



Induction of broadly neutralizing antibodies using a secreted form of the hepatitis C virus E1E2 heterodimer as a vaccine candidate

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Hepatitis C virus (HCV) is a global disease burden, and a preventive vaccine is needed to control or eradicate the virus. Despite the advent of effective antiviral therapy, this treatment is not accessible to many patients and does not prevent reinfection, making chronic hepatitis C an ongoing global health problem. Thus, development of a prophylactic vaccine will represent a significant step toward global eradication of HCV. HCV exhibits high genetic variability, which leads frequently to immune escape. However, a considerable challenge faced in HCV vaccine development is designing an antigen that elicits broadly neutralizing antibodies. Here, we characterized the immunogenicity of a vaccine based on a soluble, secreted form of the E1E2 envelope heterodimer (sE1E2.LZ). Sera from mice immunized with sE1E2.LZ exhibited an anti-E1E2-specific response comparable to mice immunized with membrane-bound E1E2 (mbE1E2) or a soluble E2 ectodomain (sE2). In competition-inhibition ELISA using antigenic domain-specific neutralizing and nonneutralizing antibodies, sera from sE1E2.LZ-immunized mice showed nearly identical or stronger competition toward neutralizing antibodies when compared with mbE1E2. In contrast, sera from mice immunized with sE2, and to a lesser extent mbE1E2, competed more effectively with nonneutralizing antibodies. An assessment of neutralization activity using both HCV pseudoparticles and cell culture-derived infectious HCV showed that immunization with sE1E2.LZ elicited the broadest neutralization activity of the three antigens, and sE1E2.LZ induced neutralization activity against all genotypes. These results indicate that our native-like soluble glycoprotein design, sE1E2.LZ, induces broadly neutralizing antibodies and serves as a promising vaccine candidate for further development.

hepatitis C virus | E1E2 envelope glycoproteins | secreted | vaccine

Hepatitis C virus (HCV) is a global disease burden, with an estimated 71 million people infected worldwide (1, 2). Roughly 75% of HCV infections become chronic (3–5), and in severe cases can result in cirrhosis or hepatocellular carcinoma (6). Viral infection can be cured at high rates by direct acting antivirals (DAAs), but several issues have blunted their effectiveness in eradicating HCV. In particular, multiple public health and financial barriers (7, 8) restrict access to DAAs in areas with high incidence of infection and DAAs do not prevent reinfection. Moreover, HCV infection is largely asymptomatic and often does not generate sterilizing immunity, thereby contributing to reinfection or continued disease progression (7, 9, 10). Collectively, these issues have resulted in a continued rise in HCV infections.

Acute HCV infections can be cleared by host immunity in ~25% of cases. Among individuals who clear their first infection, the rate of clearance rises to 80% for subsequent infections, indicating an effective immune memory response (11–14). This type of natural protective immunity to HCV requires the induction of broadly neutralizing antibodies to E1E2 ectodomains and T cell responses to the structural and nonstructural proteins (15–17). The above clinical observations suggest that, if a vaccine candidate could induce broadly neutralizing antibody and cell-mediated immune responses equivalent to that seen in spontaneous clearance, such a vaccine would be highly effective at preventing HCV infection. An HCV vaccine therefore remains an essential proactive measure to protect against viral spread, yet vaccine developments against the virus have been unsuccessful to date (17, 18).

A number of challenges exist that have thus far limited progress toward developing a prophylactic vaccine against HCV. One major challenge in developing a successful vaccine for HCV has been the remarkable genetic diversity of the virus which has six major genotypes (genotypes 1 to 6), in addition to two less-common genotypes (19)

Significance

Hepatitis C virus chronically infects approximately 1% of the world's population, making an effective vaccine for hepatitis C virus a major unmet public health need. The membrane-associated E1E2 envelope glycoprotein has been used in clinical studies as a vaccine candidate. However, limited neutralization breadth and difficulty in producing large amounts of homogeneous membrane-associated E1E2 have hampered efforts to develop an E1E2-based vaccine. Our previous work described the design and biochemical validation of a native-like soluble secreted form of E1E2 (sE1E2). Here, we describe the immunogenic characterization of the sE1E2 complex. sE1E2 elicited broadly neutralizing antibodies in immunized mice, with increased neutralization breadth relative to the membrane-associated E1E2, thereby validating this platform as a promising model system for vaccine development.

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The authors declare no competing interest.

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(genotypes 7 and 8), and intragenotypic diversity resulting in 90 total subtypes (20). Moreover, shielding of important neutralizing epitopes with glycans (21, 22), and the presence of immunodominant nonneutralizing epitopes (23–26) deflect the immune response from conserved regions that mediate virus neutralization. Multiple studies in chimpanzees and humans have used E1E2 formulations to induce a humoral immune response, but their success in generating high titers of broadly neutralizing antibody (bnAb) responses has been limited. In particular, immunological assessment in chimpanzees of an E1E2 vaccine produced superior immune responses as compared with E2 administered alone and resulted in sterilizing immunity against homologous virus challenge (27, 28), but with less cross-neutralization capacity against heterologous isolates (29). In addition, an E1E2 formulation tested in humans is well-tolerated (30). However, due to the limited neutralization breadth observed in the human clinical trial (31, 32), using native E1E2 as a vaccine is not likely to provide sufficient protection from HCV infection. Rather, optimization of E1E2 to improve its immunogenicity and capacity to elicit bnAbs through rational design appears to be the preferred path for developing an effective B cell-based vaccine (33).

An additional bottleneck contributing to the difficulty in generating protective B cell immune responses required for an effective HCV vaccine is preparation of a homogeneous E1E2 antigen. HCV envelope glycoproteins E1 and E2 form a heterodimer on the surface of the virion (34–36). Furthermore, E1E2 assembly has been proposed to form a trimer of heterodimers (37) mediated by hydrophobic C-terminal transmembrane domains (TMDs) (36, 38, 39) and interactions between E1 and E2 ectodomains (40–42). These glycoproteins are necessary for viral entry and infection, as E2 attaches to the CD81 and scavenger receptor type B class I (SR-B1) coreceptors as part of a multistep entry process on the surface of hepatocytes (43–46). Neutralizing antibody (nAb) responses to HCV infection target epitopes in E1, E2, or the E1E2 heterodimer (25, 47–52). A significant impediment to the uniform production of an immunogenic E1E2 heterodimer that could be utilized for vaccine development is the association of the antigen with the membrane via the TMDs (36, 53). Progress has been made in the production and purification of the membrane-bound E1E2 complex via immunoaffinity purification (54, 55) or the use of tags that allow protein A (56) or anti-Flag (57) chromatography. While these methods produce high-quality samples, they all involve harsh elution conditions. How such conditions might influence sample quality at a scale required for vaccine trials is unclear. Furthermore, intracellular expression and membrane extraction limits the ability to produce large quantities of sufficient homogeneity required for both basic research and vaccine production.

In contrast, viral glycoproteins of influenza hemagglutinin (58), respiratory syncytial virus (RSV) (59), SARS-CoV-2 (60), and others (61, 62) have been stabilized in soluble form using a C-terminal attached foldon trimerization domain to facilitate assembly. In addition, HIV gp120-gp41 proteins have been designed as soluble SOSIP trimers in part by introducing a furin cleavage site to facilitate native-like assembly when cleaved by the enzyme (63, 64). Recent efforts have made strides toward liberating the E1E2 complex from the membrane in its native form (65, 66). In particular, our previous work (66) showed that a soluble E1E2 (sE1E2) using the Fos/Jun leucine zipper (LZ) coiled-coil as a scaffold (sE1E2.LZ) is antigenically intact, as the protein is recognized by E1E2-specific mAbs AR4A and AR5A (67). Moreover, sE1E2.LZ

elicited nAbs in mice immunized with the antigen, making this scaffold a promising potential platform for engineering of additional HCV vaccine candidates.

