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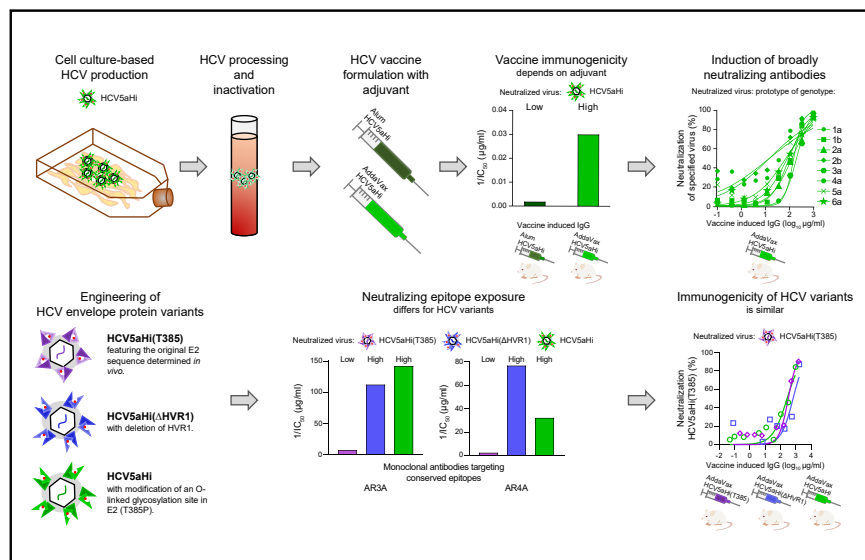
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# Inactivated whole hepatitis C virus vaccine employing a licensed adjuvant elicits cross-genotype neutralizing antibodies in mice

## Graphical abstract



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## Lay summary

A vaccine against hepatitis C virus (HCV) is needed to prevent the estimated 2 million new infections and 400,000 deaths caused by this virus each year. We developed inactivated whole HCV vaccine candidates using adjuvants licensed for human use, which, following immunization of mice, induced antibodies that efficiently neutralized all HCV genotypes with recognized epidemiological importance. HCV variants with modified envelope proteins exhibited similar immunogenicity as the virus with the original envelope proteins.

## Highlights

- Efficient production of inactivated whole virus antigen for HCV vaccine candidates.
- Whole inactivated HCV vaccines induce broadly neutralizing antibodies in mice.
- Among adjuvants, AddaVax, analogue of licensed MF-59, shows the highest immunogenicity.
- Modifications of HCV envelope proteins increase neutralization epitope exposure.
- HCV with modified and original envelope proteins has similar immunogenicity.



# Inactivated whole hepatitis C virus vaccine employing a licensed adjuvant elicits cross-genotype neutralizing antibodies in mice

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**Background & Aims:** A prophylactic vaccine is required to eliminate HCV as a global public health threat. We developed whole virus inactivated HCV vaccine candidates employing a licensed adjuvant. Further, we investigated the effects of HCV envelope protein modifications (to increase neutralization epitope exposure) on immunogenicity.

**Methods:** Whole virus vaccine antigen was produced in Huh7.5 hepatoma cells, processed using a multistep protocol and formulated with adjuvant (MF-59 analogue AddaVax or aluminium hydroxide). We investigated the capacity of IgG purified from the serum of immunized BALB/c mice to neutralize genotype 1-6 HCV (by virus neutralization assays) and to bind homologous envelope proteins (by ELISA). Viruses used for immunizations were (i) HCV5aHi with strain SA13 envelope proteins and modification of an O-linked glycosylation site in E2 (T385P), (ii) HCV5aHi(T385) with reversion of T385P to T385, featuring the original E2 sequence determined *in vivo* and (iii) HCV5aHi( $\Delta$ HVR1) with deletion of HVR1. For these viruses, epitope exposure was investigated using human monoclonal (AR3A and AR4A) and polyclonal (C211 and H06) antibodies in neutralization assays.

**Results:** Processed HCV5aHi formulated with AddaVax induced antibodies that efficiently bound homologous envelope proteins and broadly neutralized cultured genotype 1-6 HCV, with half maximal inhibitory concentrations of between 14 and 192  $\mu$ g/ml (mean of 36  $\mu$ g/ml against the homologous virus). Vaccination with aluminium hydroxide was less immunogenic. Compared to HCV5aHi(T385) with the original E2 sequence, HCV5aHi with a modified glycosylation site and HCV5aHi( $\Delta$ HVR1) without HVR1 showed increased neutralization epitope exposure but similar immunogenicity.

**Conclusion:** Using an adjuvant suitable for human use, we developed inactivated whole HCV vaccine candidates that

induced broadly neutralizing antibodies, which warrant investigation in further pre-clinical studies.

**Lay summary:** A vaccine against hepatitis C virus (HCV) is needed to prevent the estimated 2 million new infections and 400,000 deaths caused by this virus each year. We developed inactivated whole HCV vaccine candidates using adjuvants licensed for human use, which, following immunization of mice, induced antibodies that efficiently neutralized all HCV genotypes with recognized epidemiological importance. HCV variants with modified envelope proteins exhibited similar immunogenicity as the virus with the original envelope proteins.

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## Introduction

Worldwide ~2 million acute HCV infections occur yearly, leading to chronic infection in ~75% of cases.<sup>1</sup> More than 70 million individuals are estimated to be chronically infected, resulting in ~400,000 annual deaths, mostly due to cirrhosis and hepatocellular carcinoma.<sup>1</sup> Given the asymptomatic nature of HCV infection prior to development of severe (and often irreversible) liver damage and the lack of screening programs, it is estimated that less than 20% of infections are diagnosed. Further, not all diagnosed individuals receive treatment with direct-acting antivirals, partly due to their high cost.<sup>1</sup> Moreover, treatment does not protect against reinfection, can be associated with severe side effects in hepatitis B virus co-infected individuals, and does not always eliminate the risk of hepatocellular carcinoma following HCV clearance. Finally, observed emergence of antiviral resistance could compromise future treatment efficacy. Therefore, a prophylactic vaccine is essential to achieve the World Health Organization's objectives for HCV elimination as a major public health threat.<sup>2</sup>

Ideally, a vaccine should protect against different HCV variants.<sup>3</sup> Among the 8 reported major HCV genotypes, genotype 1-6 are epidemiologically significant. There are various subtypes. Genotypes and subtypes differ in ~30% and ~20% of their sequence, respectively, and show differential sensitivity to neutralizing antibodies (nAbs).<sup>4-9</sup> The HCV envelope (E)

Keywords: hepatitis C virus; hepatitis; neutralizing antibodies; HCV vaccine; whole viral particle vaccine; HCV inactivation; HCV downstream processing; adjuvant; envelope glycoprotein; hypervariable region; glycosylation.

