in HCV as determined by the analysis of patient sequences (80). This is likely due to additional roles played by most HCV glycans in protein folding and/or in other unidentified functions.

However, while conferring resistance to AP33 and HCV1 neutralizing antibodies and a greater *in vitro* fitness, the glycan shift observed in N417T and N417S led to greater sensitivity to neutralization by other antibodies targeting amino acids 412–423 (76, 77). Notably, HC33.1 antibody, which was isolated from an HCV-infected blood donor, could neutralize HCVcc bearing E2 N417T and N417S adaptive mutations more efficiently than HCVcc wild type (wt) (81). The structure of HC33.1 in complex with E2 412–423 epitope revealed a different conformation of the epitope than the beta-hairpin conformation observed in the epitope-AP33 or HCV1 complexes. In this structure, N415 residue was surface exposed such that its glycosylation would not impair antibody binding. Thus, crystallography studies have highlighted the structural flexibility of E2 412–423 epitope that exhibited three different conformations depending on the matched antibody (23, 65–67, 82). The structural flexibility of this peptide may contribute to reduce its immunogenicity in HCV-infected individuals. Such structural flexibility was also reported for E2 epitope 427–446 that presents different structures when bound to neutralizing or non-neutralizing antibodies (83). Moreover, the crystal structure of E2 core revealed that 60% of all residues are either disordered or in loops implying considerable

overall flexibility (20, 21). Hence, as proposed for HIV and influenza viruses, conformational flexibility seems to be an additional mechanism used by HCV to evade humoral immunity (84).

Modulation of Immunogenicity by N-Glycosylation

Since glycans represent one-third of E1E2 heterodimers molecular weight, they are likely to impact the immunogenicity of the envelope proteins. Several data argue in favor of this hypothesis. For instance, removal of E1N4 glycosylation site improved the anti-E1 humoral immune response (85, 86). In a similar way, mutation of E2N9 glycosylation site improved the immunogenicity of E2 in a DNA-based vaccination approach (87). Indeed, E2N9 mutant elicited higher E2-specific cytotoxic T lymphocytes activities, T lymphocyte proliferation, and expression of IFN gamma producing T cells. More recently, mice vaccination with CpG coupled E1E2 DNA containing mutated E1N2 and E2N3 glycosylation sites induced a higher cellular immune response than wt E1E2. Furthermore, the corresponding serum presented broad neutralizing activity (88). Thus, in this DNA vaccination assay, the naturally poor immunogenicity of E1E2 could be enhanced by deletion of N-glycans combined with the addition of immune activator CpG. Recently, HCV E1E2 heterodimer and a mutant form lacking E2N6 glycosylation site were transiently expressed in an edible crop, lettuce, using *Agrobacterium*. Produced antigens were used for oral vaccination of mice. The follow up of the immune response induced by HCV heterodimers revealed improved immunogenic properties for the N-glycosylation mutant compared to wt E1E2 (89).

Interestingly, the type of glycans associated with HCV envelope glycoprotein E2 can also affect its immunogenicity. Indeed, as compared to its expression in mammalian cells, E2 produced in insect cells exhibits different glycosylation patterns, which also lead to increased immunogenicity, as evidenced by the induction of higher titers of broadly neutralizing antibodies (90).

CONCLUSION

In conclusion, recent studies have greatly contributed to increase the knowledge of the mechanisms used by HCV to evade humoral immune response. The high genetic variability of the virus favors the emergence of neutralization escape variants. Furthermore, the envelope proteins associated glycans and the virus-associated lipoproteins protect conserved immunogenic epitopes from neutralizing antibodies. Although HCV, HIV, and influenza virus share the common feature of shielding neutralizing epitope with glycans, N-glycosylation sites in HCV E1E2 are far less variable than in HIV and influenza, suggesting a different contribution to HCV immune escape (91). Furthermore, the influence of HCV glycans on anti-HCV immune response makes them essential parameters to take into account in the design of an HCV vaccine based on HCV envelope glycoproteins. Indeed, while the removal of N-glycans seems to improve the envelope proteins immunogenicity, the contribution of these carbohydrates to E1 and E2 folding makes them essential components for the induction of a biologically relevant E1E2-specific antibody response. One needs, therefore, to keep a good balance between these two functions to optimize the design of a vaccine based on HCV envelope glycoproteins.

Finally, answering questions that remain on the role played by N-glycan in modulation of the humoral immune response will facilitate the design of an effective HCV vaccine.

AUTHOR CONTRIBUTIONS

ML wrote the manuscript. XH and JD revised the manuscript. XH also constructed the structural model.

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REVIEW
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Hepatitis C Virus (HCV)–Apolipoprotein Interactions and Immune Evasion and Their Impact on HCV Vaccine Design

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With more than 71 million people chronically infected, hepatitis C virus (HCV) is one of the leading causes of liver disease and hepatocellular carcinoma. While efficient antiviral therapies have entered clinical standard of care, the development of a protective vaccine is still elusive. Recent studies have shown that the HCV life cycle is closely linked to lipid metabolism. HCV virions associate with hepatocyte-derived lipoproteins to form infectious hybrid particles that have been termed lipo-viro-particles. The close association with lipoproteins is not only critical for virus entry and assembly but also plays an important role during viral pathogenesis and for viral evasion from neutralizing antibodies. In this review, we summarize recent findings on the functional role of apolipoproteins for HCV entry and assembly. Furthermore, we highlight the impact of HCV–apolipoprotein interactions for evasion from neutralizing antibodies and discuss the consequences for antiviral therapy and vaccine design. Understanding these interactions offers novel strategies for the development of an urgently needed protective vaccine.

Keywords: hepatitis C virus, apolipoproteins, neutralizing antibodies, lipo-viro-particle, viral evasion, ApoE

INTRODUCTION

With more than 71 million people chronically infected (1, 2), hepatitis C virus (HCV) is one of the leading causes of liver disease and hepatocellular carcinoma (3). The recent development of direct acting antivirals with sustained virological response rates of over 90% has revolutionized HCV therapy. However, several limitations remain: high treatment costs, emergence of resistant variants, difficult-to-treat patients with significantly decreased sustained virological response rates, and the possibility of reinfection highlight the urgent need for a protective HCV vaccine (4).

Despite the combined efforts of the HCV research community, HCV vaccine design has been hampered by the ability of HCV to rapidly mutate and escape from protective immune responses (5). This is partly due to the intimate relationship of HCV with the host lipid metabolism. All steps of the HCV life cycle are dependent on the interaction with lipoproteins and apolipoproteins. Moreover, the interaction of HCV with lipoproteins leads to the formation of lipo-viro-particles (LVPs), which is critical for HCV infectivity and evasion from neutralizing antibodies. Thus, understanding the

role of these interactions is crucial for future vaccine research. Here, we review recent findings on HCV–apolipoprotein interactions, highlight their role for viral escape, and discuss their implications for HCV antiviral therapies and vaccine design.

THE FUNCTIONAL ROLE OF APOLIPOPROTEINS IN THE HCV LIFE CYCLE

Structure of the LVP, the Infectious HCV Particle

Hepatitis C virus is an enveloped positive-stranded RNA virus belonging to the *Flaviviridae* family. The viral particle consists of a nucleocapsid containing the viral RNA surrounded by an endoplasmic reticulum (ER)-derived envelope in which viral E1 and E2 glycoproteins are embedded as heterodimers (6) (**Figure 1**). Over the past years, several studies strongly demonstrated the tight link between HCV and lipid metabolism (7, 8). A hallmark of the virus is its association with host lipoproteins. Indeed, highly infectious HCV particles circulate in patient serum in association with very-low-density lipoproteins (VLDL) or low-density lipoproteins (LDL), to form LVPs (9–11). Consequently, LVPs share several biophysical properties with the VLDL. Infectious LVPs have a low density (between 1.03 and 1.10 g/ml), are rich in cholesterol and triglycerides, and contain apolipoproteins (Apo) such as ApoB, ApoA-I, ApoE, and ApoCs (12–15) (**Figure 1**). Characterization of HCV particles produced in cell culture (HCVcc) has confirmed these properties (16–18). Interactions of HCV particles with lipoprotein components play a critical role in the viral life cycle and contribute to viral persistence and development of chronic liver diseases (19).

Apolipoproteins represent the protein moiety of the lipoproteins. Physiologically, they have three major functions in the lipoprotein metabolism: (i) they stabilize the lipoprotein structure and solubilize the lipid fraction, (ii) they interact with lipoprotein receptors and participate in lipoprotein clearance, and (iii) they act as cofactors for specific enzymes involved in lipoprotein metabolism (20, 21) (**Table 1**). In many aspects, HCV takes advantage of host apolipoproteins for efficient propagation in hepatocytes (22). The role of apolipoproteins in the HCV life cycle is highlighted in **Table 1** and **Figures 2** and **3**.

Role of Apolipoproteins in HCV Entry

The initiation of HCV entry is triggered by LVP binding to cell surface heparan sulfate proteoglycans (HSPGs). Interestingly, it was demonstrated that ApoE plays a role in this process by interacting with syndecan 1 and syndecan 4 HSPGs (23–27). Other groups have highlighted the key role of ApoE in HCV entry. Indeed, HCV infectivity can be efficiently blocked by anti-ApoE antibodies or human synthetic peptides derived from the ApoE receptor-binding domain (18, 25, 28, 29). Moreover, Owen and collaborators observed that ApoE facilitates HCV entry by interacting with the LDL receptor (30). The scavenger receptor class B type I (SR-BI) is another lipoprotein receptor identified as a HCV receptor involved in different steps of HCV entry (31, 32). During the early steps, LVP attachment to SR-BI is mediated by ApoB-100 and ApoE (33, 34). This interaction is thought to induce lipoprotein–HCV dissociation and to expose the viral glycoprotein E2 for direct interaction with SR-BI and the tetraspanin CD81 (32). Of note, it was reported that high-density lipoprotein (HDL)-associated ApoC-I, a natural ligand of SR-BI, improves this step by increasing the fusion rates between viral and target membranes through direct interaction with the LVP (35).

Lipo-viro-particle entry into hepatocytes is also influenced by changes in lipoprotein composition. One of the best examples is the action of the lipoprotein lipase (LPL) on lipoprotein-associated triglycerides. By hydrolyzing triglycerides, LPL decreases the size of the particles and induces a loss of LVP-associated ApoE. The loss of ApoE results in a strong decrease in LVP infectivity (36, 37). Consistent with this finding, it was demonstrated that ApoC-II, the natural activator of the LPL, is an anti-HCV factor, whereas ApoC-III, an LPL inhibitor, facilitates chronic HCV infection (38, 39) (**Figure 2**).

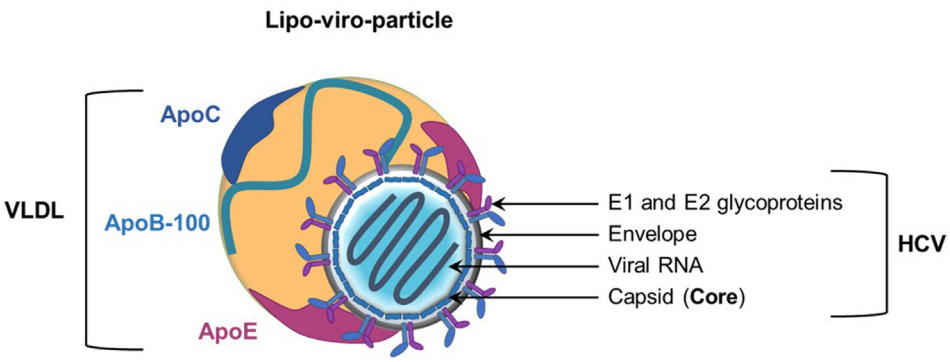

FIGURE 1 | Model of the hepatitis C virus (HCV) lipo-viro-particle (LVP). The HCV particle consists of an icosahedral capsid, formed by the viral core protein, containing the positive-stranded viral RNA. The nucleocapsid is surrounded by an endoplasmic reticulum-derived envelop in which E1 and E2 glycoproteins are embedded. The highly infectious HCV particle corresponds to a hybrid particle composed of very-low-density lipoprotein (VLDL) components and viral components named LVP. The different apolipoproteins classically associated with VLDL and LVP are illustrated on this picture (ApoB-100 and the exchangeable apolipoproteins ApoE and ApoCs).

TABLE 1 | Role of the major apolipoproteins in the HCV life cycle.