Here, we describe the immunogenicity of our native-like secreted E1E2 construct sE1E2.LZ and compare it with the membrane-bound E1E2 complex (mbE1E2) and a secreted form of the E2 ectodomain (sE2). Immunization of mice with sE1E2.LZ produced sera possessing anti-E1E2 antibodies at levels comparable to mice immunized with mbE1E2 or sE2. Moreover, the antibody response in sE1E2.LZ-immunized mice is skewed more toward nAbs relative to non-nAbs than the other two antigens. Remarkably, sera from sE1E2.LZ-immunized mice exhibited broader neutralization activity than either mbE1E2 or sE2 when assessed using both pseudotyped HCV particles (HCVpp) and cell culture-derived HCV (HCVcc), suggesting that this sE1E2 platform represents a favorable starting point for developing scaffolded E1E2 vaccine candidates.

Results

Expression, Purification, and Immunization of Mice. We recently reported the design and preliminary in vivo assessment of a native-like secreted E1E2 heterodimeric glycoprotein assembly, sE1E2.LZ using the ectodomains from genotype 1a (H77C) E1E2 (66). Those preliminary results showed that sE1E2.LZ elicits robust nAbs in vivo against pseudoparticles representing the homologous virus (H77C). To build on those promising results, we undertook a comparative assessment of neutralization breadth and assessed the polyclonal response to key conserved regions on E1E2. To compare and evaluate the antigenicity and immunogenicity of sE1E2.LZ (66) in vivo, we conducted a study in which CD1 mice were immunized with purified mbE1E2, sE1E2.LZ, and sE2 (HCV E2 residues 384 to 661). Using the methods described previously (66), the three constructs were cloned, expressed, and purified, and SDS/PAGE and Western blot analyses performed to confirm the quality and quantity of antigen prior to formulation and injection into mice (Fig. 1). Three groups of mice ($n = 6$ per group) were immunized with mbE1E2, sE1E2.LZ, and sE2, which were formulated into nanoscale size supramolecular assemblies with a polyphosphazene adjuvant (PCPP-R) (68–70). Blood samples were collected prior to each vaccination on days 0 (prebleed), 14, 28, and 42, with a terminal bleed on day 56 (Fig. 2A).

Evaluation of Anti-E1E2 Serological Responses by ELISA. Day 56 serum samples from the three groups of mice were individually tested for anti-E1E2 antibody titers in which the ELISA plates were coated with mbE1E2 (Fig. 2B), sE1E2.LZ (Fig. 2C), or sE2 (Fig. 2D). As shown, sera from mice immunized with sE1E2.LZ were able to induce an anti-E1E2-specific response comparable to mice immunized with mbE1E2 or sE2. Because the E1E2 transmembrane regions were replaced by regions of the human c-Jun/c-Fos LZ, biotinylated peptides from c-Fos and c-Jun were used to evaluate the degree of antibody responses to the c-Jun/c-Fos heterodimer scaffold by ELISA (55). Next, 2 $\mu\text{g/mL}$ of c-Jun/c-Fos peptides were mixed and coated on streptavidin plates. Endpoint titer values indicate that sE1E2.LZ induced a specific anti-Jun/Fos peptide response, and no detectable binding was observed in the mbE1E2 and sE2 immunized groups (Fig. 2E). Dimerization of the mixed c-Jun/c-Fos peptides were confirmed using circular dichroism spectroscopy (*SI Appendix, Fig. S1*).

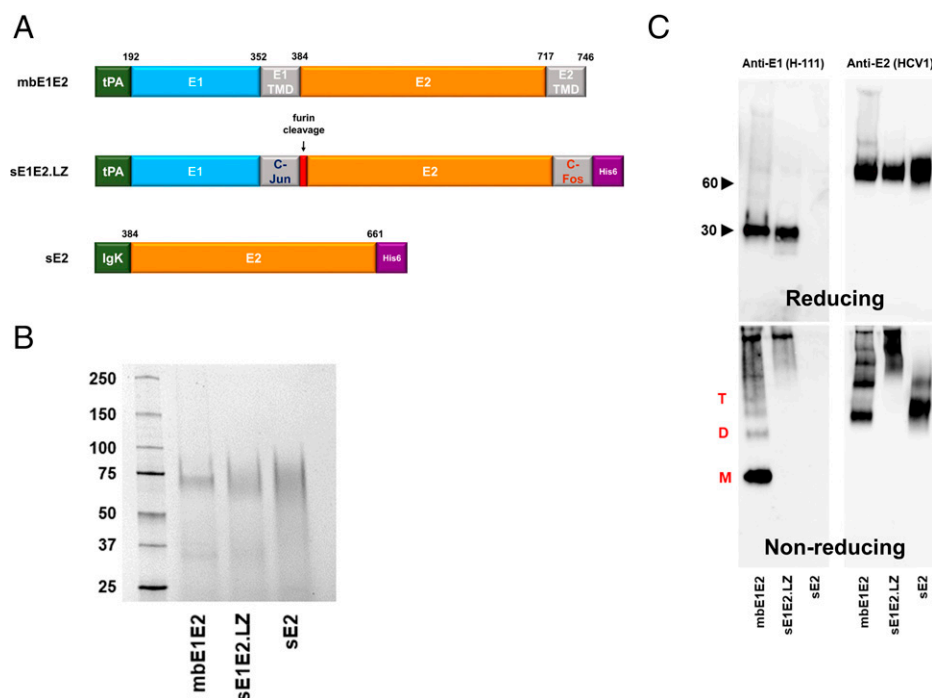


Fig. 1. Construction and characterization of mbE1E2, sE1E2.LZ, and sE2. (A) Schematic diagram of full-length mbE1E2, sE1E2 with c-Fos/c-Jun and furin cleavage sites (red), and sE2. Signal sequences (tPA and IgK) and 6× His tags are shown. (B) SDS/PAGE analysis of purified mbE1E2, sE1E2.LZ, and sE2 under reducing conditions. (C) Western blot detection of the purified proteins under reducing and nonreducing conditions using anti-E2 mAb (HCV1) and anti-E1 mAb (H-111) as probes. D, dimer; M, monomer; T, trimer.

To further evaluate epitope-specific E1E2 antibody responses, peptides representing E2 antigenic domain D, E2 domain E, E2 hypervariable region one (HVR1) and domain E (i.e., combined), the E1 N terminus, and an E1 ectodomain nAb epitope were synthesized and the relative ELISA responses were compared with the LZ peptides across the three antigen groups. These peptides were chosen to provide an approximate baseline reactivity to epitopes that elicit antibodies that exhibit some neutralization potency in either E1 or E2, along with a peptide that contains a known immunodominant decoy epitope (HVR1). Within the sE1E2.LZ group, sera exhibited the strongest relative responses to peptides corresponding to the LZ scaffold, followed by E2 HVR1 and E2 domain D (Fig. 2E). As expected, sera from sE2-immunized mice exhibited no reactivity to the E1 epitope peptides. Across the three groups, sera from sE1E2.LZ-immunized mice exhibited nearly identical responses to peptides corresponding to the E1 ectodomain nAb epitope relative to sera from mbE1E2-immunized mice and roughly equivalent responses to E2 domains D and E compared with sera from mice immunized with mbE1E2 and sE2. Remarkably, sera from sE1E2.LZ immunized mice showed an 11-fold higher response to a peptide corresponding to the E1 N terminus relative to sera from mbE1E2-immunized mice and a 3- to 4-fold lower response to a peptide corresponding to the E2 decoy epitope HVR1 and domain E, relative to sera from mice immunized with mbE1E2 and sE2. Finally, pooled sera from each group were used to examine the kinetics of the anti-HCV E1E2 antibody response by assessing the overall response at each collection point. As shown in Fig. 2F, the antibody responses can be detected beginning on day 14 after the primary immunization among all three groups. Prior to day 42, antibody titers for mice immunized with sE1E2.LZ were lower than those for mice immunized with mbE1E2 and sE2. However, the anti-E1E2 specific antibody titers reached a peak at day 42 and day 56, with similar overall titers for all three groups.

Evaluation of bnAb Responses by Competition Inhibition Analysis.