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glycoproteins (gp) E1 and E2 are the main targets of nAbs.<sup>10</sup> The highly variable 27 amino acid motif hypervariable region 1 (HVR1) at the E2 N-terminus mediates HCV evasion from nAbs; it acts as immunological decoy that induces nAbs, which are rendered inefficient due to mutational escape, and facilitates closed envelope protein conformational states, which restrict nAb access to conserved neutralization epitopes.<sup>4,11,12</sup> Further, HCV envelope protein glycosylation induced closed envelope protein states that protect conserved epitopes.<sup>12,13</sup> Thus, *in vitro* deletion of HVR1 and mutation of glycosylation sites increased HCV sensitivity to nAbs.<sup>4,11–14</sup>

Protective immunity against HCV is achievable as ~25% of acute infections are cleared, likely by nAbs and T cells.<sup>15,16</sup> In a vaccine setting, nAbs might be sufficient for protection, as most licensed viral vaccines protect by nAbs.<sup>16–18</sup> Further, early development of nAbs was predictive of HCV clearance in humans and passive immunization with nAbs prevented HCV infection in chimpanzees.<sup>19–23</sup> Moreover, nAbs induced by a recombinant gpE1/gpE2 vaccine had protective effects in chimpanzees.<sup>24,25</sup> In contrast, a viral vector-based vaccine inducing HCV-specific T cells did not protect against chronic HCV infection in chimpanzees and humans.<sup>26,27</sup>

In humans, the gpE1/gpE2 vaccine yielded robust nAbs in <50% of immunized individuals.<sup>28,29</sup> Compared to such subunit vaccines, whole virus vaccines show superior immunogenicity owing to a more native envelope protein conformation, a broader epitope array and a denser epitope presentation.<sup>30,31</sup> The development of a whole virus HCV vaccine only became feasible following the development of infectious cell culture systems for HCV production.<sup>32</sup> However, proof of the immunogenicity of cell-culture-produced HCV was only obtained with non-licensed adjuvants.<sup>31,33</sup> Additionally, it remains an unresolved question whether envelope protein modifications designed to expose conserved neutralization epitopes improve HCV immunogenicity in the context of a whole virus vaccine.

We aimed to develop inactivated HCV vaccine candidates employing adjuvants licensed for human use and to investigate their immunogenicity in mice. We evaluated the capacity of vaccine-induced antibodies to broadly neutralize cell-culture-infectious HCV genotype 1-6 recombinants. Finally, we evaluated the impact of envelope protein modifications on vaccine immunogenicity by deleting HVR1 and mutating a glycosylation site.

## Materials and methods

### HCV recombinants

Recombinants for vaccine production and/or neutralization assays were: (i) High titer HCV5aHi<sup>34</sup> with modification of an O-linked glycosylation site in E2 (T385P) in comparison to the reference strain SA13 virus HCV5a,<sup>35</sup> (ii) HCV5aHi(T385) differing from HCV5aHi by reversion of T385P to T385,<sup>34</sup> and (iii) HCV5aHi(ΔHVR1), newly engineered by deletion of HVR1 from HCV5aHi. For neutralization assays, HCV with genotype 1-6 envelope gp were used.<sup>8,9,35–37</sup> Also see Fig. S1.

### HCV cell culture

Huh7.5 cells were maintained as described.<sup>38,39</sup> Generation of HCV virus stocks and further cell-culture-adaptation of HCV5aHi(ΔHVR1) by serial passage are described in the [supplementary materials and methods](#).

### Production of HCV for vaccine generation

HCV-infected Huh7.5 cells were seeded in 10-layer cell factories (Thermo Fisher Scientific) and maintained in serum-free Adenovirus Expression Medium (ThermoFisher Scientific)<sup>40</sup> during the HCV production phase. For details see the [supplementary materials and methods](#).

### Evaluation of HCV-infected cell cultures

Details regarding the evaluation of the percentage of HCV antigen-positive cells in infected cell cultures by immunostaining,<sup>37,39</sup> as well as HCV infectivity,<sup>38,41,42</sup> RNA<sup>39</sup> and core titers<sup>43</sup> in culture supernatants, are provided in the [supplementary materials and methods](#).

### HCV sequencing

Sanger sequencing of the HCV sequence of DNA maxi-preparations or of amplicons of the HCV RNA genome of cell-culture-derived HCV generated by reverse-transcription PCR was carried out at MacroGen Europe as described<sup>38,44</sup> and as detailed in the [supplementary materials and methods](#).

### HCV concentration

HCV in serum-free culture supernatant was concentrated using a multistep process consisting of tangential flow filtration, ultracentrifugation and chromatography steps, as specified in the [supplementary materials and methods](#).

### Immunization of mice

BALB/c mice (Taconic Farms, Denmark) were vaccinated with concentrated and inactivated HCV or EndoFit™Ovalbumin (Invivogen) formulated with aluminium hydroxide (Alum)+monophosphoryl lipid A (MPLA) (Invivogen), AddaVax™ (Invivogen) or Freund's adjuvant (Invivogen) according to Danish regulations, as specified in the [supplementary materials and methods](#).

### Mouse serum IgG purification and quantification

IgG was purified (Amicon® Pro Affinity Concentration Kit Protein G, Millipore), concentrated (Vivaspin® 500, 30,000 molecular weight cut-off (MWCO), GE Lifesciences) and quantified (IgG (total) Mouse Uncoated ELISA Kit, ThermoFisher or Cedex Bio Analyzer, Roche) according to the manufacturer's instructions and as specified in the [supplementary materials and methods](#).

### *In vitro* neutralization assays

Neutralization assays for characterization of HCV with envelope protein modifications were performed as described,<sup>35</sup> using human monoclonal antibodies (mAbs: AR3A,<sup>45</sup> AR4A<sup>46</sup>) or human polyclonal IgG preparations (C211<sup>12</sup> or H06<sup>47</sup>). Neutralization assays with purified mouse IgG were carried out in a smaller volume than assays for characterization of HCV. The E1/E2 sequence of viruses used in neutralization assays was sequence confirmed to be identical to the plasmid sequence. For details see the [supplementary materials and methods](#).

### E1/E2 complexes and soluble E2 (sE2) ELISA

Binding of mouse serum IgG to recombinantly expressed native HCV5a (strain SA13) E1/E2 complexes derived from cell lysates of transfected HEK293T cells or his-tag purified HCV5a SA13 sE2 derived from supernatant of transfected HEK293T cells was

evaluated by ELISA, as described in the [supplementary materials and methods](#).

