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Inactivated genotype 1a, 2a and 3a HCV vaccine candidates induced broadly neutralising antibodies in mice

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ABSTRACT

Objective A prophylactic vaccine is needed to control the HCV epidemic, with genotypes 1–3 causing >80% of worldwide infections. Vaccine development is hampered by HCV heterogeneity, viral escape including protection of conserved neutralising epitopes and suboptimal efficacy of HCV cell culture systems. We developed cell culture-based inactivated genotype 1–3 HCV vaccine candidates to present natively folded envelope proteins to elicit neutralising antibodies.

Design High-yield genotype 1a, 2a and 3a HCV were developed by serial passage of TNcc, J6cc and DBN3acc in Huh7.5 cells and engineering of acquired mutations detected by next-generation sequencing. Neutralising epitope exposure was determined in cell-based neutralisation assays using human monoclonal antibodies AR3A and AR4A, and polyclonal antibody C211. BALB/c mice were immunised with processed and inactivated genotype 1a, 2a or 3a viruses using AddaVax, a homologue of the licenced adjuvant MF-59. Purified mouse and patient serum IgG were assayed for neutralisation capacity; mouse IgG and immune-sera were assayed for E1/E2 binding.

Results Compared with the original viruses, high-yield viruses had up to ~1000 fold increased infectivity titres (peak titres: 6–7 log10 focus-forming units (FFU)/mL) and up to ~2470 fold increased exposure of conserved neutralising epitopes. Vaccine-induced IgG broadly neutralised genotype 1–6 HCV (EC50: 30–193 µg/mL; mean 71 µg/mL), compared favourably with IgG from chronically infected patients, and bound genotype 1–3 E1/E2; immune-sera endpoint titres reached up to 32 000.

Conclusion High-yield genotype 1–3 HCV could be developed as basis for inactivated vaccine candidates inducing broadly neutralising antibodies in mice supporting further preclinical development.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ A prophylactic vaccine is required to control the ongoing HCV epidemic.
⇒ HCV shows large genetic heterogeneity, with genotypes 1, 2 and 3 causing >80% of infections worldwide.
⇒ Neutralising antibodies are key immune correlates for protection in vaccine settings and can protect against chronic HCV infection.
⇒ Such protection is associated with antibodies broadly neutralising different HCV genotypes and targeting conserved neutralising epitopes.
⇒ For HCV, a whole virus inactivated vaccine strategy is attractive because of its ability to present the HCV envelope glycoprotein complex in its native conformation and to induce neutralising antibodies.
⇒ Development of such a vaccine is hampered by low HCV yields in currently available cell culture systems.

WHAT THIS STUDY ADDS

⇒ Based on previously developed cell culture infectious HCV recombinants, we developed high-yield genotype 1, 2 and 3 HCV recombinants growing to high titres in cell culture.
⇒ Compared with the original viruses, high-yield viruses showed increased exposure of conserved epitopes targeted by neutralising antibodies important for protection against HCV infection.
⇒ Vaccine candidates based on inactivated genotype 1, 2 or 3 HCV, respectively, and an adjuvant analogue to the licenced MF-59 adjuvant had the capacity to induce antibodies broadly neutralising HCV of all major genotypes with recognised epidemiological importance.

INTRODUCTION

Hepatitis C virus (HCV) is a highly prevalent, blood-borne enveloped positive-sense single strand RNA virus of the *Flaviviridae* family.[1] In contrast to the non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B), the structural proteins, capsid protein core and envelope glycoproteins E1 and E2 constitute the viral particle. The E1/E2 heterodimer is the main target for neutralising antibodies (nAb).[2] Among eight major genotypes differing in ~30% of their sequence, genotypes 1, 2 and 3 cause >80% of infections worldwide. Genotypes 4, 5 and 6 show a more restricted geographic

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study is a milestone in the preclinical development of an inactivated HCV vaccine candidate.
⇒ Following finalisation of preclinical development, including optimisation of vaccine production conditions, the identified vaccine candidates could enter clinical development.
⇒ A prophylactic HCV vaccine is an essential tool to prevent the approximately 1.5 million new infections and 290 000 deaths attributed to this virus every year.

localisation in the Middle East/Africa, South Africa and South-east Asia, respectively, while genotypes 7 and 8 were reported in few individuals.[1] Subtypes (a, b, c, etc) differ in ~20% of their sequence.

