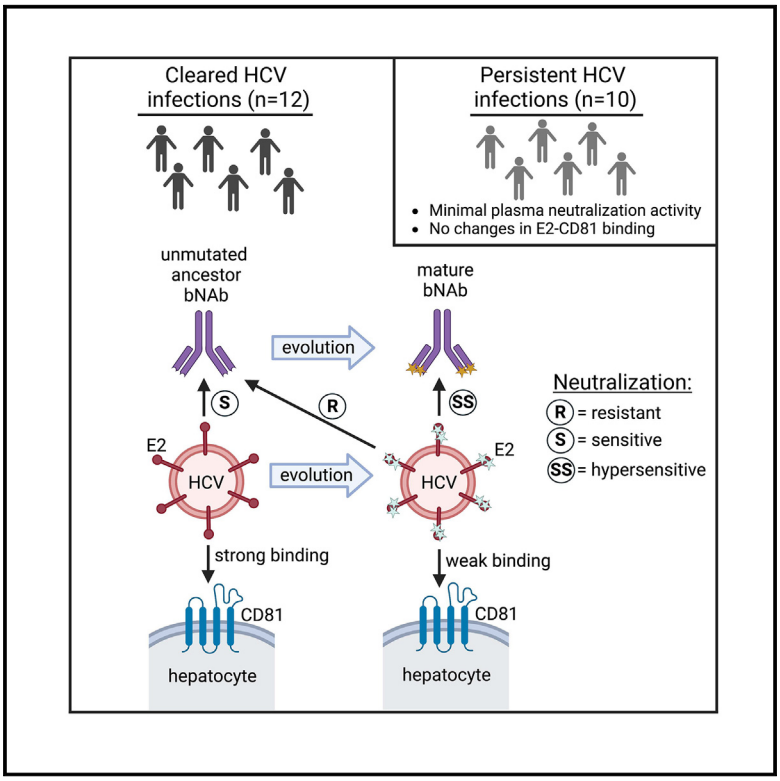
Article

Immunity

Neutralizing antibodies evolve to exploit vulnerable sites in the HCV envelope glycoprotein E2 and mediate spontaneous clearance of infection

Graphical abstract



Authors

Nicole Frumento, Ariadne Sinnis-Bourozikas, Harry T. Paul, ..., George M. Shaw, Andrea L. Cox, Justin R. Bailey

Correspondence

jbailey7@jhmi.edu

In brief

Frumento et al. identify a mechanism of antibody-mediated antiviral protection by which neutralizing antibodies evolve to select and then exploit deleterious escape mutations in the HCV envelope glycoprotein E2. These mutations selected by unmutated ancestor broadly neutralizing antibodies (bNAbs) can confer hypersensitivity to mature bNAbs and loss of viral replication fitness, contributing to repeated immune-mediated clearance of HCV infection.

Highlights

- Public substitutions in HCV E2 arose independently in multiple cleared infections
- E2 substitutions selected by NAbs led to viral fitness loss
- These substitutions conferred hypersensitivity to some broadly neutralizing antibodies
- NAbs targeting these vulnerable E2 sites should be a goal of HCV vaccine development

Frumento et al., 2024, Immunity *57*, 40–51
January 9, 2024
https://doi.org/10.1016/j.immuni.2023.12.004

CellPress

CellPress
OPEN ACCESS

Immunity

Article

Neutralizing antibodies evolve to exploit vulnerable sites in the HCV envelope glycoprotein E2 and mediate spontaneous clearance of infection

Nicole Frumento,[1] Ariadne Sinnis-Bourozikas,[1] Harry T. Paul,[1] Georgia Stavrakis,[1] Muhammad N. Zahid,[2] Shuyi Wang,[3] Stuart C. Ray,[1] Andrew I. Flyak,[4] George M. Shaw,[3] Andrea L. Cox,[1] and Justin R. Bailey[1,5,*]

[1]Division of Infectious Diseases, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA
[2]University of Bahrain, Department of Biology, College of Science, Sakhir Campus, Sakhir, Bahrain
[3]Department of Medicine and Department of Microbiology, University of Pennsylvania, Philadelphia, PA, USA
[4]Department of Microbiology and Immunology, Cornell University, Ithaca, NY, USA
[5]Lead contact
*Correspondence: jbailey7@jhmi.edu
https://doi.org/10.1016/j.immuni.2023.12.004

SUMMARY

Individuals who clear primary hepatitis C virus (HCV) infections clear subsequent reinfections more than 80% of the time, but the mechanisms are poorly defined. Here, we used HCV variants and plasma from individuals with repeated clearance to characterize longitudinal changes in envelope glycoprotein E2 sequences, function, and neutralizing antibody (NAb) resistance. Clearance of infection was associated with early selection of viruses with NAb resistance substitutions that also reduced E2 binding to CD81, the primary HCV receptor. Later, peri-clearance plasma samples regained neutralizing capacity against these variants. We identified a subset of broadly NAbs (bNAbs) for which these loss-of-fitness substitutions conferred resistance to unmutated bNAb ancestors but increased sensitivity to mature bNAbs. These data demonstrate a mechanism by which neutralizing antibodies contribute to repeated immune-mediated HCV clearance, identifying specific bNAbs that exploit fundamental vulnerabilities in E2. The induction of bNAbs with these specificities should be a goal of HCV vaccine development.

INTRODUCTION

Hepatitis C virus (HCV) infection can cause hepatocellular carcinoma (HCC), liver failure, and liver-related mortality.[1,2] Despite the advent of direct-acting antiviral (DAA) therapy, the incidence of new HCV infections continues to rise[3,4]; therefore, a prophylactic HCV vaccine is needed to achieve the World Health Organization's goal of eliminating HCV as a public health problem by 2030.[5] Despite this urgent need, the development of an effective HCV vaccine has been difficult. Two major challenges are the extraordinary genetic diversity of the virus and its rapid evolution in infected people.[6–10]

Spontaneous clearance of primary HCV infection occurs in about 25% of infected individuals.[11,12] We and others have found that clearance of primary HCV infection is associated with the early development of broadly neutralizing antibodies (bNAbs), which are capable of blocking infection by genetically diverse viral variants.[13–21] BNAbs target the viral envelope glycoproteins E1 and E2, and we previously showed in two individuals that bNAbs selected substitutions in E2 that caused a loss of viral fitness prior to spontaneous clearance of their primary infections. Although limited to a small number of study participants, these data suggested that bNAbs can play an important role in clearance of primary HCV infection.[22] However, an important unanswered question is how HCV-Ab coevolution leads to persistent E2 recognition by bNAb lineage antibodies and selection for less fit E2s.

Interestingly, and for reasons that remain unclear, immune responses mediating clearance of primary infection do not always provide sterilizing immunity but lead to enhanced virus clearance rates as high as 80%. These reinfections are associated with a rapid rise in neutralizing antibody (NAb) titers, expansion of B cell and CD8+ T cell responses, shorter duration of infection, and lower peak viremia, demonstrating protective adaptive immunity that can serve as a model for a protective vaccine response.[12,23,24] We and others have isolated bNAbs from a small number of individuals who cleared multiple infections,[19,21,22] but defining mechanisms by which bNAbs lead to viral clearance has been challenging.

In this study, we investigated the humoral immune mechanism of repeated immune-mediated clearance of HCV reinfections in a prospective, longitudinal cohort of persons who inject drugs (PWIDs). We sequenced the viral quasispecies at longitudinal time points in individuals with repeated HCV clearance (n = 8) or persistent infection (n = 8) to characterize the evolution of the envelope glycoprotein E2 genetically, antigenically, and functionally. We assessed the global prevalence of substitutions

Table 1. Participant demographic and HCV infection data

	Reinfection clearance	Reinfection persistence	Clearance 1 infection	Persistence 1 infection
No of subjects	5	2	1	8
Age[a]	23.4 (1.5)	26.5 (3.5)	28	24.1 (3.1)
% female	40	50	100	25
% Caucasian	100	100	100	100
No of infections	9	4	1	8
HCV subtype (%)				
1a	55.5	100.0	100.0	100.0
1b	1.1	0.0	0.0	0.0
2b	1.1	0.0	0.0	0.0
3a	22.2	0.0	0.0	0.0

[a]At seroconversion, mean years (SD).

arising in E2, and we measured the effect of these E2 substitutions on virus sensitivity to neutralization by longitudinal autologous plasma samples, bNAbs, and bNAb unmutated ancestors. We also measured the effect of these substitutions on E2 binding to CD81, the primary HCV receptor, and on the efficiency of virus entry into cells. We used these data to infer a mechanism by which repeated virus infection results in enhanced bNAb-mediated clearance of HCV reinfection.

RESULTS

Selection of study participants

Study participants were PWIDs enrolled in the Baltimore Before and After Acute Study of Hepatitis (BBAASH) cohort who were followed prospectively with regular blood sampling, beginning while they were HCV seronegative, through acute infection, and then during HCV clearance or chronic infection. We selected study participants who spontaneously cleared at least one HCV infection (n = 8) or who had a single, persistent HCV infection (n = 8) (Figure S1; Table 1). Five participants cleared multiple infections with periods of aviremia between each infection (Figure S1A). Two participants cleared their primary infections spontaneously and, after a period of aviremia, were reinfected with a different virus that was not cleared (Figure S1B). One participant cleared one infection spontaneously with no documented reinfection (Figure S1C). As controls, we selected eight participants with incident HCV infection who remained persistently infected with a single HCV strain over many years of follow-up (Figure S1D). All primary infections and reinfections were grouped into cleared (n = 12) or persistent (n = 10) categories. Duration of infection at the time of sampling, participant age at the time of infection, sex, race, and HCV infection genotype were not different between cleared and persistent infections (Figure S1E; Table 1).

Distinct substitutions developed in E2 in cleared or persistent infections

We performed RT-PCR amplification and sequencing of 5′ hemigenomes (Core-NS4A) of plasma viruses obtained at longitudinal time points during each infection. An average of 29 (2–66) clonal sequences were examined from each plasma time point, and the most abundant viral variant at the earliest and latest available viremic time point for each cleared infection was selected for further analysis (Table S1). For persistent infections, we selected the most abundant virus at the earliest available viremic time point and at a median of 6 months after initial infection (Table S1). We identified multiple amino acid substitutions arising over time in each infection (Figure 1A). Notably, in cleared infections ($p < 0.0001$), but not in persistent infections ($p > 0.999$), substitutions were significantly enriched in E2 relative to the remainder of the hemigenome (Figure 1B). We also compared the nonsynonymous and synonymous mutation rates (dN-dS) in the 5′ hemigenomes between the early and late viral populations for each infection (Figure S2). Overall, cleared infections showed positive dN-dS in E2, which is evidence of selective pressure in E2, whereas most persistent infections showed lower dN-dS values, consistent with reduced or absent selective pressure. These data suggest that substitutions developed in E2 in cleared infections due to selective pressure rather than by chance. Therefore, we analyzed E2 amino acid substitutions in greater depth.

We found that amino acid substitutions arising over time in both cleared and persistent infections were present in all domains of E2. We also assessed the global prevalence of these substitutions by analyzing subtype-matched sequences from the HCV-Genes Linked by Underlyng Evolution (GLUE) database, which incorporates HCV sequences from around the world.[25,26] In both cleared and persistent infections, substitutions ranged in prevalence from very common (frequency > 0.9 in subtype-matched sequences) to extremely uncommon (frequency 0.0 to <0.1 in subtype-matched sequences) (Figures 2A and 2B; Tables S2 and S3).