## Results

### Production of HCV vaccine antigen

HCV for immunizations was produced in Huh7.5 cells in 10-layer cell factories under serum-free conditions and processed using a multistep protocol. A representative production with the cell-culture-infectious HCV recombinant HCV5aHi<sup>34</sup> (Fig. S1) is shown in Fig. 1A-E. Following UV inactivation, the HCV antigen was formulated with adjuvant and used for 4 subcutaneous immunizations of BALB/c mice at 3-week intervals (Fig. 1F).

### Immunization of mice with an inactivated whole virus HCV vaccine formulated with Alum+MPLA elicited nAbs with limited efficacy

First, mice were immunized with inactivated HCV5aHi formulated with Alum+MPLA, licensed for human use. Following sacrifice, serum IgG was purified and tested for nAbs against homologous HCV5aHi, showing dose-dependent neutralization, however, with limited efficacy. The half maximal inhibitory concentration (IC<sub>50</sub>) ranged from 64 to 589 µg/ml (mean 313 µg/ml) for 4 animals, while for 2 animals 50% neutralization was not observed at 1,000 µg/ml, the highest IgG concentration used (Fig. 2A). Near complete neutralization was only observed for 1 animal, while 18–72% was achieved for the other animals at the highest IgG concentration. No HCV-specific nAbs were detected in control mice immunized with ovalbumin and Alum+MPLA (Fig. S2). Vaccine-induced IgG specifically bound to homologous E1/E2 complexes and less strongly to sE2 (Fig. S3). Immunization with HCV5aHi formulated with the widely used experimental Freund's adjuvant induced slightly better nAb responses, with mean IC<sub>50</sub> at 286 µg/ml IgG and mean maximum neutralization of 83% against HCV5aHi (Fig. S4).

### Immunization of mice with an inactivated whole virus HCV vaccine formulated with AddaVax elicited potent cross-genotype nAb responses

Next, we immunized mice with inactivated HCV5aHi formulated with AddaVax, an analogue of MF-59; MF-59 is licensed for human use. IgG purified from 9 vaccinated animals neutralized homologous HCV5aHi at IC<sub>50</sub> of 15–66 µg/ml (mean 36 µg/ml) and near complete neutralization at the highest IgG concentration (Fig. 2B). Thus, AddaVax showed superior efficacy, with mean IC<sub>50</sub> more than 15-fold lower than that of the Alum+MPLA vaccination group (Fig. 2C). Further, mean maximum neutralization at the highest IgG concentration was 97% vs. 61% for IgG induced by AddaVax vs. Alum+MPLA (Fig. 2D). Importantly, IgG pooled from these 9 animals efficiently cross-neutralized HCV genotypes 1–6 at IC<sub>50</sub> of 14–192 µg/ml, with near complete neutralization at the highest IgG concentration (Fig. 2E). No HCV-specific nAbs were detected in control mice (Fig. S2). Further, IgG purified from pooled serum of these 9 animals after the 2<sup>nd</sup> and 3<sup>rd</sup> immunization neutralized HCV5aHi at IC<sub>50</sub> of 50 and 76 µg/ml, respectively, and with near complete neutralization at the highest IgG concentration (Fig. 2F). Moreover, vaccine-induced IgG specifically bound to homologous E1/E2 complexes and sE2 individually (Fig. 3), showing stronger binding than IgG induced by Alum+MPLA (Fig. S3). IgG induced following 3 and 4 immunizations showed similar, concentration-dependent binding.

Slightly lower binding was observed for IgG derived following 2 immunizations (Fig. 3).

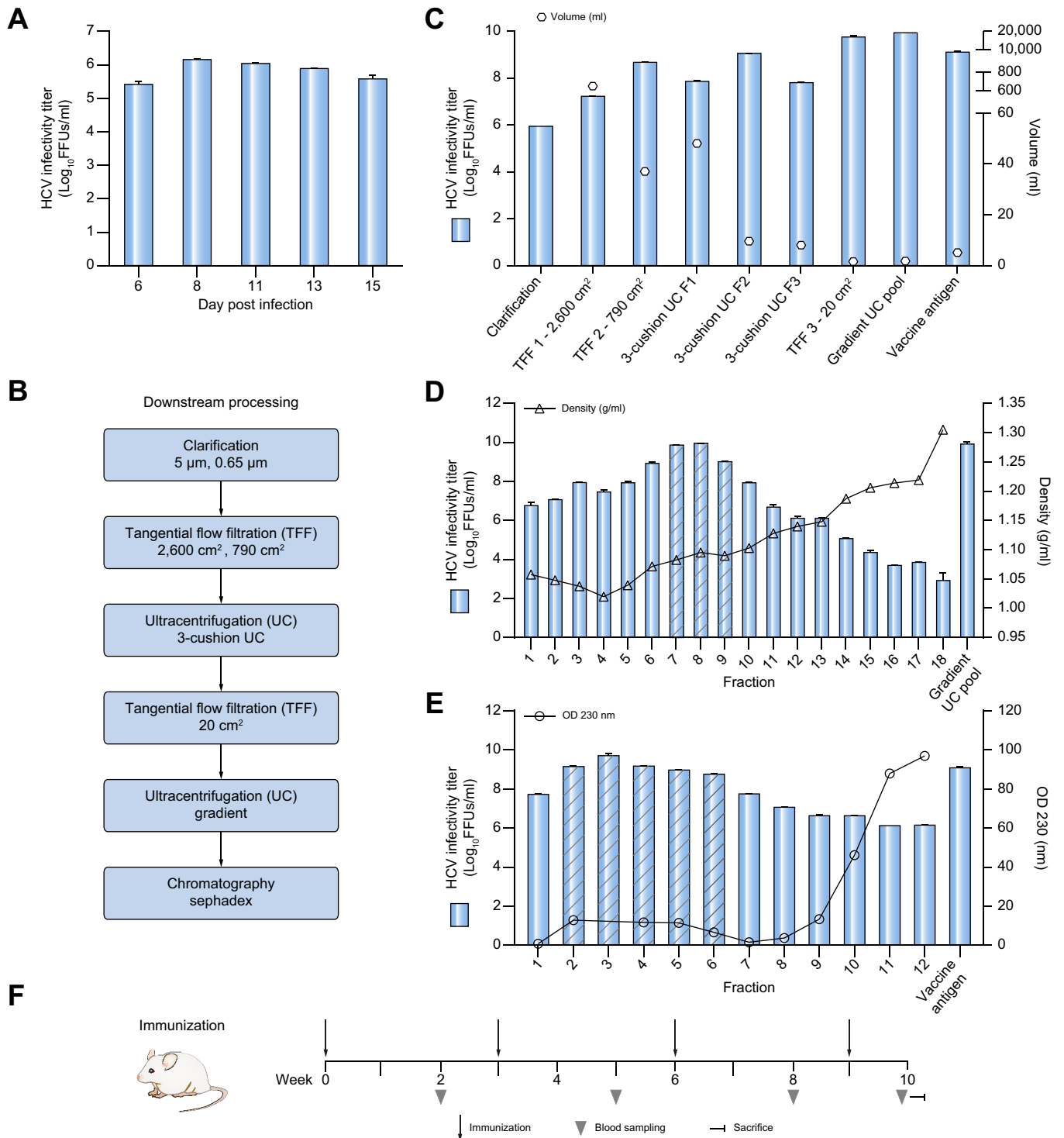
### HCV with modified envelope proteins showed differential sensitivity to nAbs