Name	Physiological role	Role in HCV life cycle	Reference
ApoA-I (exchangeable apolipoprotein)	*Structural role*: major component of HDL *HDL metabolism*: involved in HDL maturation by activating LCAT *Reverse cholesterol transport*: from peripheral tissues to liver through interaction with SR-BI and ABCA1 (cholesterol efflux)	*Structural role*: component of the LVP *HCV morphogenesis*: redundantly participate in the production of infectious HCV particles	(12, 15, 19, 49, 50)
ApoB-100 (non exchangeable apolipoprotein)	*Structural role*: major component of VLDL and LDL *Triglyceride transport*: involved in VLDL synthesis and clearance through interaction with LDLR *Cholesterol transport*: transfer of LDL-cholesterol in cells through LDLR	*Structural role*: major component of the LVP *HCV entry*: mediates LVP binding through interaction with SR-BI *HCV morphogenesis*: LVP synthesis and secretion	(12, 15, 19, 33, 34, 41, 42, 49)
ApoC-I (exchangeable apolipoprotein)	*Structural role*: component of VLDL and HDL *HDL metabolism*: LCAT activator	*Structural role*: component of the LVP *HCV entry*: enhance HCV infectivity through complex interaction with SR-BI	(12, 15, 19, 35)
ApoC-II (exchangeable apolipoprotein)	*Structural role*: component of VLDL and HDL *Triglyceride metabolism*: LPL activator	*Structural role*: component of the LVP *HCV entry*: physiological HCV entry inhibitor by activating LPL *HCV morphogenesis*: redundantly participate in the production of infectious HCV particles	(12, 15, 19, 37)
ApoC-III (exchangeable polipoprotein)	*Structural role*: component of VLDL and HDL *Triglyceride metabolism*: LPL inhibitor	*Structural role*: component of the LVP *HCV entry*: enhance HCV entry by inhibiting LPL *HCV morphogenesis*: redundantly participate in the production of infectious HCV particles	(12, 15, 19, 38)
ApoE (exchangeable apolipoprotein)	*Structural role*: major component of VLDL and HDL *Triglyceride transport*: involved in VLDL synthesis and clearance trough interaction with HSPG, LRP1, and LDLR *HDL metabolism*: involved in reverse cholesterol transport	*Structural role*: major component of the LVP *HCV entry*: mediates LVP binding through interaction with HSPG, LDLR, and SR-BI. Involved in cell-to-cell transmission *HCV morphogenesis*: crucial role in HCV assembly by interaction with NS5A, E1, and E2, necessary for the production and maturation of infectious HCV particles	(12, 15, 19, 23–34, 43–61)

ABCA1, ATP-binding cassette A1; Apo, apolipoprotein; HDL, high-density lipoprotein; HSPG, heparan sulfate proteoglycan; LCAT, lecithin cholesterol acyltransferase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; LRP1, LDLR-related protein 1; LVP, lipo-viro-particle; SR-BI, scavenger receptor class B type I; VLDL, very-low-density lipoprotein; HCV, hepatitis C virus.

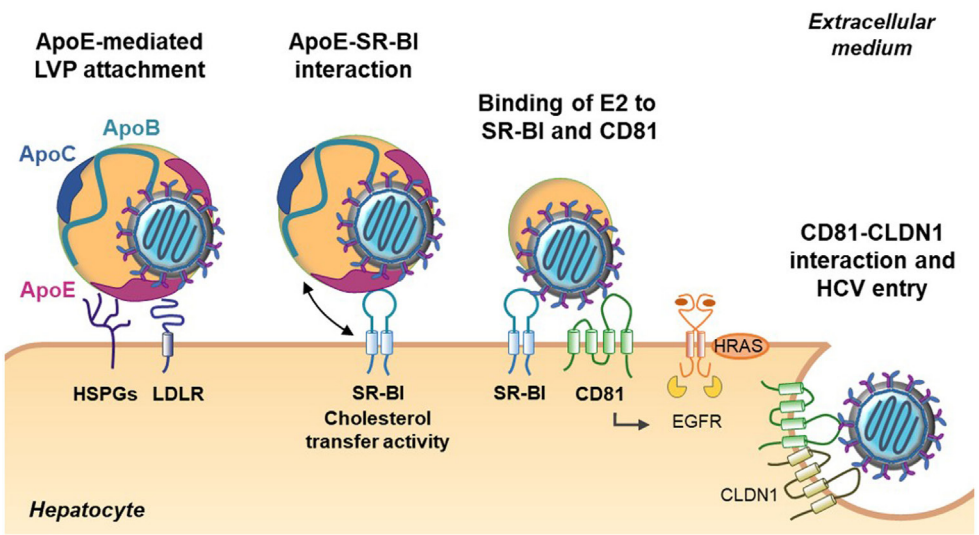

FIGURE 2 | Role of apolipoproteins during early steps of hepatitis C virus (HCV) entry. The first step of HCV entry consists of the interaction between lipo-viro-particle (LVP)-associated ApoE, the heparan sulfate proteoglycans (HSPGs), and the low-density lipoprotein receptor (LDLR). Subsequently, the LVP interacts with the scavenger receptor class B type I (SR-BI) through ApoE and ApoB (not illustrated). The cholesterol transfer activity of SR-BI allows E2 exposure and binding of E2 to SR-BI and the tetraspanin CD81. Binding on CD81 activates the epithelial growth factor receptor (EGFR) signaling pathway and interaction between CD81 and claudin 1 (CLDN1) that triggers HCV entry.

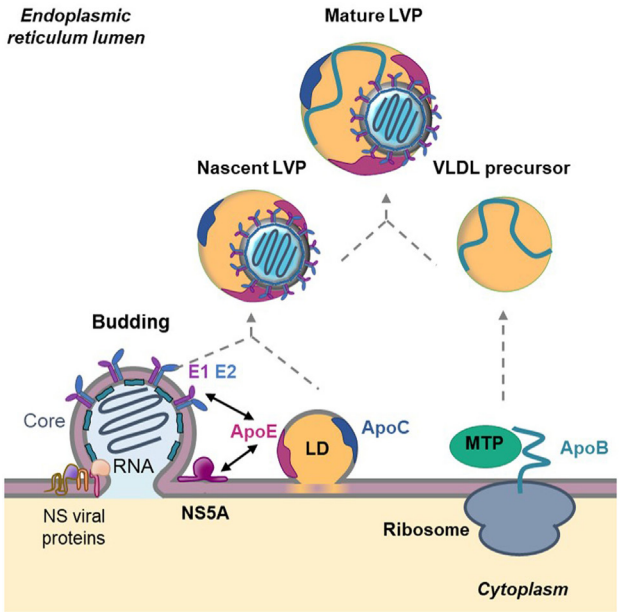

FIGURE 3 | Role of apolipoproteins in hepatitis C virus (HCV) morphogenesis. HCV assembly takes place at the surface of endoplasmic reticulum (ER)-derived membranes in close proximity to lipid droplets (LD). Core protein associates with viral RNA to form the nucleocapsid. The nucleocapsid buds at the ER membrane where E1 and E2 glycoproteins are anchored and afterward associate with nascent LD to acquire ApoE and ApoC. This step is facilitated by the interaction between ApoE and the non-structural (NS) viral protein NS5A as well as by the interaction between ApoE and the glycoproteins E1 and E2. In parallel, ApoB is lipidated by the microsomal triglyceride transfer protein (MTP) to generate very-low density lipoprotein (VLDL) precursors. The nascent HCV particle associates with these precursors by an unknown mechanism to generate mature lipo-viro-particles (LVPs).

Role of Apolipoproteins in HCV Morphogenesis and Maturation

Following HCV entry and viral RNA replication, virions are assembled in a coordinated and complex process (39) (**Figure 3**). As mentioned above, LVP share numerous properties with VLDL suggesting that HCV coopts the VLDL machinery for its own morphogenesis. In hepatocytes, the VLDL production is divided in two steps. First, ApoB-100 is lipidated by the microsomal triglyceride transfer protein (MTP) to form a VLDL precursor. Second, the precursor is enriched in lipids and acquires ApoE and ApoCs in the ER by a mechanism not fully understood (40). Interestingly, it was shown that the impairment of VLDL synthesis, through MTP inhibitors or siRNA-mediated knockdown of ApoB expression, leads to a decrease in HCV production (12, 41, 42). However, the functional importance of ApoB in HCV assembly remains controversial. Other studies revealed that HCV assembly is independent on ApoB expression but is rather highly dependent on ApoE (43, 44). The different observations could be due to the use of different models and to a defect of Huh7 cells in producing authentic VLDL (45). The hypothesis is supported by studies showing that there is no correlation between the ability to generate VLDL and the production of infectious viral particles. Indeed, the ectopic expression of ApoE but not ApoB is necessary to produce infectious HCV particles in human non-liver cells (46, 47). Recently, two studies demonstrated that ApoE but not ApoB is required for HCV cell-to-cell transmission (47, 48). Finally, by using Huh7 cells knockout for either or both *APOB* and *APOE*, the Matsuura group revealed that ApoB and ApoE redundantly participate in the formation of infectious HCV particles (49). Of note, not only the expression of ApoE but also of other exchangeable apolipoproteins belonging to the ApoA and ApoC family rescued the production of infectious virions, indicating that exchangeable apolipoproteins possess redundant roles in HCV assembly (49, 50). ApoA, ApoC, and ApoE are exchangeable apolipoproteins that are able to dissociate from one lipoprotein and reassociate with another due their high content in α-helical structures (20). Accordingly, two research groups highlighted the role of α-helical structures in HCV morphogenesis. The authors demonstrated that expression of short sequences containing amphipathic α-helices derived from apolipoproteins but also of other proteins such as the human cathelicidin antimicrobial peptide is sufficient to rescue the production of infectious HCV particles in apolipoprotein knockout cells (49–51). Of note, a recently published paper showed that α-helices found in host-derived apolipoproteins and in NS1 of other *Flaviviridae* may have overlapping roles in the formation of infectious flaviviral particles (52).

Despite the redundant role of exchangeable apolipoproteins in HCV morphogenesis, ApoE remains critical for HCV assembly and infectivity. The role of ApoE was reinforced by a study showing that all the main HCV genotypes (from genotypes 1 to 7) are strictly ApoE dependent, regardless of ApoE isoforms. Indeed, the three main ApoE isoforms, ApoE3, ApoE2, and ApoE4, differing at only two amino acid positions (residues 112 and/or 158) seem to complement HCV production to a comparable degree.

The molecular mechanism by which ApoE associates with HCV particles was recently highlighted. Indeed, ApoE was found to interact with the viral protein NS5A through its C-terminal α-helix domain (53, 54). Furthermore, two other studies evidenced the interaction between ApoE and the HCV glycoproteins E1 and E2 in the ER but also at the LVP surface. Association of ApoE with the viral proteins NS5A, E1 and E2 would trigger LVP morphogenesis (55, 56). Finally, it was shown that extracellular ApoE play a role in LVP maturation. Mature LVP are highly enriched in ApoE compared with normal VLDL (18, 57). Recent studies related that ApoE exchange occurs between LVP and circulating lipoproteins. This process is important to maintain a high ApoE level on the LVP surface that is required for an efficient infectivity and facilitates escape host immunity (57–59). Indeed, a study performed in our lab demonstrated that association of ApoE with E2 helps the virus to escape from patient neutralizing antibodies (60). These observations are of utmost importance for vaccine development: design of immunogens mimicking the E2/ApoE interface might help to achieve an efficient neutralizing humoral immune response against HCV (60).

APOLIPOPROTEINS AND VIRAL PATHOGENESIS

Clinical evidence indicates that chronic HCV infection is associated with dysregulated circulating lipoproteins and

apolipoproteins within the HCV-infected hepatocytes. HCV infection induces the accumulation of lipoproteins and apolipoproteins by upregulation of genes involved in lipid synthesis (61, 62). Disturbed lipoprotein and Apo homeostasis may not only contribute to clinical progression of HCV-induced liver diseases but also represent important risk factors for cardiovascular disease (63).

Hepatitis C virus infection appears to disturb serum apolipoprotein levels depending on the genotype of the virus. Seki et al. reported that infection with genotype 1b was associated with elevated serum levels of ApoA-II and ApoE and reduced levels of ApoC-II and ApoC-III. By contrast, genotype 2 infection only reduced ApoC-II and ApoC-III serum levels. In infected patients, a reduction in ApoC-II and ApoC-III serum levels may enhance HCV infection. In particular, low ApoC-II serum levels were found to be associated with advanced liver fibrosis, which indicate an important role in liver pathogenesis (64). A similar effect has been observed for the HCV core protein induced upregulation of ApoC-IV that has been also reported to induce hepatic steatosis (65).

Finally, it has been observed that single-nucleotide polymorphisms (SNPs) in apolipoproteins are associated with HCV infection and alteration of lipid metabolism. Two studies reported that SNP rs12979860 near the IL28B gene, which encodes for interferon-λ-3, is associated with the response to IFN treatment (66, 67). A follow-up study reported that the rs12979860 CC responder genotype was associated with higher serum levels of ApoB, suggesting that alteration of ApoB levels are part of the IFN response (68). Moreover, a recent study showed that the ApoB polymorphism rs1042034 is significantly associated with the HCV infection status (69). The AA allele, which was characterized by significantly lower serum levels of LDL-cholesterol, might contribute to facilitating serum LDL uptake into human hepatocytes. Consequently, individuals carrying the polymorphism might be more susceptible to HCV infection, indicating a direct influence of the polymorphism on the low-density lipoprotein receptor-mediated host cell entry of HCV.

MECHANISMS OF NEUTRALIZING ANTIBODIES TARGETING HCV INFECTION

Neutralizing antibodies to HCV are mainly directed at the E2 glycoprotein with a wide range of specificity and degree of conservation. The majority of broadly neutralizing antibodies mediate neutralization by blocking virus binding to CD81, a tetraspanin HCV co-receptor (70). Fine epitope mapping shows that these antibodies are directed at clusters of overlapping epitopes that include key residues that also participate in virus interaction with CD81 (71, 72). Thus, the binding of these antibodies to the viral surface prevents virus interaction to this required co-receptor during viral entry. These antibody clusters are designated as antigenic domains B, D, and E or antigenic region 3 (AR3) (see Keck et al. in this issue). Note that domains B, D, and AR3 are clusters of overlapping conformational epitopes, while domain E has mainly overlapping linear epitopes. Two other clusters of broadly neutralizing antibodies are directed at conformational epitopes formed by key residues on both E1 and E2 glycoproteins, designated as AR4 and AR5. These antibodies do not block virus binding to CD81 and are thought to mediate neutralization by inhibiting E1E2 heterodimer conformational change during the entry process (73).

