Functional and immunogenic characterization of diverse HCV glycoprotein E2 variants

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Abstract

Background and Aims Induction of cross-reactive antibodies targeting conserved epitopes of the envelope proteins E1E2 is a key requirement for an HCV vaccine. Conserved epitopes like the viral CD81-binding site are targeted by rare broadly neutralizing antibodies. However, these viral segments are occluded by variable regions and glycans. We aimed to identify antigens exposing conserved epitopes and to characterize their immunogenicity.

Methods We created HCV variants with mutated glycosylation sites and/or hypervariable region 1 (HVR1). Exposure of the CD81 binding site and conserved epitopes was quantified by soluble CD81 and antibody interaction and neutralization assays. E2 or E1-E2 heterodimers with mutations causing epitope exposure were used to immunize mice. Vaccine-induced antibodies were examined and compared with patient-derived antibodies.

Results Mutant viruses bound soluble CD81 and antibodies targeting the CD81 binding site with enhanced efficacy. Mice immunized with E2 or E1E2 heterodimers incorporating these modifications mounted strong, cross-binding, and non-interfering antibodies. E2-induced antibodies neutralized the autologous virus but they were not cross-neutralizing.

Conclusions Viruses lacking the HVR1 and selected glycosylation sites expose the CD81 binding site and cross-neutralization antibody epitopes. Recombinant E2 proteins carrying these modifications induce strong cross-binding but not cross-neutralizing antibodies.

Lay summary

Conserved viral epitopes can be made considerably more accessible for binding of potently neutralizing antibodies by deletion of HVR1 and selected glycosylation sites. Recombinant E2 proteins carrying these mutations are unable to elicit cross-neutralizing antibodies suggesting that exposure of conserved epitopes is not sufficient to focus antibody responses on production of cross-neutralizing antibodies.

Highlights

- High resolution mapping of the impact of HVR1 and glycosylation sites on CD81 binding, antibody binding and virus neutralization
- Viral mutants lacking HVR1 and selected glycosylation sites are functional and they expose the viral CD81 binding site and conserved cross-neutralization epitopes
- E2 proteins with these mutations induce cross-binding and non-interfering antibodies in mice

Introduction

Hepatitis C virus (HCV) is a global health burden affecting approximately 71 million people worldwide [1]. Infection often leads to chronic hepatitis, with the subsequent risk for liver cirrhosis and hepatocellular carcinoma. Persistent HCV infection is now curable with the introduction of direct-acting antivirals (DAAs). However, a prophylactic HCV vaccine is not available. Since viral re-infection is possible and as many HCV infected individuals are not diagnosed, a vaccine against HCV would facilitate global HCV eradication programs.

The extreme diversity of HCV is a major obstacle for vaccine development [2]. The HCV E1E2 proteins are essential for viral cell entry, they bind the HCV receptor CD81 and they are targets for neutralizing antibodies. Hence, immunogens based on E1E2 represent one major branch of vaccine development [3, 4] and numerous approaches to induce E1E2-targeting broadly neutralizing antibodies (bNabs) have been explored [5]. For recombinant E1E2, the most advanced HCV subunit vaccine candidate, induction of robust cross-binding and cross-neutralizing antibody responses was observed in multiple animal models and in humans [4, 6, 7, 8].

HCV has evolved mechanisms to evade humoral immune responses including high functional flexibility and variability of immunogenic portions of its envelope proteins [9]. The highest sequence variability occurs in the first 27 amino acids of the N-terminus of E2, which is referred to as the hypervariable region 1 (HVR1), and which is dispensable for HCV infection *in vitro* [10, 11]. The HVR1 is immunogenic and most patients mount antibodies targeting this region [12]. However, these antibodies rapidly select resistant viral variants [13]. Deletion of the HVR1 renders HCV more susceptible to antibody neutralization, and it increases virus binding to soluble CD81, suggesting that this region occludes key neutralization epitopes and the viral CD81 binding site [10, 11]. HCV E1E2 heterodimers are also heavily glycosylated at multiple sites both within E1 and E2 and glycans modulate glycoprotein function and antibody neutralization [14, 15, 16]. Structural analyses of the E2 core domain show that the conserved CD81 binding site is surrounded by several glycosylation sites and that it overlaps with the epitopes of bNabs isolated from patients like for instance HC1 and HC11 [17, 18, 19, 20] (Fig. 1). Strikingly, *in vitro* HCV is unable to escape antibody pressure by HC-1 and HC-11 suggesting that immune responses targeting these epitopes may confer robust protection [21, 22].