Each year, ~1.5 million new infections occur. Of these, ~80% proceed to chronic infection resulting in a total of ~58 million chronically infected individuals with increased risk for liver cirrhosis and hepatocellular carcinoma, causing ~290 000 deaths annually.[3] So far antiviral treatment did not have a major impact on this epidemic, mainly due to lack of symptoms prior to severe liver damage, lack of screening programmes and high treatment costs in many countries.[1 3] A prophylactic vaccine will be required to reach the WHO aim to eliminate hepatitis as a major public health threat.

During natural infection, T and B cells appear to contribute to protective immunity. A T cell vaccine using a viral vector approach did not protect against chronic infection in chimpanzees and humans.[4 5] In contrast, a B cell vaccine based on E1/E2 glycoprotein heterodimers showed protective effects in chimpanzees and induced nAb in non-human primates, chimpanzees and humans, even though nAb were induced in <50% of human vaccine recipients with limited capacity to neutralise different HCV genotypes.[6–10] Induction of nAb correlates with efficacy of other viral vaccines.[11 12] Moreover, during natural HCV infections induction of broadly nAb mediated protection.[13–15] Protective nAb targeted conserved conformational neutralising epitopes in E2 and E1/E2 localising to antigenic regions 3 and 4 (AR3 and AR4)[2 14–16] also targets of well-defined human monoclonal antibodies (mAb).[17 18] For efficacy against different HCV genotypes, a future vaccine should target such epitopes which, however, appear to be hidden by closed envelope protein conformational states (E1/E2 states).[19 20] Another approach might be a multivalent vaccine based on different viral variants.

For HCV, in mice whole virus vaccines showed a higher capacity to induce nAb than protein-based vaccines,[21] presumably due to higher density and more native conformation of the envelope proteins. Indeed, many licenced viral vaccines are based on whole viruses or virus-like particles.[12 22] However, application of this technology in HCV vaccine development was hampered by relatively low viral yields in cell culture systems for production of HCV. In 2005, the first systems were developed based on a single genotype 2a isolate (JFH1),[23 24] followed by JFH1-based systems expressing genotype specific proteins[25] and full-length systems not depending on JFH1.[26–28] These systems typically yield 10^3–10^5 infectious viruses per mL, considered suboptimal for vaccine development. Nevertheless, proof of concept for immunogenicity of a JFH1-based genotype 2a recombinant was obtained in mice and non-human primates.[21 29] However, efficient nAb were only induced with adjuvants not licenced for human use.

Aims of this study were to: (1) develop high-yield culture systems for production of genotype 1a, 2a and 3a HCV, (2) characterise neutralising epitope exposure of high-yield HCV focusing on conserved epitopes associated with protection; and (3) obtain proof of concept for immunogenicity of each high-yield HCV in mice using an adjuvant applicable for human use focusing on detection of antibodies broadly neutralising genotype 1–6 HCV.

MATERIAL AND METHODS

Most sections are further detailed in online supplemental materials.

HCV recombinants

Original TNcc, J6cc and DBN3acc recombinants were developed previously.[26–28] High-yield HI-recombinants were engineered using subclones of cell culture derived reverse transcription PCR (RT-PCR) amplicons and In-Fusion technology. Recombinants with genotype(isolate) 1a(TN), 1b(J4), 2a(J6), 2b(J8), 3a(S52), 3a(DBN), 4a(ED43), 5a(SA13) and 6a(HK6a)[24 25 30] specific core-NS2 and remaining sequences of genotype 2a isolate JFH1 were used in in vitro HCV neutralisation assays.