Deletion of HVR1 or mutation of glycosylation sites has been reported to influence the neutralization sensitivity of cell-culture-derived HCV, presumably by influencing exposure of neutralization epitopes.<sup>4,11–14</sup> However, how such modifications influence immunogenicity in the context of a whole virus vaccine has not been studied. HCV5aHi harbored the E2 substitution T385P, which has been shown to increase neutralization sensitivity.<sup>34</sup> To investigate the influence of T385P on immunogenicity, we produced HCV5aHi(T385) with the substitution reverted to T385,<sup>34</sup> yielding 5.6 log<sub>10</sub> focus-forming units (FFUs)/ml in pooled supernatant from cell factories. Further, to study the influence of HVR1 on immunogenicity we developed HCV5aHi(ΔHVR1) by deleting HVR1 from HCV5aHi and by further culture adaptation, carrying out 18 passages in Huh7.5 cells to compensate for fitness impairment. The resulting polyclonal virus had acquired 3 additional dominant substitutions based on Sanger sequencing: N532D and L735I in E2 as well as K1609R in non-structural protein 3 (amino acid positions are related to the polyprotein of the 1a H77 reference sequence (GenBank accession no. AF009606)) (Fig. S1). Pooled passage 20 supernatant from cell factories inoculated with a passage 19 seed stock yielded 5.8 log<sub>10</sub> FFUs/ml.

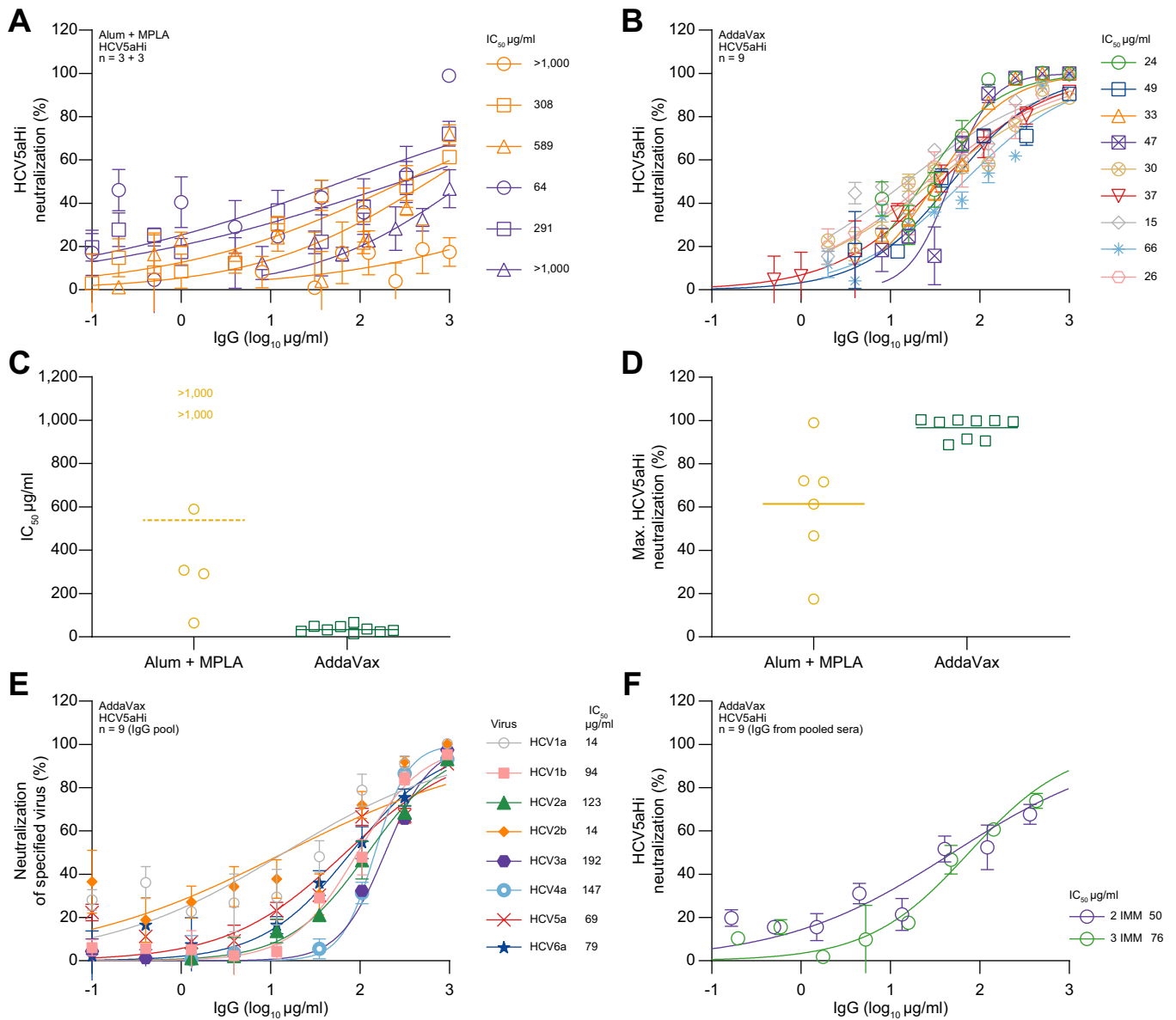
We characterized neutralization sensitivity of HCV5aHi(T385), HCV5aHi(ΔHVR1) and HCV5aHi in comparison to the reference virus HCV5a with the original envelope protein sequence<sup>35</sup> using well characterized human mAbs (AR3A<sup>45</sup> and AR4A<sup>46</sup>) and human polyclonal IgG (C211 and H06) (Fig. 4A–D).<sup>12,47</sup> In line with previous results, HCV5aHi showed 6- to 18-fold increased neutralization sensitivity compared to HCV5a<sup>34</sup> (Fig. 4E and F). HCV5aHi(ΔHVR1) showed a similar (6- to 22-fold) increase in neutralization sensitivity. In contrast, HCV5aHi(T385), also harboring the original E2 sequence, showed similar neutralization sensitivity as HCV5a. Similar differences in neutralization profiles were observed for processed HCV5aHi, HCV5aHi(ΔHVR1) and HCV5aHi(T385) (Fig. S5).