We hypothesized that the HVR1 and protein glycosylation limits access to these conserved viral epitopes. In turn, immunogenicity of these epitopes may be low and antibodies targeting these viral regions may arise only very infrequently. To overcome this limitation and to focus immune responses to conserved

viral epitopes we combined targeted protein deglycosylation with deletion of HVR1 to create viruses and proteins with increased exposure of the crucial CD81 binding site and possibly superior immunogenicity.

Results

Inactivation of glycosylation sites and deletion of HVR1 increase exposure of conserved neutralization epitopes and the CD81 binding site

To generate an improved HCV vaccine antigen, we aimed to increase the exposure of the CD81-binding site by combined mutation of the HVR1 and selected glycosylation sites. Jc1 reporter viruses expressing the J6 (GT2a) E1E2 proteins [23, 24] were constructed harboring N to A substitutions at glycosylation sites N417 (417) (numbering according to the H77 reference stain; J6 numbering in brackets), N423 (423), N448 (448), N532 (534) and N645 (649) (Fig. 1A). A previous report had shown that mutations at these residues still allowed viral entry of JFH1 and can therefore be studied and further evaluated *in vitro* [15]. Molecular modelling of the glycans associated with the HCV E2 core structure revealed that some were proximal to the CD81 binding site and conserved neutralization epitopes (Fig. 1B). Therefore, to further increase exposure of the CD81 binding site, we combined these mutations with the deletion of the HVR1. Fitness of mutant viruses was examined by transfection of Huh7-Lunet N#3 cells that do not express CD81 [25], and therefore do not permit reinfection of the cells. Replication of all viral mutants was comparable to wild type (WT) Jc1 (Fig. 1C). Huh-7.5 cells were inoculated with virus stocks after normalization to viral core protein that acts as a marker for virus particle release. In line with our previous report [10], deletion of HVR1 resulted in a 5-fold reduction of specific infectivity of Jc1 (Fig. 1D). Likewise, point mutations of individual glycosylation sites were well tolerated as specific infectivity was unaffected or at most reduced by ca. 5-fold.

To examine the impact of these mutations on the exposure of conserved neutralization epitopes within the CD81 binding site, we used the soluble, large-extracellular loop of CD81 (CD81-LEL) and two potent bNabs (HC-1 and HC-11) that target the CD81 binding site [17, 18, 19, 20, 21], for precipitation of the indicated viral mutants (Fig. 2A and B). Deglycosylation of specific residues in the parental Jc1 virus slightly increased binding of HC-1 in particular for mutant N448A and N534A as is evidenced by enhanced core protein precipitation compared to Jc1, without being statistical significant (Fig. 2A). However, the deletion of HVR1 significantly enhanced precipitation by this antibody, which did not further increase by additional point mutations of glycosylation sites at indicated positions (Fig. 2A). Similar results were observed when the HC-11 antibody was used: mutation of individual glycosylation sites increased antibody binding (e.g. N423A, N448A). Deletion of HVR1 had the greatest effect, which was not further boosted by mutation of the glycosylation sites (Fig. 2B). Comparable results were obtained

when we used CD81-LEL for precipitation. In the context of parental Jc1, inactivation of individual glycosylation sites modestly increased precipitation (Fig. 2C). As may be expected from the available structural information (Fig. 1B) [17, 18], the effect was greatest for mutants N423A and N534A, as these glycosylation sites are directly adjacent to the CD81 binding site. Deletion of HVR1 had the greatest effect and precipitation of viruses lacking HVR1 was not further boosted by deglycosylation of specific residues.