The relative magnitude of domain-specific serological responses to conserved, continuous and discontinuous epitopes were analyzed by competition inhibition ELISA using a panel of broadly neutralizing human monoclonal antibodies (HMAbs) derived from HCV-infected individuals (67, 71–75). Pooled sera (day 56) from each group were used to compete with a pair of HMAbs from the following antigenic domains of E2: domain B (AR3A/HEPC74), domain D (HC84.26.WH.5DL/HC84.1), and domain E (HCV1/HC33.3); to the E1E2 heterodimer, AR4A and AR5A; to E1-specific antibodies, H-111 and IGH526; and to nonneutralizing E2 antibodies (CBH-4B, CBH-4G) (Fig. 3). (Hereafter, we will refer to HMAb HC84.26.WH.5DL as HC84.26 for brevity.) While sera of all immunized mice were able to compete for the binding to E1E2-specific antibodies, sE1E2.LZ-immunized mice showed nearly identical or stronger inhibitory activities in competing with antibodies corresponding to domain B (AR3A, HEPC74), domain D (HC84.26, HC84.1), domain E (HC33.1, HCV1), E1E2 heterodimer (AR4A and AR5A), and E1 (H-111). In contrast, sera from mice immunized with sE2 and mbE1E2 had higher level of competition with the nonneutralizing antibodies, CBH-4B and CBH-4G.

In order to further analyze the epitope-specific responses, competition ELISA was performed using individual mouse serum (day 56) on a select group of antibodies for statistical comparison (Fig. 4). Based on these results, there was a trend toward a higher level of competition in sE1E2.LZ-immunized mice but no statistically significant difference among the groups, except the anti-E1 antibody, H-111. While cohorts immunized with mbE1E2 and sE1E2.LZ elicited antibodies corresponding to the E1E2 heterodimer antibodies, AR4A and AR5A, the serum from the sE2-immunized group also showed competition with these antibodies. This result is consistent with previous studies from the Law group (67), in which they showed that AR5A competes with the E2 domain C antibody,

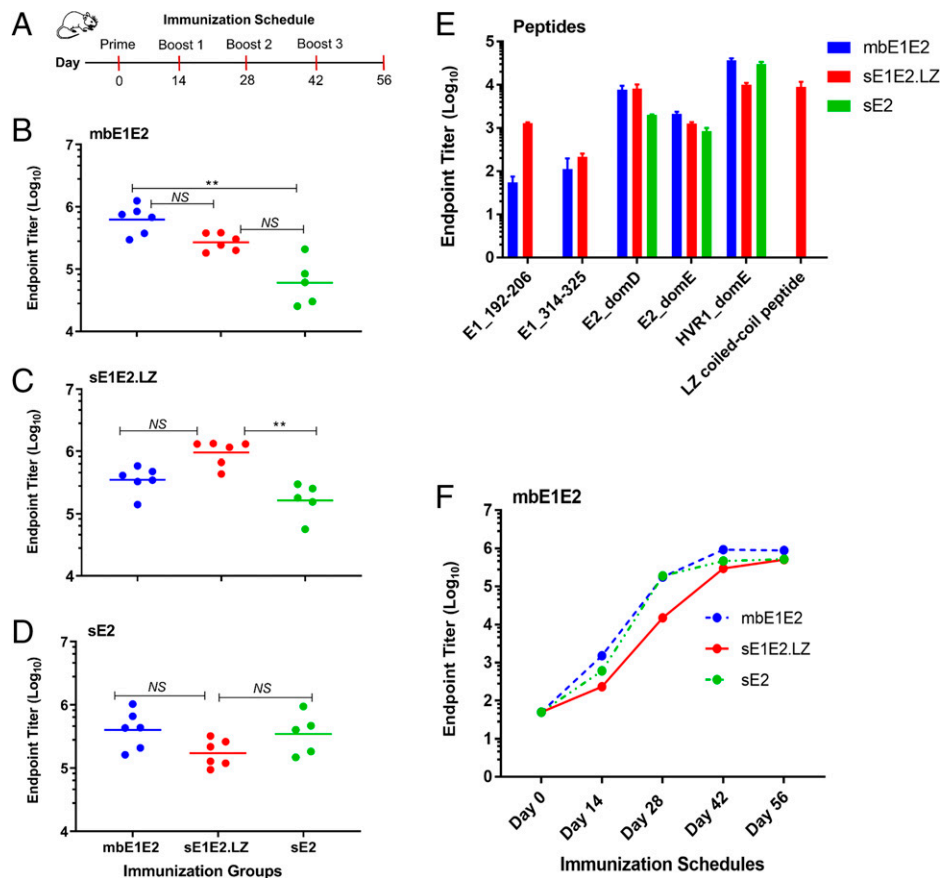


Fig. 2. Immunogenicity assessment of antibody induced in immunized mice at day 56 by ELISA. (A) Mouse immunization schedule. (B) Anti-mbE1E2 titer. (C) Anti-sE1E2.LZ titer. (D) Anti-sE2 titer. (E) Binding to E1 peptides, E2 peptides, and c-Fos/c-Jun. The numbers presented in D represent the average of duplicate experiments. (F) Anti-mbE1E2 titers at different days postimmunization. In B–D, sera from individual mice immunized with the indicated antigen are analyzed. In E and F, pooled sera from the indicated groups are analyzed. Endpoint titers were calculated by curve fitting in GraphPad Prism software with endpoint OD defined as four times the highest absorbance value of day 0 sera. In D, peptide binding endpoint titers were calculated by curve fitting in GraphPad Prism software with endpoint OD defined as seven times the highest absorbance value of day 0. *P* values were calculated using Kruskal–Wallis analysis of variance with Dunn’s multiple comparison test, and significant *P* values are shown (**P* < 0.05). B represents previously published data from our team (66), shown here for comparison.

CBH-7, for E1E2 binding. However, AR4A does not compete with CBH-7 and binds E1E2 utilizing D698 as a key binding residue in the highly conserved E2 membrane proximal external region. Thus, as a control, we performed antibody competition experiments in the presence of increasing amounts of sE2 (1, 5, and 50 μ g/mL) for the antibodies AR3A, HC84.26, AR4A, and AR5A. As shown in *SI Appendix, Fig. S2*, the controls indicate that added sE2 can eliminate the ability of HC84.26 and

AR3A to compete for binding to themselves, but the added antigen did not affect AR4A and AR5A self-competition, as expected. However, the serum competition experiments show a dose-dependent effect of added sE2 on competition potency for all four antibodies. Since added sE2 only depletes E2-specific antibodies, these data indicate that there are E2-specific antibodies present in the polyclonal sera that compete for AR4A and AR5A binding, albeit with less potency than for the

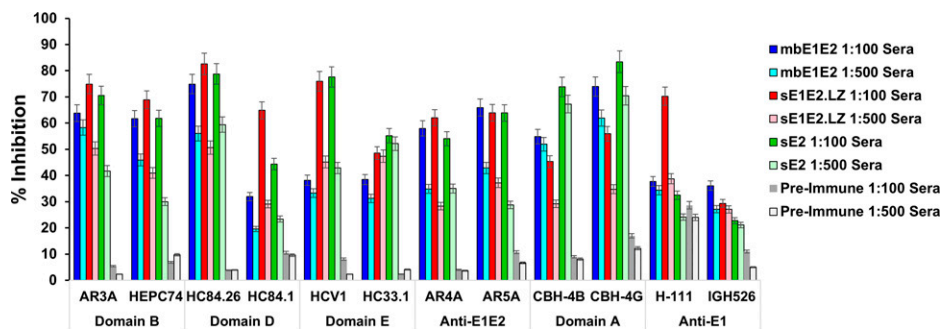


Fig. 3. Competition ELISA using day 56 pooled serum with paired domain specific antibodies of E2 at domain B (AR3A and HEPC74), domain D (HC84.26 and HC84.1), domain E (HCV1 and HC33.1), and anti-E1E2 antibodies (AR4A and AR5A). Serum competition with HCV E1-specific antibodies (H-111 and IGH526) and nonneutralizing antibodies (CBH-4B and CBH-4G) was also analyzed. The value shown is the percentage inhibition relative to the antibody in the absence of serum.

control mAbs AR3A and HC84.26. While unexpected, these data provide a possible rationale for our observations in Figs. 3 and 4 in which sE2 polyclonal sera exhibits competition for AR4A and AR5A binding. It is plausible that polyclonal E2-specific antibodies can compete with AR4A via steric hindrance, shared binding residues near the E1–E2 interface, or via induction of a conformational change that weakens E1E2 binding.