### HCV vaccine candidates with modified envelope proteins induced similar nAb responses in mice

To evaluate if differences in neutralization sensitivity affected immunogenicity, mice were immunized with HCV5aHi, HCV5aHi(ΔHVR1) and HCV5aHi(T385) equivalent to 7.5 log<sub>10</sub> FFUs using AddaVax. First, we proved that serum IgG purified from each animal neutralized HCV5aHi with similar efficacy (Fig. 2B and S6). Then, for each group, IgG pools were generated and used to neutralize HCV5aHi, HCV5aHi(ΔHVR1), HCV5aHi(T385) and the HCV5a reference virus (Fig. 5A–D). In these assays, IC<sub>50</sub> ranged from 17 to 654 µg/ml. The HCV5aHi IgG pool showed somewhat higher neutralization efficacy than the other pools with IC<sub>50</sub> of 17, 267, 126 and 69 µg/ml against HCV5aHi, HCV5aHi(T385), HCV5aHi(ΔHVR1) and HCV5a, respectively. Further, the IgG pools neutralized HCV1a at IC<sub>50</sub> of 14–143 µg/ml (Fig. 5E) and HCV3a at IC<sub>50</sub> of 192–295 µg/ml (Fig. 5F), with HCV5aHi-induced IgG showing the highest efficacy. Finally, IgG pools showed similar concentration-dependent binding to E1/E2 complexes and sE2 (Fig. 6). Overall, while modifications of the envelope proteins did not have major effects on immunogenicity, we observed a trend



**Fig. 1. Production and downstream processing of cell-culture-derived HCV.** (A) Example of production of HCV5aHi in 1 cell factory yielding 5 harvests of 800 ml serum-free supernatant with the indicated infectivity titers. (B) Schematic overview of the downstream process. (C-E) Example of processing ~16l HCV5aHi-containing supernatant pooled from 4 cell factories. (C) HCV infectivity titers and volumes of material from specified process steps: clarification; TFF 1 and TFF 2; 3-cushion UC with collection of 3 fractions (F1, F2, F3) and F2 being processed further; TFF 3; gradient UC pool (pool of 3 fractions); size exclusion Sephadex chromatography (vaccine antigen, pool of 5 fractions). (D) HCV infectivity titers and buoyant densities for gradient UC fractions; fraction 7, 8 and 9 (striped bars) were pooled (gradient UC pool). (E) HCV infectivity titers and OD at 230 nm for size exclusion chromatography fractions; fractions 2-6 (striped bars) were pooled (vaccine antigen). (A, C, D, E) HCV infectivity titers are means of triplicates with SEM. (F) Timeline for animal experiments. FFUs, focus-forming units; OD, optical density; TFF, tangential flow filtration; UC, ultracentrifugation. (This figure appears in color on the web.)



**Fig. 2. Immunization of BALB/c mice with HCV formulated with a licensed adjuvant induced broadly neutralizing antibodies.** Neutralization of HCV5aHi with purified serum IgG from mice immunized with HCV5aHi equivalent to (A) 8.1 log<sub>10</sub> FFUs using Alum+MPLA or (B) 7.5 log<sub>10</sub> FFUs using AddaVax. (A) data are from 2 independent experiments, indicated by different colors. (C) IC<sub>50</sub> and (D) maximum % neutralization for mice immunized with Alum+MPLA vs. AddaVax. Lines, means; broken line, least possible mean calculated by setting IC<sub>50</sub> for the 2 mice for which 50% neutralization was not achieved to 1,000 µg/ml. (E) Neutralization of specified genotype 1-6 HCV with pool of equal amounts of purified serum IgG from mice in (B) following 2 and 3 IMM. (A, B, E, F) Datapoints are means of triplicates with SEM. Alum, aluminium hydroxide; FFUs, focus-forming units; IC<sub>50</sub>, half maximal inhibitory concentration; IMM, immunizations; MPLA, monophosphoryl lipid A.

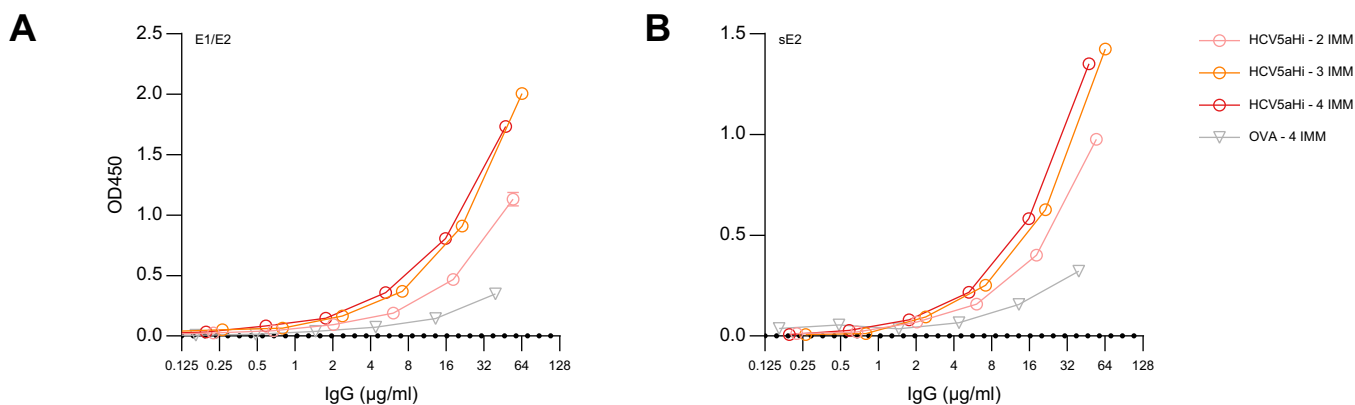
towards superior induction of nAbs by the HCV5aHi virus antigen.

**Discussion**

We provide proof-of-concept for immunogenicity of a whole virus inactivated HCV vaccine employing an adjuvant analogue of MF-59, which is licensed for human use. Immunizations resulted in induction of potent antibodies broadly neutralizing all major HCV genotypes with recognized epidemiological importance. HCV envelope protein variants with deletion of HVR1 and mutation of a putative O-linked glycosylation site

showed differential neutralization sensitivity but overall similar immunogenicity.