Deletion of HVR1 and glycosylation sites enhances virus neutralization

To characterize the accessibility of HCV antibody epitopes, we performed neutralization assays with a panel of bNAbs (Fig. 3A and C and supplementary table 1). Moreover, we quantified virus neutralization by CD81-LEL (Fig. 3B and C and supplementary table 1). Dose dependence of neutralization for selected viruses is displayed in figure 3C. Radar plots indicating the inhibitory concentration 90% (IC_{90}) of different antibodies or the CD81-LEL against parental HCV and all mutant viruses is displayed in figure 3A and 3B. Each plot shows a specific virus and its sensitivity towards given antibodies (Fig. 3A), whereas figure panel 3B highlights the susceptibility of each virus to competition by CD81-LEL. In the context of parental Jc1, deletion of glycans at position N423 and N448 increased the susceptibility to all tested antibodies, whereas removal of glycosylation at residues N417, N534, and N649 had little effect (Fig. 3A upper panels). As expected, deletion of HVR1 enhanced neutralization by all antibodies tested. Combination of deletion of HVR1 with deletion of specific N-glycosylation sites modified neutralization by these antibodies, and the Jc1-ΔHVR1-N534A mutant exhibited a further enhanced susceptibility to neutralization by HC-1 and HC-11 (Fig. 3A and 3C). Removal of glycosylation at the N423, N534, and N417 site in the context of WT Jc1 enhanced inhibition of infection by CD81-LEL, whereas mutation of the N417 and N649 sites had no effect (Fig. 3B). Deletion of HVR1 increased neutralization by CD81-LEL and this was further enhanced ca. 5-10-fold by addition of the N534 mutation. Therefore, combined deletion of HVR1 and inactivation of glycosylation site N534 enhanced antibody and CD81 binding to virus particles and it had the most drastic effect on neutralization by bNabs and soluble CD81.

Next, we evaluated neutralization of Jc1, Jc1-N534A, Jc1-ΔHVR1and Jc1-ΔHVR1-N534A by sera from individuals chronically infected with HCV GT 1 and 2 (Fig. 4A-C). Similar to the results of neutralization with monoclonal antibodies and CD81-LEL (Fig. 3), the sera poorly neutralized Jc1. Neutralization was enhanced by elimination of the N534 site in Jc1, further boosted by deletion of HVR1, and it was maximal when the deletion of HVR1 was combined with the inactivation of the N534 glycosylation site with minor interpatient variation, independent of the patients` HCV genotype (Fig. 4A-C). Thus, combined deletion of HVR1 and inactivation of glycosylation site N534 enhanced antibody and CD81 binding to virus particles and it had the most drastic effect on neutralization by bNabs and soluble CD81 and patient sera. As these

viruses are infectious they present well-folded and functional viral envelope proteins that display conserved epitopes in a more accessible manner. Thus, envelope proteins carrying these modifications, particularly the deletion of HVR1 combined with the N534A mutation, may constitute good candidates for focusing antibody responses on conserved viral epitopes.

Immunogenicity of recombinant E2 proteins with and without HVR1 and glycosylation

To test this, we prepared soluble, truncated HCV J6 E2 protein variants in 293T cells and immunized BALB/c mice. These proteins have been previously reported to inhibit virus infection indicating that they are properly folded [26]. Supplementary figure 1 shows the immunization scheme. Briefly, animals received 30 µg of soluble E2 injected together with bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP) as adjuvant and were boosted thrice with the same formulation. As control, animals were vaccinated with PBS and adjuvant only. We also used two vaccination series where we changed antigens in each boost in order to promote the stimulation of antibodies specific for the conserved epitopes. In sequence A animals were primed using WT J6 E2 (GT2a), followed by three boosters with E2ΔHVR1 (J6 GT2a derivative), E2ΔHVR1/N534A (J6 GT2a derivative), and WT Con1 E2 (GT1b), respectively. In sequence B mice were primed with E2ΔHVR1/N534A, followed by three booster immunizations with E2ΔHVR1 (J6 GT2a derivative), WT Con1 (GT1b) E2, and WT J6 (GT2a) E2, respectively. Finally, to examine the relevance of global glycosylation for antigenicity, we vaccinated with WT J6 (GT2a) or with J6 (GT2a) E2ΔHVR1 that had been deglycosylated by PNGaseF treatment prior to vaccination.