Induction of bnAb Responses. The ability of mbE1E2, sE1E2.LZ, and sE2 immunized mice sera to inhibit HCV infection in vitro was tested against a panel of HCVpp covering the structural proteins of the major HCV genotypes. HCVpp packaged with the E1E2 glycoproteins of seven antigenically distinct HCV genotypes (GT)—GT1a (H77C, AF011751) and GT1b (UKNP1.18.1), GT2a (J6) and GT2b (UKNP2.5.1), GT3a (UKNP3.2.2), GT4a (UKNP4.2.1), GT5a (UKNP5.1.1), GT6a (UKNP6.1.1), and GT7a (QC69)—were produced in HEK293T cells (*SI Appendix*, (10) and used for neutralization assays (see Fig. 6 and Table 1). Preimmune and day 56 serum samples were used at twofold serial dilutions starting at 1:64 (*SI Appendix*, Fig. S3) and inhibition values (ID₅₀) are expressed as the serum dilution level corresponding to 50% neutralization (ID₅₀). As a control, we assessed the neutralization of each HCVpp strain by a panel of nAbs (*SI Appendix*, Fig. S5 and Table S1) to ensure the expected neutralization behavior. Known bnAbs, such as AR3A, HC84.26, and AR4A, potently neutralized each of the strains used in this study (IC₅₀ values ranging from 0.02 to 3.1 μ g/mL).

Serum neutralization activities were first assessed using time-point collected pooled sera at days 0, 14, 28, 42, and 56 (Fig. 5). Similar ID₅₀ titers were identified in the homologous GT1a neutralization among three groups at each time-point. In the heterologous GT2a (J6) neutralization assay, sE1E2.LZ-immunized mice showed measurable ID₅₀ values at the last three time points, whereas neutralization was only detectable at day 42 for

mbE1E2-immunized mice and day 56 for sE2-immunized mice. The sE1E2.LZ immunized group was the only one to exhibit neutralization activity at day 28 (prime and one boost), despite the fact that the anti-E1E2 antibody endpoint titers were similar across the three groups on that day (Fig. 2E), suggesting a qualitative difference in the neutralization capacity of the polyclonal antibody response in mice immunized with sE1E2.LZ. For day 56, the highest levels of neutralization were detected against homologous H77C HCVpp (GT1a) by all three immunized groups. No statistically significant differences were found among these three groups against HCVpp GT1a.

In general, for all of the heterologous genotypes, sera from sE1E2.LZ-immunized mice exhibited equal or higher ID₅₀ values than sera from mice immunized with mbE1E2 or sE2. However, a cross-genotype statistical assessment indicates that the differences between serum neutralization titers is not statistically significant. In the case of GT2b (Fig. 6D), the observed difference in neutralization between sera from sE1E2.LZ-immunized mice and mice immunized with sE2 is statistically significant when solely comparing serum neutralization for that genotype. Moreover, the trend among the HCVpp data also shows that sera from sE1E2.LZ-immunized mice exhibit significant neutralization against all strains tested and a higher ID₅₀ against the two resistant strains tested. In contrast, sera from mice immunized with sE2 failed to significantly neutralize (i.e., ID₅₀ < 100) 3 of the 11 HCVpp strains tested (Table 1 and *SI Appendix*, Table S2). If one views the trend of ID₅₀ values at successively higher cutoff values, the sera from mice immunized with sE1E2.LZ consistently outperform the other sera as judged by the number of strains for which the ID₅₀ is above the threshold (*SI Appendix*, Table S2), indicating a greater breadth of neutralization.

Assessment of Homologous Neutralization and Breadth Using the HCVcc System. To assess the efficacy of the hyperimmune sera from the vaccinated mice to block entry of infectious

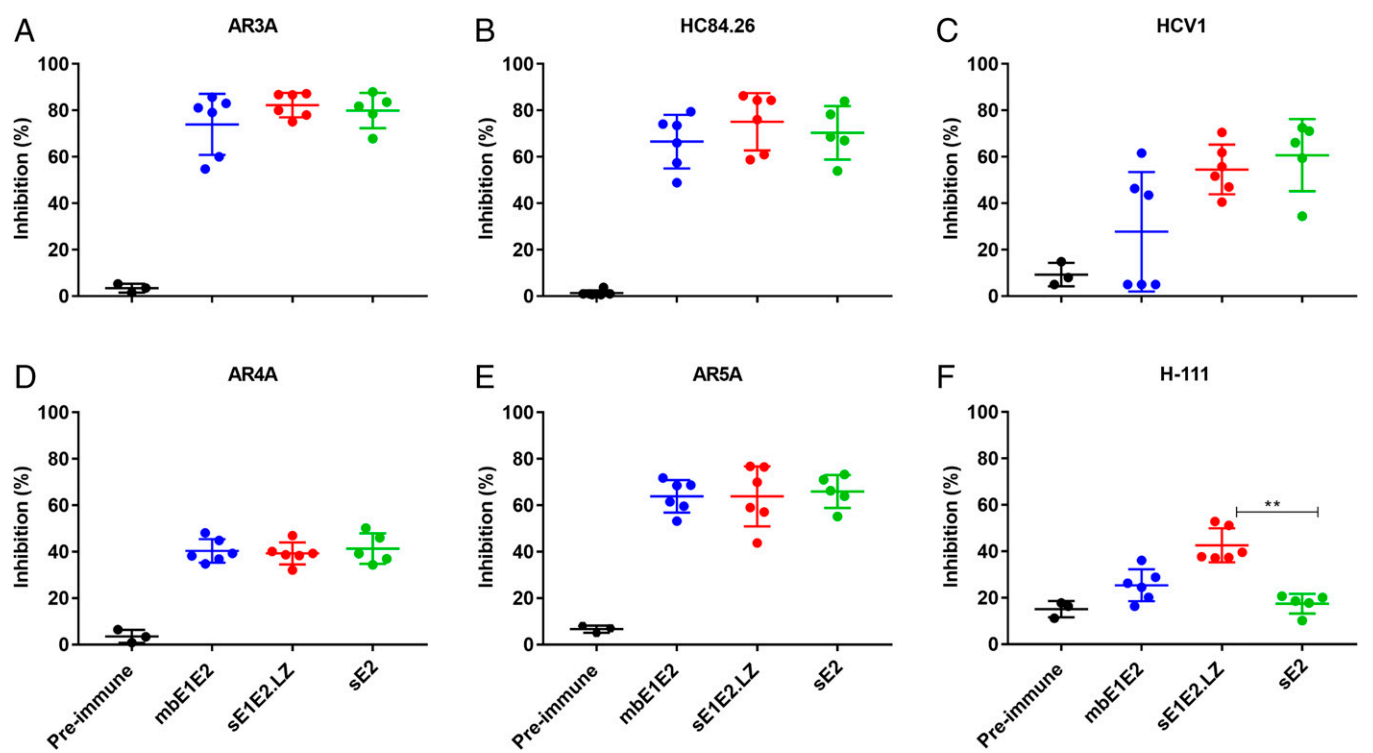


Fig. 4. Competition ELISA of individual mouse Day 56 serum at 1:60 dilutions with anti-E2 antibodies: domain B AR3A (A), domain D HC84.26 (B), domain E HCV1 (C); anti-E1E2 antibodies: AR4A (D), AR5A (E); and anti-E1 antibody H-111 (F). *P* values were calculated using Kruskal-Wallis ANOVA with Dunn's multiple comparison test and significant *P* values are shown (***P* < 0.01).