To further characterize these substitutions, we compared the number of E2 amino acid changes and the global prevalence of substitutions arising in cleared or persistent infections. We found that there was a trend toward a higher frequency of substitutions in cleared infections (Figure 2C, $p = 0.05$). Most substitutions in cleared infections arose in hypervariable region 1 (HVR1), the front layer, and VR2. In persistent infections, substitutions also arose most commonly in HVR1, but the remainder of substitutions were more evenly distributed across E2. We also observed a trend toward lower prevalence substitutions arising in cleared infections (Figure 2D, $p = 0.05$). In particular, multiple low prevalence substitutions were observed in HVR1, the front layer, and VR2 in cleared infections that were not observed in persistent infections. Taken together, these data showed that amino acid substitutions developed in E2 in both cleared and persistent infections, but E2 substitutions arising in cleared infections were more frequent and less prevalent in a worldwide database relative to substitutions arising in persistent infections.

E2 substitutions in cleared infections were selected by the early plasma NAb response and led to decreased E2 function

To better understand the role of NAbs in the selection of E2 substitutions, we measured the neutralization of HCV pseudoparticles (HCVpp)-expressing dominant early and late E1E2 variants from each infection by autologous plasma from the same infections (Figure 3A). We tested autologous, early plasma from a visit at a median of 1 month after the first viremic time point and autologous, late plasma from the latest viremic time point

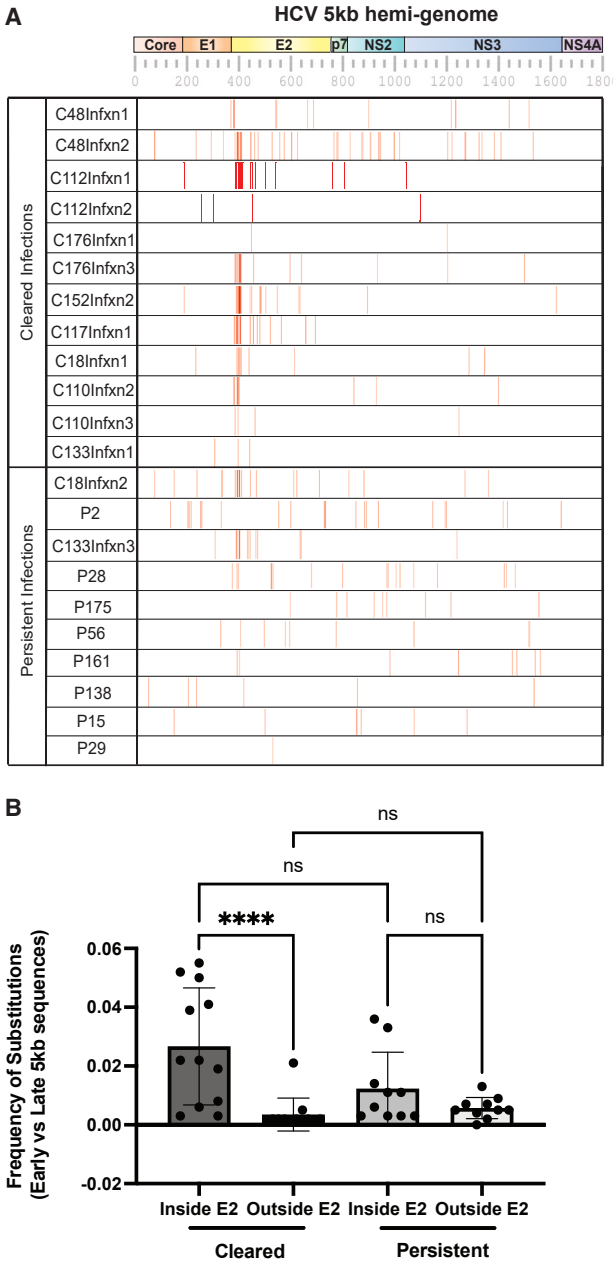


Figure 1. Amino acid substitutions are enriched in E2 in cleared but not in persistent infections

(A) Amino acid sequence evolution of 5 kb hemi-genome sequences in cleared and persistent infections. Mismatches between the dominant early sequence and the dominant late sequence for each infection are shown in red. An average of 29 clonal sequences (2–66) per time point were used to identify the dominant sequences. Amino acid positions (top) were based on the H77 sequence. Plots were generated using the HIV Sequence Database Pixel tool. (B) Frequency of amino acid substitutions within E2 vs. outside of E2 in cleared or persistent HCV infections. Kruskal-Wallis test was conducted. Means with SD are shown. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. See also Figures S1 and S2.

(contemporaneous to the late E1E2). For cleared infections, autologous, early plasma was able to neutralize HCVpp-expressing early E1E2s, whereas HCVpp-expressing late, peri-clearance E1E2s from the same infection were resistant to neutralization by the early plasma ($p = 0.011$). This finding indicates that the substitutions observed in E2 of cleared infections were likely selected by early plasma NAbs. Unexpectedly, late, peri-clearance plasma regained capacity to neutralize HCVpp-expressing late, autologous, contemporaneous E1E2 ($p = 0.021$), indicating that the NAb response "caught up" to the mutated virus immediately prior to clearance of infection. In contrast with cleared infections, we observed minimal neutralization of autologous HCVpp by either early or late plasma from persistent infections, indicating that plasma NAbs were less likely responsible for selection of the substitutions appearing over time in the persistent infections.

To further characterize the phenotypic effects of the amino acid substitutions arising in E2, we measured binding of soluble forms of the early and late E2 proteins (sE2) to CD81 or scavenger receptor-B1 (SR-B1), the two principal HCV cell surface receptors,[27] expressed on the surface of Chinese hamster ovary (CHO) cells. We also measured binding of sE2 to the CD81 long extracellular loop (CD81 LEL) in an ELISA. We validated this method by testing binding to CD81 LEL by a wild type, functional sE2 protein (strain 1a157), or a negative control 1a157 sE2 protein with mutations introduced to the front layer at CD81 binding residues (1a157 FRLYKO)[28] (Figure S3A). As expected, we saw strong binding of wild-type 1a157 sE2 and complete loss of CD81 LEL binding by 1a157 FRLYKO.

We observed a significant decrease in CD81 binding from early to late E2 for cleared infections measured using either CHO-CD81 or CD81 LEL binding ($p = 0.007$ and $p = 0.044$), whereas there is no change in CD81 binding over time for sE2 variants from persistent infections (Figures 3B and S3B). We also tested E2 binding to SR-B1 expressed on CHO cells and observed a nonsignificant trend of decreased binding from early to late E2 from cleared infections (Figure 3B). We have previously shown that differences in E2 binding to CD81 expressed on CHO cells correlate positively with differences in viral fitness measured using chimeric replication competent (HCVcc) viruses with the same E2 sequences.[22] To further validate sE2 binding to CD81 as a relevant measure of viral fitness, we generated chimeric HCVcc using early and late E1E2 sequences from one cleared and one persistent infection. We observed a greater drop in HCVcc infectivity between early and late E1E2 for the cleared infection than for the persistent infection, and we also observed a greater decrease in CD81 binding of the corresponding early and late sE2 from the cleared infection relative to the persistent infection (Figures S3C–S3F). Overall, these studies demonstrated that NAb resistance substitutions arising over time in cleared infections led to reduced E2 binding capacity for CD81.

To determine whether there was also a difference in the magnitude of sE2 binding to CD81 between cleared and persistent infections, we compared early or late sE2 binding with CD81 LEL between cleared and persistent infections (Figure S4). We detected no difference in early sE2 binding between the two groups, indicating that participants who cleared HCV infections were not infected with a defective virus relative to those with persistent infections. Rather, the difference in outcome between groups appeared to be driven by differences in the immune response. Taken together, these data showed that clearance of infection was associated with early selection of NAb

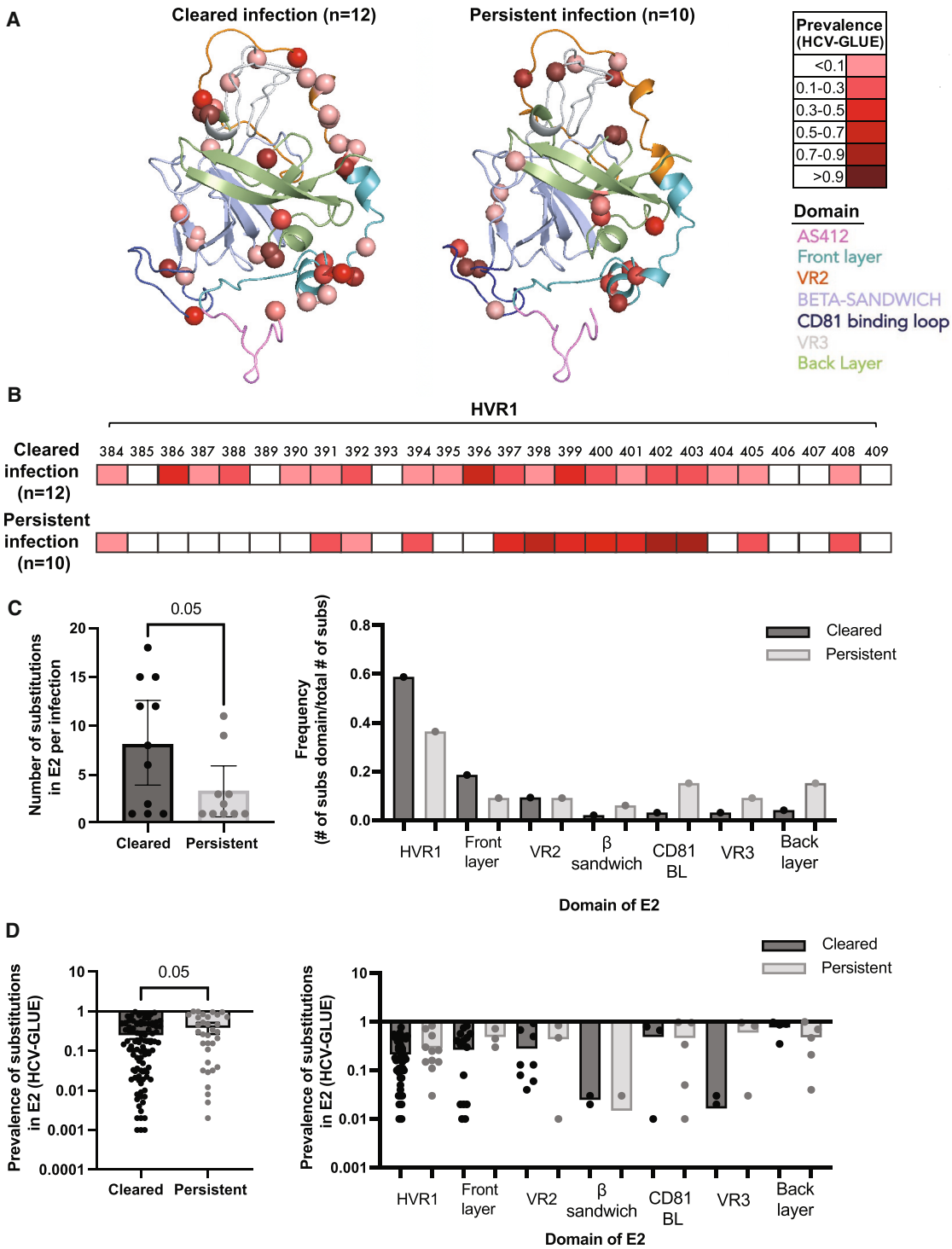


Figure 2. Cleared infections have a greater number of lower prevalence E2 amino acid substitutions relative to persistent infections

(A and B) Substitutions in E2 in cleared or persistent infections, with positions numbered based on the H77 sequence and indicated with (A) spheres superimposed on the HEPC3-E2ecto1b09 (aa 410–647) structure (PDB: 6MEE) or (B) colored blocks in HVR1, which was unresolved in the crystal structure (aa 384–409). Substitutions are colored with a red heatmap to indicate their global prevalence in the HCV-GLUE alignment (n_{gt1a} = 15,060; n_{gt3a} = 3,464; n_{gt2b} = 506). Regions of E2 are indicated with different colors.