Broadly nAbs are associated with protection from chronic HCV infection.<sup>16,20,23,48</sup> Our results suggest that induction of broadly nAbs by a single virus antigen is possible. This is in line with proof-of-concept findings in chronically HCV-infected patients and in humans immunized with the gpE1/gpE2 vaccine, where cross-nAb targeting conformational epitopes were elicited by single isolates.<sup>7,28,49</sup> HCV5aHi vaccine-induced nAbs exhibited slight differences in efficacy against different HCV isolates, overall reflecting differences previously observed using other nAbs, such as the relatively low efficacy against the HCV3a



**Fig. 3. Immunization of BALB/c mice with HCV and AddaVax induced IgG binding to HCV5a E1/E2 complexes and sE2.** Binding to HCV5a (A) E1/E2 complexes and (B) sE2 in ELISA was tested for pooled purified serum IgG from 9 mice immunized 4 times with HCV5aHi (Fig. 2B) and 3 mice immunized with OVA (Fig. S2) using AddaVax. Furthermore, IgG purified from pooled sera from 9 mice following 2 and 3 IMM was tested (Fig. 2F). Datapoints are means of duplicates with SEM. E1/E2, HCV envelope glycoprotein heterodimer; IMM, immunizations; OD, optical density; OVA, ovalbumin; sE2, soluble HCV envelope glycoprotein E2.

virus.<sup>4,8,9,24,49,50</sup> These observations are in line with the hypothesis that conformational epitopes targeted by potent cross-genotype neutralizing mAbs, such as AR3A and AR4A, are conserved among HCV isolates, and that different neutralization sensitivity is mainly caused by isolate-specific epitope protection by HVR1- and glycan-dependent closed envelope protein conformational states.<sup>4,7,12,51</sup>

The highest IgG concentrations used in *in vitro* neutralization assays, yielding close to complete neutralization, were ~10-fold lower than mean IgG concentrations in human serum. IC<sub>50</sub> were comparable to those achieved in previous immunogenicity studies using whole inactivated genotype 2a HCV, however, formulated with non-licensed adjuvants; in these studies cross-neutralization of genotype 1a/b and 3a was observed, while genotypes 4-6 were not tested.<sup>31,33</sup> Moreover, IC<sub>50</sub> were in the range of IC<sub>50</sub> of IgG in chimpanzees protected from HCV challenge following vaccination with the gpE1/gpE2 vaccine<sup>24,25</sup> and at least comparable to that of IgG induced by vaccines based on sE2 in small animal models.<sup>14,52-54</sup>

Future development of an inactivated HCV vaccine candidate will be facilitated by development of optimized serum-free bioreactor-based upstream and downstream processes.<sup>38,40,55</sup> Future immunogenicity studies should aim at defining an optimal immunization schedule and HCV antigen dose. For production of whole virus vaccines, amounts of viral particles required to achieve a given immune response are of interest. In this study, antigen doses were defined by infectious unit equivalents, as infectivity titrations were used to monitor viral processing, providing evidence for the presence of intact particles, and as reported by others.<sup>56,57</sup> This might not allow for an optimal comparison of amounts of particles for viruses showing different specific infectivities. Therefore, we retrospectively determined amounts of HCV core and genome copies in vaccine preparations. This analysis suggested that 3- to 6-fold higher amounts of viral particles might have been contained in the HCV5aHi(ΔHVR1) vaccine compared to the HCV5aHi and HCV5aHi(T385) vaccines (Table S1). This could explain the slightly higher E1/E2 binding by HCV5aHi(ΔHVR1)-induced IgG compared to IgG induced by the other 2 viruses. Based on core determinations, vaccine doses used in this study were comparable to doses used in previous inactivated HCV

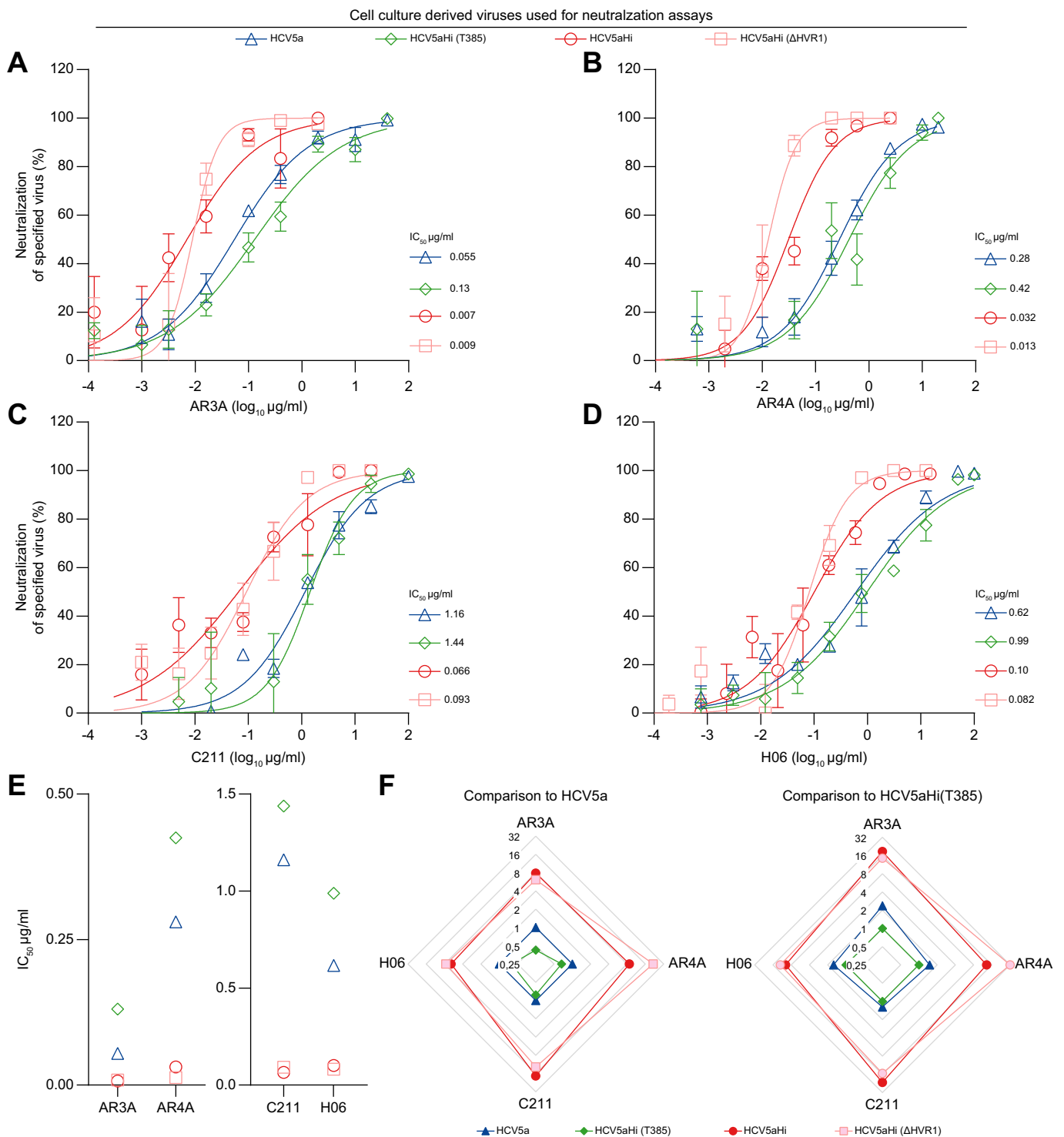
vaccine studies.<sup>31,33</sup> A future research focus should be establishment of assays to quantify the amount of HCV envelope proteins, being the main antigenic proteins, in vaccine preparations.