Table 1. Comparison of ID₅₀ between HCVcc (pooled serum) and HCVpp (mean ID₅₀)

HCV genotypes		ID ₅₀		
Genotypes	Isolates	mbE1E2	sE1E2.LZ	sE2
GT1a	H77C (HCVpp)	9,607	9,032	9,739
	H77C (HCVcc)	141	741	562
GT1b	UKNP1.20.3 (HCVpp)	311	284	122
	J4 (HCVcc)	177	154	426
	Con1/jc1 (HCVcc)	23	199	30
GT2a	J6 (HCVpp)	294	533	468
GT2b	UKNP2.5.1 (HCVpp)	233	489	83
	J8 (HCVcc)	115	363	550
GT3a	UKNP3.2.2 (HCVpp)	163	344	212
	S52 (HCVcc)	0	218	0
GT4a	UKNP4.2.1 (HCVpp)	157	288	153
	ED43 (HCVcc)	95	645	112
GT5a	UKNP5.1.1 (HCVpp)	504	107	715
	SA13 (HCVcc)	1,698	1,000	660
GT6a	UKNP6.1.1 (HCVpp)	140	107	42
	HK (HCVcc)	229	251	776
GT7a	QC69 (HCVpp)	410	544	92
	QC69 (HCVcc)	62	794	19
GT1b (resistant)	UKNP1.18.1 (HCVpp)	429	589	337
GT2b (resistant)	UKNP2.4.1 (HCVpp)	297	433	222

HCV, we performed in vitro neutralization assays using antigenically diverse HCVcc. The development of the genotype 2a JFH1 cell culture system (76), and the more efficient J6/JFH1 system with the Core-NS2 region from another 2a isolate (77), has enabled the study of the entire viral life cycle in vitro. Subsequent generation of intergenotypic chimeras harboring the structural proteins of antigenically diverse HCV genotypes has been very useful to assessing the breadth of neutralizing antibody responses to the virus. We utilized bicistronic versions of H77C(1a)/JFH (T2700C, A4080T), Con1 (1b)/Jc1 (G2833C, T2910C, A4274G, A6558G, A7136C), J4(1b)/JFH (T2996C, A4827T), J8(2b)/JFH, ED43(4a)/JFH1 (A2819G, A3269T), SA13(5a)/JFH1 (C3405G, A3696G), HK(6a)/JFH (T1389C/ A1590C), and QC69(7a)/JFH (T2985C, C8421T) (78–82) expressing Gaussia luciferase (Gluc) (*SI Appendix*). These genomes are replication competent in Huh7.5 cells and produce infectious virions. We have previously used these genomes to determine the in vitro neutralization capacity of bnAbs in mouse sera (83). As a control, we assessed the neutralization of each HCVcc strain by a panel of nAbs (*SI Appendix*, Fig. S6 and Table S1) to ensure the expected neutralization behavior. Our results largely mirror those observed in a previous study (67). In particular, for the strains that are identical in two studies [H77 (1a), S52 (3a), ED43 (4a), SA13 (5a), and HK6a (6a)], the relative sensitivity of neutralization we observe closely matches that observed by Giang et al. (67), with SA13 (5a) and HK (6a) being the most sensitive. Accordingly, the serum ID₅₀ values for those two strains were the highest among the heterologous strains (Table 1).

Pooled sera collected 56 d following immunization with mbE1E2, sE1E2.LZ, or sE2 inhibited infections with the HCV intergenotypic chimeras with varying efficiencies depending both on the antigen and HCV genotype (Fig. 7, Table 1, and *SI Appendix*, Fig. S4). The ID₅₀ values we observe fall within or exceed the range of values observed previously in similar studies (31, 84, 85). Sera derived from mbE1E2-

vaccinated mice neutralized most efficiently the SA13 (GT5a) strain and slightly less efficiently, H77C (1a), J4 (1b), J8 (2b), ED43 (4a), and HK (6a). sE1E2.LZ serum showed high neutralization activity against all strains. In particular, GT7a (QC69) was the most difficult to neutralize using mAbs (*SI Appendix*, Table S1), but was neutralized very effectively by sE1E2.LZ serum, and not at all by the other two groups. sE2 serum showed high neutralization activity against H77C, J4, J8, SA13, and HK strains, and moderate or low neutralization activity against the ED43 strain. Similar to the HCVpp data, sera from sE1E2.LZ-immunized mice neutralized significantly against all HCVcc strains tested (i.e., ID₅₀ > 100). Sera from mice immunized with mbE1E2 or sE2 failed to significantly neutralize four and three of the nine HCVcc strains tested, respectively (Table 1 and *SI Appendix*, Table S2). As with HCVpp, the sera from mice immunized with sE1E2.LZ consistently outperform the other sera at higher ID₅₀ cutoffs (*SI Appendix*, Table S2). Thus, immunization by sE1E2.LZ and sE2 showed broad neutralization activity compared with full-length mbE1E2, and sE1E2.LZ in particular induced neutralization activity against all genotypes. These results indicate that broadly neutralizing antibodies were induced efficiently by the soluble glycoprotein design, sE1E2.LZ. To compare breadth of

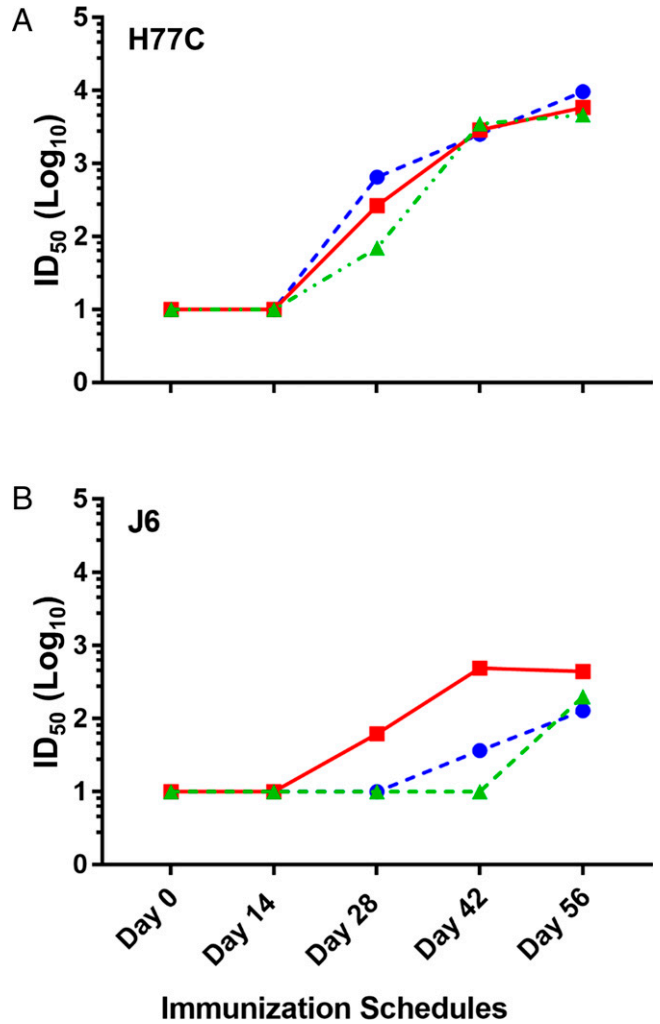


Fig. 5. Kinetics of HCVpp neutralization. (A) Neutralization titers (ID₅₀s) against homologous isolate (H77C, GT1a). (B) The same shown against heterologous isolate (J6, GT2a). Serial dilutions of pooled sera from the indicated days were used, and titers were calculated as serum dilution levels reached at 50% neutralization (ID₅₀) by curve fitting in GraphPad Prism software.