The N-terminal region of E2 (amino acid 384–410) is hypervariable and some antibodies to this region, designated as HVR1, do mediate virus neutralization. These antibodies are directed at epitopes located at the C-terminal portion of HVR1 that includes key residues that are also found to be involved in the initial attachment step of virus entry to heparan sulfate and subsequent interaction with SR-BI (74–76). While the majority of antibodies to HVR1 are isolate specific, several described antibodies exhibit broad virus neutralization and block virus binding to SR-BI (77, 78).

APOLIPOPROTEINS AND VIRAL EVASION DURING VIRUS NEUTRALIZATION

With about 75–80% of all HCV infections progressing to chronic disease, it is clear that evasion from the neutralizing antibody response is a key feature of HCV: although patients during the chronic phase often have high levels of serum neutralizing antibodies, in most cases, the immune system is not able to control the infection. A potential determinant for viral escape is the close association of HCV with lipid metabolism (79).

Early indications that HCV-lipoprotein interactions are involved in viral escape from the neutralizing antibody response stem from reinfection experiments in chimpanzees, where infection could only be transmitted by low-density fractions of serum-derived HCV (80).

A very similar effect was observed in virus derived from humanized mice: those particles displayed lower density and higher infectivity than cell culture-derived viruses. However, this effect was lost after a single passage in cell culture, indicating the responsibility of host-derived factors (17). Indeed, highly infectious particles were associated with ApoB and E, forming LVPs with a buoyant density of 1.06 mg/ml while poorly infectious LVPs of buoyant densities around 1.25 mg/ml were linked to immunoglobulins (81). Furthermore, Thomssen et al. reported that virus-bound ApoB-100 excluded binding of neutralizing antibodies (82), indicating a negative correlation of apolipoprotein content and binding of neutralizing antibodies.

The development of cell culture-derived HCV (HCVcc) that displays a similar lipid composition as native serum-derived HCV particles allowed for a more detailed analysis of the involved mechanism. Immature intracellular HCVcc that are characterized by a lower lipoprotein content, when compared with released HCVcc were more sensitive to neutralization by anti-E2 antibodies and less sensitive to anti-ApoE antibodies than mature HCVcc (83), indicating a shielding function of the lipoproteins. In addition, a cell culture adaptive mutation in E2 (I414T) that decreased the dependency on the host factors SR-BI and CD81 also led to reduced lipoprotein content in combination with increased susceptibility to neutralizing anti-E2 antibodies (83). A similar mechanism was observed for mutation G451R that also decreased the dependency of HCV on SR-B1 and CD81 and altered the relationship of infectivity and density with peak infectivity

occurring at higher density, meaning lower lipid content, than the wild-type virus (84). This was associated with a drastic increase in the sensitivity of the virus to neutralizing antibodies targeting E2 or soluble CD81 protein, indicating that lipoprotein content directly affects the binding efficiency of neutralizing antibodies (84). Bankwitz et al. recently confirmed that physiological levels of ApoE, which are much higher than those found in cell culture (10–60 μg/ml compared with 0.3 μg/ml) directly enhanced HCV particle infectivity across all genotypes (59). Furthermore, the overall ApoE capacity of serum-derived HCV particles was higher than cell culture-derived HCV, indicating that not only the higher concentration of the serum but also apolipoprotein incorporation during the assembly process is responsible for the elevated apolipoprotein levels of native HCV particles. Enhancement of infection was independent of HVR1 and SR-BI but was reliant on HSPGs. Removal of HSPGs abrogated the enhancement of infection by ApoE, indicating that incorporated ApoE mediated the binding to cell surface proteoglycans (59).

A recent publication showed that ApoE levels in HCV-producing cells directly determined the ability of HCV to evade the neutralizing antibody response (60). Viruses that were produced in hepatoma cells expressing only low amounts of ApoE were more susceptible to neutralizing antibodies directed against the envelope proteins. Utilization of ApoE to escape from neutralizing antibodies was pan-genotypic; however, it was most exploited by variants that were characterized by most efficient viral escape (60). Functional studies with different monoclonal antibodies revealed that E2 domains B and C were exposed after ApoE deletion, confirming the shielding mechanism of ApoE. In variants that were selected post liver transplantation, a mutation on E2 residue 447 appeared to modify the E2–ApoE interaction that altered the sensitivity to neutralization by both ApoE and E2-specific neutralizing antibodies, despite comparable incorporation of ApoE in wild-type and mutant viruses (60), indicating that viral evasion mediated by ApoE is determined both by incorporation and conformation of incorporated ApoE. A study by Weller et al. demonstrated that usage of ApoE was strain dependent, indicating that ApoE might contribute to strain-dependent differences in neutralization (85). Shielding of antigenic sites on the envelope proteins, however, is not the only mechanism by which apolipoproteins contribute to viral escape.

It has been shown that lipoproteins attenuated antibody binding to HCVpp and HCVcc by augmenting virus entry in an SR-BI-dependent fashion (35). HDL activation of target cells accelerated virus entry by removing a 1-h lag during virus internalization. This augmentation of virus entry resulted in decreased binding of neutralizing antibodies to the CD81 binding site on E2, potentially due to limited exposure time of these epitopes. Antibodies targeted to E1E2 complex epitopes were not affected (35). This effect was mediated by the lipid transfer function of SR-BI, as inhibitors of SR-BI mediated lipid transfer fully restored the neutralizing ability of antibodies targeting the CD81 binding site. Part of the accelerated entry efficiency is potentially due to the enhancing ability of ApoC-I which has been shown to be affected by SR-BI mediated lipid transfer (86). Incorporation of ApoC-I increased the infectivity of HCV pseudoparticles after incubation with old world nonhuman primate or human sera. Antibodies against ApoC-I abrogated the enhancing activity of human serum showing that ApoC-I was indeed responsible for the enhancement of infectivity (87). In contrast to ApoE, the enhancement of infectivity by ApoC-I was dependent on HVR1 (35, 86) and its interaction with SR-BI. It remains to be determined whether enhancement of infection and escape from the neutralizing antibody response are two completely independent mechanisms or whether faster virus entry limits the exposure time to neutralizing antibodies and thus mediates escape from the neutralizing antibody response.

In addition, apolipoproteins might not only be involved in the escape from adaptive immune responses. Experimental evidence suggests that ApoE3 also mediates escape from the innate effector molecule Ficolin-2 that blocks HCV entry at an early time point during infection (88). ApoE3 indirectly blocked the interaction of Ficolin-2 and E2, even when HCVcc were preincubated with Ficolin-2, potentially due to the higher affinity of ApoE3 for the viral envelope protein (88). This underlines the important function of apolipoproteins for the evasion from the host immune response.

Taken together, apolipoproteins contribute to viral escape by two different mechanisms. Association of HCV particles with lipid components in LVPs directly inhibits neutralization by anti-envelope antibodies. In addition, interaction with apolipoproteins enhances viral entry, which limits the exposure of the virus to neutralizing antibodies. Understanding the mechanisms by which HCV usurps apolipoproteins for viral escape might offer new strategies for antiviral intervention and could pave the way toward the development of a protective vaccine.

IMPACT FOR ANTIVIRAL THERAPIES AND VACCINE DESIGN

The close association of HCV with the host lipid metabolism has several important implications for HCV treatment and vaccine design. Modulation of the apolipoprotein–HCV interaction may open new opportunities for antiviral therapies and vaccines: targeting the interaction sites of apolipoproteins and viral envelope proteins could be an approach to block HCV infection and at the same time to restrict its capacity to evade from the neutralizing antibody response. In particular, viral variants isolated from patients undergoing liver transplantation are characterized by efficient viral escape (89, 90), which has been partially attributed to their incorporation of ApoE (60). Supporting this concept, Avasimibe, a clinically approved inhibitor of lipid transportation that leads to decreased ApoB and ApoE serum secretion showed broad pan-genotypic inhibition of HCV infection (91). Second, the interaction of apolipoproteins and HCV proteins provides an opportunity to identify epitopes for broadly neutralizing antibodies. Antibodies that target conserved conformational structures are less prone to mutations. However, antibodies directed against host epitopes might also open the risk for autoimmune diseases, as it has been reported for the development of autoimmune-antibodies directed against ApoA-I during HCV infection (92). This also has to be considered while choosing the correct system for vaccine production. While production in hepatic

cell lines, in particular in HepG2 cells would lead to correctly lipidized LVPs that might be the best option to generate a suitable immune response, masked epitopes or the risk for autoimmune diseases, as discussed earlier, might favor different production systems such as CHO cells or yeast, as for the hepatitis B virus and human papillomavirus vaccines. It remains to be determined which production system is suitable to generate a correct immune response against HCV. Studies on chronic viral infection in the lymphocytic choriomeningitis mouse model revealed that chronic infection and the associated chronic inflammation resulted in the formation of persistent immune complexes. The complexes resulted in a dampened Fc-mediated effector activity, potentially impacting antibody-based treatment options also for HCV and other chronic viral infections (93). Furthermore, HIV vaccine trials showed that post-translational modifications, such as glycosylations that greatly depend on the production system are of utmost importance for vaccine efficacy (94–96).

Another promising approach might be the development of monoclonal antibodies targeting E1 since the shielding function of apolipoproteins was primarily directed against epitopes located on the envelope protein E2. Finally, a detailed understanding of the HCV–lipoprotein–antibody interactions may help to design immunogens inducing broadly neutralizing antibodies for protection of infection and may guide the way toward the development of a protective HCV vaccine.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Defining Breadth of Hepatitis C Virus Neutralization

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Extraordinary genetic diversity is a hallmark of hepatitis C virus (HCV). Therefore, accurate measurement of the breadth of antibody neutralizing activity across diverse HCV isolates is key to defining correlates of immune protection against the virus, and essential to guide vaccine development. Panels of HCV pseudoparticle (HCVpp) or replication-competent cell culture viruses (HCVcc) can be used to measure neutralizing breadth of antibodies. These *in vitro* assays have been used to define neutralizing breadth of antibodies in serum, to characterize broadly neutralizing monoclonal antibodies, and to identify mechanisms of HCV resistance to antibody neutralization. Recently, larger and more diverse panels of both HCVpp and HCVcc have been described that better represent the diversity of circulating HCV strains, but further work is needed to expand and standardize these neutralization panels.

Keywords: hepatitis C virus, Flaviviridae, antibodies, neutralizing breadth, viral diversity

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HEPATITIS C VIRUS (HCV) VIRAL DIVERSITY AND THE NEUTRALIZING ANTIBODY RESPONSE

Interferon-free therapies for HCV have revolutionized treatment of those infected with the virus, but a vaccine to prevent infection is needed (1). The extensive genetic diversity of HCV has been a major obstacle to vaccine development. HCV is genetically heterogeneous with seven genotypes and more than 80 subtypes (**Figure 1**). Within the envelope genes (E1 and E2), approximately 30% of amino acids differ between strains from different genotypes, while strains from different subtypes within each genotype differ at approximately 20% of their E1E2 amino acids (2–5). Even viral strains within the same subtype differ at up to 10% of their E1E2 amino acids. Within an infected individual, error-prone replication by HCV and immune selection lead to the generation of a viral swarm made up of many distinct strains, providing opportunities for expansion of antibody resistant variants (6–8). Therefore, induction of high-titer cross reactive antibodies that are capable of neutralizing diverse viruses within subtypes and across multiple genotypes may be required for an effective vaccine.

Significant effort has been devoted to development of vaccines intended to induce protective antibodies against HCV, but most vaccines tested to date in nonhuman primates or humans have induced humoral responses with relatively poor activity against heterologous HCV strains [reviewed in Ref. (11, 12)]. For example, a vaccine composed of recombinant full-length E1E2 protein from a single HCV strain has been tested in both chimpanzees and humans. Vaccination was protective against homologous HCV challenge in chimpanzees (12). However, the same vaccine reduced rates of persistence but did not prevent infection in chimpanzees after challenge with a heterologous virus (13), and neutralizing antibodies against heterologous HCV strains were induced in only three of 16 vaccines in a phase 1a human trial (14). Overall, these and other vaccine trials suggest that induction of antibodies with broad neutralizing activity across diverse HCV strains may be a critical challenge for HCV vaccine development.

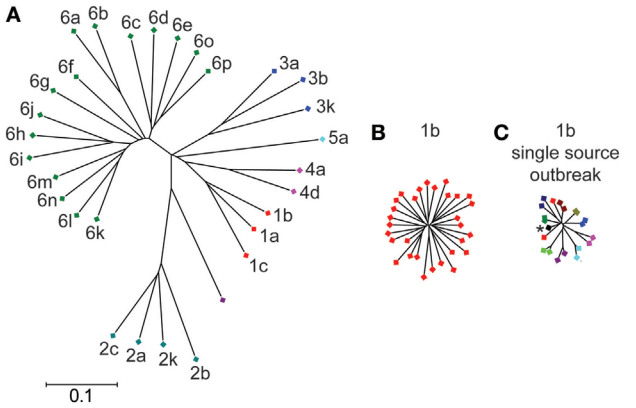

FIGURE 1 | Genetic diversity of hepatitis C virus (HCV) E1E2 across subtypes and within a single subtype. Phylogenetic trees of E1E2 amino acid sequences. E1E2 amino acid sequences from multiple subtypes **(A)** or a single subtype **(B)** were downloaded from the LANL HCV sequence database. Not all known subtypes are shown. **(C)** E1E2 amino acid sequences of 10 individuals from the Irish anti-D cohort, who were all infected from the same inoculum (asterisk) between 1977 and 1978 (9). Sequences of virus from each individual are indicated with a different color. Trees were inferred using the Neighbor-joining method, with branch lengths drawn to scale. Subtypes are labeled. All three trees are on the same scale. Distances were computed using the JTT method. Analyses were performed in MEGA7 (10).