An important achievement of our study is the development of a whole inactivated HCV vaccine candidate employing an analogue of a licensed adjuvant, facilitating its use in humans. In line with previous findings, MF-59 analogue AddaVax, also used in the gpE1/gpE2 vaccine,<sup>29</sup> was superior in inducing binding Abs and nAbs compared to Alum used in most human vaccines and the experimental golden standard Freund's adjuvant.<sup>56,58</sup> Further, whole inactivated genotype 2a HCV formulated with Alum yielded nAbs showing <20% neutralization.<sup>33</sup> Nevertheless, comparatively high neutralization sensitivity of our vaccine antigens might have contributed to comparatively high immunogenicity. Thus, compared to the genotype 2a (isolate J6) HCV,<sup>31,33</sup> HCV5aHi showed at least 200-fold higher sensitivity to the human antibodies used for characterization of HCV neutralization sensitivity (unpublished results).<sup>12</sup>

For other viruses, increased neutralization epitope exposure resulted in enhanced induction of nAbs following vaccination.<sup>59-61</sup> According to assays applied in this study, increased HCV neutralization epitope exposure mediated by deletion of HVR1 did not result in increased immunogenicity, while modification of an O-linked glycosylation site in HCV5aHi resulted in somewhat increased induction of nAbs. While this question had not been addressed using whole HCV vaccines that would be expected to show close to native envelope conformations, similar findings were reported using envelope protein subunit vaccines. Thus, gpE1/gpE2 heterodimers<sup>50</sup> or sE2<sup>14</sup> without HVR1, with or without an additional modification of a glycosylation site, enzymatically deglycosylated sE2,<sup>14</sup> or insect cell produced sE2<sup>52,54</sup> showed no or slightly increased immunogenicity. Somewhat increased immunogenicity was observed for sE2 lacking all 3 variable regions.<sup>53</sup>

Future vaccine studies with HCV showing greater differences in neutralization sensitivity than the viruses used in this study might further clarify the potential of envelope protein engineering to increase immunogenicity. Compared to the reference virus HCV5a, both HCV5aHi and HCV5aHi(ΔHVR1) showed up to ~20-fold increased neutralization sensitivity,