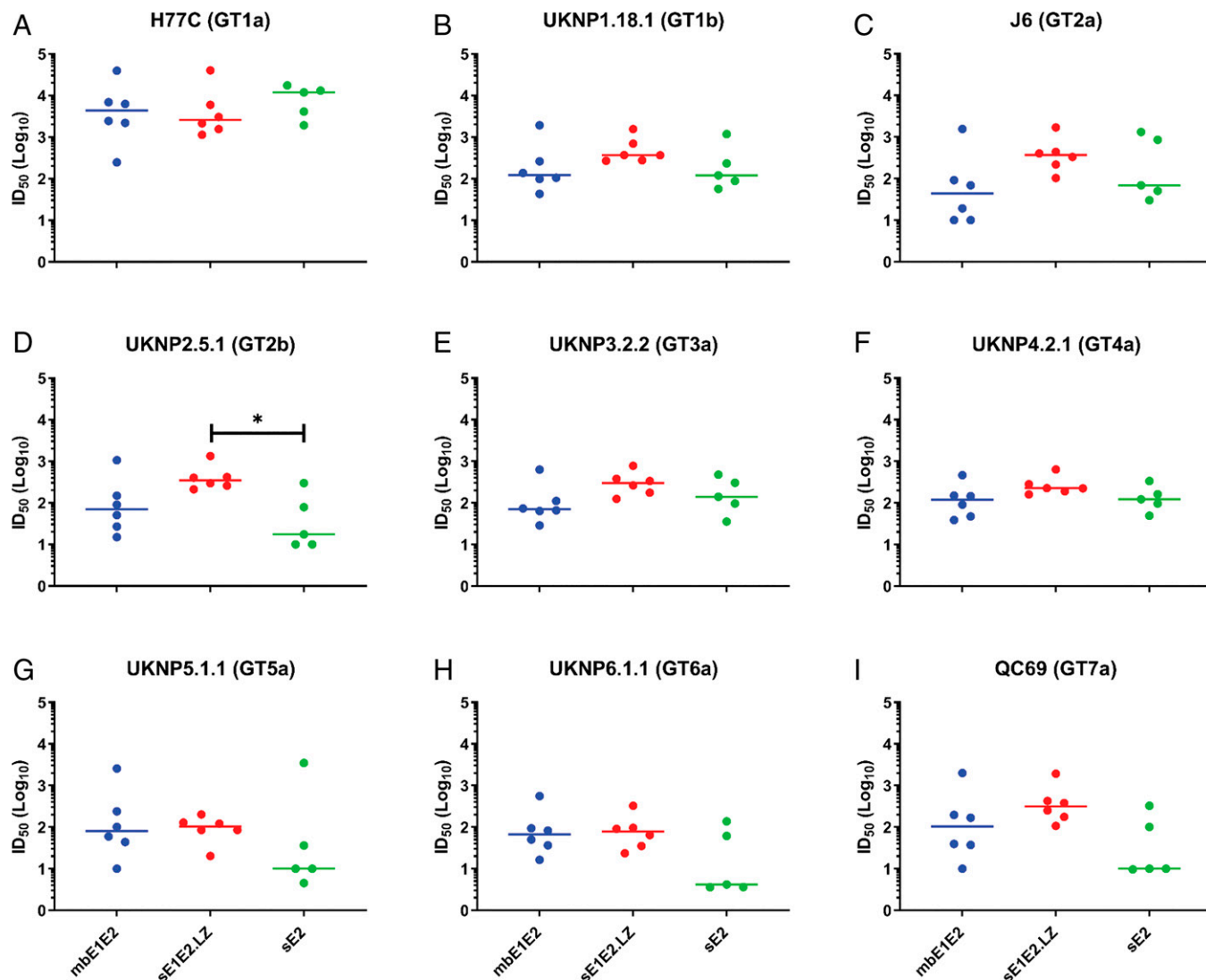


Fig. 6. Breadth of neutralization against all HCV genotypes with HCVpp. (A–I) Individual immunized mice sera were assessed for neutralization activities at day 56 and day 0 against seven genotypes of HCVpp. Neutralization titers were calculated as serum dilution levels reached at 50% neutralization (ID_{50}) by curve fitting in Graphpad Prism software. Serum dilutions were performed as two-fold dilutions starting at 1:64 for HCVpp neutralization. ID_{50} values are plotted on a \log_{10} scale on the y axis. P values were calculated using Kruskal–Wallis analysis of variance with Dunn's multiple comparison test, and significant P values are shown (* $P < 0.05$). The H77C neutralization data (A) were previously published by our team (66) and are shown here for comparison.

nAb responses elicited by mbE1E2, sE1E2.LZ, and sE2 measured by HCVpp and HCVcc systems, we represented group ID_{50} s on a heatmap (Fig. 8). This clearly shows that sE1E2.LZ elicits broader nAb responses than mbE1E2 and sE2 in mice, and supports the use of this class of scaffold for HCV vaccine development.

Discussion

A major challenge in developing an E1E2-based vaccine is producing homogeneous amounts of this complex membrane-associated protein in large quantities that reflects the native form found on the surface of the virus. Part of the difficulty stems from the fact that mbE1E2 undergoes a complex folding and processing pathway in which E1 and E2 mutually assist each other in achieving their native forms (37, 86, 87). An additional complication arises due to the membrane anchoring TMDs on E1 and E2, which makes membrane extraction required for mbE1E2 purification and sets an inherent limit on the amount of protein that can be produced per volume of cell culture. Recent efforts have made strides in liberating E1E2

from the membrane (65, 66, 88) and our group in particular recently developed heterodimeric coiled-coil LZ scaffolded secreted E1E2 (sE1E2.LZ) that retains native-like antigenicity and elicits neutralizing mAbs in mice (66). In this study, we assessed the quality of sE1E2.LZ as an immunogen.

Based on the immunological response to sE1E2.LZ in a mouse model observed here, as well as our previous biophysical characterization of sE1E2.LZ, the soluble heterodimeric coiled-coil appears to be a bona fide functional replacement for the E1 and E2 TMDs, and thus this platform provides an opportunity for further development of a soluble E1E2-based vaccine candidate. In particular, the overall antibody titers elicited by sE1E2.LZ were equal or superior to those elicited by mbE1E2 or sE2 (Fig. 2). Moreover, sE1E2.LZ was competent to elicit antibodies that target important neutralizing domains (B, D, E, E1E2) to the same extent as its membrane-bound counterpart (Figs. 3 and 4). Domains B and D are of particular importance for vaccine development because they elicit bnAbs (89, 90) and domain D in particular has a low propensity to accumulate mutants that allow viral escape (91). Finally, the antibodies elicited by sE1E2.LZ are broadly neutralizing (Figs. 5–8 and

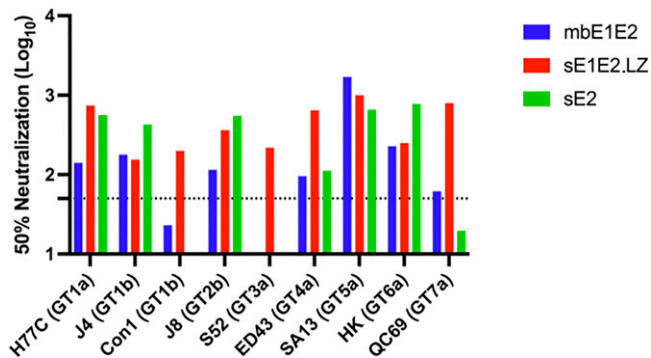


Fig. 7. Breadth of neutralization against all HCV genotypes with HCVcc. Day 56 immunized mice pooled sera were analyzed for neutralization using chimeric HCVcc with H77C (GT1a), J4 (GT1b), Con1 (GT1b), J8 (GT2b), S52 (GT3a), ED43 (GT4a), SA13 (GT5a), HK (GT6a), QC69 (GT7a). Percent neutralization was calculated using RLU normalized to RLU of supernatant cultured without HCVcc nor serum (100%) and RLU of supernatant cultured with HCVcc without serum (0%). ID₅₀ neutralization was calculated from the sigmoid curve. Dotted line indicates highest concentration of serum.

Table 1). These properties of sE1E2.LZ persist despite the fact that it is purified in a manner that disadvantages it relative to mbE1E2. mbE1E2 is purified using an HC84.26 immunoaffinity column which, since HC84.26 is both conformation-specific and affinity matured (92), selects a population of mbE1E2 that has its domain D conformationally intact. sE1E2.LZ is purified using immobilized metal affinity chromatography, which is insensitive to the integrity of neutralizing epitopes.

It should be noted that sE2 is purified in the same manner, yet the neutralization breadth of sE2-immunized mice is not superior to that of mbE1E2. Using the LZ scaffold as a starting point, additional stabilization of important neutralizing domains as a next step could result in an improved E1E2-based immunogen. Structural data pertaining to the LZ scaffolded sE1E2s would greatly accelerate such efforts. Another area for potential development is the scaffold itself. In Fig. 2E we observe a significant immunological response to the LZ scaffold. Since c-Fos and c-Jun are of human origin, incorporation of structurally homologous scaffolds that are either of bacterial origin or rationally designed and lack any sequence homology with human proteins is an important next step. The LZ was chosen as a scaffold in

part because the structure is well-characterized, making such a transition potentially straightforward.