NEUTRALIZING ANTIBODY MEDIATED PROTECTION AGAINST HCV INFECTION

Antibodies capable of neutralizing HCV infection (NAbs) target the viral envelope glycoproteins, E1 and E2, which are membrane-anchored proteins believed to form a heterodimer on the surface of viral particles. E2 interacts with multiple cell surface receptors, including but not limited to CD81 and scavenger receptor B1 (SR-B1), while the function of E1 remains unclear (15). Most HCV-infected individuals develop strain-specific NAbs against hypervariable region 1 (HVR1), a 27-amino acid region at the amino-terminus of E2, but viral escape mutations generally confer resistance to these antibodies (6, 16–20). In contrast, NAbs capable of neutralizing infection by multiple diverse HCV strains *in vitro*, commonly called broadly neutralizing antibodies (bNAbs), develop in some infected individuals. These bNAbs generally bind to relatively conserved epitopes at the CD81 receptor binding site of E2 (CD81bs) or to the E1E2 heterodimer [reviewed in Ref. (21, 22)].

There is evidence from studies of HCV infection of humans and animal models that bNAbs can be protective. Notably, early-developing HCV-specific bNAb responses are associated with clearance of primary human HCV infection (23–26). Pestka et al. demonstrated that women infected during a single-source outbreak of HCV were more likely to clear the virus if they developed serum antibodies early in infection that were capable of neutralizing at least one heterologous HCV strain *in vitro*. Similarly, Osburn et al. demonstrated that plasma isolated immediately prior to clearance of HCV infection neutralized a median of 6 of 19 heterologous HCV strains, while acute infection plasma of control subjects with subsequent persistence of infection neutralized a median of only 1 of 19 heterologous strains. In addition, individuals who clear one infection clear subsequent infections more than 80% of the time, and clearance of reinfection is associated with rapid induction of antibodies capable of neutralizing heterologous HCV strains *in vitro* (27).

There is also evidence of protection in animal models of HCV infection. Infusion of immunoglobulin isolated from the serum of a chronically infected human prior to challenge with homologous virus from the same donor prevented infection of most human liver chimeric mice. Infusion of this chronic-phase human immunoglobulin prior to challenge of a chimpanzee prevented infection with homologous, but not heterologous HCV strains (28–30). In contrast, infusion of monoclonal bNAbs prior to challenge with heterologous viruses prevented infection in most human liver chimeric mice (31–33) and chimpanzees (34), and combinations of monoclonal bNAbs also abrogated established HCV infection in a human liver chimeric mice (35). Together, these studies demonstrate that induction of bNAbs may be necessary to prevent infection by diverse, heterologous HCV strains.

EXPERIMENTAL SYSTEMS FOR QUANTITATION OF ANTIBODY NEUTRALIZING ACTIVITY

Assessment of antibody neutralization of HCV relies largely upon two different *in vitro* systems: the HCV pseudoparticle (HCVpp) system and the replication-competent HCV cell culture (HCVcc) system. HCVpp are retroviral particles with HCV envelope glycoproteins (E1 and E2) on their surface. These particles can be produced by transfection of HEK-293T cells with an E1E2-expressing plasmid and a plasmid expressing an envelope-defective HIV-1 genome with a luciferase reporter. Alternatively, cells can be transfected with an E1E2-expressing plasmid, a murine leukemia virus (MLV) Gag/Pol packaging construct, and a luciferase-encoding reporter plasmid. In either case, after transfection, enveloped particles bearing HCV E1E2 proteins on their surface are released through a retroviral budding process into culture supernatant, enabling the measurement of single rounds of viral entry into hepatoma cells or primary human hepatocytes. HCVpps were used to identify many of the cell surface receptors required for HCV entry (36–40). Unlike HCVpp, HCVcc reproduce the full replication cycle of HCV *in vitro* (41–45) and in animal models (46–48). HCVccs are produced through transfection of *in vitro* transcribed full-genomic HCV RNA into hepatoma cells. Relatively few HCV strains are capable of replication in *in vitro*, but recently strains from genotypes 1–3 have been adapted to allow *in vitro* replication (49–53). After initial transfection, culture supernatants are infectious and can be passaged serially *in vitro*. The HCVcc system has been used to further define the viral entry pathway (54–57).

Both HCVpp and HCVcc panels have been developed to express diverse E1E2 strains, including strains from genotypes 1–6 (HCVpp) (58, 59) and genotypes 1–7 (chimeric HCVcc) (42, 60), enabling the assessment of breadth of antibody neutralizing activity. Notably, HCVpps were used in studies described earlier in this review which demonstrated that the early development of bNAbs was associated with clearance of HCV infection

(23–26). HCVccs were used in the studies described earlier which demonstrated that antibodies with broad *in vitro* neutralizing activity can prevent HCV infection in animal models (31–34).

The development of diverse panels of HCVpp or HCVcc has been complicated by the relatively high frequency with which primary E1E2 isolates are poorly functional or nonfunctional *in vitro*, producing HCVpp that do not mediate detectable entry into hepatoma cells, or chimeric HCVcc that do not replicate in *in vitro* (61, 62). Recently, efforts have been made to address these technical obstacles. E1E2 clones that are poorly functional when pseudotyped with HIV-1 Gag protein may produce functional HCVpp when pseudotyped with MLV Gag protein, or vice versa. The mechanisms explaining this phenomenon are unknown. In addition to pseudotyping with either HIV-1 or MLV, optimization of ratios of MLV and HCV E1E2 plasmids during transfection can also improve the function of some E1E2 strains in HCVpp (63). Along with technical improvements in HCVpp production, technology for production of HCVcc has also advanced. The development of chimeric HCVcc based on the genotype 2a JFH1 strain with Core-NS2 genes from multiple genotypes has expanded the number of diverse HCVcc available for neutralization studies. These chimeric HCVccs have an advantage relative to some full-length HCVcc strains, since adaptive mutations required for efficient replication *in vitro* are outside of E1E2 in most cases. Therefore, the E1E2 genes expressed accurately represent the sequences of naturally circulating viruses. Two recent studies described cloning of dozens of naturally occurring E1E2 genes into HCVcc chimeras, generating replication-competent viruses (61, 64). Wasilewski et al. used an E1E2-deleted H77/JFH-1 chimeric construct with an introduced restriction site to facilitate high-throughput insertion of diverse E1E2 genes.

As more E1E2-matched HCVpp and HCVcc are produced, some phenotypic differences between E1E2 expressed using the two systems have been identified. For reasons that are still unknown, some E1E2 strains that produce replication-competent HCVcc chimeras produce poorly functional HCVpp, and, conversely, some E1E2 strains that are functional in HCVpp do not produce replication-competent HCVcc chimeras (62). In addition, HCVpp tend to be generally more neutralization sensitive than HCVcc (61, 62), perhaps due to structural differences between HCVcc and HCVpp, including the association of apolipoproteins with HCVcc but not HCVpp (65, 66). Fortunately, despite these differences, multiple studies have demonstrated concordance between the neutralization results of identical E1E2 clones expressed in either HCVpp or HCVcc, including concordance in the rank order of neutralization sensitivity of different E1E2 strains and concordance in resistance phenotypes of specific mutations (61, 62, 64, 67). These results suggest that, for most experiments, either HCVpp or HCVcc can be used to measure antibody neutralizing activity, expanding the diversity of E1E2 isolates available for neutralization assays.

HCV PANELS USED TO QUANTITATE NEUTRALIZING BREADTH

Defining the neutralizing breadth of anti-HCV monoclonal antibodies and immune sera is critical to understand the antibody response required to protect against circulating HCV strains. Due to the previously limited availability of diverse E1E2 isolates in HCVcc and HCVpp, the neutralizing breadth of anti-HCV antibodies has traditionally been measured using relatively small panels of HCVcc or HCVpp (31, 36, 42, 59, 68, 69). As described earlier in this review, these smaller panels have greatly advanced understanding of virus neutralization, but they do not express many of the polymorphisms common in natural HCV isolates, and, therefore, they may overestimate neutralizing breadth of antibodies or sera. In addition, many of these studies have measured neutralization of historically important reference strains of HCV (31, 36, 42, 59, 68–70), such as the genotype 1a isolate, H77, which is highly sensitive to neutralization (69).

Recently, panels of HCVpp and HCVcc have been expanded to encompass more of the diversity of circulating HCV strains (**Table 1**). A panel of HCVpp from the University of Nottingham includes 78 HCVpp predominantly from genotype 1, but also including some isolates from genotypes 2–6 (61). A second panel developed at Johns Hopkins University comprises natural genotype 1a and 1b E1E2 isolates from cohorts in the US and Ireland. This panel includes 113 HCVpp and expresses 97% of amino acid polymorphisms present at greater than 5% frequency in a reference set of 643 genotype 1 HCV isolates submitted to GenBank from around the world (71). Sensitivity of HCVpp in this panel to neutralization by two bNAbs varied by more than 100-fold (67). A 19-HCVpp subset of this panel expresses 94% of amino acid polymorphisms present at greater than 5% frequency in worldwide genotype 1 sequence (72). This 19 HCVpp genotype 1 panel has been used extensively to define neutralizing breadth of mAbs and sera (9, 23, 27, 62, 64, 72, 73). While significantly fewer replication-competent HCVcc strains are available, progress has been made in expanding the number of isolates (61–63, 74). Most recently, Carlsen et al. applied a panel of chimeric HCVcc constructs expressing 16 E1E2 genes from genotypes 1–3. Like the diverse HCVpp in the Nottingham and Hopkins panels, these HCVcc also varied widely in sensitivity to neutralization by monoclonal antibodies (74).

NEUTRALIZATION SENSITIVITY VARIES BY HCV STRAIN, NOT BY GENOTYPE

Heterologous neutralizing activity of HCV-infected human sera is not primarily dictated by the infecting viral genotype. Some studies demonstrated that sera from genotype 1 or 4-infected

TABLE 1 | HCV cell culture (HCVcc) and HCV pseudoparticle (HCVpp) panels for neutralization breadth testing.

Source	Assay system	Genotypes/subtypes represented	Number of isolates	Reference
Copenhagen hepatitis C program	HCVcc	1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a, and 7a	20	(42, 44, 60, 74)
Johns Hopkins	HCVcc	1a and 1b	13	(62)
University	HCVpp	1a and 1b	113	(25, 67)
University of	HCVcc	1, 2, and 3	49	(75)
Nottingham	HCVpp	1a, 1b, 2, 3, 4, 5, and 6	78	(58, 61, 69)

individuals neutralized genotype 1, 4, 5, and 6 viruses more efficiently than viruses from genotypes 2 and 3 (59, 76). However, these findings may be attributable to strain-specific, rather than genotype-specific effects, since IgG isolated in a different study from serum of subjects infected with either genotype 1, 2, or 3 viruses showed similar neutralization of genotype 1, 2, and 3 HCVpp. There was no relationship between infecting genotype and the genotype of HCVpp that was neutralized by each sample (69). Similarly, in a third study, neutralizing breadth of plasma samples measured using a diverse panel of genotype 1 HCVpp did not differ between individuals infected with genotype 1, 2, or 3 viruses (25). Together, these studies suggest that genetic subtypes of HCV are not neutralization serotypes.

Several recent studies have also demonstrated that neutralization sensitivity varies by HCV strain, rather than by genotype. In a study by Carlsen et al., which measured neutralization of 16 HCVcc from genotypes 1–3 by 10 mAbs, 50% inhibitory concentrations (IC_{50}) of the same mAb across different HCVcc isolates varied by more than 1,000-fold, with many of the mAbs failing to neutralize all strains in the panel. Notably, this variation in neutralization sensitivity was discernable between isolates from the same subtype as well as between isolates from different genotypes (74). Another study by Urbanowicz et al. measured neutralization of more than 70 HCVpp from genotypes 1–6 by 5 monoclonal antibodies, and also observed wide variation in neutralization sensitivity (61). Again, this neutralization sensitivity varied widely across isolates within each of the subtypes tested. Notably, neutralizing breadth of 7 mAbs was similar when measured in 2 independent studies using either 16 genotype 1–3 HCVcc from the Copenhagen Hepatitis C Program panel (74) or the Johns Hopkins University panel of 19 genotype 1 HCVpp (72), further supporting the concept that neutralization sensitivity to many bNAbs varies by HCV strain, rather than by genotype (**Figure 2**).