(C) Comparison of number of substitutions per infection anywhere in E2 (left) or by E2 domain (right) between cleared (dark gray) and persistent (light gray) infections. Unpaired t test was conducted. Mean with 95% confidence interval (CI) is shown.

(D) Comparison of global prevalence of substitutions per infection anywhere in E2 (left) or by E2 region (right) between cleared (dark gray) and persistent (light gray) infections. Mann-Whitney test was conducted. Mean with 95% CI is shown.

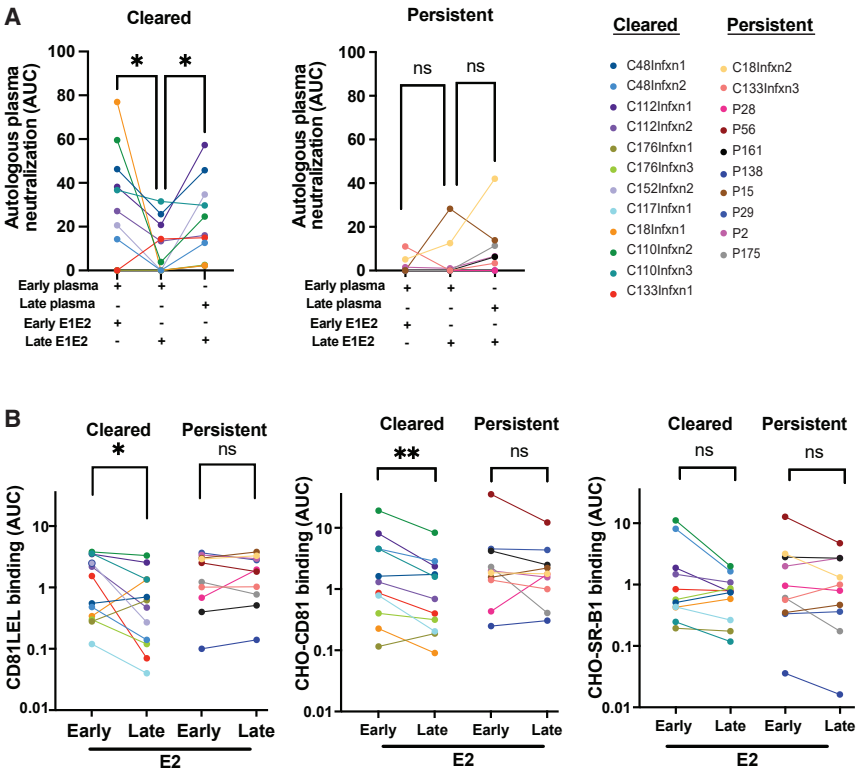


Figure 3. Naturally selected substitutions in cleared infections confer resistance to early autologous plasma antibodies and lead to loss of E2 function

(A) Neutralization of HCVpp expressing dominant early or late E1E2 variants from cleared infections (left) or persistent infections (right) by autologous plasma of the study participants. Friedman test and Šidák's multiple comparisons test were conducted. The area under the curve (AUC) was calculated based on three 2.5-fold serial dilutions of plasma starting at 1:50. Early E1E2 was isolated at the earliest available viremic time point, early plasma at a median of 1 month after the first viremic time point; late E1E2 and late plasma were contemporaneous. Each AUC was derived from one experiment, performed in duplicate.

(B) Binding of sE2 to soluble recombinant human CD81 large extracellular loop (CD81 LEL) was measured by ELISA (left) and binding of sE2 to full-length, cell-surface-expressed human CD81 (middle) or SR-B1 (right) on Chinese hamster ovary (CHO) cells was measured by flow cytometry. The normality of data was tested by Shapiro-Wilk test, with Wilcoxon matched-pairs signed rank test. Each infection is displayed in different colors. Each AUC was derived from one experiment, performed in duplicate. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. See also Figures S3 and S4.

resistance substitutions that reduced E2 binding to CD81, with later, peri-clearance plasma samples regaining neutralizing capacity against these variants.

Public E2 substitutions arising in multiple participants with HCV clearance led to reduced E2 function

To identify specific amino acids targeted by protective NAbs, we investigated whether single substitutions arising in natural variants could recapitulate the phenotype observed for those variants. To do so, we selected a subset of E2 substitutions based on whether they met four selection criteria. First, we chose substitutions that arose exclusively in either cleared or persistent infections. Second, we chose two substitutions representative of each domain of E2 to avoid overrepresenting domains that have a higher frequency of substitutions, such as HVR1 and the front layer (Figure 2B). Third, we chose substitutions that were shared by the greatest number of infections. Fourth, we chose substitutions that were the least prevalent in HCV-GLUE because we predicted they would have a more profound effect on E2 function. By doing so, we maintained the same relative prevalence between clearance and persistence subsets of substitutions chosen for further analysis and all substitutions arising in natural variants (Figure S5). The substitutions selected based on these four selection criteria were introduced individually in subtype-matched genotype 1a or 3a transmitted/founder viruses ("reference E2") via site-directed mutagenesis (SDM) (Figures 4A and 4B).

When we tested binding of the sE2 mutants to CD81, we saw that most E2 substitutions arising in cleared infections reduced CD81 binding relative to the reference E2 (Figures 4C, 4D, and S6, $p = 0.04$). Although some substitutions arising in persistent infections also conferred a reduction in CD81 binding, the majority (60%) had no change or even increased binding to the receptor. We designated substitutions in sE2 leading to decreased or increased CD81 binding as loss of function (LOF) or gain of function (GOF), respectively. To further understand the effect of each substitution on E2 function, we examined each infection individually and found that most of the SDM substitutions were co-expressed in late E2 variants (Figures 4E, S7A, and S7B). Notably, the two GOF substitutions from cleared infections were co-expressed in natural E2 variants with multiple other substitutions that conferred LOF. We also observed a correlation between lower prevalence of substitutions and greater LOF, indicating that less globally prevalent substitutions led to greater loss in fitness (Figure S7C).

LOF and GOF substitutions in sE2 conferred resistance to unmutated ancestor bNAbs and sensitivity to mature bNAbs

Next, we investigated whether neutralization by bNAbs would be affected by these same LOF and GOF substitutions by introducing each substitution into reference E1E2s and generating HCVpp for neutralization assays. We tested four bNAbs (HEPC74, HEPC108, HEPC146, and AR4A) in both their inferred reverted unmutated ancestor (RUA) form and their mature form, with all naturally acquired somatic mutations present. HEPC74 targets the E2 front layer,[29] and AR4A targets the E2 stalk.[30] HEPC146 and HEPC108 have not been structurally characterized; therefore, their epitopes are less well defined, but available data indicate that they also target the E2 front layer (see STAR Methods and Colbert et al.[18]). We hypothesized that these

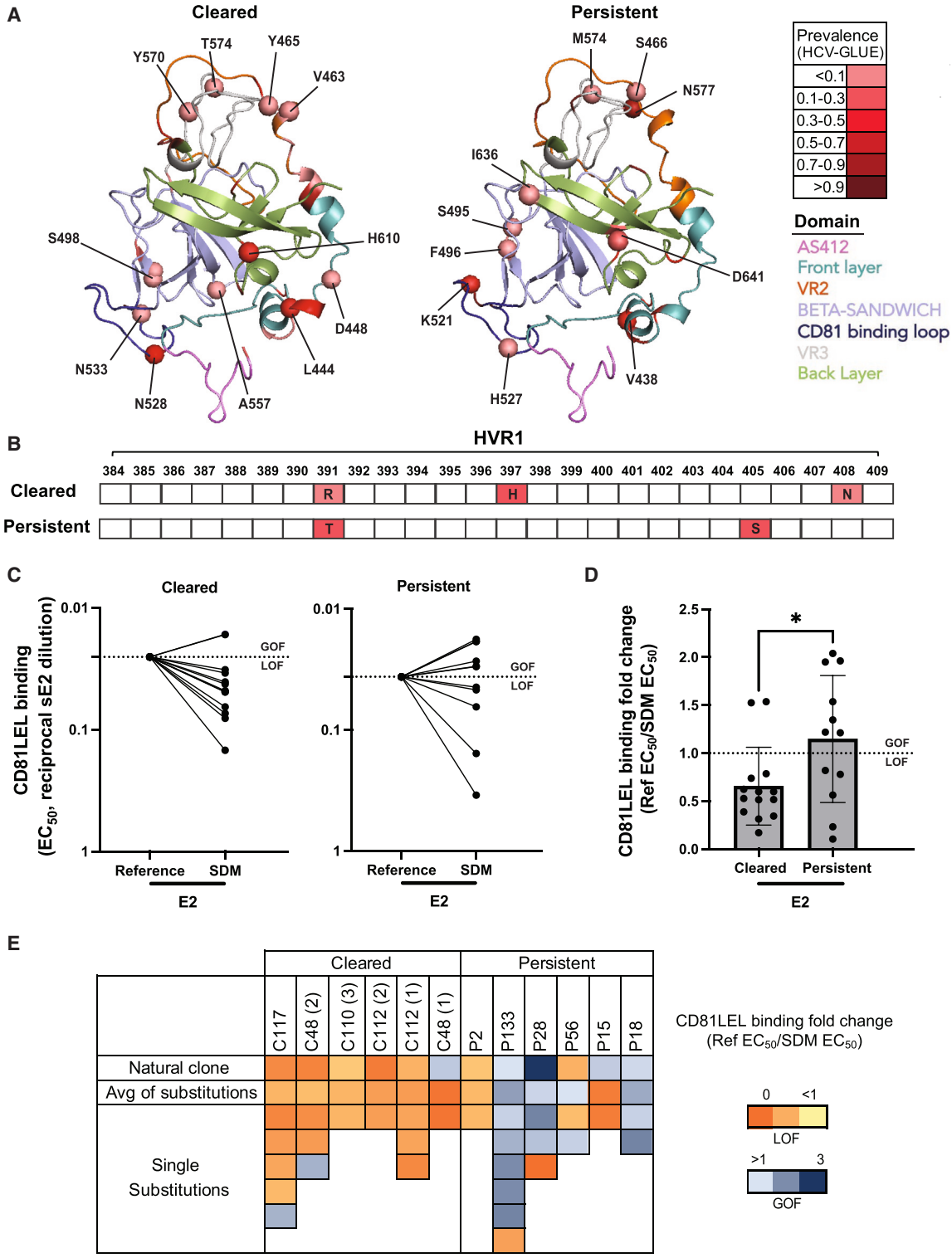


Figure 4. Individual E2 substitutions arising in cleared infections reduces E2 function

(A and B) Substitutions arising in cleared or persistent infections, which were introduced into reference E2s (gt1a strain C110 D0 or gt3a strain UKNP3.1.2) by site-directed mutagenesis (SDM) are indicated by residue number (H77 reference) and the letter corresponding to the introduced amino acid. Amino acid substitutions are indicated with (A) spheres on the HEPC3-E2ecto1b09 (aa 410–647) structure (PDB: 6MEE) or (B) with colored blocks in HVR1 (aa 384–409). Substitutions are colored with a red heatmap to indicate their global prevalence in the HCV-GLUE alignment (n_{gt1a} = 15,060; n_{gt3a} = 3,464; n_{gt2b} = 506). E2 regions are indicated with different colors on the ribbon structure.