Given the potential of this approach, it is important to consider the possible origins of improved neutralization breadth as these considerations will inform future designs. One advantage of the sE1E2.LZ platform is that it maintains neutralizing epitopes on E1, E2, and those that require the E1E2 complex in a soluble antigen. That these epitopes are intact is borne out by both our previous biochemical analysis and the immunological response observed here. An additional factor that might contribute to increased neutralization breadth is lower immunoreactivity to nonneutralizing epitopes. Based on our peptide ELISA data (Fig. 2E), sera from sE1E2.LZ-immunized mice exhibit three- to fourfold lower reactivity to a peptide containing the sequence of HVR1. HVR1 is an immunodominant region in patients infected with HCV (93, 94). As such, HVR1 provides many opportunities for viral escape as the region readily undergoes sequence changes during the course of an infection (95). Moreover, of the three antigens, sE1E2.LZ exhibits the weakest competition with nonneutralizing antigenic domain A mAbs in competition ELISA experiments. This suggests that among the polyclonal sera, those from sE1E2.LZ-immunized mice contain fewer mAbs that recognize domain A than sera from either mbE1E2 or sE2. sE2-immunized mouse sera contained the most domain A mAbs, consistent with our previous observation that sE2 binds CBH-4D and CBH-4G (96) 15- to 30-fold tighter than sE1E2.LZ or mbE1E2 (66).

A final potential contributor to increased neutralization breadth is increased homogeneity of the sE1E2.LZ antigen relative to mbE1E2. Our previous biophysical analysis indicated that, while sE1E2.LZ is not a single, homogeneous species in solution, it is more homogeneous than mbE1E2 (66). It is possible that cellular quality checks on the secreted complex, such as the ER-associated degradation pathway, contribute to homogeneity. Perhaps differences in the pathways that check the quality of membrane-bound versus secreted proteins (97), combined with the fact that mbE1E2 extracted from the membrane is likely to be a mix of proteins at various stages of the quality control pathways, results in a more heterogeneous mbE1E2 preparation. For sE1E2.LZ, only protein that has completed the checks by the ER-associated degradation will be secreted from cells and ultimately purified, thereby limiting the number

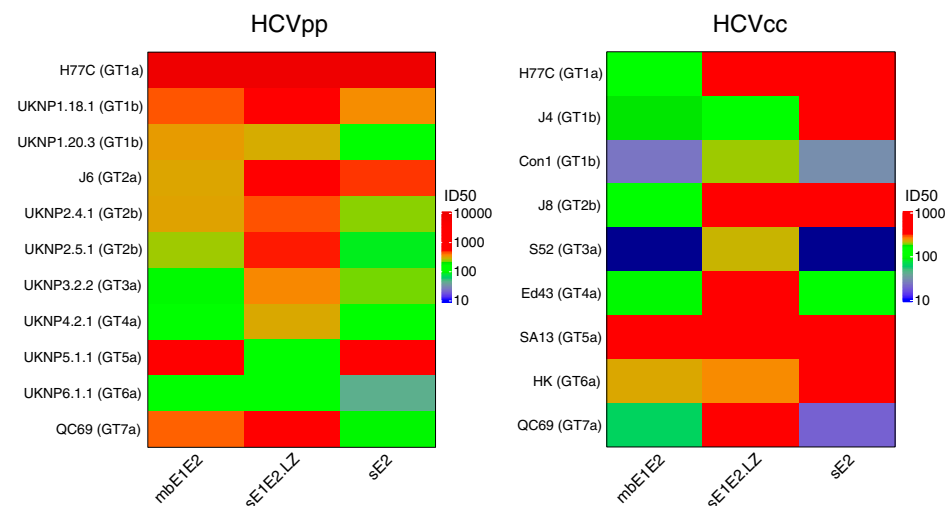


Fig. 8. Heat map ID₅₀ showing heterologous neutralization for three immunized groups. HCVpp neutralization (Left) and HCVcc neutralization (Right). Each row corresponds to an HCV genotype represented as HCVpp or HCVcc, and cell colors represent mean group ID₅₀ values from Fig. 6, or pooled serum ID₅₀ values from Fig. 7.

of species in solution. Further research in this area would highlight cellular factors that might allow production of more uniform sE1E2 preparations.

In summary, the immunological response to the sE1E2.LZ validates the heterodimeric coiled-coil LZ scaffold as a platform for rational design of E1E2 immunogens capable of eliciting bnAbs outside of a membrane or detergent environment. While rational design has led to significant successes for viruses, such as influenza (98, 99), HIV (100, 101), and RSV (102, 103), similar efforts in HCV have been more limited in scope (55). Moreover, these efforts have largely been focused on the E2 ectodomain alone. Since the effect of design changes observed in the isolated E2 ectodomain might not translate directly in the context of the E1E2 heterodimer, having a validated, native-like secreted E1E2 will allow a more thorough exploration of rationally designed E1E2 vaccine candidates. Finally, validation of the LZ platform allows the use of high-yield production systems that were previously only available for sE2 production, thereby making the transition to eventual clinical scale manufacturing of E1E2 vaccine antigens more feasible.

Materials and Methods

Plasmid Construction. In order to express the proteins of membrane-bound E1E2 (mbE1E2), the native-like and secreted form of E1E2 (sE1E2.LZ), and the secreted E2 (sE2) (HCV E2 residues 384 to 661), the human codon-optimized cDNA sequences encoding the proteins of mbE1E2, sE1E2.LZ, and sE2 were synthesized by GenScript and then cloned into pcDNA3.1 (+) and pSecTag2, respectively, as described in a previous study (1). The tissue plasminogen activator (tPA) leader sequence was used to replace the native lead sequences in the pcDNA3.1-based mbE1E2 and sE1E2 constructs, and the signal peptides from the mouse Ig κ -chain (IgK) was used for pSecTag2-based sE2 construct. A C-terminal 6xHis tag was added to both soluble sE1E2.LZ and sE2 constructs. In the sE1E2.LZ construct, the TMDs of E1E2 were replaced by human c-Fos/c-Jun LZ. A hexaarginine furin cleavage site was also incorporated between E1 and E2 to facilitate polyprotein processing.

Protein Expression and Purification. Expression of recombinant mbE1E2, sE1E2.LZ, and sE2 were performed in a transient expression in human Expi293 cells using the Expi293 Expression System by following the manufacturer's protocols (Thermo Fisher Scientific). Briefly, Expi293 cells were cultured in Expi293 Expression Medium in the shaker incubator at 37 °C, with 120 rpm and 8% CO₂. When the cells reached a density of 2.0×10^6 cells/mL, Expi293 cells were transfected using proper amounts of plasmid DNA. For the furin-cleavable polyprotein expression, sE1E2.LZ construct was cotransfected with the furin construct (kindly provided by Yuxing Li, Institute for Bioscience and Biotechnology Research University of Maryland, Rockville, MD) at a 2:1 ratio. Culture supernatants of sE1E2.LZ and sE2 were harvested at 72 h after transfection, clarified by centrifugation at 10,000 rpm for 10 min, and filtered by a 0.22- μ m filters. Protein was then purified from the supernatant by sequential HisTrap Ni²⁺-NTA and Superdex 200 size-exclusion chromatography as described previously (55, 66). Expi293 cells transfected with recombinant mbE1E2 were collected 72 h after transfection and the cell pellets were lysed using 1% NP-9 cell lysis buffer (55). Recombinant mbE1E2 was then purified by sequential Fractogel EMD TMAE (Millipore), Fractogel EMD SO3⁻ (Millipore), HC84.26 immunoaffinity (92), and Galanthus Nivalis Lectin (GNL, Vector Laboratories) affinity chromatography, as described previously (55).

SDS/PAGE and Western Blot. Purified proteins of mbE1E2, sE1E2.LZ, and sE2 were separated by a precast, 4-20% Mini-PROTEAN TGX stain-free gels on a Mini-PROTEAN Tetra cell electrophoresis instrument (Bio-Rad). In reducing conditions, each sample was incubated with loading dye (4 \times Laemmli buffer + 10% β -mercaptoethanol) (Bio-Rad) and heated to 95 °C. In nonreducing conditions, each sample was incubated with Laemmli buffer and heated to 37 °C. For Western blot detection, the purified protein samples on SDS/PAGE were transferred onto Trans-Blot Turbo Mini nitrocellulose membranes (Bio-Rad). The membranes were then probed using the anti-HCV E2 mAb HCV1 at 5 μ g/mL and anti-HCV E1 mAb

H-111 at 10 μ g/mL followed by detection using a secondary goat anti-human IgG-HRP conjugate (Invitrogen) at a 1:5,000 dilution and the Western ECL substrate (Bio-Rad). All gels were imaged using the ChemiDoc system (Bio-Rad).