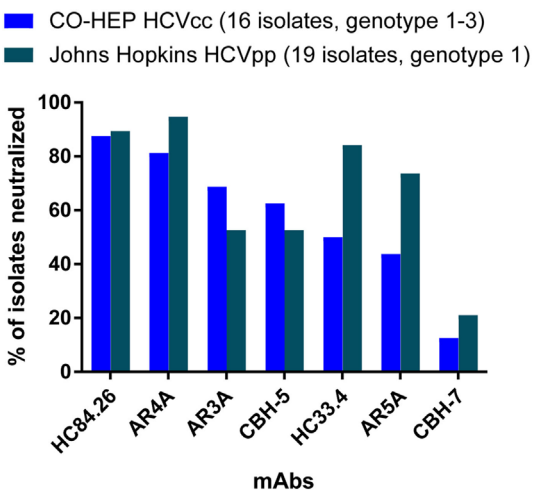

FIGURE 2 | Neutralizing breadth of seven monoclonal antibodies measured using HCV cell culture (HCVcc) and HCV pseudoparticle (HCVpp) panels. Values shown are the percentage of 16 genotype 1–3 HCVcc isolates from the Copenhagen Hepatitis C Program (CO-HEP) neutralized with an IC_{50} less than 10 μg/mL (74), or the percentage of 19 genotype 1 HCVpp isolates from Johns Hopkins University neutralized more than 50% by 10 μg/mL of mAb (72).

Together, these studies suggest that it may be most important for neutralization panels to include many diverse E1E2 strains, even if they represent a single genotype, rather than fewer strains from multiple genotypes.

RESISTANCE IS CONFERRED BY POLYMORPHISMS WITHIN AND DISTANT FROM BNAB EPITOPES

Studies using genetically diverse HCVpp and HCVcc have identified multiple mechanisms of HCV resistance to bNAbs. Polymorphisms have been identified in primary HCV isolates that confer resistance to many individual bNAbs and groups of bNAbs. Some bNAb resistance mutations fall within identified bNAb epitopes. When given monotherapy with the monoclonal bNAb designated HCV1, HCV-infected liver transplant recipients developed neutralization resistance mutations at polyprotein positions 415 and 417, within the HCV1 binding epitope (77). Similarly, a naturally occurring F442I polymorphism within the binding epitope of another bNAb, designated HC84.26, confers resistance to neutralization by that bNAb (72). Notably, however, polymorphisms distant from bNAb epitopes can also confer resistance to neutralization. Several studies have demonstrated no association between the level of bNAb resistance of natural HCV strains and mutations within the epitopes of those bNAbs (25, 72). Other studies have identified few mutations in bNAb epitopes in HCV-infected individuals (78), even though bNAbs commonly develop during chronic infection. This observation may be explained in part by the discovery that mutations outside of bNAb epitopes are capable of conferring bNAb resistance (67, 72, 79). Mutations in the central beta sheet of E2, distant from known binding epitopes, can confer resistance to antibodies binding at the CD81 binding site of E2 (72, 79), and a mutation in HVR1 that modulates E2-scavenger receptor-B1 interaction confers resistance to bNAbs AR4A and HC33.4, even if their binding epitopes are fully conserved (67). Prentoe et al. also demonstrate the role of HVR1 in modulating sensitivity of different HCV strains to bNAbs targeting conserved epitopes (80). Although bNAbs in the study did not bind to HVR1, deletion of HVR1 reduced differences in bNAb sensitivity between many HCV strains. Together, these studies suggest that neutralizing breadth of an antibody cannot be predicted solely from the level of conservation of the binding epitope of that antibody.

ACCURATE ASSESSMENT OF NEUTRALIZING BREADTH INFORMS VACCINE DEVELOPMENT

Given the extraordinary genetic and phenotypic diversity of HCV, accurate measurement of antibody neutralizing breadth is critical to guide vaccine development. Some studies have suggested that very high titers of bNAbs, as measured using *in vitro* neutralization assays, may be necessary for protection in *in vivo*. Bukh et al. demonstrated that IgG with high cross-neutralizing titers *in vitro* protected chimpanzees against homologous, but not heterologous challenge with HCVcc expressing E1E2 strains

identical to those used for *in vitro* testing (28). In addition, measurement of neutralizing breadth of bNAbs using diverse HCVpp and HCVcc panels has shown that no bNAb or serum sample identified to date potently neutralizing all HCV strains (25, 61, 72, 74). These studies suggest that vaccine induction of bNAbs targeting a single HCV epitope may be insufficient for protection. Importantly, neutralizing breadth of bNAb combinations can exceed that of individual bNAbs. In one study, multiple bNAbs, including AR3A and HC84.26, displayed enhanced neutralization against multiple HCV strains when combined with a second bNAb, designated AR4A (74). In another study, bNAbs targeting distinct epitopes displayed enhanced neutralizing breadth when used in combination (73). Two NAbs described in the Mankowski et al. study, designated HEPC74 and HEPC98, displayed both enhanced neutralizing breadth and enhanced potency (synergy) when used in combination. HEPC74 binds at the CD81 binding site of E2 and acts primarily by blocking E2 binding to the CD81 cell surface receptor. In contrast, HEPC98 binds to HVR1 of E2 and acts primarily by blocking E2 binding to the co-receptor SR-B1. Together, these studies suggest that vaccine induction of multiple bNAbs targeting distinct epitopes may be desirable.

FUTURE DIRECTIONS

Diverse panels of HCVpp and HCVcc have been used to identify bNAbs, to define neutralizing breadth of antibodies in serum, and to identify mechanisms of HCV resistance to antibody neutralization. However, methods used to define neutralizing breadth can be improved. The vast genetic diversity of HCV is well-described, but the phenotypic diversity of the virus as it relates to neutralization sensitivity remains incompletely understood. Recently, larger and more diverse panels of both HCVpp and HCVcc have been described to better represent the diversity of circulating HCV strains. Currently, available genotype 1 panels encompass most commonly occurring polymorphisms of genotype 1 that isolates worldwide, but fewer isolates are available from genotypes 2 through 7. The expansion of HCVpp and HCVcc panels has identified natural E1E2 strains with resistance to even the most broadly neutralizing mAbs. It is not known whether further expansion of HCVpp and HCVcc panels will identify additional resistant clones, or if strains currently in use accurately represent worldwide phenotypic diversity. Given this limited understanding of the relationship between genetic and phenotypic differences between E1E2 strains, comparison of antibody responses induced by different vaccines has been hampered by the use of different neutralization panels in different laboratories. Therefore, future goals for the field should include optimization and standardization of a genotype 1 neutralization panel representing the many diverse E1E2 isolates already available, along with expansion and standardization of neutralization panels encompassing other genotypes. These standardized panels could be used to accurately evaluate and compare neutralizing antibodies and post-vaccination immune sera tested in different laboratories, accelerating development of an HCV vaccine.

AUTHOR CONTRIBUTIONS

VK wrote the manuscript. JB edited the manuscript and prepared figures.

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REVIEW
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Animal Models to Study Hepatitis C Virus Infection

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With more than 71 million chronically infected people, the hepatitis C virus (HCV) is a major global health concern. Although new direct acting antivirals have significantly improved the rate of HCV cure, high therapy cost, potential emergence of drug-resistant viral variants, and unavailability of a protective vaccine represent challenges for complete HCV eradication. Relevant animal models are required, and additional development remains necessary, to effectively study HCV biology, virus–host interactions and for the evaluation of new antiviral approaches and prophylactic vaccines. The chimpanzee, the only non-human primate susceptible to experimental HCV infection, has been used extensively to study HCV infection, particularly to analyze the innate and adaptive immune response upon infection. However, financial, practical, and especially ethical constraints have urged the exploration of alternative small animal models. These include different types of transgenic mice, immunodeficient mice of which the liver is engrafted with human hepatocytes (humanized mice) and, more recently, immunocompetent rodents that are susceptible to infection with viruses that are closely related to HCV. In this review, we provide an overview of the currently available animal models that have proven valuable for the study of HCV, and discuss their main benefits and weaknesses.

Keywords: hepatitis C virus, animal models, humanized mice, homologs, vaccine, antiviral therapy

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INTRODUCTION

The worldwide prevalence of hepatitis C virus (HCV) infection is 3% with an estimated 71 million people who are persistently infected. The severity of HCV infection ranges from mild symptoms to serious illness with chronic hepatitis. Chronic infection may lead to liver cirrhosis and eventually hepatocellular carcinoma (HCC) (1). In recent years, new direct acting antivirals (DAAs) have first supplemented the treatment combination of ribavirin and pegylated interferon alpha (IFNα), reaching cure rates of up to 90% in genotype 1 infected patients. The latest DAA combinations are even more effective and do not require additional ribavirin or interferon administration. Despite these recent advances, significant concerns remain about drug resistance, high cost, and worldwide accessibility of these new antivirals. Besides, DAAs do not necessarily ameliorate the long-term effects of chronic infection and predisposition for liver disease (2). In addition, since therapy-induced HCV clearance does not provide immunity to a new infection, an effective preventive vaccine remains an important need (3).

The first accessible system to study HCV replication in cell culture was the sub-genomic replicon system (4). This approach allows efficient viral replication in human hepatoma (Huh7) cells, transfected with sub-genomes that contain a selectable marker linked to the non-structural region (NS2-NS5B) of HCV (4, 5). Using this system, HCV RNA replication and cellular immunity (6, 7) can be studied

and novel antiviral compounds (8) can be evaluated. Important to note is that no infectious viral particles are produced using this sub-genomic replicon system. In parallel, *in vitro* systems for the study of viral entry were developed. Virus-like particles, produced in a baculovirus system and containing the structural proteins core, E1 and E2, resemble HCV virions and are capable of inducing humoral immune responses against HCV (9). However, these particles are not secreted and have no infectious potential. The first infectious systems consisted of pseudotyped vesicular stomatitis virus or influenza virus containing chimeric E1 and/or E2 glycoproteins (10–13). However, due to modifications that allow assembly at the cell surface, the conformation and functions of the E1/E2 complexes are disturbed (13). The development of infectious HCV pseudo-particles (HCVpp), which consist of defective retroviral particles expressing HCV E1 and E2 glycoproteins on their surface, represented a major breakthrough for investigating the HCV entry process (14–16). More specifically, the role of putative HCV (co-) receptors, the host range, and the E1 and E2 glycoproteins can be examined. This system also allows screening of potential entry inhibitors. In this way, the HCVpp are shown to be hepatotropic and can specifically be neutralized by anti-E2 monoclonal antibodies and HCV-infected patient sera (15). Further steps in the HCV life cycle are not supported by HCVpp and can, therefore, not be explored using HCVpp (15). In 2005, transfection of *in vitro* transcribed full-length genotype 2a HCV (JFH1) isolate and chimeric derivatives thereof into Huh7 cells was described, showing RNA replication and secretion of infectious viral particles (17–20). In contrast to the HCVpp system, this cell culture-derived HCV (HCVcc) system allows the study of all aspects of the viral life cycle and still plays a major role in the identification and evaluation of novel antivirals (19, 20).

Cell culture systems are very useful for initial studies of different aspects of HCV. However, culture conditions are artificial; hence, *in vivo* studies are required to more closely mimic the natural situation. Due to the narrow tropism of HCV, *in vivo* studies were long restricted to chimpanzees. Over the years, other animal species have been evaluated for their susceptibility to HCV infection, although most of them seemed resistant. Therefore, several modified models have been developed in recent years, which allow either complete or partial study of HCV infection. In this review, we provide an overview of currently existing *in vivo* models for HCV infection. We will also discuss their applicability, major advantages, and limitations (**Table 1**; **Figure 1**).

HOST SYSTEM REQUIREMENTS FOR HCV REPLICATION

As with any experimental system for human disease, a model for HCV infection should mimic as many, if not all, relevant clinical features as observed in human patients. Desirably, the model should be susceptible to all HCV genotypes with resulting persistent viremia in the majority of exposed animals. The ideal model should also be fully immunocompetent in order to study protective immunity, persistence, and immune-mediated pathogenesis. From a practical point of view, the animal model for HCV infection should be cheap, highly reproducible, easy to propagate and high in throughput (21). Finally, the ethical impact should be as minimal as possible. Up to this day, no such model exists.

Since the number of unmodified hosts perceptive to HCV infection is limited, extensive research is performed to create a suitable model by modifying existing models. From all animal models used in research, rodents are currently the most popular species for genetic modifications and are therefore highly explored, also in the field of HCV research. Genetic manipulation of the host can be applied to knock down certain host factors that interfere with viral replication or on the other hand, to complement the host with human factors that are essential for this process. The propagation of HCV in rodent cells is inefficient, presumably due to genetic incompatibility of rodent cofactors and/or due to suppression of HCV replication by rodent innate immune defenses. Thus, engineering mice expressing the relevant human genes and/or with deleted mouse restriction factors may permit HCV propagation (22).

A large number of human factors have been determined to be involved in the uptake of HCV into human hepatocytes: glycosaminoglycans (23), low density lipoprotein receptor (24), CD81 (25), scavenger receptor class B type 1 (SR-BI) (26), tight junction proteins claudin-1 (CLDN1) (27) and occludin (OCLN) (28, 29), the receptor tyrosine kinases epidermal growth factor receptor and ephrin receptor A2 (30), the cholesterol transporter Niemann-Pick C1-like 1 (31), transferrin receptor 1 (32), cell death-inducing DFFA-like effector b (33), and E-cadherin (34). The entry of HCV into primary hepatocytes is mediated by CD81, OCLN, CLDN1, and SR-BI. To our current knowledge, CD81 and OCLN comprise the minimal human factors required for HCV uptake by rodent cells (35). However, these animals do not sustain viral replication and chronic infection. Finally, it is still not entirely clear which host factors should be humanized, because there is little knowledge about the specific host factors that cause inhibition of HCV RNA replication or host factors that determine species tropism.