(legend continued on next page)

RUA and mature bNAbs would be representative of the NAb repertoire present in the early and late plasma of the study participants because we previously showed that combinations of these four bNAb-types were present in the late plasma of the same participants.[31] Overall, we observed that most LOF and GOF substitutions conferred increased resistance to most unmutated ancestor bNAbs, as well as mature HEPC146, and increased sensitivity to mature HEPC74, HEPC108, and AR4A (Figure 5A). We found that the fold changes in neutralization sensitivity after introduction of LOF substitutions were significantly different between AR4A RUA and mature AR4A ($p = 0.0008$), HEPC74 RUA and mature HEPC74 ($p = 0.0071$), and HEPC108 RUA and mature HEPC108 ($p = 0.0008$). For each mAb pair, LOF substitutions conferred resistance to the RUA form and increased sensitivity to the mature form. Fold changes in sensitivity to HEPC146 RUA and mature HEPC146 did not differ significantly ($p = 0.37$) because most LOF substitutions conferred resistance to both RUA and mature forms. In contrast, GOF substitutions had significantly different effects on RUA and mature bNAbs only for AR4A RUA and mature AR4A ($p < 0.0001$) or HEPC108 RUA and mature HEPC108 pairs ($p = 0.04$), with increased sensitivity to both HEPC74 RUA and mature HEPC74 and increased resistance to both HEPC146 RUA and mature HEPC146 (Figure 5B). When we quantitatively compared the effects of LOF and GOF substitutions, we found that both sets of substitutions were equally resistant to the RUA antibodies (Figure 5C), but GOF substitutions conferred a greater increase in sensitivity than LOF substitutions to mature bNAbs AR4A, HEPC74, and HEPC108 ($p = 0.004$, 0.0009, and 0.017, respectively). Overall, we identified a subset of bNAbs (AR4A, HEPC74, and HEPC108) for which LOF E2 substitutions increased resistance to RUA forms but increased sensitivity to mature forms. GOF substitutions conferred even more dramatic increases in sensitivity to the mature forms of these bNAbs relative to LOF substitutions. Notably, although HEPC146 is also a bNAb, both LOF and GOF substitutions conferred resistance to both the RUA and mature forms of this antibody.

Next, we mapped LOF substitutions that increased resistance to one or more RUA bNAbs and increased sensitivity to the corresponding mature bNAb(s) onto the AR4A Fab-E1E2 structure recently determined by de la Peña et al.[32] We superimposed the AR4A Fab-E1E2 structure along with the HEPC74-E2 and CD81 LEL-E2 structures on their E2 ectodomains[29,33] (Figures 6A and 6B). Of the 14 LOF substitutions, half (S391R, K408N, N448D, S496F, Y527H, M574M, and D577N) conferred resistance to all three RUA bNAbs and increased sensitivity to the corresponding mature bNAb(s). Four substitutions (P498S, S528N, D533N, and S557A) conferred resistance only to AR4A RUA and increased sensitivity to mature AR4A. Two substitutions (T463V and V636I) conferred resistance to both HEPC108 RUA and HEPC74 RUA and increased sensitivity to the corresponding mature bNAbs. One substitution (N570Y) conferred resistance to HEPC108 RUA and increased sensitivity to mature HEPC108. Overall, we observed that these LOF substitutions were distributed across E2, often in domains far removed from the bNAb binding or contact residues, as well as the CD81 binding site (Figure 6C).

DISCUSSION

Mechanisms of immune-mediated clearance of HCV reinfections remain poorly understood. Because reinfections are generally cleared very efficiently, these immune responses can serve as a model for responses that a vaccine should induce. Here, we identified amino acid substitutions in E2 that were shared across multiple cleared infections. We determined that these substitutions were selected by early autologous plasma NAbs, and they reduced E2 binding to CD81. Later, peri-clearance plasma samples regained neutralizing capacity against these variants. We concluded that the combination of reduced viral fitness due to selection pressure from early antibodies and simultaneously the neutralization by late antibodies likely contributed to viral clearance. We then identified a subset of bNAbs (AR4A, HEPC74, and HEPC108) for which these same loss-of-fitness E2 substitutions conferred resistance to unmutated bNAb ancestors but increased sensitivity to mature bNAbs.

The observation that naturally occurring plasma NAb resistance substitutions in E2 conferred resistance to some unmutated ancestor bNAbs but increased sensitivity to the corresponding mature bNAbs was unexpected and potentially important for vaccine development. If a vaccine could induce these bNAb-types, responses would presumably include a mixture of immature and mature bNAb variants. The immature bNAbs might select both LOF and GOF substitutions in E2, but the mature bNAbs would neutralize these LOF E2 variants effectively and GOF E2 variants even more effectively. It is important to note that although HEPC146 is also a bNAb, which is thought to target the front layer of E2 similar to HEPC74 and HEPC108,[18] both LOF and GOF substitutions conferred resistance to both HEPC146 RUA and mature HEPC146. Although the epitope of HEPC146 has not been fully defined, available data indicate that this bNAb primarily binds at the CD81 binding loop of E2.[18] In contrast, HEPC74 targets a wider range of residues distributed across the E2 front layer, which may explain why GOF substitutions in E2 confer resistance to HEPC146 but not to HEPC74. Therefore, if HEPC146-like bNAbs were induced by a vaccine, they might not be protective because they could select escape mutants that also confer E2 GOF. Overall, these findings suggest that bNAb responses to particular E2 epitopes are likely to be protective, whereas bNAb responses to other epitopes are not. Therefore, quantitation of neutralizing breadth of serum or even delineation of the antigenic regions targeted by

(C) Comparison of binding (50% effective concentration [EC_{50}]) to CD81 LEL between the reference sE2 and reference sE2 after introduction of single substitutions from cleared (left) or persistent (right) infections. EC_{50}s were derived from one experiment, performed in duplicate. GOF, gain of function, LOF, loss of function.

(D) Fold change in CD81 LEL binding after SDM introducing individual substitutions was calculated from EC_{50} values of the reference sE2 divided by EC_{50} values of SDM sE2. Normality of data was tested by Shapiro-Wilk test with Kolmogorov-Smirnov test for significance. Means with SD are indicated.

(E) Each column in the heatmap illustrates the fold difference in binding to CD81 LEL by early vs. late natural E2 clones from each infection, and by reference E2 vs. E2 with individual substitutions that arose in that infection. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

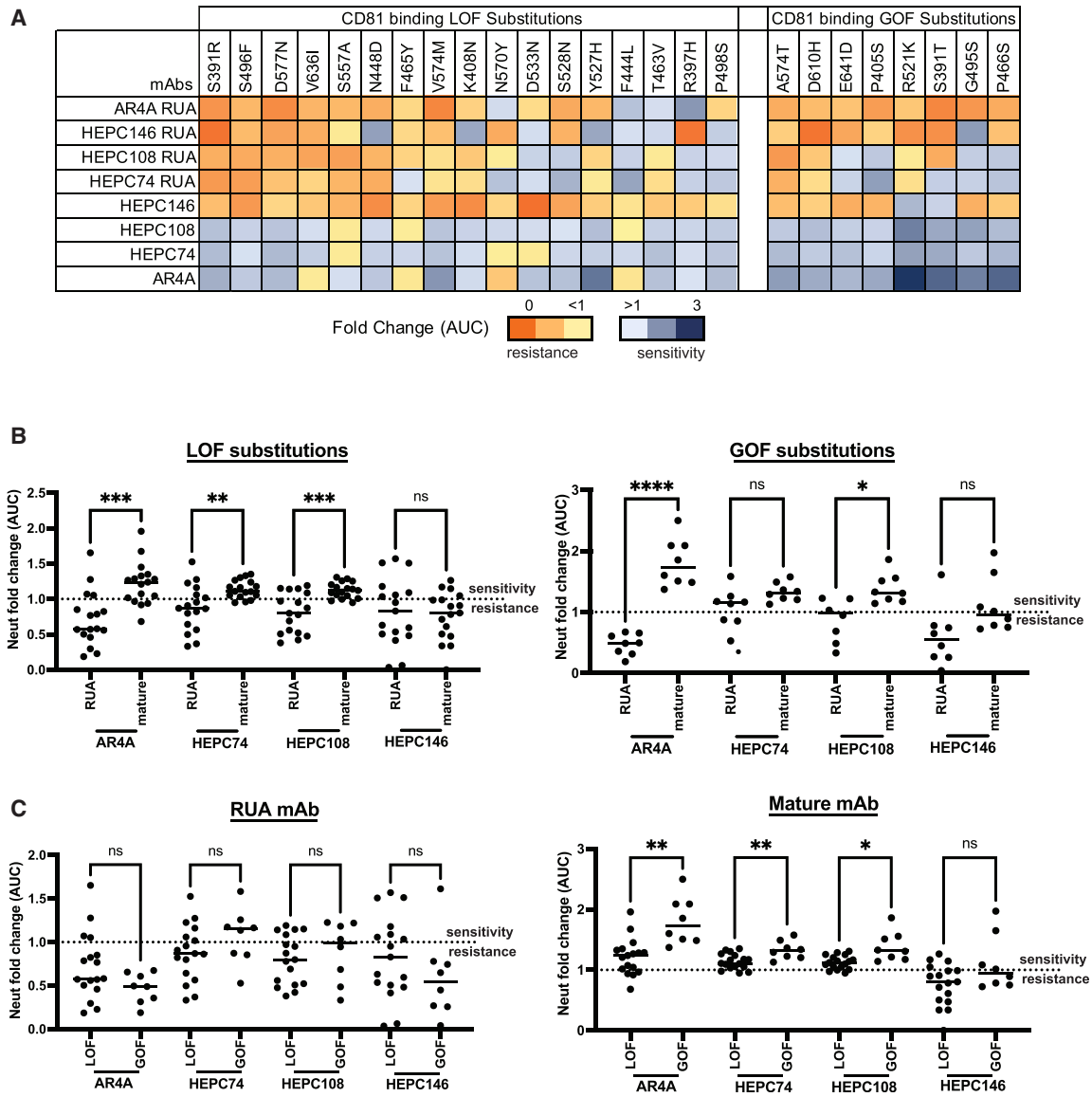


Figure 5. Naturally selected LOF and GOF substitutions confer resistance to unmutated ancestor bNAbs and increase sensitivity to most mature bNAbs

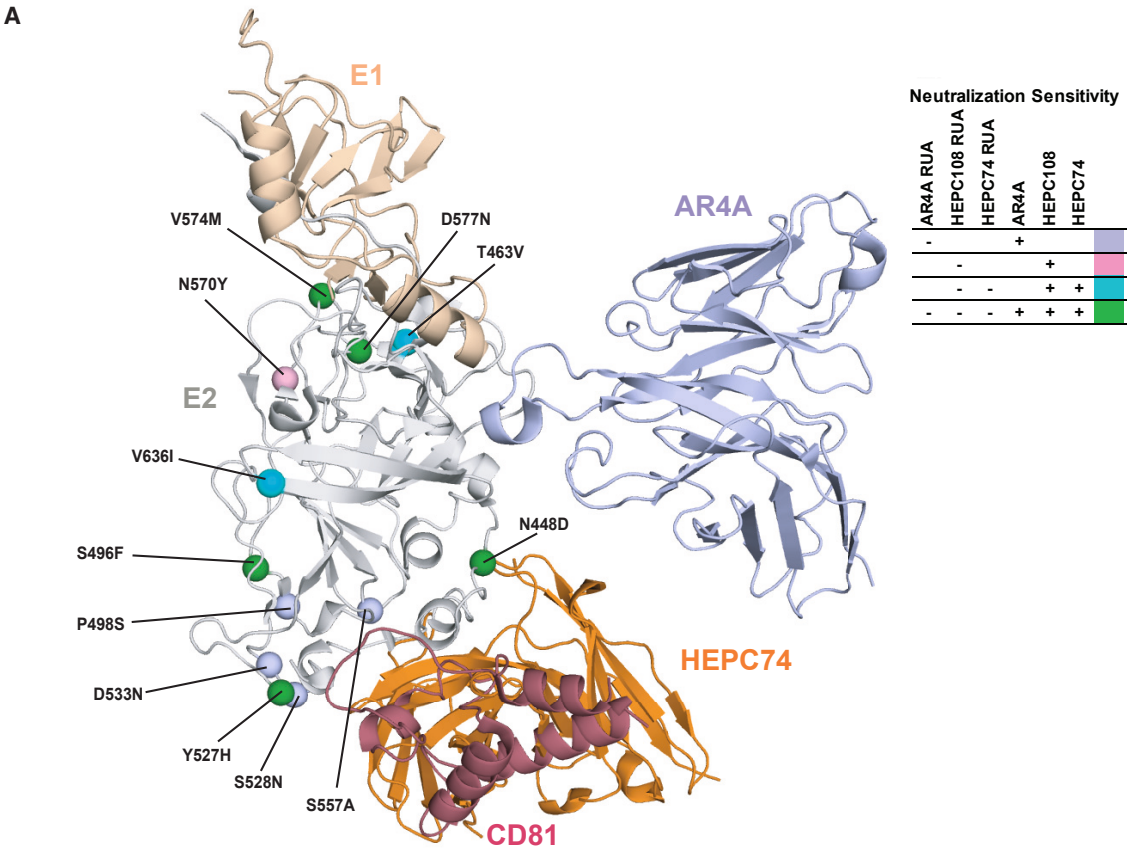
(A) Heatmap illustrating the fold change in neutralization sensitivity of reference HCVpp (strain C110 D0 or strain UKNP3.1.2) after introduction by SDM of individual E2 substitutions, tested using reverted unmutated ancestor (RUA) bNAbs or mature bNAbs. Fold change was calculated as AUC of the reference HCVpp divided by AUC of SDM HCVpp. Substitutions are segregated based on whether they led to LOF or GOF in CD81 LEL binding.