The binding of vaccine-induced antibodies to 293T cell derived recombinant E2 proteins or E1-E2 heterodimers from HCV infected cells was determined by ELISA (Fig. 5 and supplementary figure S2). Fig 5A shows end point titers of sera from vaccinated animals reacting with H77 (GT1a), Con1 (GT1b), J6 (GT2a) or J6ΔHVR1 (GT2a) recombinant proteins. High binding titers across these GT1 and GT2 antigens were observed in all vaccinated groups up to a maximal end point dilution approaching 10E6 (Fig. 5A). Moreover, irrespectively of which protein was used for vaccination, most of these antibodies targeted binding sites outside of the HVR1, as end point titers were comparable between J6 (GT2a) and J6ΔHVR1 (GT2a) ELISA antigens. Cross-binding activity to these recombinant E2 proteins was clearly enhanced for vaccination with E2ΔHVR compared to wildtype recombinant E2 (Fig.5C left panel). Moreover, vaccination according to sequence A and B resulted in greater cross-binding to recombinant E2 proteins. To evaluate the binding of these antibodies to heterodimeric E1-E2 protein complexes from different HCV strains, we used plates coated with galanthus nivalis lectin (GNA) to capture HCV E1E2 complexes expressed in Huh-7.5 cells transfected with infectious HCVcc chimeras. Subsequently, these plates were incubated with sera from our vaccinated animals and end point binding titers were calculated

(Fig. 5B). In each group of vaccinated animals, anti-E2 antibodies binding to all examined E1-E2 protein complexes were detectable. Thus, each vaccination approach induced broadly cross-binding antibodies that recognize E2 proteins of all major HCV GTs. With exception of the vaccination with J6ΔHVR-534 E2 protein, however, all vaccination approaches induced antibodies with cross-binding activity to these E1-E2 heterodimers indistinguishable from the ones induced by parental J6 E2 (Fig. 5B and 5C right panel). Taken together, all vaccination approaches induced strong cross-binding antibodies to E2 and E1-E2 heterodimers. Vaccination with different E2 proteins, most notably with E2ΔHVR1, enhanced cross-binding when judged by ELISAs involving recombinant E2. However, this difference was not detected when cross-binding was quantified with ELISAs based on E1-E2 heterodimers from cells replicating infectious virus.

Evaluation of neutralizing activity of vaccine induced antibodies

To examine autologous virus and virus cross-neutralizing responses we incubated Jc1 and GT5a reporter viruses, respectively, with increasing doses of purified IgG from vaccinated animals or from patient sera (Fig. 6A). The autologous virus Jc1 was neutralized by antibodies from the animals vaccinated with deglycosylated E2, with deglycosylated ΔHVR1 or the series A antigen combinations. The strength of these autologous neutralizing antibody responses was at least as strong as the neutralization by polyclonal human antibodies collected from a panel of GT1, 2 and 3 chronically infected individuals. This correlated with comparable binding of patient-derived and vaccine-induced antibodies to recombinant E2 and virus particle associated E2, quantified by surface plasmon resonance and virus particle precipitation (supplementary figure S4). However, none of the vaccine-induced antibodies were able to cross-neutralize the GT5a reporter virus which was at least partially neutralized by the majority of patient derived antibodies.

It has been reported that HCV induces antibodies that bind to E2 and that interfere with the activity of neutralizing antibodies, thereby masking vaccine-induced antibody neutralization [27, 28]. To explore whether our vaccine candidates induced such interfering antibodies, we used pooled polyclonal mouse IgGs from each vaccinated group and mixed them with polyclonal IgGs from a patient that displays strong neutralizing activity. The neutralization efficacy was not decreased by addition of IgG vaccinated mice, suggesting that the vaccines did not induce antibodies interfering with neutralization (Fig. 6B).