NON-RODENT MODELS

The chimpanzee (*Pan troglodytes*) played an important role in the discovery of HCV. In fact, the viral genome of HCV was cloned from a chimpanzee that was experimentally infected with non-A, non-B hepatitis (36). For a long time, the chimpanzee was the only available model to study HCV, and their use has greatly advanced our knowledge on this virus. Humans and chimpanzees share more than 98% of their genome sequence. Despite this high genomic homology, there are some clear differences between the two which makes that the disease pattern and outcome in chimpanzees does not necessarily reflect that in humans. Whereas only a minority of humans spontaneously clear an acute infection (15%), few chimpanzees evolve to chronicity (30–40%) (37), and to date, no fibrosis and only one HCC case has been observed in this model (38). Nevertheless, the chimpanzee proved very valuable for the study of the molecular, immunological, and clinical aspects of HCV infection. Furthermore, while it is very difficult to study the acute phase of HCV infection in humans because specific symptoms are usually absent during that phase, experimental infection of chimpanzees

TABLE 1 | Characteristics of hepatitis C virus (HCV) animal models and HCV homologs.

Animal model	Complete viral life cycle	Viremia	Liver disease	DAA testing	Passive immunization	Vaccine development	Availability	Reference
Non-rodent models								
Chimpanzee	Yes	High	Acute, chronic[a]	Yes	Yes	Yes	Very low	(37, 39, 45)
Tree shrew	Yes	Low	Fibrosis, cirrhosis	No	Yes	No	Low	(54, 55)
Zebrafish	Replication	Not relevant	Virus–host interaction	Yes	No	No	High	(56)
Viral protein transgenic mouse models								
Inducible transgene expression	Not relevant	Not relevant	Virus–host interaction	Not relevant	Not relevant	No	High	(58, 67, 68)
Full HCV genome	Not relevant	Not relevant	Fibrosis, HCC	Not relevant	Not relevant	No	High	(70)
Immunocompromised human liver xenograft mouse models								
Trimera mouse	Yes	Low	No	Yes	Yes	No	Low	(78, 80)
Alb-uPA-SCID mouse with humanized liver	Yes	High	No	Yes	Yes	No	Low	(79, 83, 88, 90–92)
FRG mouse	Yes	High	No	Yes	Yes	No	Low	(96, 130, 133, 134, 136, 137)
Immunocompetent xenograft mouse models								
Tolerized rat	Yes	Very low	No	Yes	Yes	Yes	Very low	(139, 141)
AFC8-hu HSC/Hep mouse	Yes	Only in liver	Inflammation, fibrosis	No	No	Yes	Very low	(142)
HIL mouse	Yes	Very low	Inflammation, fibrosis	No	No	Yes	Very low	(155, 156)
Viral adaptation								
	Entry	No	No	No	Yes	No	High	(157, 158)
Genetically humanized mouse models								
Rosa26-Fluc mouse	Yes	Persistent viremia	No	Yes	Yes	Yes	High	(159, 160)
ICR-C/OTg mouse	Yes	Persistent viremia	Fibrosis	Yes	Yes	Yes	High	(161)
HCV homologs in natural host								
GB-virus	Yes	Acute	No	Yes[b]	Yes[b]	Yes[b]	Low	(165)
NPHV in horses	Yes	Persistent viremia, acute	Inflammation	Yes[b]	Yes[b]	Yes[b]	Low	(168, 169)
NrHV in rats	Yes	Acute, chronic	Inflammation	Yes[b]	Yes[b]	Yes[b]	High	(172–174)

DAA, direct-acting antiviral; HCC, hepatocellular carcinoma; uPa, urokinase-type plasminogen activator; HSC, hematopoietic stem cell; HIL, human immune system and liver; NPHV, non-primate hepacivirus; NrHV, Norway rat hepacivirus.

[a]*Chimpanzees are not necessarily good models of chronic liver disease. They do not develop fibrosis or HCC.*

[b]*These models can be used to evaluate the efficacy of DAAs and vaccine candidates against the homolog virus, not HCV itself.*

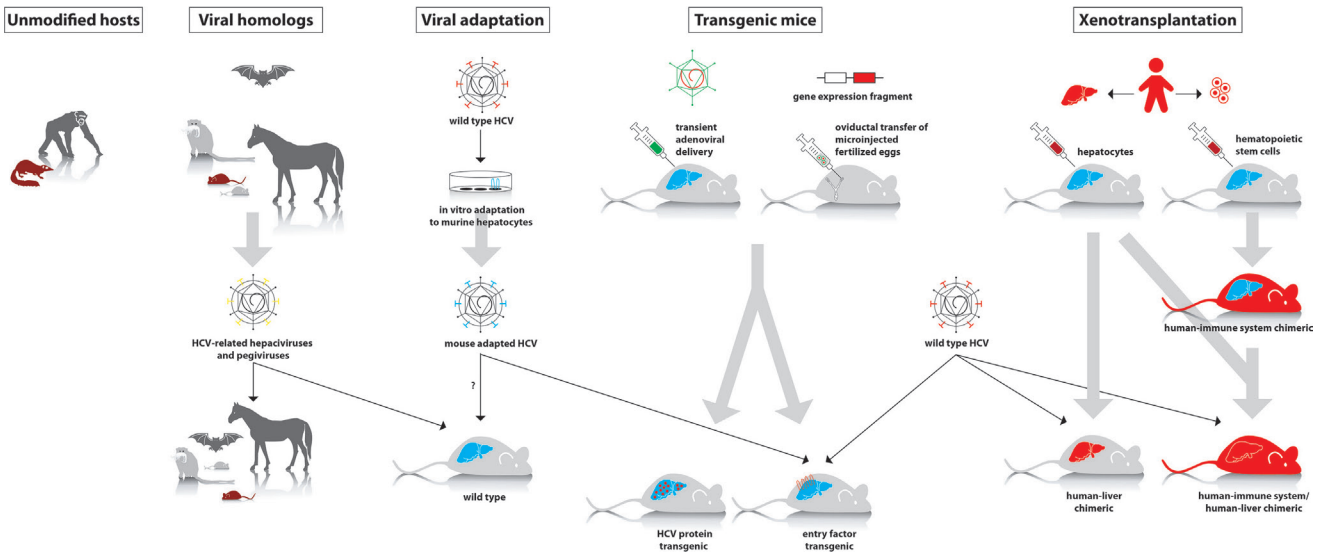

FIGURE 1 | Different approaches to study hepatitis C virus (HCV) in animal models. First panel: animal species that can be experimentally infected with wild-type HCV (chimpanzee and tree shrew). Second panel: hepaciviruses and pegiviruses that infect animal species such as wild mice, rats, tamarins, bats, and horses. These viruses can be studied in their natural host, where they cause a HCV-like infection. Third panel: *in vitro* adaptation of HCV to mouse hepatocytes may allow the isolation of viral variants that can establish an infection in wild-type mice. Fourth panel: transient or stable expression of human factors that are essential to support infection of wild-type HCV or transgenic expression of viral proteins. Fifth panel: in xenotransplantation models, either the liver alone or both the liver and immune system are humanized.

allows close monitoring of viral kinetics, host immune response, disease manifestation, and outcome in a highly controlled manner (39–43). Immunological studies in chimpanzees have also led to the development and evaluation of several candidate vaccines (44, 45). Moreover, in the context of antiviral efficacy studies, they have been successfully used to track resistance associated with the use of entry (46), protease, NS5A (47), and polymerase (48, 49) inhibitors and combinations thereof (50).

The chimpanzee model fulfills many of the requirements for a good animal model. However, limited availability and ethical and financial constraints associated with these studies are major drawbacks. Recently, the National Institute of Health of the United States Department of Health and Human Services decided to effectively end its support for invasive research on chimpanzees. Other primates have been tested for their susceptibility to HCV infection, with little success. Although HCV can infect induced pluripotent stem cells derived from hepatocyte-like cells from pigtail macaques (51, 52), HCV does not seem to be able to establish persistent infection in non-human primates except for chimpanzees (53).

In addition, several other non-primate species have been tested for their susceptibility to HCV. The tree shrew (*Tupaia belangeri*) is for example a non-rodent squirrel-like mammal that is permissive for persistent low-level HCV viremia, including HCV-related liver disorders (54, 55). Still, limited availability and incompatibility of the *Tupaia* host environment with robust HCV replication limits the use of this animal for the study of HCV pathogenesis and vaccine development.

Recently, Ding et al. (56) developed a zebrafish model for sub-genomic HCV replication. The zebrafish is often used as a model organism for human diseases, including liver disease (57). The sub-replicon is created using two vectors: one containing HCV NS5B and the other containing the minus strand of HCV 5′UTR, core, and 3′UTR, under the control of the mouse hepatocyte nuclear factor 4 promoter. These vectors are then co-injected into zebrafish zygotes. The sub-replicon is able to replicate in the liver and causes alterations in the expression of certain genes, which is similar to HCV infection in human liver cells. Administration of ribavirin and oxymatrine significantly inhibits the replication of the HCV sub-replicon in the larvae (56). To conclude, the zebrafish is small, easy to handle experimentally, and useful for investigating mechanisms of HCV replication and liver pathology *in vivo*. Also, this model may aid in drug evaluation studies and thus the discovery of new anti-HCV drugs.

VIRAL PROTEIN TRANSGENIC MICE

Mice that transgenically express viral proteins have been created to study the *in vivo* interactions between these viral proteins and the host cell. Transgenic mice, containing the genetic code for HCV structural proteins E1, E2 or core (or combinations thereof); or the non-structural NS3/4A protein, show conflicting results in the development of liver pathologies. Some reports do not show any evidence of hepatocellular damage (58–61), while other groups describe progressive hepatic steatosis and HCC (62–65). These discrepancies may be explained by the relationship between inflammation-associated hepatocarcinogenesis and the host genetic background (66). A major drawback of these HCV-transgenic mice is that the transgene integrates randomly and in high copy numbers. Consequently, the viral proteins are highly

overexpressed, often in an uncontrolled manner. Certain aspects of the HCV-transgenic mouse phenotype may be attributed to the artificial overexpression and/or interference with the regulation of genes located near the integration site. If the expression of viral proteins can be controlled and fine-tuned, the limitations of these models may be overcome. The Cre/Lox system (67) or hydrodynamic injection (68) allows inducible expression of the transgene. Using the murine major urinary protein (MUP)-promoter, the expression can be delayed (58). The immune system of this model closely resembles that of a chronically infected patient. Hence, it allows the evaluation of potential therapeutic vaccine strategies (69). Lerat et al. (70) created a transgenic FL-N/35 mouse model expressing the full HCV genome at levels corresponding to natural human infection (70, 71). The FL-N/35 mouse model is certainly the most relevant transgenic mouse model available at this time, especially for investigating hepatic steatosis, fibrosis, and HCC.

IMMUNOCOMPROMISED (HUMAN) LIVER XENOGRAFT MOUSE MODELS

Because mice are naturally not susceptible to HCV infection, an interesting approach to overcome the species barrier is by humanizing the liver *via* transplantation of primary human hepatocytes. In this way, mice can not only be infected with HCV but also with other human hepatotropic pathogens. However, if immunocompetent rodents are transplanted with xenogeneic hepatocytes, rejection of donor cells by the host cellular immune system is observed (72–74). In order to prevent this rejection, mice need to be immunocompromised. In addition, recipients must suffer from some type of liver disease to ablate murine hepatocytes and to allow proliferation of donor hepatocytes in the mouse liver parenchyma. This liver injury can be generated in three ways: chemically, surgically, or genetically (75). Several humanized mouse models have been developed and explored for HCV infection during the past 20 years.

The Trimera Mouse Model

The Trimera mouse was the first chimeric model and is composed of three genetically disparate sources of tissue (i.e., recipient mouse, bone marrow donor mouse, and human liver tissue), hence its name (76). After the recipient mouse is preconditioned by lethal total body irradiation, it is radioprotected by immediate injection of bone marrow cells from an immunodeficient SCID mouse (76). Then, human liver fragments, infected *ex vivo* with hepatotropic virus, are transplanted in ectopic sites of the recipient mouse such as the ear pinna or under the kidney capsule (76–78). Using this method, Ilan et al. (77, 78) were able to generate mice that can be infected with HBV and HCV. Higher serum HCV loads are obtained when pre-infected liver fragments from HCV-positive patients are employed compared to *ex vivo* infected liver fragments (78). HCV viremia persists for approximately 1 month and declines thereafter as a result of fibrosis and necrosis of the human graft (78). These observed histological abnormalities of the transplant can be attributed to their transplantation at an extrahepatic location (79). Also, *de novo* infection of Trimera mice, transplanted with healthy liver grafts, has so far not been achieved. This means that viral entry or neutralization studies cannot be performed using this model (79). Nevertheless, the 1-month time window may be sufficient for the evaluation of certain anti-HCV therapeutics or HCV vaccines (78). In fact, an HCV internal ribosomal entry site inhibitor was successfully tested in the Trimera mouse model (78, 80).