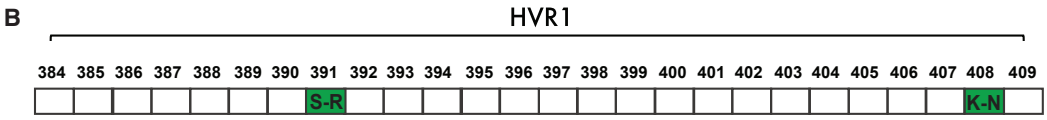
(B) Fold change in neutralization sensitivity of HCVpp by an RUA bNAb vs. the corresponding mature bNAb after introduction of individual LOF (left) or GOF substitutions (right). Normality of data was tested by Shapiro-Wilk test. One-Way ANOVA and Šidák's multiple comparisons tests were conducted. Medians are indicated. LOF, loss of function; GOF, gain of function.

(C) Fold change in neutralization sensitivity of HCVpp by RUA bNAbs (left) or mature bNAbs (right) after introduction of LOF vs. GOF substitutions. AUCs were derived from one experiment performed in duplicate. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. See also Figures S5–S7.

NAbs might not provide the high-resolution information needed to fully assess the potential for protection by candidate vaccine responses. In addition to these standard assays, new approaches, such as high-resolution structural studies of RUAs and matured bNAbs, should be optimized to identify the specific bNAb-types induced by vaccine candidates.

In this study, we identified public LOF E2 substitutions arising primarily in cleared infections, and GOF substitutions arising primarily in persistent infections. Interestingly, we observed two instances where different substitutions that arose in E2 at the same amino acid positions (391 and 574) led to opposite E2 phenotypes: S391R and V574M (arising in cleared infections) conferred a loss of E2 function, whereas S391T and A574T (arising in persistent infections) led to a gain in E2 function. These data highlight the important effect of single substitutions on E2-CD81 binding, confirming that both the position and the specific amino acid change should be considered when assessing the functional impact of substitutions in E2.





C

	Amino Acid Position													
E2 Sequence	391	408	448	463	496	498	527	528	533	557	570	574	577	636
Founder	S	K	N	T	S	P	Y	S	D	S	N	A	D	V
CD81 binding LOF Substitution	R	N	D	V	F	S	H	N	N	A	Y	M	N	I
HEPC74 contact residue			**				*	*	*					
AR4A contact residue														
HEPC108 binding residue							*	*	*					
CD81 contact residue			*											

Figure 6. Loss-of-function substitutions that confer resistance to unmutated ancestor bNAbs and increase sensitivity to corresponding mature bNAbs are distributed across E2

(A and B) LOF substitutions that conferred resistance to one or more reverted unmutated ancestor (RUA) bNAbs and increased sensitivity to the corresponding mature bNAb(s) displayed as spheres on the E1E2-AR4A-AT12009-IGH505 cryoelectron microscopy (cryo-EM) structure (PDB: 7T6X) (A) or as colored blocks on the HVR1 cartoon (B). Substitutions are colored based on the individual bNAb or combination of bNAbs to which they confer RUA bNAb resistance and mature bNAb sensitivity. (–) = decreased neutralization sensitivity, (+) = increased neutralization sensitivity. E1E2-AR4A-AT12009-IGH505 (PDB: 7T6X), HEPC74 Fab-E2 (PDB: 6MEH), and CD81-LEL-E2 (PDB: 7MWX) structures were superimposed on their E2 ectodomains. The AT12009 Fab, IGH505 Fab, 2A12 Fab, AR4A-LC, and HEPC74-LC are not shown for clarity. E1 is tan, E2 is gray, AR4A-VH is purple, HEPC74-VH is orange, and CD81 LEL is green.

(C) Most E2 substitutions depicted in (A) and (B) do not fall at known bNAb-E2 or CD81-E2 contact and/or binding residues. HEPC108 binding residues were determined by alanine scanning mutagenesis. Amino acid residues are indicated that fall at contact or critical binding residues (**) or within three amino acids of a contact or critical binding residue (*). LOF, loss of function. GOF, gain of function.

We found that the loss-of-fitness E2 substitutions that conferred resistance to unmutated bNAb ancestors, but increased sensitivity to mature bNAbs, were distributed across E2 and mostly did not fall at known bNAb-E2 or CD81-E2 binding residues.[18,29,32,33] We observed a loss in CD81 binding when individual substitutions are introduced outside and far away from the CD81 binding site, which is located at the front layer and the CD81-binding loop of E2.[33] One possible explanation for this result is that these substitutions triggered global conformational changes in the E2 that reduced both CD81 binding and binding of unmutated ancestor bNAbs, which target conformational epitopes. Further studies are needed to determine whether the observed increased sensitivity to mature bNAbs was the result of increased mature bNAb-E2 binding affinity, or simply that reduced CD81 affinity made viral entry less efficient and thus more susceptible to mature bNAb neutralization.

Several substitutions that arose over time in E2 in cleared infections in this study were previously identified as bNAb resistance substitutions by other groups (Table S2).[34] Of these, L438I, N434D, F442I, H444Y, N445H, R461L, A466D, F465Y, P498S, D533N, and D610H were found to be resistant to bNAbs targeting the front layer or CD81 binding loop of E2 (aa 424–459 and 519–535).[22,35–38] Another study found that L403F, G401S, and T404S, which we found in cleared infections, conferred resistance to several bNAbs targeting multiple E2 and E1E2 sites, potentially by modulating a shift between closed and open E1E2 conformations.[39] L403F was also previously found to modulate resistance to neutralization by both HC33.4 and AR4A in a separate study.[40] Although not all of these substitutions were individually characterized via SDM in this study, their prior identification as bNAb resistance substitutions supports our conclusion that NAbs selected viruses with these substitutions prior to HCV clearance.

In this study, we investigated the related effects of viral bNAb sensitivity and viral fitness on infection outcomes. We observed that in participants who repeatedly cleared infections, naturally selected substitutions in E2 that conferred resistance to early autologous plasma antibodies also led to the loss of E2 function. Viruses containing the same substitutions were sensitive to later, peri-clearance plasma antibodies and to mature bNAbs. In contrast, in participants who remained persistently infected with HCV, NAb pressure was minimal and substitutions in E2 favored enhanced function. From this, we conclude that NAbs targeting specific sites of vulnerability in E2 significantly contribute to clearance of HCV infections. We also identified a set of bNAbs for which these same loss-of-fitness E2 substitutions conferred resistance to unmutated bNAb ancestors but increased sensitivity to mature bNAbs. Induction of these specific bNAb-types should be a goal of HCV vaccine development.

Limitations of the study

One limitation of this study is the relatively small number of study subjects, necessitated by the extraordinary challenge of identification and frequent, longitudinal sampling of individuals with incident HCV infection followed by reinfections. Our results should be confirmed in additional participants and other cohorts. However, despite this limitation, results were quite consistent across participants. The small sample size makes it particularly remarkable that we identified public NAb resistance, LOF substitutions across multiple participants with cleared infections, suggesting that targeting these specific sites of vulnerability in E2 may be important for HCV control. Although we did not differentiate between primary infections and reinfections in this analysis, given the limited number of reinfection episodes, it is worth noting that most of the reinfection participants were repeatedly infected with antigenically related viruses belonging to antigenic clade 1 (Table S4) and developed progressively greater plasma neutralization breadth and potency with each reinfection.[31] Although clearance of established infection is dictated to a greater extent by neutralization of autologous viruses, these observations regarding plasma neutralization breadth and potency might further explain the development of selective pressure driving viral evolution and clearance of reinfections. In addition to the humoral response, T cell responses are also important for HCV control.[41–43] A recent study by Mazouz et al. demonstrated expansion of both anti-HCV CD8+ T cell and B cell responses after reinfection, along with increased NAb titers, suggesting that both humoral and T cell immunity are important in combination for clearance of reinfection.[24] Therefore, further work needs to be done to assess the potential contribution of T cell responses to viral evolution and clearance of infection in these subjects.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Human participants
  - Cell lines
- METHOD DETAILS
  - Source of bNAbs
  - Epitope mapping of HEPC108
  - HCV Viral Load and Serology Testing
  - Amplification and Cloning of the 5′ Hemigenome
  - E1E2 clone selection
  - HCVpp Production, Infectivity, and Neutralization Assays
  - Site Directed Mutagenesis
  - Expression of Soluble E2
  - Quantitation of Relative sE2 Protein Concentration
  - sE2 Binding to CHO Cells
  - sE2 Binding to CD81 LEL
  - Generation of HCVcc Chimeras
  - HCVcc Infectivity Assays
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.immuni.2023.12.004.

ACKNOWLEDGMENTS

We thank Desiree Wright and Jessica Mimms for technical support. This research was supported by the National Institutes of Health grants R01 AI127469 (to J.R.B. and A.I.F.) and U19 AI159822 (to J.R.B., A.I.F., and A.L.C.). Content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

AUTHOR CONTRIBUTIONS

J.R.B. and A.L.C. conceived the study. M.N.Z., S.W., and G.M.S. performed viral sequencing and sequence analysis. N.F., A.S.-B., and H.T.P. performed binding, neutralization, and viral function experiments. A.I.F. provided antibodies. N.F., A.L.C., G.M.S., G.S., S.C.R., and J.R.B. analyzed the data. N.F. and J.R.B. wrote the original draft, and all authors reviewed and edited the manuscript.

DECLARATION OF INTERESTS

A.I.F. and J.R.B. are inventors of patents submitted pertaining to some of the antibodies presented in this paper.