The role of glycans and the HVR1 for immunogenicity was also assessed using protein from a different genotype (GT1a H77), as well as in the context of E1E2 heterodimers purified from CHO cell extracts. A detailed description of the purification procedure is provided in the materials and methods section and the quality control of the purification is given in supplementary figure S5A. The binding of E1E2 (WT), E1E2

(N417Q) and E1E2 (N532Q) antigens to CD81-LEL was examined by ELISA and revealed enhanced binding for E1E2 (N532Q) compared to parental E1E2 and the N417Q mutant (Fig. 7A and supplementary figure S5B). These GT1a antigens were used to immunize mice resulting in the induction of E2 specific antibody responses comparable between all groups (Fig. 7B). As shown in figure 7C all three vaccine candidates induced antibodies competing with binding of the known monoclonal antibodies AR3B, 1:7, HC33.4 and AP33 as assessed by a competition assay. Finally, we used GT1a H77 HCVpp assays to examine neutralization efficacy of vaccine induced antibodies. Each vaccine candidate induced HCVpp neutralizing antibodies whereby no significant differences between the vaccine candidates were observed (Fig. 7D).

Discussion

Here we provide a high resolution map of viral determinants that govern exposure of the CD81 binding site and of conserved neutralization epitopes. We show that these changes are well tolerated indicating that they do not abrogate the functioning of E1/E2. The most global change in antibody binding/neutralization and CD81 binding/neutralization was accomplished by deletion of HVR1 combined with inactivation of glycosylation site 534. Thus, we chose this combined modification for vaccination approaches involving recombinant proteins. To rule out strain-specific differences between immunogens, we created these variants in the background of J6 (GT2a) E2 and in the context of the H77 (GT1a) strain. We prepared H77 E1E2 heterodimers from CHO cell extracts and recombinant E2 protein secreted from 293T cells to examine the importance of producer cells and the relevance of E1. To globally assess the relevance of glycosylation in our vaccine candidates we also examined the immunogenicity of 293T cell-derived proteins that had been enzymatically deglycosylated prior to vaccination. Finally, we included a vaccination protocol involving variable immunogens and in each case at least one immunogen from an alternative viral GT.

All vaccination approaches induced robust cross-binding antibody responses as determined by ELISA assays involving recombinant proteins or cell extracts expressing E1-E2 heterodimers from all major HCV GTs (Fig. 5). Vaccination with E1-E2 heterodimers induced antibodies that competed with the binding of previously described bNAbs AR3B, 1:7, HC33.4 and AP33 and we detected virus neutralization in HCVpp assays (Fig. 7). However, inactivation of N417 or N532 glycosylation sites in the context of H77 E1E2 heterodimers did not grossly affect these responses although the mutation N532Q mediated increased binding of the recombinant E1E2 complex to CD81. Among vaccinations with 293T-derived E2 proteins immunization with J6-E2ΔHVR1 and the two immunization series A and B mounted superior cross-binding antibodies compared with J6-E2 and the other examined approaches. However, this was only detected when recombinant E2 proteins from 293T cells were used as ELISA antigens and not when E1-E2 heterodimers partially purified from HCV replicating cells were employed. Thus, some of our E2-protein based vaccination approaches successfully improved production of cross-binding antibodies to the recombinant immunogens but not to the E1-E2 heterodimer expressed in infected cells. Thus, apparently there are structural differences between these proteins and E1-E2 heterodimers. These differences could impact on vaccine efficacy and they should be considered when standardizing tests between laboratories and when estimating cross-binding between different studies. It is also possible that such structural differences were responsible that vaccination with 293T-derived E2 proteins triggered only modest autologous neutralizing antibodies and no cross-neutralization. It has been shown that purified, UV-inactivated HCVcc particles induce cross-neutralizing antibodies in mice and non-human primates [29,

30]. Thus, it will be interesting to explore if vaccination with the HCVcc variants characterized here induces a focused and enhanced neutralizing antibody response.