Cells

Human hepatoma Huh7.5 cells were used for propagation of HCV. Human embryonic kidney HEK293 cells were used for production of HCV E1/E2 complexes.

Transfection of HCV in vitro RNA transcripts in Huh7.5 cells

Transcripts were produced using T7 RNA polymerase (Promega); transfections were done using Lipofectamine2000 (Invitrogen).[25]

Infection of Huh7.5 cells with HCV

Cells were inoculated with supernatants derived from transfection experiments at the peak of infection.[25]

Serial passage of HCV in Huh7.5 cells

Cells were inoculated with culture supernatant derived from the previous passage at the peak of infection.[31]

Generation of virus stocks in Huh7.5 cells

Cells were inoculated with culture supernatants derived at the peak of infection. Supernatants collected around the peak of infection were pooled. For virus stocks used in neutralisation assays, the HCV envelope protein sequence was confirmed by Sanger sequencing. For virus seed stocks for vaccine virus production, the complete HCV open reading frame (ORF) was analysed by next-generation sequencing (NGS).

Immunostaining of HCV antigens in cell culture

Percentage of HCV infected cells was monitored by immunostaining using primary antibodies monoclonal anti-HCV core antibody C7-50 (EnzoLifeSciences, Farmingdale, New York, USA) diluted 1:5000 and monoclonal anti-HCV NS5A antibody 9E10[24] diluted 1:5000 as well as secondary antibody Alexa Fluor 488 goat antimouse IgG (H+L) (Invitrogen) diluted 1:500.[25]

Determination of HCV infectivity titres

HCV infectivity titres in culture supernatants were determined as focus-forming units (FFU)/mL by titration on 96-well plates and subsequent immunostaining using primary antibodies anti-HCV core antibody C7-50 (EnzoLifeSciences) diluted 1:1000 and

anti-HCV NS5A antibody 9E10 diluted 1:3000 as well as secondary antibody ECL sheep antimouse IgG diluted 1:500, followed by visualisation and automated counting of FFU.[32]

Sequencing of cell culture derived HCV

HCV RNA was extracted from culture supernatants, and either the complete ORF (serial passage experiments, first passage kinetic experiments and virus seed stocks) or E1/E2 (virus stocks for neutralisation assays) were amplified by RT-PCR using specific primers (online supplemental tables 1 and 2) followed by NGS (ORF amplicons) or Sanger sequencing (E1/E2 amplicons).[32 33]

Subclonal analysis

Selected RT-PCR ORF amplicons were subcloned using the TOPO-XL Cloning kit (Invitrogen) followed by Sanger sequencing.

HCV production for vaccine generation

HCV was produced in serum-free medium in 10-layer cell factories.[34 35]

Processing of HCV for vaccine generation

HCV was clarified using 5 µm and 0.65 µm filters and concentrated by tangential flow filtration (TFF) with molecular weight cut-off (MWCO) 500 kDa,[36] followed by two ultracentrifugation steps using Optiprep Density Gradient Medium (Sigma) for formation of three density cushions and a continuous gradient, respectively, separated by an intermediate TFF step (MWCO 500 kDa). Following size exclusion chromatography using Sephadex G-100 (Sigma Aldrich), HCV was UV irradiated with a UVG-54 Handheld UV lamp (240 nm UV, 6 watt) (Analytik Jena).[35]

Immunisation of mice

BALB/c mice aged 6–8 weeks (Taconic Farms, Denmark) were subcutaneously immunised four times every 3 weeks with HCV or ovalbumin (OVA) formulated with adjuvant AddaVax 50%/50% (v/v).

Patient samples

Sera or plasma from patients with chronic hepatitis C (CHC) were collected between May 2011 and August 2021 in biobanks attached to the Danish Database for Hepatitis B and C and the HCV Tandem cohort at the Department of Infectious Diseases, Copenhagen University Hospital-Hvidovre. Patients were ≥18 years, had no previous history of treatment for CHC, no coinfection with human immunodeficiency virus (HIV) or hepatitis B virus and no recent intravenous drug use.