Received: July 14, 2023
Revised: September 28, 2023
Accepted: December 6, 2023
Published: January 2, 2024

REFERENCES

1. van der Meer, A.J., Veldt, B.J., Feld, J.J., Wedemeyer, H., Dufour, J.F., Lammert, F., Duarte-Rojo, A., Heathcote, E.J., Manns, M.P., Kuske, L., et al. (2012). Association between sustained virological response and all-cause mortality among patients with chronic hepatitis C and advanced hepatic fibrosis. JAMA *308*, 2584–2593.
2. Davis, G.L., Alter, M.J., El-Serag, H., Poynard, T., and Jennings, L.W. (2010). Aging of hepatitis C virus (HCV)-infected persons in the United States: a multiple cohort model of HCV prevalence and disease progression. Gastroenterology *138*, 513–521.
3. Centers for Disease Control and Preventions (2018). Viral hepatitis surveillance report 2018 — Hepatitis C. https://www.cdc.gov/hepatitis/statistics/2018surveillance/index.htm.
4. Suryaprasad, A.G., White, J.Z., Xu, F., Eichler, B.A., Hamilton, J., Patel, A., Hamdounia, S.B., Church, D.R., Barton, K., Fisher, C., et al. (2014). Emerging epidemic of hepatitis C virus infections among young non-urban persons who inject drugs in the United States, 2006–2012. Clin. Infect. Dis. *59*, 1411–1419.
5. WHO (2017). Global Hepatitis Report (World Health Organization).
6. Smith, D.B., Bukh, J., Kuiken, C., Muerhoff, A.S., Rice, C.M., Stapleton, J.T., and Simmonds, P. (2014). Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology *59*, 318–327.
7. Smith, D.B., Becher, P., Bukh, J., Gould, E.A., Meyers, G., Monath, T., Muerhoff, A.S., Pletnev, A., Rico-Hesse, R., Stapleton, J.T., and Simmonds, P. (2016). Proposed update to the taxonomy of the genera Hepacivirus and Pegivirus within the Flaviviridae family. J. Gen. Virol. *97*, 2894–2907.
8. Bukh, J. (2016). The history of hepatitis C virus (HCV): basic research reveals unique features in phylogeny, evolution and the viral life cycle with new perspectives for epidemic control. J. Hepatol. *65*, S2–S21.
9. Zein, N.N., Perez, R.G., and Wiesner, R.H. (2000). Hepatitis C virus infection and lymphoproliferative disorders after liver transplantation. Hepatology *31*, 808–809.
10. Martell, M., Esteban, J.I., Quer, J., Genescà, J., Weiner, A., Esteban, R., Guardia, J., and Gómez, J. (1992). Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. J. Virol. *66*, 3225–3229.
11. Micallef, J.M., Kaldor, J.M., and Dore, G.J. (2006). Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. J. Viral Hepat. *13*, 34–41.
12. Osburn, W.O., Fisher, B.E., Dowd, K.A., Urban, G., Liu, L., Ray, S.C., Thomas, D.L., and Cox, A.L. (2010). Spontaneous control of primary hepatitis C virus infection and immunity against persistent reinfection. Gastroenterology *138*, 315–324.
13. Lanford, R.E., Guerra, B., Chavez, D., Bigger, C., Brasky, K.M., Wang, X.H., Ray, S.C., and Thomas, D.L. (2004). Cross-genotype immunity to hepatitis C virus. J. Virol. *78*, 1575–1581.
14. Osburn, W.O., Snider, A.E., Wells, B.L., Latanich, R., Bailey, J.R., Thomas, D.L., Cox, A.L., and Ray, S.C. (2014). Clearance of hepatitis C infection is associated with the early appearance of broad neutralizing antibody responses. Hepatology *59*, 2140–2151.
15. Pestka, J.M., Zeisel, M.B., Bläser, E., Schürmann, P., Bartosch, B., Cosset, F.L., Patel, A.H., Meisel, H., Baumert, J., Viazov, S., et al. (2007). Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proc. Natl. Acad. Sci. USA *104*, 6025–6030.
16. Logvinoff, C., Major, M.E., Oldach, D., Heyward, S., Talal, A., Balfe, P., Feinstone, S.M., Alter, H., Rice, C.M., and McKeating, J.A. (2004). Neutralizing antibody response during acute and chronic hepatitis C virus infection. Proc. Natl. Acad. Sci. USA *101*, 10149–10154.
17. Raghuraman, S., Park, H., Osburn, W.O., Winkelstein, E., Edlin, B.R., and Rehermann, B. (2012). Spontaneous clearance of chronic hepatitis C virus infection is associated with appearance of neutralizing antibodies and reversal of T-cell exhaustion. J. Infect. Dis. *205*, 763–771.
18. Colbert, M.D., Flyak, A.I., Ogega, C.O., Kinchen, V.J., Massaccesi, G., Hernandez, M., Davidson, E., Doranz, B.J., Cox, A.L., Crowe, J.E., Jr., and Bailey, J.R. (2019). Broadly neutralizing antibodies targeting new sites of vulnerability in hepatitis C virus E1E2. J. Virol. *93*. e02070–e02018.
19. Keck, Z.Y., Pierce, B.G., Lau, P., Lu, J., Wang, Y., Underwood, A., Bull, R.A., Prentoe, J., Velázquez-Moctezuma, R., Walker, M.R., et al. (2019). Broadly neutralizing antibodies from an individual that naturally cleared multiple hepatitis C virus infections uncover molecular determinants for E2 targeting and vaccine design. PLoS Pathog. *15*, e1007772.
20. Walker, M.R., Leung, P., Eltahla, A.A., Underwood, A., Abayasingam, A., Brasher, N.A., Li, H., Wu, B.R., Maher, L., Luciani, F., et al. (2019). Clearance of hepatitis C virus is associated with early and potent but narrowly-directed, Envelope-specific antibodies. Sci. Rep. *9*, 13300.
21. Bailey, J.R., Flyak, A.I., Cohen, V.J., Li, H., Wasilewski, L.N., Snider, A.E., Wang, S., Learn, G.H., Kose, N., Loerinc, L., et al. (2017). Broadly neutralizing antibodies with few somatic mutations and hepatitis C virus clearance. JCI Insight *2*, e92872.
22. Kinchen, V.J., Zahid, M.N., Flyak, A.I., Soliman, M.G., Learn, G.H., Wang, S., Davidson, E., Doranz, B.J., Ray, S.C., Cox, A.L., et al. (2018). Broadly neutralizing antibody mediated clearance of human hepatitis C virus infection. Cell Host Microbe *24*, 717–730.e5.
23. Bailey, J.R., Barnes, E., and Cox, A.L. (2019). Approaches, progress, and challenges to hepatitis C vaccine development. Gastroenterology *156*, 418–430.
24. Mazouz, S., Salinas, E., Bédard, N., Filali, A., Khedr, O., Swadling, L., Abdel-Hakeem, M.S., Siddique, A., Barnes, E., Bruneau, J., et al. (2022). Differential immune transcriptomic profiles between vaccinated and resolved HCV reinfected subjects. PLoS Pathog. *18*, e1010968.
25. Singer, J.B., Thomson, E.C., McLauchlan, J., Hughes, J., and Gifford, R.J. (2018). GLUE: a flexible software system for virus sequence data. BMC Bioinformatics *19*, 532.
26. Singer, J.B., Thomson, E.C., Hughes, J., Aranday-Cortes, E., McLauchlan, J., da Silva Filipe, A., Tong, L., Manso, C.F., Gifford, R.J., Robertson, D.L., et al. (2019). Interpreting viral deep sequencing data with GLUE. Viruses *11*, 323.

27. Zeisel, M.B., Fofana, I., Fafi-Kremer, S., and Baumert, T.F. (2011). Hepatitis C virus entry into hepatocytes: molecular mechanisms and targets for antiviral therapies. J. Hepatol. *54*, 566–576.

28. Weber, T., Potthoff, J., Bizu, S., Labuhn, M., Dold, L., Schoofs, T., Horning, M., Ercanoglu, M.S., Kreer, C., Gieselmann, L., et al. (2022). Analysis of antibodies from HCV elite neutralizers identifies genetic determinants of broad neutralization. Immunity *55*, 341–354.e7.

29. Flyak, A.I., Ruiz, S., Colbert, M.D., Luong, T., Crowe, J.E., Jr., Bailey, J.R., and Bjorkman, P.J. (2018). HCV broadly neutralizing antibodies use a CDRH3 disulfide motif to recognize an E2 glycoprotein site that can be targeted for vaccine design. Cell Host Microbe *24*, 703–716.e3.

30. de la Peña, A.T., Sliepen, K., Eshun-Wilson, L., Newby, M., Allen, J.D., Koekkoek, S., Zon, I., Chumbe, A., Crispin, M., Schinkel, J., et al. (2021). Structure of the hepatitis C virus E1E2 glycoprotein complex.

31. Frumento, N., Figueroa, A., Wang, T., Zahid, M.N., Wang, S., Massaccesi, G., Stavrakis, G., Crowe, J.E., Jr., Flyak, A.I., Ji, H., et al. (2022). Repeated exposure to heterologous hepatitis C viruses associates with enhanced neutralizing antibody breadth and potency. J. Clin. Invest. *132*, e160058.

32. Torrents de la Peña, A., Sliepen, K., Eshun-Wilson, L., Newby, M.L., Allen, J.D., Zon, I., Koekkoek, S., Chumbe, A., Crispin, M., Schinkel, J., et al. (2022). Structure of the hepatitis C virus E1E2 glycoprotein complex. Science *378*, 263–269.

33. Kumar, A., Hossain, R.A., Yost, S.A., Bu, W., Wang, Y., Dearborn, A.D., Grakoui, A., Cohen, J.I., and Marcotrigiano, J. (2021). Structural insights into hepatitis C virus receptor binding and entry. Nature *598*, 521–525.

34. Frumento, N., Flyak, A.I., and Bailey, J.R. (2021). Mechanisms of HCV resistance to broadly neutralizing antibodies. Curr. Opin. Virol. *50*, 23–29.

35. Keck, Z.Y., Saha, A., Xia, J., Wang, Y., Lau, P., Krey, T., Rey, F.A., and Foung, S.K. (2011). Mapping a region of hepatitis C virus E2 that is responsible for escape from neutralizing antibodies and a core CD81-binding region that does not tolerate neutralization escape mutations. J. Virol. *85*, 10451–10463.

36. Velázquez-Moctezuma, R., Galli, A., Law, M., Bukh, J., and Prentoe, J. (2019). Hepatitis C virus escape studies of human antibody AR3A reveal a high barrier to resistance and novel insights on viral antibody evasion mechanisms. J. Virol. *93*, e01909–e01918.

37. Keck, Z.Y., Olson, O., Gal-Tanamy, M., Xia, J., Patel, A.H., Dreux, M., Cosset, F.L., Lemon, S.M., and Foung, S.K. (2008). A point mutation leading to hepatitis C virus escape from neutralization by a monoclonal antibody to a conserved conformational epitope. J. Virol. *82*, 6067–6072.

38. Bailey, J.R., Wasilewski, L.N., Snider, A.E., El-Diwany, R., Osburn, W.O., Keck, Z., Foung, S.K., and Ray, S.C. (2015). Naturally selected hepatitis C virus polymorphisms confer broad neutralizing antibody resistance. J. Clin. Invest. *125*, 437–447.

39. Augestad, E.H., Castelli, M., Clementi, N., Ströh, L.J., Krey, T., Burioni, R., Mancini, N., Bukh, J., and Prentoe, J. (2020). Global and local envelope protein dynamics of hepatitis C virus determine broad antibody sensitivity. Sci. Adv. *6*, eabb5938.

40. El-Diwany, R., Cohen, V.J., Mankowski, M.C., Wasilewski, L.N., Brady, J.K., Snider, A.E., Osburn, W.O., Murrell, B., Ray, S.C., and Bailey, J.R. (2017). Extra-epitopic hepatitis C virus polymorphisms confer resistance to broadly neutralizing antibodies by modulating binding to scavenger receptor B1. PLoS Pathog. *13*, e1006235.

41. Chang, K.M., Thimme, R., Melpolder, J.J., Oldach, D., Pemberton, J., Moorhead-Loudis, J., McHutchison, J.G., Alter, H.J., and Chisari, F.V. (2001). Differential CD4 and CD8 T-cell responsiveness in hepatitis C virus infection. Hepatology *33*, 267–276.

42. Rehermann, B. (2009). Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. J. Clin. Invest. *119*, 1745–1754.

43. Schulze Zur Wiesch, J., Ciuffreda, D., Lewis-Ximenez, L., Kasprowicz, V., Nolan, B.E., Streeck, H., Aneja, J., Reyor, L.L., Allen, T.M., Lohse, A.W., et al. (2012). Broadly directed virus-specific CD4+ T cell responses are primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. J. Exp. Med. *209*, 61–75.