The Alb-uPA-SCID Mouse Model With Humanized Liver and Variants

The Alb-uPA mouse model was initially designed to study the pathophysiology of plasminogen hyperactivation and to evaluate new therapy regimens for bleeding disorders (81). These transgenic mice carry a tandem repeat of four murine urokinase-type plasminogen activator (uPA) genes under the control of a mouse albumin (Alb) promoter/enhancer (Alb-uPA mice) (81, 82). The hepatic uPA transgene overexpression results in elevated uPA plasma levels, but also leads to accelerated hepatocyte death, hypofibrinogenemia, and serious hemorrhagic events such as intra-abdominal and intestinal bleedings in neonatal transgenic mice (81–83). However, the high uPA concentration gradually returns to normal levels by the age of 2 months (82). This is probably due to somatic deletions of (parts of) the transgene construct within hepatocytes (82). Consequently, these transgene-deficient cells can selectively proliferate and regenerate the diseased liver tissue (82). On the other hand, when newborn Alb-uPA mice are transplanted with healthy donor hepatocytes, their functional liver deficit is also restored by the transplanted cells that repopulate the diseased liver (82, 84). In order to prevent rejection of hepatocyte transplants of xenogeneic origin, Alb-uPA mice should be backcrossed to an immunotolerant genetic background (84).

Mouse, rat, and woodchuck hepatocytes can be successfully transplanted into immunodeficient Alb-uPA mice using intrasplenic injection (84–86). Mouse livers are chimerically composed of both donor-derived and host-derived cells, the latter having a survival advantage by deletion of (parts of) the transgene (84–86). This transgene inactivation occurs less frequently in homozygous uPA animals compared to their hemizygous counterparts, because in the former two transgene arrays must be inactivated which is less likely to occur (82, 86). Accordingly, liver chimerism can be sustained for a much longer period and at higher levels in homozygous mice (86). Up to 90% of the liver may be reconstituted with donor hepatocytes and initially these cells appear to grow in a nodular fashion (84, 85).

By extension, this model is suitable for evaluating the susceptibility of donor hepatocytes to liver infections with a specific tropism for the donor species. Petersen et al. (85) were able to detect persistent non-cytotoxic woodchuck hepatitis virus infection in chimeric livers of uPA/recombination activating gene 2 (RAG2) mice transplanted with woodchuck hepatocytes (85). Accordingly, the same group (87) was able to transplant adult human hepatocytes early after birth in immunotolerant uPA/RAG2 mice. Up to 15% of the livers consist of human hepatocytes and homozygosity of the Alb-uPA transgene is also required to ensure sustained human engraftment (83, 87). The human hepatocytes seem to repopulate the liver in a well-organized fashion

with preservation of normal cell function and pharmacological responses (88, 89). In addition, human albumin, which indicates the functionality of the chimeric liver, is detected in plasma for at least 2 months after transplantation (87). Besides mature human hepatocytes, also hepatic progenitor cells are observed in these livers (90). Better humanization is obtained using commercially available, cryopreserved human hepatocytes (91). Remarkably, after inoculation with human HBV infectious serum, productive infection is initiated (87). Mercer et al. (83) showed for the first time that chimeric immunotolerant uPA-SCID mice were susceptible to HCV infection, thereby permitting the *in vivo* study of HCV biology and the evaluation of different antivirals. Efficient infection is independent from HCV genotype, but human albumin plasma levels exceeding 1 mg/ml are required for a consistent HCV infection in chimeric mice, whereas infectivity criteria for HBV infection are clearly less stringent (91, 92).

However, several shortcomings can be highlighted regarding the uPA-SCID mouse model: high neonatal lethality, a tendency to develop kidney disorders, lower body size, reduction of donor hepatocytes (even in homozygotes), less efficient breeding, technically challenging surgical manipulation in young and fragile mice, and finally the inability to expand engrafted hepatocytes (83, 88, 93–96). Tateno et al. (93) hypothesized that the first four mentioned limitations are caused by inadequate transgene structure and/or very high expression levels of the uPA gene before or after birth. Therefore, they produced chimeric mice using embryonic stem cell techniques in order to generate a number of transgenic lines. In addition, transgenic lines with the most appropriate uPA expression for a damaged, but not a detrimental liver were selected (93). This variant is called the hemizygous cDNA-uPA-SCID mouse model (93). More albumin-positive human hepatocytes are present compared to the original model, potentially due to an overgrowth of mouse hepatocytes in the uPA-SCID mouse by somatic deletion of uPA genes (97). After HBV infection, high titers of HBV viremia that persisted for at least 34 weeks are found in cDNA-uPA-SCID mice, but entecavir treatment results in a similar viremia decline in both models (97). HCV viremia is significantly more observed in cDNA-uPA-SCID mice in comparison with uPA-SCID mice, but not one mouse remains HCV-positive 8 weeks post-inoculation (97). Finally, fewer kidney disorders, higher body weight, and a higher survival rate are observed in the cDNA-uPA-SCID model (93, 97). Taken together, the cDNA-uPA-SCID mouse model may be preferred over the original uPA-SCID model for the study of HBV and HCV biology and by extent for the evaluation of anti-HBV/HCV drugs.

A second variant consists of transgenic mice carrying the uPA gene driven by the MUP promoter (98). These mice can be backcrossed onto a SCID/Beige background, resulting in the MUP-uPA SCID/Bg mouse model (99). The initial purpose of this model was to study liver regeneration after repopulation of the diseased liver, but Tesfaye et al. (100) were able to show that, upon humanization, these mice are susceptible to infection with HBV, genotypes 1–6 of HCV and tissue culture-derived virus (98, 99). Interestingly, these mice are in better health compared to the classical uPA-SCID mouse model and they offer a longer time window (up to 4–12 months of age) for transplantation of human hepatocytes (100). The same group (101) reported successful HCV infection after engraftment with hepatocyte-like cells, generated from both human embryonic stem cells and patient-specific human-induced pluripotent stem cells. Finally, this model is also valuable for the study of HCV-associated HCC and for the analysis of tumor-promoting factors in liver cancer (102).

As a third uPA-based variant, the non-obese diabetic (NOD)/Shi-scid IL2Rg^{null} (NOG) background is employed, resulting in the uPA/NOG mouse model (94). Donor hepatocytes can be transplanted in 6-week-old uPA/NOG mice which enable easier surgical manipulation and moreover an improved survival rate of the transplanted mice (94). In addition, absence of neonatal lethality increases the efficacy of homozygote production by mating and finally, the severely immunocompromised NOG background allows higher xenogeneic cell engraftment (94). Another advantage is that a relatively low frequency of physical loss of the transgene is observed (94). However, HCV infection is not reported in this model yet. Importantly, Hasegawa et al. (103) generated another model by using an alternative strategy for the endogenous liver injury: targeting the expression of herpes simplex virus type 1 thymidine kinase to the liver of the NOG mice. Hepatocytes that express this transgene can be ablated after brief exposure of a non-toxic dose of ganciclovir (103). Thereby, mouse livers can be stably replaced with mature and functional human liver tissue at a chosen time (103). This model can be successfully infected with HBV and HCV and is therefore useful to test different antiviral agents (104).

Taken together, the chimeric human liver uPA-SCID mouse model or discussed variants thereof have proven valuable for *in vivo* metabolism studies, basic biology research of HCV infection, and the evaluation of different antiviral therapies and passive immunization strategies (79, 105–113). Our group also contributed by demonstrating the prophylactic effect of monoclonal and polyclonal antibodies, isolated from a chronically infected patient, against challenge with different HCV genotypes (107, 108, 114). In addition, anti-receptor antibodies are shown to protect these mice from a subsequent challenge with HCV of different genotypes (106, 115–117). Next to chimpanzees, this human liver chimeric mouse model is also attractive for monitoring HCV drug resistance (118). Our laboratory has particular interest in this matter and showed that the combination of DAAs with entry inhibitors restricts the breakthrough of DAA-resistant viruses (119, 120). Finally, the uPA-SCID mouse model is also applicable for studies concerning malaria, which is caused by the parasite *Plasmodium falciparum*, and the study of the hepatitis E virus (HEV) (121–125).

The FRG Mouse Model With Humanized Liver

In the original uPA-SCID mouse model, hepatocyte transplantation needs to be performed very shortly after birth (i.e., in very fragile and small animals) (126). Because of this practical inconvenience, other models were explored in which liver injury can be induced at a later age, such as in certain earlier discussed variants of the uPA-SCID model. Another example is based on mice

that have a genetic knockout for fumarylacetoacetate hydrolase ($Fah^{-/-}$), a metabolic enzyme that catalyzes the last step of the tyrosine catabolism pathway (127, 128). This knockout results in an accumulation of toxic compounds (e.g., fumarylacetoacetate and maleylacetoacetate), which in turn leads to liver dysfunction and lethality, unless mice are rescued by the protective drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) (127–129). NTBC blocks the enzyme hydroxyphenylpyruvate dioxygenase upstream of FAH, thus preventing the accumulation of hepatotoxic metabolites (130). Using this approach, Grompe et al. (129) showed that liver injury can be induced at any desired time point when NTBC is withdrawn. The resulting prolonged lifespan of these $Fah^{-/-}$ mice resembles a phenotype of humans suffering with hereditary tyrosinaemia type I (HT1), which is an inborn error of metabolism caused by deficiency of the Fah enzyme (127–129). As a result, the adult $Fah^{-/-}$ mouse, when removed from NTBC treatment, is a valuable model for studying the pathophysiology and evaluating the treatment options of HT1 and by extension hepatic cancer (128, 129). In the immunodeficient FRG mouse, the aforementioned Fah, RAG2, and common γ-chain of the interleukin receptor (Il2rg) are knocked out. The livers of these mice can be successfully repopulated with human hepatocytes after NTBC withdrawal (95, 130). In support of enhanced engraftment, Azuma et al. (95) administered an uPA-expressing adenovirus before transplantation which induces cell-autonomous hepatotoxicity rendering a more favorable niche for transplantation (131). In later experiments, Bissig et al. (96) showed higher transplantation rates (up to 95%) using an increased human hepatocyte dose per mouse. They also demonstrated successful infection of the FRG mouse with HBV and HCV, however, infection with HCV is only achieved in mice with a high human liver chimerism (96).

The FRG mouse model is in some ways favorable over the uPA-SCID mouse model. First, the deficiency caused by the Fah deletion cannot revert back to its wild type form, as seen in the uPA-SCID model (95). As a result, serial transplantations are possible and transplantation can be performed in adult animals (at any age) which simplifies surgery (95). Second, mutant breeders are completely viable and finally, there is no renal disease observed (95). Unfortunately, there are also drawbacks for such a model. First, primary engraftment does not occur in 100% of the recipients, even when the aforementioned urokinase-expressing adenovirus is administered (95). Second, the growth disadvantage of mouse hepatocytes in the FRG mouse depends on the absorbed tyrosine and the use of NTBC, whereas the growth advantage in uPA-SCID mice is sustained as long as the transgene is expressed (130).

Applications of this FRG mouse model with humanized liver are wide-ranging. First, human lipid and bile acid metabolism can be studied, next to the metabolism of candidate pharmaceuticals or toxicity of drug metabolites (95, 132). Second, after inoculation with pathogens that are dependent on human liver cells for replication such as HBV, HCV, and HEV, the life cycle can be studied, but also experimental treatment options can be evaluated (95, 133–137). Finally, because the FRG mouse model also supports complete *P. falciparum* liver stage development, this model is suitable for evaluating existing drugs and screening of candidate antimalarials (138).

IMMUNOCOMPETENT XENOGRAFT MOUSE MODELS

The human liver xenograft mouse models are very valuable as challenge models for HCV or other human hepatotropic pathogens, but their major drawback is the lack of a functional immune system. As a consequence, they cannot be used for the study of HCV-specific immune responses or HCV immunopathogenesis after infection, nor for HCV vaccine studies (79). Second, histopathology such as fibrosis, cirrhosis, or HCC has not been reported, in contrast to what is seen in humans that are chronically infected with HCV (75). In human patients, an ongoing inflammatory response is probably responsible for disease progression, so the presence of a functional human immune system in HCV mouse models is highly demanded and explored (75).

The Tolerized Rat Model

Another way to avoid rejection of allogeneic transplants, in addition to generalized immunosuppression or the use of genetically immunodeficient animals, is by induction of immunological tolerance to transplanted cells in immunocompetent animals (76, 77, 85, 139, 140). Therefore, Huh7 cells can be injected *in utero* into the peritoneal cavity of fetal rats (139). In this time frame, the immune system is still in development, so tolerance toward engrafted hepatocytes can be established (139, 140). Corresponding cells are then intrasplenically injected into the newborn rats within the first 24 h after birth (139). The major benefit of this model is that there is no need for genetic or pharmacological immunosuppression (139). However, engraftment rates are much lower compared to the uPA-SCID model for example, because there is no mechanism for host hepatocyte depletion (141). The use of hepatoma cells instead of primary hepatocytes also limits further applications. Another drawback is the mismatch between the human major histocompatibility complex (MHC) antigens on the transplanted cells and the rat immune system, so there will be no recognition of HCV antigens by the immune cells of the rat (141). Despite these limitations, HCV gene expression, viral replication, and hepatitis symptoms can be observed when these tolerized immunocompetent rats are intrasplenically injected with HCV inocula 1 week after transplantation (141). Unfortunately, HCV viremia is low (peak at 22,500 copies/ml) and the observed inflammation is probably due to cytokine-mediated effects (141).