It is surprising that vaccination with E2 from 293T cells induced very robust, cross-binding antibodies that, however, did not cross-neutralize. One possible explanation was that these immunogens induced antibodies that interfere with cross-neutralization as has been described previously [27, 28]. However, the vaccine induced antibodies did not interfere with cross-neutralization of HCV patient derived polyclonal antibodies (Fig. 6). Thus, we believe that the 293T-derived E2 vaccines examined here efficiently induced antibodies that target conserved binding epitopes, but that targeting these epitopes is not cross-neutralizing. It is possible that this reflects in part a species-specific limitation of immunogenicity of recombinant E2 proteins in mice as recent results by other groups and us suggest that anti-HCV neutralizing antibody responses are more readily induced in guinea pigs [31, 32]. Although neutralizing antibodies were elicited, those were primarily strain-specific, thus explaining the neutralization of autologous Jc1 particles. Notably, the conserved CD81 binding site seems to be structurally highly flexible, and it was proposed that this flexibility may favor induction of non-neutralizing antibodies [33]. In line with this, it is possible that the recombinant E2 proteins used here preferentially adopt a conformation that induces binding, but non-neutralizing antibodies and that the conformation inducing neutralizing antibodies may be under represented in these recombinant proteins. Thus, epitope exposure alone may be insufficient and should be complemented by vaccine design aiming at rigidifying this region.

The lack of robust immunocompetent animal models permissive for HCV makes it hard to assess if the quantity and quality of vaccine-induced immunity is sufficient to confer protection. Thus, it is difficult to predict to which extent antibody responses induced by current vaccination approaches, including the ones presented here, contribute to protection. *In vivo*, antibody functions independent of direct virus particle neutralization could facilitate protection. For instance antibody dependent cytotoxicity (ADCC) may contribute to clearance of HCV infected cells. In this regard, binding, but non-neutralizing antibodies may contribute to HCV protection, as has been recently reported for an HIV infection *in vivo* [34]. Therefore, development of vaccination approaches inducing strong T cell responses as well as nNAbs and bNAbs and involving all antibody effector functions is likely of key importance for induction of robust protection.

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Materials and Methods

Plasmid constructs

The renilla-luciferase harboring Jc1 [23] and Jc1-ΔHVR1 [10] viral cDNA clones with or without alanine substitutions within N-linked glycosylation-sites of E2 (N417A, N423A, N448A, N534A and N649A) were constructed by standard PCR-based techniques and verified by sequencing.

HCVcc neutralization assay

For inhibition of HCV infection, 200 µl of a Huh7.5 cell suspension (5×10^4 cells per ml) was seeded into each well of a 96-well plate 24 h prior to inoculation. Luciferase reporter viruses were mixed with serial dilutions of indicated serum/antibody concentrations and pre incubated for 1 h. This mixture was used to inoculate cells for 4 h in triplicates per dilution. Thereafter, 170 µl of DMEM was added onto the cells. Viral infection was determined 48 or 72 h after infection by removing the supernatant and lysing of the cells by addition of passive lysis buffer or water and measurement of RLU using a 96-well plate reader (Berthold).

HCV pseudotyped viruses (HCVpp) neutralization assay

HCV pseudotyped viruses (HCVpp) expressing a luciferase reporter were generated as described [35]. For neutralization assays, Huh7.5 cells were plated on poly-lysine coated 96-well plates 1 day prior to infection. HCVpp were diluted 1:10 and premixed with heat inactivated diluted sera (1:100) for 1 h at 37°C followed by addition to Huh 7.5 cells. Six hours post-infection, the antibody-virus inoculum was replaced with fresh culture medium. Cells were processed 48 h post-infection using the Bright-glo luciferase assay system (Promega, Madison, WI, USA). Luminescence was measured using an Enspire plate reader (Perkin-Elmer,Waltham, MA, USA). The neutralization activity was calculated using the following formula: % neutralization = (pre-post)/pre × 100 where pre/post represent the luciferase activity done after incubating with either the pre- or post-vaccination sera.

Molecular modeling

Modelling of the glycosylated core structure (PDB 4MWF) was performed using Glycoprotein Builder (www.glycam.org). Glycans are depicted as wireframes; protein as a surface. The predicted CD81 binding site and mAb HC1/HC11 epitopes (highlighted in green) were annotated using UCSF Chimera (www.cgl.ucsf.edu/chimera) using a space fill model. Other amino acid side chains were coloured by hydrophobicity (red = hydrophobic; blue = hydrophilic as determined by the Kyte-Doolittle scale).