44. Lindenbach, B.D., Evans, M.J., Syder, A.J., Wölk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., and Rice, C.M. (2005). Complete replication of hepatitis C virus in cell culture. Science *309*, 623–626.

45. Giang, E., Dorner, M., Prentoe, J.C., Dreux, M., Evans, M.J., Bukh, J., Rice, C.M., Ploss, A., Burton, D.R., and Law, M. (2012). Human broadly neutralizing antibodies to the envelope glycoprotein complex of hepatitis C virus. Proc. Natl. Acad. Sci. USA *109*, 6205–6210.

46. Liu, L., Fisher, B.E., Thomas, D.L., Cox, A.L., and Ray, S.C. (2012). Spontaneous clearance of primary acute hepatitis C virus infection correlated with high initial viral RNA level and rapid HVR1 evolution. Hepatology *55*, 1684–1691.

47. Cox, A.L., Netski, D.M., Mosbruger, T., Sherman, S.G., Strathdee, S., Ompad, D., Vlahov, D., Chien, D., Shyamala, V., Ray, S.C., and Thomas, D.L. (2005). Prospective evaluation of community-acquired acute-phase hepatitis C virus infection. Clin. Infect. Dis. *40*, 951–958.

48. Kalemera, M.D., Cappella-Pujol, J., Chumbe, A., Underwood, A., Bull, R.A., Schinkel, J., Sliepen, K., and Grove, J. (2021). Optimised cell systems for the investigation of hepatitis C virus E1E2 glycoproteins. J. Gen. Virol. *102*, jgv001512.

49. Urbanowicz, R.A., McClure, C.P., Brown, R.J., Tsoleridis, T., Persson, M.A., Krey, T., Irving, W.L., Ball, J.K., and Tarr, A.W. (2015). A diverse panel of hepatitis C virus glycoproteins for use in vaccine research reveals extremes of monoclonal antibody neutralization resistance. J. Virol. *90*, 3288–3301.

50. Blight, K.J., McKeating, J.A., and Rice, C.M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. *76*, 13001–13014.

51. Sabo, M.C., Luca, V.C., Prentoe, J., Hopcraft, S.E., Blight, K.J., Yi, M., Lemon, S.M., Ball, J.K., Bukh, J., Evans, M.J., et al. (2011). Neutralizing monoclonal antibodies against hepatitis C virus E2 protein bind discontinuous epitopes and inhibit infection at a postattachment step. J. Virol. *85*, 7005–7019.

52. Kong, L., Giang, E., Nieusma, T., Kadam, R.U., Cogburn, K.E., Hua, Y., Dai, X., Stanfield, R.L., Burton, D.R., Ward, A.B., et al. (2013). Hepatitis C virus E2 envelope glycoprotein core structure. Science *342*, 1090–1094.

53. Li, Y.P., Ramirez, S., Gottwein, J.M., Scheel, T.K., Mikkelsen, L., Purcell, R.H., and Bukh, J. (2012). Robust full-length hepatitis C virus genotype 2a and 2b infectious cultures using mutations identified by a systematic approach applicable to patient strains. Proc. Natl. Acad. Sci. USA *109*, E1101–E1110.

54. Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C.M., and McKeating, J.A. (2003). Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. USA *100*, 7271–7276.

55. Dowd, K.A., Netski, D.M., Wang, X.H., Cox, A.L., and Ray, S.C. (2009). Selection pressure from neutralizing antibodies drives sequence evolution during acute infection with hepatitis C virus. Gastroenterology *136*, 2377–2386.

56. Salas, J.H., Urbanowicz, R.A., Guest, J.D., Frumento, N., Figueroa, A., Clark, K.E., Keck, Z., Cowton, V.M., Cole, S.J., Patel, A.H., et al. (2022). An antigenically diverse, representative panel of envelope glycoproteins for hepatitis C virus vaccine development. Gastroenterology *162*, 562–574.

57. Wasilewski, L.N., El-Diwany, R., Munshaw, S., Snider, A.E., Brady, J.K., Osburn, W.O., Ray, S.C., and Bailey, J.R. (2016). A hepatitis C virus envelope polymorphism confers resistance to neutralization by polyclonal sera and nroadly neutralizing monoclonal antibodies. J. Virol. *90*, 3773–3782.

58. Wasilewski, L.N., Ray, S.C., and Bailey, J.R. (2016). Hepatitis C virus resistance to broadly neutralizing antibodies measured using replication-competent virus and pseudoparticles. J. Gen. Virol. *97*, 2883–2893.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
6x-His Tag Monoclonal Antibody (HIS.H8)	Thermo Fisher Scientific	Cat#MA1-21315, RRID: AB_557403
AF647 6x-His Tag Monoclonal Antibody (HIS.H8)	Thermo Fisher Scientific	Cat#MA1-21315-AF647, RRID: AB_2610647
Goat anti-human IgG secondary antibody (HRP)	Vector Laboratories	Cat#PI-3000, RRID: AB_2336151
Nonspecific human IgG	Sigma-Aldrich	Cat# I4506, RRID:AB_1163606
IgG1 Fc Mouse anti-Human antibody	R&D Systems™	Cat#110-HG, RRID:AB_276244
Ms x polyHis-HRP-conjugated antibody	R&D Systems™	Cat#MAB050, RRID:AB_357353
Primary anti-NS5A antibody 9E10	Lindenbach et al.[44]	N/A
Dylight 488–conjugated goat anti-mouse IgG	Abcam	Cat# ab96879, RRID:AB_10687475
HRP-conjugated goat anti-mouse IgG secondary antibody	Abcam	Cat# ab97023, RRID:AB_10679675
HEPC74	Bailey et al.[21]	N/A
HEPC146	Bailey et al.[21]	N/A
HEPC108	Bailey et al.[21]	N/A
AR4A	Giang et al.[45]	N/A
Bacterial and virus strains		
max efficiency DH5a competent cells	Thermo Fisher Scientific	Cat#18258012
HCV viral strains S110	Kinchen et al.[22]	Genbank: MH_834892 -GenBank: MH_835192 *, GenBank: OK_582746 - GenBank: OK_583164
HCV viral strains S152	Frumento et al.[31]	GenBank: OL_332220 - GenBank: OL_332312, GenBank: MZ_556841 - GenBank: MZ_556946
HCV viral strains S18	Frumento et al.[31]	GenBank: OK_553726 - GenBank: OK_554430
HCV viral strains S133	Frumento et al.[31]	GenBank: OK_583165 - GenBank: OK_583829
HCV viral strains S48	Frumento et al.[31]	GenBank:OK_502877 - GenBank: OK_503334
HCV viral strains S112	Frumento et al.[31]	GenBank: OK_503618 - GenBank: OK_504315
HCV viral strains S176	Frumento et al.[31]	GenBank: OK_582292 - GenBank: OK_582745
HCV viral strains S29	Kinchen et al.[22]	GenBank: OK_503335 - GenBank: OK_503617
HCV viral strains S117	Bailey et al.[21]	GenBank: KY_965445-GenBank: KY_965807, GenBank: MH_834650 - GenBank: MH_834671 *, GenBank: OL_332047-GenBank: OL_332219
HCV viral strains S2, S15, S56, S161, S175, S138, S28	Liu et al.[46]	GenBank: DQ_061308 - GenBank: DQ_061310, GenBank: DQ_061312, GenBank: DQ_061323 - GenBank: DQ_061326, GenBank: FJ_828967 - GenBank: FJ_828969, GenBank: HM_000514, GenBank: HM_000520, GenBAnk: HM_000521, GenBank: HM_000529, GenBank: HM_000538, GenBank: HM_000543 - GenBank: HM_000562, GenBank: HM_000939-GenBank: HM_000960, GenBAnk: HM_001118 - GenBank: HM001137, and GenBank: JQ_343222 - GenBank: JQ_343826.
Biological samples		
Human plasma	Cox et al.[47]	N/A
Chemicals, peptides, and recombinant proteins		
Lipofectamine 2000 transfection reagent	Thermo Fisher Scientific	Cat#11668019
3 30 5 50-tetamethylbenzidine (TMB) liquid	Sigma Aldrich	Cat#T4319
Pure Galanthus nivalis lectin (Snowdrop Bulb)	EY Labs	Cat#L74015
CD81 LEL Fc Chimera protein	R&D Systems™	Cat#9144-CD

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Ortho HCV version 3.0 ELISA Test System	Ortho Clinical Diagnostics	Cat#930740
In-Fusion HD Cloning Plus	Clontech	Cat#638909
HCV Real-time Assay	Abbot	Cat#01N30-090
QIAamp viral RNA mini column	Qiagen	Cat# 52904
Luciferase Assay System	Promega	Cat#E1500
QuikChange Lightning Multi Site-Directed Mutagenesis Kit	Agilent	Cat#210513
T7 MEGAscript kit	Ambion	Cat#AM1334
Nucleofector Kit T	Amaxa	Cat#VCA-1002
Experimental models: Cell lines		
Human: Hep3B2.1-7	ATCC	Cat# HB-8064, RRID: CVCL_0326
Human: HEK293T/17	ATCC	Cat# CRL-11268, RRID: CVCL_1926
Human: CD81-knockout HEK293T	Kalemera et al.[48]	N/A
Human: Huh7	Urbanowicz et al.[49]	N/A
Human: Huh7.5.1	Blight et al.[50]	N/A
CHO-CD81	Sabo et al.[51]	N/A
CHO-SR-B1	Sabo et al.[51]	N/A
Recombinant DNA		
pNL4-3.Luc.R-E-	NIH AIDS Reagent	Cat# 3418
pAdvantage	Promega	Cat#E1711
phCMV3-Ig Kappa-HIS	Kong et al.[52]	N/A
Software and algorithms		
Prism v10	GraphPad	RRID: SCR_002798
Pixel	HIV Sequence Database	https://www.hiv.lanl.gov/
The PyMOL Molecular Graphics System v2.0	Schrödinger, LLC	RRID:SCR_000305
FlowJo v10	Tree Star	RRID: SCR_008520)
Other		
Immulon 2HB strips	Thermo Fisher Scientific	Cat# 14-245-81

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Justin Bailey (jbailey7@jhmi.edu).

Materials availability

E1E2 and E2 expression plasmids generated in this study are available from the lead contact upon request.

Data and code availability

- E1E2 nucleotide sequences have been deposited in GenBank and are publicly available from the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report any additional code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human participants

Plasma was obtained from the BBAASH cohort.[47] Details of the cohort are provided in Table 1. All samples were collected prior to availability of direct acting antiviral therapy for HCV, and none of the participants in the study pursued interferon-based HCV

treatment. The protocol was approved by the Institutional Review Board of the Johns Hopkins Hospital, and written informed consent was obtained from all study participants.

Cell lines

HEK293T/17 cells (sex: female) were obtained from ATCC (cat# CRL-11268), used for production of sE2, and maintained in Dulbecco's Modified Eagle Medium and supplemented with sodium pyruvate, 10% heat inactivated fetal bovine serum, and glutamine. CD81-knockout HEK293T cells[48] (Dr Joe Grove, University of Glasgow, Glasgow, United Kingdom) were used for production of HCVpps, and maintained in the same media as HEK293T/17 cells. HEP3B cells (sex: male), obtained from the ATCC (cat # HB-8064), and Huh7 cells[49] were used for neutralization assays. HEP3B cells were maintained in Modified Eagle Medium, supplemented with sodium pyruvate, 10% heat inactivated fetal bovine serum, nonessential amino acids, penicillin-streptomycin, and glutamine. Huh7 cells were maintained in Modified Eagle Medium, supplemented with 10% heat inactivated fetal bovine serum, and nonessential amino acids. Huh7.5.1 cells[50] (sex: male) were obtained from Charles Rice (The Rockefeller University, New York City, New York, USA), maintained in Dulbecco's Modified Eagle Medium supplemented with 10% heat inactivated fetal bovine serum, and nonessential amino acids, and used for HCVcc generation and infectivity. All cells were cultured at 37°C in a humidified incubator with 5% CO_2, and monolayers were disrupted at 80% to 100% confluence with Trypsin-EDTA.