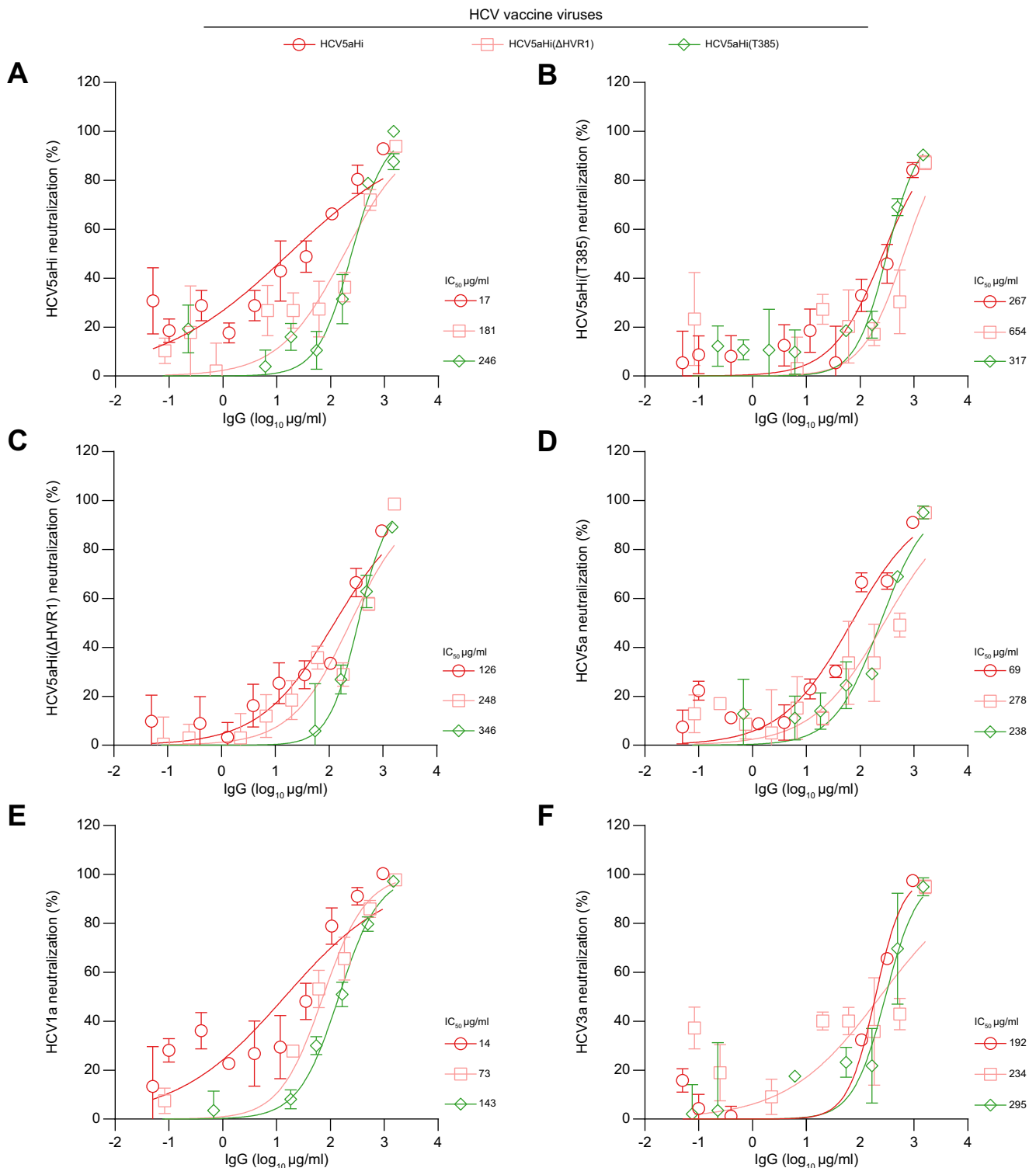




**Fig. 4. HCV with different envelope proteins showed differential sensitivity to neutralizing antibodies.** HCV5a, HCV5aHi(T385), HCV5aHi and HCV5aHi(ΔHVR1) were used in neutralization assays with human monoclonal antibodies – (A) AR3A<sup>45</sup> and (B) AR4A<sup>46</sup> – and human polyclonal IgG (C) C211<sup>12</sup> and (D) H06.<sup>47</sup> Datapoints are means of triplicates with SEM. (E) IC<sub>50</sub> from experiments (A-D). (F) Fold differences in IC<sub>50</sub> against specified viruses in comparison to HCV5a (left) or HCV5aHi(T385) (right) shown in log<sub>2</sub> radar plots. HVR1, hypervariable region 1; IC<sub>50</sub>, half maximal inhibitory concentration.

while for other HCV recombinants, HVR1 deletion and modifications of glycosylation sites increased neutralization sensitivity by up to 5 orders of magnitude.<sup>4,12,14,51</sup> This phenomenon is likely explained by the inherent high neutralization

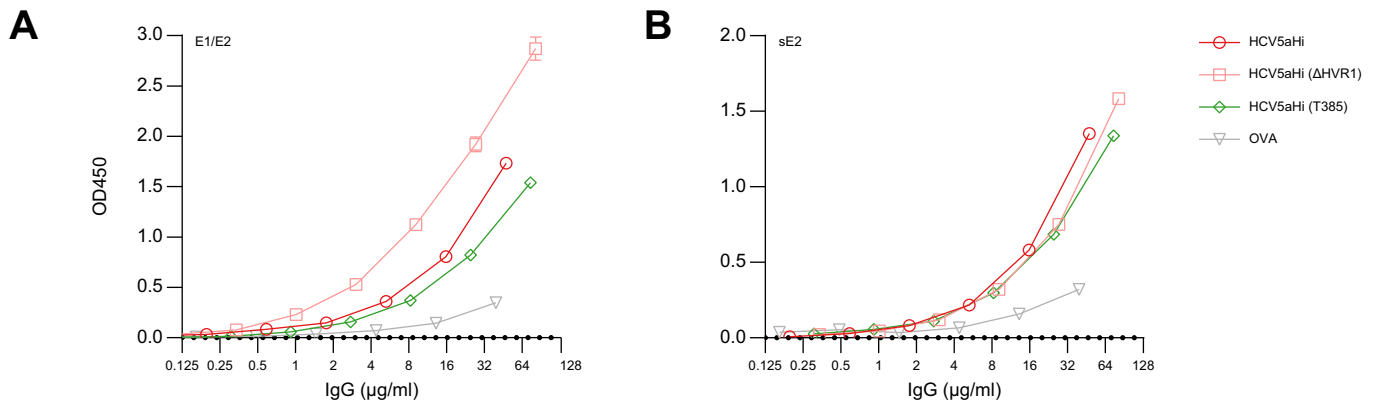
sensitivity of the HCV5a (strain SA13) reference virus, suggesting a predominantly open envelope protein conformational state with relatively high epitope exposure, which could only be somewhat increased by envelope modifications.<sup>4,12,51</sup>



**Fig. 5. Immunization of BALB/c mice with HCV5aHi variants resulted in induction of similar neutralizing antibody responses.** Pooled purified serum IgG from “n” mice immunized 4 times with an equivalent of 7.5 log<sub>10</sub> FFUs of HCV5aHi (n = 9, Fig. 2B), HCV5aHi(T385) (n = 3), or HCV5aHi(ΔHVR1) (n = 2) in parallel using AddaVax were used to neutralize (A) HCV5aHi, (B) HCV5aHi(T385), (C) HCV5aHi(ΔHVR1), (D) reference virus HCV5a, (E) HCV1a or (F) HCV3a. Datapoints are means of triplicates with SEM. FFUs, focus-forming units; HVR1, hypervariable region 1; IC<sub>50</sub>, half maximal inhibitory concentration.

In conclusion, using an analogue of the licensed adjuvant MF-59 and whole inactivated cell-culture-derived HCV, we have developed an attractive HCV vaccine approach for further pre-

clinical development. While a recently tested immunogenic T cell-based vaccine did not protect against chronic HCV infection in a phase I/II study,<sup>27</sup> we here describe a vaccine platform



**Fig. 6. Immunization with HCV5aHi variants resulted in IgG with similar binding to HCV5a E1/E2 complexes and sE2.** Binding to HCV5a (A) E1/E2 complexes and (B) sE2 in ELISA was tested for pooled purified serum IgG from mice immunized with HCV5aHi (Fig. 2B), HCV5aHi(T385) (Fig. 5), HCV5aHi(ΔHVR1) (Fig. 5), or OVA (Fig. S2) using AddaVax. Data from HCV5aHi and OVA immunizations are reproduced from Fig. 3 for comparison. Datapoints are means of duplicates with SEM. E1/E2, HCV envelope glycoprotein heterodimer; OD, optical density; OVA, ovalbumin; sE2, soluble HCV envelope glycoprotein E2.

inducing potent nAbs. In the future, following optimization of the vaccine preparation processes, clinical studies will be needed to elucidate if this vaccine approach will confer protection against chronic HCV infection.

**Abbreviations**

Alum, aluminium hydroxide; E1, HCV envelope glycoprotein E1; E2, HCV envelope glycoprotein E2; E1/E2, HCV envelope glycoprotein heterodimer; FFUs, focus-forming units; gp, glycoprotein; HCV, hepatitis C virus; HVR1, hypervariable region 1; IC<sub>50</sub>, half maximal inhibitory concentration; mAbs, monoclonal antibodies; MPLA, monophosphoryl lipid A; nAbs, neutralizing antibodies; sE2, soluble HCV envelope glycoprotein E2.

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**Conflict of interest**

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

**Author contributions**

AFP, SF, AO, JPC, JB and JG designed the study. AFP, SF, AO, GPA, EHA, CKM, TBJ, HK and JPC performed the experiments. AFP, SF, AO, GPA, EHA, CKM, TBJ, ML, JP, JPC, JB and JMG analyzed and interpreted the data. AFP and JMG wrote the manuscript. JP, JPC, JB and JMG supervised the study. All authors reviewed the manuscript.

**Data availability statement**

All data needed to evaluate the conclusions in the paper are present in the paper and/or the [supplementary information](#).

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**Supplementary data**

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2021.12.026>.

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*Author names in bold designate shared co-first authorship*

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