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Figure Legends

Figure 1: Schematic and structure of HCV E2 and characterization of mutant viruses. (A) N-linked glycosylation sites (NGS) in the HCV E2 ectodomain are indicated by inverted open triangles. For positional referencing, a graphic chart of the E2 protein is located directly below, with two hypervariable regions and the intergenic variable region (HVR1, HVR2 and igVR) highlighted, and the stem region of E2 marked in light grey. All numbering is relative to the full-length ORF position in the H77 (GT1a) reference strain (accession number NC_004102) while numbers in brackets indicate the homologous position in the J6 (GT2a) E2 ectodomain. The HVR1 region and glycan sites, which were deleted, are indicated in red. Zoomed in region highlights the viral CD81 binding site with key contact residues displayed in blue [36, 37]. Amino acid important for binding of bNAbs HC1, HC11, and HC84-like antibodies are highlighted with colored inverted triangles (pink, HC1; green HC11, orange HC84-series). ** indicates indels responsible for length differences between H77 (GT1a) and J6 E2 (GT2a). Note that HC84.20 also binds to Y613 and W616 and HC84.22 also binds to W616. **(B)** Structural modelling of the E2 core domain with the CD81, HC1 and HC11 binding sites highlighted in green. Glycosylation was modelled using Glycoprotein Builder and are shown and numbered according to the H77 (GT1a) reference strain. The E2 protein is colored by hydrophobicity of aa side chains (red and blue represent hydrophobic and hydrophilic residues, respectively) and green shows antibody or CD81 contact residues. **(C)** Replication of mutant viruses with alanine substitutions at indicated positions with or without deletion of the HVR1 within the E2 protein. Viral replication was determined by renilla-luciferase activity of cell lysates 48 h after transfection (n=3, duplicates each; SD). **(D)** Specific infectivity of particles. Released virus was normalized to equal amounts of core and was used to infect Huh-7.5 cells. Infection is displayed as RLU per well (n=3, measured in triplicates each; SD). The dotted line depicts the background of the assay as determined by measurements of uninfected cell lysates.

Figure 2: Precipitation of mutant viruses with monoclonal antibodies and with GST-CD81-LEL. Mutant viruses were normalized to equal amounts of core and incubated with either HC-1 **(A)**, HC-11 (**B)** or with GST-C81-LEL **(C)**, respectively. Wild-type mutants are depicted in light blue dots, ΔHVR1 mutants in black. Virus-antibody or virus-CD81-LEL-GST complexes were precipitated by addition of protein G or glutathione coated beads. Viral core protein was determined to measure bound viral particles. Data are expressed as fold over control (antibody RO4 or GST) and compared to WT virus (n=4, ANOVA, Friedman, Dunn's multiple comparison, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***)). Symbols represent individual IPs.

Figure 3: Neutralization of Jc1 and mutant viruses by antibodies or by receptor competition with GST-CD81LEL. Viruses were incubated with serial dilutions of given antibodies **(A)** or GST-CD81-LEL **(B)** and infection efficiency was quantified by luciferase assays. Selected dose-response curves are plotted in panel **(C)**. Radar plots in **(A)** highlight IC_{90} values (µg/ml) for antibody neutralization, and IC_{90} values of GST-CD81-LEL receptor competition in panel **(B)**, respectively. The IC_{90} values were calculated using non-linear regression method on graph pad version 6. Wild-type mutants are depicted in light blue lines, ΔHVR1 mutants in black. Abbreviations: RLU, Relative light units.

Figure 4: Neutralization of viruses by GT1 and GT2 patient sera. Indicated viruses were mixed with serial dilutions of sera of uninfected **(A, #1-3)**, GT1 **(B, #1-3)** or GT2 infected **(C, #1-3)** individuals. Infection efficiency was quantified by inoculation of Huh-7.5 cells and subsequent luciferase assays. It is plotted relative to control infections in the absence of human serum. Means ± SD (n=2(A/B/C #1-2) or 3(A/B/C #3)).