METHOD DETAILS

Source of bNAbs

HEPC74, HEPC146, and HEPC108 were isolated in the James Crowe laboratory.[18,21] AR4A was a kind gift of Dr. Mansun Law (Scripps Research Institute, La Jolla, California).[45] Reverted unmutated ancestor (RUA) variants of HEPC74, HEPC146, HEPC108, and AR4A were inferred with IMGT/V-QUEST using complete sequences of heavy- and light-chain variable domains as previously described.[29]

Epitope mapping of HEPC108

The epitope of HEPC108 was partially mapped in a prior publication.[18] For this study, to improve epitope mapping resolution, ELISA binding of HEPC108 was measured with wild-type variant H77 E1E2 protein or a curated set of 25 H77 E1E2 single alanine mutant variants, selected based on binding epitopes of all published bNAbs (a gift of Dr. Mansun Law at The Scripps Research Institute). E1E2 lysates were generated following lipofectamine transfection of HEK293T cells. The single substitutions were as follows: V246A, R259A, H316A, M323A, M324A, L413A, N417A, G418A, W420A, T425A, N428A, G436A, L441A, F442A, G523A, W529A, G530A, T534A, D535A, P545A, G547A, W549A, R639A, R657A, and D698A. Critical binding residues were defined as positions where alanine mutation reduced HEPC108 binding by at least 50% (L441, F442, G523, G530, D535, N540, P545).

HCV Viral Load and Serology Testing

HCV viral loads (IU/mL) were quantified after RNA extraction from serum with commercial real-time reagents (Abbot HCV Real-time Assay) migrated onto a research-based real-time PCR platform (Roche 480 LightCycler). This assay has a lower limit of detection of 50 IU/mL.

HCV seropositivity was determined using the Ortho HCV version 3.0 ELISA Test System (Ortho Clinical Diagnostics).

Amplification and Cloning of the 5′ Hemigenome

All HCV strains from clearance participants and P29 were amplified by RT-PCR from plasma of HCV-infected individuals after limiting dilution to ensure single-genome amplification, using previously described methods.[53] PCR products were gel extracted and directly Sanger sequenced. E1E2 was PCR amplified from single-genome amplification amplicons of interest and cloned as previously described.[14] For each subject, E1E2 genes were cloned and expressed from transmitted/founder strains and strains isolated longitudinally over the course of infection. Sequences of all E1E2 clones were confirmed after cloning. HCV-RNA from persistence participants (except P29) were extracted, reverse transcribed, and nested-PCR amplified by Liu et al.[46] Nucleotide sequences described in this report were previously described[21,22,31,46] and have GenBank accession numbers: OK553726–OK554430, OK583165 –OK583829, MZ834892–MZ835192, OK582746–OK583164, OL332220–OL332312, MZ556841–MZ556946, OK502877–OK503334, MZ457964–MZ458098, OK503618–OK504315, OK582292–OK582745, MZ834892–MZ835192, DQ061308-DQ061310, DQ061312, DQ061323-DQ061326, FJ828967-FJ828969, HM000514,HM000520, HM000521, HM000529, HM000538,HM000543-HM000562,HM000939-HM000960,HM001118-HM001137, and JQ343222-JQ343826.

E1E2 clone selection

Clones from earliest viremic and last viremic timepoints for each infection were selected. In infections with multiple viremic timepoints, when possible, clones from the same phylogenetic lineage spanning multiple timepoints were selected. When clones from the same lineage could not be selected, a dominant clone was selected from the dominant clade (clade with most clones) from each timepoint. Within a clade, the most abundant clone was selected, or if there was no most abundant clone, the clone closest to the most recent common ancestor for that clade was selected. For infections that were sequenced at a single timepoint, the most abundant clone was selected. A mean of 29 SGA sequences (2-66) per timepoint were used to identify the dominant sequences.

The second infection of subject C133 was excluded was excluded from the analysis because the patient missed visits for 4 months after the virus was detected.

HCVpp Production, Infectivity, and Neutralization Assays

HCVpp were produced by lipofectamine-mediated transfection of HCV E1E2, pNL4-3.Luc.R-E, and pAdVantage (Promega) plasmids into CD81-knockout HEK293T cells as previously described.[16,54] For infectivity testing, HCVpp were incubated on Hep3B target cells for 5 hours before removing media. All HCVpp used in neutralization assays produced RLU values at least 10-fold above background entry by mock pseudoparticles. Only HCVpp preparations producing at least 5E5 RLU were used for neutralization experiments, and HCVpp input was normalized to 1 to 10E6 RLU. Neutralization assays were performed as described previously.[55] To assess neutralization of autologous variants, mAbs were serially diluted five-fold, starting at a concentration of 100 ug/mL. To assess neutralization of autologous variants by plasma, heat-inactivated plasma was serially diluted 2.5-fold, starting at a 1:50 dilution. For each primary infection, we tested early plasma at a median of 1 month after the earliest viremic timepoint (when the early E1E2 was isolated) by preferentially choosing the earliest plasma with some neutralization activity or at a timepoint prior to longitudinal substitutions appearing in the E1E2, when such information was available. For each reinfection, early plasma was contemporaneous to the early E1E2 (from the earliest viremic timepoint). For all infections, late plasma was contemporaneous to the late E1E2 (from the last viremic timepoint prior to clearance or at a median of 6 months for persistent infections). Heat-inactivated pre-immune plasma at 1:50 (mean AUC=4.9) and nonspecific human IgG (Sigma-Aldrich) at 100 μg/mL (mean AUC=35.2) were used as a negative control in every neutralization assay.

Site Directed Mutagenesis

Substitutions were introduced into reference E1E2 and sE2 plasmids C110 D0[31] or UKNP3.1.2[49,56] using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent) and confirmed by Sanger sequencing.

Expression of Soluble E2

The E2 ectodomains were cloned into a mammalian expression vector (phCMV3-Ig Kappa-HIS, a gift of Leopold Kong, The Scripps Research Institute, La Jolla, California, USA) and sequence verified. This truncated, soluble form of E2 ectodomain (sE2) retains antigenicity and function as previously described,[52] encompassing residues 384–645. The vector allows expression of E2 protein with a C-terminal His tag as well as an N-terminal murine Ig Kappa leader signal for efficient protein secretion. Each E2 construct was co-transfected with pAdvantage (Promega) into HEK293T cells and incubated for 72 hours at 37C. Supernatant was collected at 48 and 72 hours, passed through a 0.2 mm filter, and concentrated using a regenerated cellulose centrifugal filter with a 10 kDa cutoff (Amicon).

Quantitation of Relative sE2 Protein Concentration

Serial 5-fold dilutions of each sE2 supernatant were immobilized onto ELISA wells pre-coated with 500 ng Galanthus nivalis lectin (Ey Labs) and blocked with PBS containing 0.5% Tween 20, 1% nonfat dry milk, and 1% goat serum. Wells were probed with 0.5 mg of a mouse monoclonal anti-6x His-tag antibody (Thermo Fisher Scientific) and quantified using an HRP-conjugated goat anti-mouse IgG secondary antibody (Abcam). The EC50 value for each sE2 construct was calculated by nonlinear regression analysis. Fold differences in EC50 values were used to normalize sE2 concentration in subsequent experiments.

sE2 Binding to CHO Cells

CHO-CD81 and CHO-SR-B1 binding experiments were carried out as previously described.[51] CHO cells expressing recombinant human CD81 or SR-B1 (a gift from Dr. Matthew Evans, Icahn School of Medicine, Mount Sinai, New York) were detached using PBS supplemented with 4mM EDTA and 10% FBS and washed in PBS containing 1% BSA. Cells (2 x 10^5) were pelleted in a 96-well u-bottom plate and resuspended in 2-fold serial dilutions of each normalized sE2 construct. Following a 30-min incubation on ice, the cells were washed twice and incubated with 0.5 mg of AF647-conjugated mouse anti-6x His-tag antibody (Thermo Fisher Scientific) for another 20 min on ice. After a final wash, the cells were fixed with 1% paraformaldehyde and analyzed on a LSRII flow cytometer (Becton, Dickinson).

sE2 Binding to CD81 LEL

96-well plates were coated with IgG1 Fc Mouse anti-Human antibody (R&D Systems™) at 1 μg/mL in cold PBS. After overnight incubation at 2-8 °C, plates were washed three times with ELISA wash buffer (0.05% Tween 20 in PBS, pH 7.2 – 7.4) and blocked with blocking buffer (PBS with 1% BSA) for 2 hours at 37 °C. In a separate working plate, 3-fold serial dilutions of sE2 were made in binding buffer (PBS with 0.5% BSA) starting at undiluted. CD81 LEL Fc Chimera protein (R&D Systems™) at 4 μg/mL was added to the working plate. Following a 30 min incubation on a shaker at 500 RPM at room temperature, the CD81 LEL Fc Chimera protein and sE2 mix was transferred to the coated assay plate. Assay plates were incubated at 2-8 °C overnight. After washing 3 times, Ms x polyHis-HRP-conjugated antibody (R&D Systems™) at 0.5 μg/mL in binding buffer was added. After 2 hours at room temperature, the binding was quantified. Purified strain 1a157 sE2 protein was used as a positive control for CD81 LEL binding, and 1a157 FRLYko sE2 with mutations at CD81 binding residues (T425A, L427A, N428A, S432A, G436A, W437A, G530A, and D535A) was used as a negative control.

Generation of HCVcc Chimeras

HCVcc chimeras were generated as previously described.[57,58] Briefly, after digestion of the HCVcc backbone with AfeI (New England Biolabs), E1E2 genes were inserted in frame using In-Fusion cloning (Clontech). Plasmid DNA was linearized using XbaI (New England Biolabs) and then used for in vitro RNA transcription using the T7 MEGAscript kit (Ambion). RNA clean-up was performed using RNeasy mini kit (QIAGEN), quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). 10 μg of RNA was transfected into Huh7.5.1 cells[50] (a gift of Charles Rice, The Rockefeller University, New York City, New York, USA) using Nucleofector Kit T (Amaxa) and plated in a 6-cm plate. Medium was changed at 24 hours and supernatants were collected 48 hours later. To control for transfection efficiency, FFU values were adjusted for relative input copy numbers of HCVcc.

HCVcc Infectivity Assays

8,000 Huh7.5.1 cells[50] (a gift from Charles Rice, The Rockefeller University, New York City, New York, USA) per well were plated in flat-bottom 96-well tissue culture plates and incubated overnight at 37°C. The following day, HCVcc were serially diluted 2-fold and added to Huh7.5.1 cells in triplicate and incubated overnight, after which the HCVcc were removed, and media was replaced and incubated for 24 hours at 37°C. After 24 hours, the medium was removed, and cells were fixed and stained with primary anti-NS5A antibody 9E10[44] (a gift from Charles Rice, The Rockefeller University, New York City, New York, USA) at a 1:2,000 dilution for 1 hour at room temperature, and with secondary antibody Dylight 488-conjugated goat anti-mouse IgG (Abcam) at a 1:500 dilution for 1 hour at room temperature. Images were acquired and spot-forming units (SFU) were counted using an AID iSpot Reader Spectrum operating AID ELISpot Reader version 7.0.

QUANTIFICATION AND STATISTICAL ANALYSIS

Correlations with p values < 0.05 were deemed statistically significant. Neutralization curves for HCVpp and ELISA binding curves were fit by nonlinear regression in Prism v10 (Graphpad). Flow cytometry data were analyzed using FlowJo v10 software (Tree Star). Mean fluorescence intensity (MFI) values were calculated from 10,000 events.