The Dually (Immune System and Hepatocytes) Engrafted Mouse Models

To overcome the human/rodent MHC mismatch as in the tolerized rat model, it would be favorable to introduce both human hepatocytes and human immune cells from the same donor into the same recipient animal. The first mouse model that supported this double engraftment was generated in 2011 (142). A fusion protein of the FK506 binding protein (FKBP) and caspase 8 under the control of the albumin promoter (AFC8) is therefore transgenically expressed in hepatocytes of immunodeficient Balb/C Rag2-γC^{null} mice. After administration of an FKBP dimerizer, hepatocytes that expressed the transgene are depleted (142, 143).

This induced liver-specific cell death provides a niche for engraftment with human hepatocyte progenitors (142). Moreover, after irradiation, these mice are transplanted with human CD34$^+$ hematopoietic stem cells (HSCs) from the same human fetal liver tissue, providing these AFC8-hu HSC/Hep mice with a, to some extent, functional human immune system (142, 144). Following inoculation with primary HCV isolates, HCV infection can be observed that in turn induces infiltration of human immune cells in the livers with liver inflammation and fibrosis as a result (142, 145). This model enables the study of HCV-specific immune responses (i.e., T-cell responses) and HCV immunopathogenesis (142, 145). However, HCV RNA could only be detected in the liver and not in plasma, probably due to the low level of human liver engraftment (~15%) in this model (126, 142). Another limitation is the suboptimal activity of the immune system inherent in human CD34$^+$ HSC transplanted mice and also the lack of functional B-lymphocytes that hampers the study of antibody responses and vaccine development (126, 142, 146).

A second immunocompetent model was launched by Gutti et al. (147) who used non-myeloablative conditioning with treosulfan as a safe and well-tolerated alternative to total body irradiation for HSC transplantation. Long-term dual reconstitution is achieved in uPA/NOG mice with HSCs and allogeneic mature hepatocytes (not fetal hepatoblasts) (147). Even MHC mismatched transplantation is sustained without any evidence of hepatocyte rejection by the human immune system (147). Wilson et al. (148) also accomplished double humanization of mice. Following preconditioning with a DNA-damaging chemical for enhanced HSC engraftment and an uPA-expressing adenovirus for enhanced hepatocyte engraftment, they co-transplanted adult human hepatocytes and HSCs in immunodeficient FRG mice on a NOD-strain background (FRGN mice) (95, 148). Another variant is achieved in BALB/c RAG2$^{-/-}$ IL-2Rγc$^{-/-}$ NOD.sirpα (BRGS) mice that harbor the uPA transgene (uPA/BRGS mice) (149). Irradiated newborn pups are injected with human HSCs and later implanted with human hepatocytes to generate dually engrafted mice that are not haplo-type matched. Engraftment (~20–50% of chimerism) is stable for at least 5 months and is similar as observed in the uPA/NOG and FRGN host, but higher than in the AFC8 host (142, 147–149). Interestingly, a complete viral life cycle can be observed after HBV infection in this model (150). This enables the evaluation of experimental anti-HBV therapies, but also the study of anti-HBV immune responses (150). Bility et al. (151) also reported successful HBV infection in a similar human liver progenitor cell and human CD34$^+$ HSC cell engraftment model using mice on a NOD-SCID IL2r$\gamma^{-/-}$ background (HLA-A2/NSG mice). These mice carry the human HLA-A2 transgene that enhances the development of human MHC-restricted T-lymphocytes (151, 152). To promote efficient hepatocyte repopulation, mice are first treated with an anti-Fas agonistic antibody (151, 153). Chen et al. (154) performed one-step engraftment of hepatoblasts and a matching human immune system using fetal liver-derived HSC cells in the same NSG mouse (human immune system and liver or HIL mice) and this without the need for transgenic modification or drug treatment. HIL mice support HCV infection, liver inflammation, human HCV-specific immune responses, as well as liver fibrosis, however, in a low number of hepatocytes (154, 155). This can be explained by the low human chimerism rate (<10%) (154, 155). Antiviral treatment using IFNα-2a is able to block the progression of the HCV-associated liver pathogenesis (154, 155). These HCV-infected mice also show expansion of monocytes/macrophages and (especially CD4$^+$) T-cells, suggesting exhaustion of immune cell phenotypes as seen in HCV patients (156). Unfortunately, HCV infection is not reported in every discussed dually engrafted model and this will also be challenging, especially due to the very low engraftment rates.

VIRAL ADAPTATION

Hepatitis C virus exhibits a narrow species tropism which is incompletely understood. Resistance of mice to HCV infection is determined to be at the level of viral entry and/or replication. There are two ways of surmounting this barrier: either the host can be adapted to the virus or the virus can be adapted to the host. First, utilizing the error-prone replication of RNA viruses, the HCV virus can be adapted to the murine environment. More specifically, long-term cultivation in the presence of mouse cells could allow the virus to adapt to murine entry factors (CD81, OCLN, CLDN1, and SR-BI). Bitzegeio et al. (157) attempted to adapt an HCV genotype 2a strain (Jc1) to the murine CD81. They identified three adaptive mutations in the HCV envelope proteins E1 and E2. This Jc1/mCD81 virus has increased affinity for the extracellular loops of human CD81, indicating a more accessible binding site for human CD81 (157). The uptake of this murine-tropic HCV in mouse primary hepatocytes *in vitro* and *in vivo* is rather inefficient and more modifications are required to increase efficiency. There is unfortunately no persistent infection observed, even in mice with impaired innate and adaptive immune system. To conclude, additional barriers may exist in the replication and post-entry steps (158). In addition, the applicability of such systems for the study of entry processes might be affected by the influence of the adaptive mutations on the envelope conformation and receptor usage.

GENETICALLY HUMANIZED MOUSE MODEL

Rather than to adapt the virus to a new host, an alternative strategy could be to genetically adapt the host to natural HCV isolates. Despite differences to the natural human host of HCV, an immunocompetent animal model can be generated in this way. Transient expression of the minimal human factors (CD81, OCLN, CLDN1, and SR-BI) by adenoviral delivery in Rosa26-Fluc mice allows entry of HCVcc in mouse hepatocytes (35). Furthermore, mice transgenic for these four human receptors (4hEF-mice), but with deficiencies in several innate immune signaling pathways (STAT1$^{-/-}$), support not only viral entry of HCVcc but also low-level replication and sustained HCV infection for 90 days. The infection elicits antiviral cellular and humoral responses, but does not result in development of liver disease (159).

However, these models express non-physiologically high levels of the entry factors and impair tight junction formation and B-cell development (160). Interestingly, by selectively humanizing the second extracellular loops of CD81 and OCLN, required for HCVcc entry, the chimeric alleles are expressed at physiological levels and mice support HCV uptake at similar levels as mice expressing HCV receptors using transgenical or adenoviral methods. Also, tight junctions are formed normally and the defects in B-cell development are absent (160).

However, since replication in immunocompetent mice is inefficient, the latter model does not allow a profound study of all complex virus–host interactions. Viral RNA replication in mouse cells appears to be the final hurdle to overcome in order to reconstitute the entire viral life cycle in mice. Chen et al. (161) described an immunocompetent animal model permissive for HCV infection and ensuing development of liver disease. They created transgenic mice expressing OCLN and CD81 on an outbred ICR (CD-1) background (C/OTg). These mice can be infected with serum- or cell culture-derived HCV and sustain this infection for over 12 months. Moderate hepatic inflammation, micro- and macro-vesicular steatosis, and fibrosis are observed in some of the infected animals. However, none of the animals develop HCC (161). It is rather striking that HCV can establish a persistent infection in ICR-C/OTg mice, whereas a similar approach on a C57BL/6 background fails to show sustained HCV replication. Backcrossing C/OTg to a C57BL/6 background (B6-C/OTg) significantly reduces the RNA copy number in serum and liver. ICR hepatocytes express higher levels of apolipoprotein E, which is shown to improve HCV production (162). Also, miR-122 is not upregulated upon HCV infection in B6-C/OTg (161). In conclusion, the ICR-C/OTg mouse model appears to fulfill to a large extent the criteria for a suitable HCV animal model and is therefore a valuable addition to the current pool of animal models.

HCV HOMOLOGS

Alternative models are based on the use of HCV homologs. These HCV-related viruses infect either rodents, horses or dogs and can therefore be used to study viral biology, pathogenesis, and host immune responses in an immunocompetent setting. The GB virus B has long been the only known homolog to HCV. This virus was first discovered in tamarins experimentally infected with serum of a surgeon (G.B.) suffering from acute hepatitis (163). The infected tamarins developed acute hepatitis (164) and are used together with marmosets as a surrogate model for the study of protective immunity (165) and evaluation of antivirals (166). However, persistence is rare in these animals and the natural host is yet to be identified (163, 167, 168).

By using deep sequencing virome analyses, novel HCV-related hepaciviruses and pegiviruses have been identified in dogs, horses, bats, rodents, and non-human primates (168). Several of these viruses have the potential to serve as a surrogate model for HCV. However, not all are hepatotropic or mimic the natural course of HCV infection. The non-primate hepacivirus (NPHV) was first discovered in dogs and therefore termed canine hepacivirus (169), but subsequent studies revealed that horses are the natural host for this virus (168). NPHV infection in horses greatly resembles HCV infection in humans. It is a hepatotropic virus that is able to establish a persistent infection, although the chronicity rate is considerably lower than for HCV infection. The host immune response is similar to that in humans, including the delayed seroconversion and immune-related liver pathology (168). These characteristics allow NPHV to be a valuable animal model for HCV, especially since it is immunocompetent. Drawbacks, however, are the large size and animal care costs that accompany this model. Conceivably, rodents are still the desired animal model, due to their small size, easy handling, and possibility to be genetically manipulated. Therefore, the newly discovered rodent hepaciviruses (RHV) are of particular interest (170–172). Infections of these viruses in their natural host, or possibly in immunocompetent laboratory inbred mouse strains, require further investigation.

Methodical searches for hepaciviruses in several wild rodent species have led to the identification of potential small animal models for HCV. Some of these rodents, including bank voles (*Myodes glareolus*) and rats (*Rattus norvegicus*), experience signs of liver inflammation after infection with a RHV. During a metagenomics survey in commensal Norway rats (Nr) in New York city, Firth et al. (172) also discovered some new viruses, including two novel hepaciviruses (NrHV-1 and NrHV-2) and one novel pegivirus (NrPgV). These hepaciviruses were demonstrated to be hepatotropic and are consequently the first small-mammal hepaciviruses known to replicate in the liver (172). Although rats are the natural hosts of NrHV, Billerbeck et al. (173) aimed to develop a mouse model of NrHV infection. NrHV is able to establish a persistent infection in immunocompromised mice lacking type I interferon and adaptive immunity (A129, AG129, and NRG). On the other hand, immunocompetent mice (C57BL/6J and BALB/c) clear the virus in a few weeks (173). NrHV, passaged through NRG mice, is cleared significantly slower than NrHV derived from rats, indicating possible adaptation to the mouse host. The developed immunocompetent inbred mouse model can potentially help to unravel certain mechanisms of hepacivirus host adaptation, immune activation and evasion, and development of liver disease (173). Because this inbred mouse model only results in an acute, self-limiting infection, Trivedi et al. (174) searched for a fully immunocompetent surrogate model in which a persistent infection can be established. The natural host of NrHV, the rat, was therefore further investigated. Inbred Brown Norway rats fail to even partially control the infection, while different outbred lines [Spraque–Dawley, Holtzman (HTZ), Long Evan, and Wistar Han] show limited suppression of viral replication. HTZ rats display the largest suppression of viremia and were explored in more detail. The rats exhibit hepatic inflammation characterized by dense lymphocytic aggregates focused on the portal tracts, parenchymal damage, associated with apoptotic hepatocytes and macro- and micro-vesicular steatosis, characteristic for human HCV infection (174). This model is also suitable to study the role of various interferon stimulating genes and immune responses in hepacivirus pathogenesis. Thus, NrHV infected rats can serve as an informative, fully immunocompetent surrogate to study the mechanisms of HCV persistence, immunity, and pathogenesis.

CONCLUSION

Despite extensive research, there is still no vaccine available for the prevention of HCV infection. In order to design and test new vaccines, the immunocompetent human liver xenograft mouse models are very promising. Next to the study of HCV immune responses, these models also allow investigation of disease progression. Contrary to this, the immunocompromised human liver xenograft mouse models only allow passive immunization. In this way, antibodies targeting different genotypes of HCV can be evaluated. Vaccine studies are not relevant, because these mice lack or only show limited cellular immunity. Furthermore, during the past decade, especially the uPA-based mouse with humanized liver has considerably contributed to our understanding of the HCV life cycle and the development of antiviral strategies. Alternatively, for studying the basic aspects of HCV biology, viral replication or for the evaluation of certain antiviral strategies, it may not be necessary to establish complicated dual-chimeric models. The genetically humanized models are adequate to study viral biology. However, they can only be used to evaluate prophylactic vaccines, not therapeutic vaccines. Finally, the HCV homologs, more specifically NrHV, can be used for vaccination studies and for the evaluation of both humoral and cellular immune responses. The knowledge that is built from this model can be partially transferred to the existing HCV models, but it is important to consider that HCV and hepaciviruses comprise different viruses. In conclusion, it is clear that the HCV model of choice is highly dependent upon the specific research question. The development and characterization of new HCV animal models or the improvement of existing models, especially those with a human immune system, is highly demanded to develop a potent HCV vaccine. An effective vaccine is probably the most essential key for eradication of HCV.

AUTHOR CONTRIBUTIONS

RB and LC wrote the manuscript. AM and PM revised the manuscript.

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