Animal Immunization. CD1 mice were purchased from Charles River Laboratories. Prior to immunization, sE2 and E1E2 (mbE1E2 and sE1E2.LZ) antigens were formulated with polyphosphazene adjuvant as described in previous studies (68, 69). In brief, 50 μ g PCPP was formulated with 25 μ g resiquimod, R848 in PBS (pH 7.4) to form the PCPP-R adjuvant. The resulting supramolecular complex (PCPP-R) was formulated with either E1E2 (70 μ g for prime or 15 μ g for boost immunization) or sE2 antigen (50 μ g for prime or 10 μ g for boost immunization), with antigen amounts selected to ensure approximate molar equivalence of E2 in the vaccines. Dynamic light scattering was used to confirm the absence of aggregation in adjuvanted formulations. Groups of six female CD-1 mice, aged 7 to 9 wk, were immunized via the intraperitoneal route, first with a prime as described above on day 0, then with boosts as described above on day 14, day 28, and day 42. Unvaccinated mice served as a control for later analysis. Blood samples were collected prior to each vaccination on days 0 (prebleed), 14, 28, and 42, and a terminal bleeding on day 56. The blood samples were processed for serum by centrifugation and stored at -80 °C until analysis was performed. The *in vivo* work was conducted in the AAALAC and USDA Animal Welfare Act compliant vivarium of Noble Life Sciences, Inc. (NLS, Sykesville, Maryland) and monitored by NLS IACUC (NIH OLAW Assurance # D16-00845 [A4633-01]).

ELISAs for Serum Antibody Detection. ELISA was performed to measure HCV E1E2-specific antibody responses in immunized mouse serum; 96-well plates (MaxiSorp, Thermo Fisher Scientific) were coated overnight with 5 μ g/mL Galanthus Nivalis Lectin (Vector Laboratories) at 4 °C. The next day, plates were washed with PBS containing 0.05% Tween 20 and coated with 200 ng per well antigens of mbE1E2, sE1E2.LZ, and sE2 at 4 °C. After overnight incubation, plates were washed with PBS containing 0.05% Tween 20 and blocked with Pierce Protein-Free Blocking Buffer (Thermo Fisher Scientific) for 1 h, and serially diluted mice sera samples were then added to the plates and incubated for another hour. The binding of HCV E1E2-specific antibodies was detected by a 1:5,000 dilution of HRP-conjugated anti-mouse IgG secondary antibody (Abcam) with TMB substrates (Bio-Rad Laboratories). Absorbance values at 450 nm (SpectraMax M3 microplate reader) were used to determine endpoint titers, which were calculated by curve fitting in GraphPad Prism software and defined as four times the highest absorbance value of preimmune sera. Significance comparison was performed using Kruskal-Wallis one-way ANOVA.

For peptide ELISA, 100 μ L of biotinylated peptides (2 μ g/mL) were coated on the Well-Coated Streptavidin plates (G-Biosciences) for overnight at 4 °C. Peptides included in this study were c-Fos (LTDITLQAEADQLEDKKSALQTEIANLLKEKEKLE-FILAAAY) and c-Jun (RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNY), along with peptides representing E2 domain D (NTGWLAGLFYQHK), E2 domain E (NIQ-LINTNGSWHINS), E2 HVR1 and domain E (ETHVTGGSAGRITAGLVLLTPGAKQNIQ-LINTNGSWHIN), the E1 N terminus (YQVRNSSLGYHVTND), and an E1 ectodomain nAb epitope (TGHMAWDMMMN). After washing with PBS containing 0.05% Tween 20 and blocking with Pierce Protein-Free Blocking Buffer or serially diluted pooled mice sera, ranging from a 1:150 to a 1:328,050 dilution, they were incubated at 37 °C for 1 h and detected by ELISA as described above.

Competition ELISA. The ability of antibodies in immunized mouse sera to compete with both conformation-dependent and linear HCV E1E2-specific HMABs was assessed by ELISA. The antibodies used for these experiments include AR3A and HEP74 (domain B), HC84.26 and HC84.1 (domain D), HCV1 and HC33.1 (domain E), AR4A and AR5A (anti-E1E2), CBH-4G and CBH-4B (domain A), and H-111 and IGH526 (anti-E1). mbE1E2 was captured on GNA-coated microtiter plates at 4 °C for overnight. After blocking with Pierce Protein-Free Blocking Buffer (Thermo Fisher Scientific) for 1 h followed by three-time washing using Pierce Protein-Free Blocking Buffer, diluted mouse antisera (terminal bleed) were added to each well and incubated for 1 h at room temperature. After plates were washed with PBS containing 0.05% Tween 20, HCV E1E2-specific HMABs were added at a concentration demonstrated previously to result in 70% of maximal binding and incubated for an additional hour. The HMABs used for the competition ELISA were biotinylated using an EZ-Link NHS-PEO solid-phase biotinylation kit (Thermo Fisher Scientific). Bound biotinylated

HMAb was detected using HRP-conjugated streptavidin (Abcam) at a dilution of 1:20,000. Absorbance was read at 450 nm using a SpectraMax M3 microplate reader. Percent inhibition values were calculated as the percentage of mAb binding relative to the mAb bound in the absence of serum.

HCVpp Neutralization Assay. The human hepatoma cell line, Huh7, was maintained in the DMEM supplemented with 10% FBS and 1% nonessential amino acids (NEAA) (Thermo Fisher Scientific), and used as the target cell line for neutralization assays (1, 10). To test sera and antibodies for neutralization, Huh7 cells were preseeded into 96-wells plates at a density of 1×10^4 per well. The next day, the pseudoparticles were incubated with defined concentrations of mAbs and the heat-inactivated serum at indicated dilutions for 1 h at 37 °C, and then added to each well. After the plates were incubated in a CO₂ incubator at 37 °C for 5 to 6 h, the mixtures were replaced with fresh medium and then continued to incubate for 72 h. After incubation, 100 µL Bright-Glo (Promega) was added to each well for 2 min at room temperature and the luciferase activity was measured using a FLUOstar Omega plate reader (BMG Labtech) with the MARS software. The IC₅₀ titer was calculated as the mAbs concentration that caused a 50% reduction in relative light units (RLU) compared with pseudoparticles in the control wells. nAbs titers in animal sera were reported as ID₅₀ values. All values were calculated using a dose-response curve fit with nonlinear regression in GraphPad Prism. All experiments involving the use of pseudoparticles were performed under biosafety level 2 conditions.

HCVcc Neutralization Assay. Twofold dilutions were performed starting at 1:100 for preimmune pooled serum or 1:50 for day 56 pooled serum. HCVcc was mixed with diluted serum (final multiplicity of infection = 0.1) and incubated for 1 h at 4 °C. After the incubation, the serum and virus mixture was added onto Huh7.5 cells (kindly provided by Charles Rice, The Rockefeller University, New York, NY), plated on 96-well plate for 1 d, and cultured for 4 h at 37 °C. Thereafter, the inoculum was removed, cells washed with HBSS twice, and then the cells were cultured with DMEM containing 3% fetal bovine serum (FBS, Atlanta Biologicals), NEAA (0.1 mM; Thermo Fisher Scientific), Hepes (20 mM; Thermo Fisher Scientific), polybrene (4 µg/mL; Sigma-Aldrich Chemie), and penicillin streptomycin for 72 h at 37 °C. After 72 h, supernatants were

collected and luciferase assay was performed following the manufacturer's protocol (GeneCopoeia). Percent neutralization was calculated as RLU from supernatant cultured without HCVcc nor serum was 100% neutralization and RLU from supernatant cultured with HCVcc without serum was 0% neutralization. The serum concentration of 50% neutralization was calculated from the sigmoid curve (GraphPad Prism 8).

Statistical Analysis. The differences among group endpoint titers and group ID₅₀ values were statistically compared using the nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test. $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism software.

Data Availability. All study data are included in the main text and/or *SI Appendix*.

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