Figure 5: Cross-binding of vaccine-induced antibodies to HCV GT1 to 6 glycoproteins. **(A)** Recombinant E2 proteins (2 µg/ml) from different HCV GTs were immobilized on ELISA plates. **(B)** Alternatively, E1-E2 heterodimers prepared from virus transfected cells were used to determine antibody cross binding. Plates were incubated with serial dilutions of mouse sera from vaccinated and mock treated animals, and bound antibodies were detected with a secondary anti-mouse antibody coupled to horseradish peroxidase (HRP). The background detected upon incubation with sera from mock vaccinated animals was subtracted and the end point binding titers were calculated based on the median effect method as described in the materials and methods section. Raw data are provided in the supplementary figure 2A and B. **(C)** Antibody cross-binding expressed relative to vaccination with the parental J6-E2 protein and measured by ELISAs using recombinant E2 (left panel) or E1-E2 heterodimers extracted from cells with infectious HCV (right panel). Horizontal bars represent the median value of cross binding relative to the wildtype J6-E2 vaccination.

Figure 6: Quantification of vaccine-induced neutralizing antibody responses. (A) Neutralization of autologous Jc1 and of heterologous GT5A reporter virus by vaccine induced mouse antibodies and human antibodies from HCV patients. Viruses were incubated with given doses of antibodies and infection efficiency was determined and expressed relative to infections conducted in the absence of antibodies. Means ± SD of three technical replicates are shown. **(B)** Influence of vaccine-induced mouse IgG on neutralization by human neutralizing antibodies. Purified mouse IgGs (100µg/ml; pooled from all animals in one group) were mixed with human IgG (10µg/ml) from an HCV patient, pre-incubated with GT5a reporter virus for 1h, and then used to inoculate Huh-7.5 cells. Infection efficiency was determined as above and is expressed relative to infections conducted in the absence of any IgGs. Means ± SD (n=3).

Figure 7: Vaccination with E1E2 heterodimers with or without glycosylation site mutation induces comparable neutralizing antibody responses. (A) Effect of N-glycosylation mutations on E1E2 interaction with CD81-LEL. Purified WT or N-glycosylation mutant (N417Q and N532Q) E1E2 proteins (25, 50 and 100 ng) were added to CD81-LEL coated wells or BSA coated wells (single 100 ng E1E2 dose) as a negative control. Bound E1E2 antigens were detected by anti-E2 (H53) mAb. Values are shown as a percentage of WT E1E2 binding at the highest dose tested (100 ng). (*) Designates $p<0.05$ respective to WT (100 ng) by One way ANOVA; Tukey post-hoc test. **(B)** HCV E2 binding antibodies induced by vaccination. WT recombinant E2 (384-661) H77c antigens were coated to ELISA plates in triplicate and probed with post-vaccinated mice sera. Binding of E2-specific antibodies from WT, N417Q, and N532Q (1000, 2000, 4000-fold dilutions) vaccinated animals compared to control **(C)** sera (1000-fold dilution) were detected by anti-mouse HRP conjugated secondary antibody and peroxidase substrate. The OD450-570 nm values (mean and SEM) plotted vs serum dilution. Shown is one representative of three independent experiments. **(C)** Competition of mice antisera with HCV cross-neutralizing monoclonal antibodies (mAb) to E1E2. Microtiter wells containing GNA-purified E1E2 H77c were incubated with diluted terminal antiserum (1:100) from control (C) or E1E2 (WT, N417Q or N532Q antigen) vaccinated mice. After washing, plates were incubated with anti-HCV mAbs. Bound AR3B, 1:7 and HC33.4 were detected with anti-human alkaline phosphatase-conjugated secondary antibody and biotin-AP33 detected using neutravidin-alkaline phosphatase. The percentages of mAb binding were calculated relative to the amount of mAb bound in the absence of antiserum. Shown are mean values for each group ± range from three independent experiments. (*) designates $p<0.05$ respective to the control group by One way ANOVA; Tukey post-hoc test. **(D)** Comparison of the HCVpp (H77) neutralization response from WT and N-glycosylation mutant E1E2 vaccinated mice. Neutralization assay using homologous HCVpp H77 (1a) were performed using pre- and post-vaccinated sera (1:100) and the group means with SEMs plotted from representatives of three independent experiments. Positive control: Anti-CD81 mAb [1 μg/ml]). Abbreviations: OD, optical density.