Purification, concentration and quantification of IgG

IgG was purified from mouse serum or patient serum or plasma with the Amicon Pro Affinity Concentration Kit Protein G with 50 kDa Amicon Ultra-0.5 Device (Merck Millipore) and concentrated with the Vivaspin 500, 30 000 MWCO (GE Lifescience) kit. Mouse and patient IgG was quantified with the IgG (TOTAL) mouse uncoated ELISA Kit (ThermoFisher) and the Cedex Bio Analyzer (Roche), respectively.

In vitro neutralisation assay

HCV neutralisation with mAb AR3A and AR4A[17 18] and polyclonal antibody C211[19] was done in a volume of 100 µL followed by inoculation of Huh7.5 cells plated on 96-well plates, subsequently subjected to immunostaining of HCV antigen and automated FFU counting.[31 34] HCV neutralisation with purified mouse or patient IgG was done similarly in a volume of 10 µL.[37] Percentage of neutralisation was calculated as 100-[100×(FFU count in experimental wells)/(mean FFU count in virus only wells)].

HCV E1/E2 complex ELISA

E1/E2 complexes were obtained from lysates of HEK293 cells transfected with E1/E2 expression plasmids. Binding of mouse IgG or immune-sera to E1/E2 complexes was evaluated by ELISA using secondary antibody ECL sheep antimouse IgG horseradish-peroxidase linked whole antibody (GE Healthcare) diluted 1:1000. Positive controls were mAb AP33[38] and H77.39.[39] Negative control was secondary antibody only.

Patient and public involvement statement

Patients or the public were not involved in design, conduct, reporting or dissemination plans of our research.

RESULTS

Generation of high-yield genotype 1a, 2a and 3a polyclonal HCV

To develop high-yield culture systems, we serially passaged full-length TNcc (genotype 1a),[26] J6cc (genotype 2a)[27] and DBN3acc (genotype 3a)[28] HCV recombinants in Huh7.5 hepatoma cells until peak HCV infectivity titres showed a plateau at ~6 log10 FFU/mL (figure 1). An additional criterion for termination of passaging was detection of putative cell culture adaptive substitutions in >80% of the viral population as determined by NGS. For an initial TNcc passage line, NGS suggested viral quasispecies populations with mutations at a prevalence <80%, spurring a later passage line with a total of 41 viral passages. For J6cc and DBN3acc, 43 and 22 passages were done, respectively. Compared with the initial passages, late passages showed an increase in HCV infectivity titres of up to 2.6 log10 for TNcc, 1.7 log10 for J6cc and 1.3 log10 for DBN3acc.

Genetic changes acquired by high-yield polyclonal genotype 1a, 2a and 3a HCV

To identify genetic correlates of high-yield phenotypes, we carried out NGS of the entire ORF of polyclonal passage (PP) viruses. For TNcc-PP-10 and TNcc-PP-18, derived from passage 10 and 18 of the initial passage line, NGS suggested a viral quasispecies population with most coding nucleotide changes present in <80% of viral genomes (online supplemental table 3). In contrast, for TNcc-PP-38.1 derived from the later passage line, as well as J6cc-PP-35 and DBNcc-PP-16, a more homogeneous viral population was found with most coding nucleotide changes present in ≥80% of viral genomes (figure 2 and online supplemental tables 4–6). TNcc-PP-38.1, J6cc-PP-35 and DBNcc-PP-16 had 17, 17 and 7 coding changes in ≥80% of viral genomes, among which 4, 3 and 1 localised to the envelope proteins, respectively. Of note, DBN3acc already harboured five coding changes in the envelope proteins compared with the consensus HCV sequence in the infected patient this recombinant was based on, while TNcc and J6cc did not contain coding changes in the envelope proteins.[26–28] Subclonal analysis of these PP-viruses and phylogenetic analysis of TNcc-PP subclones reflected

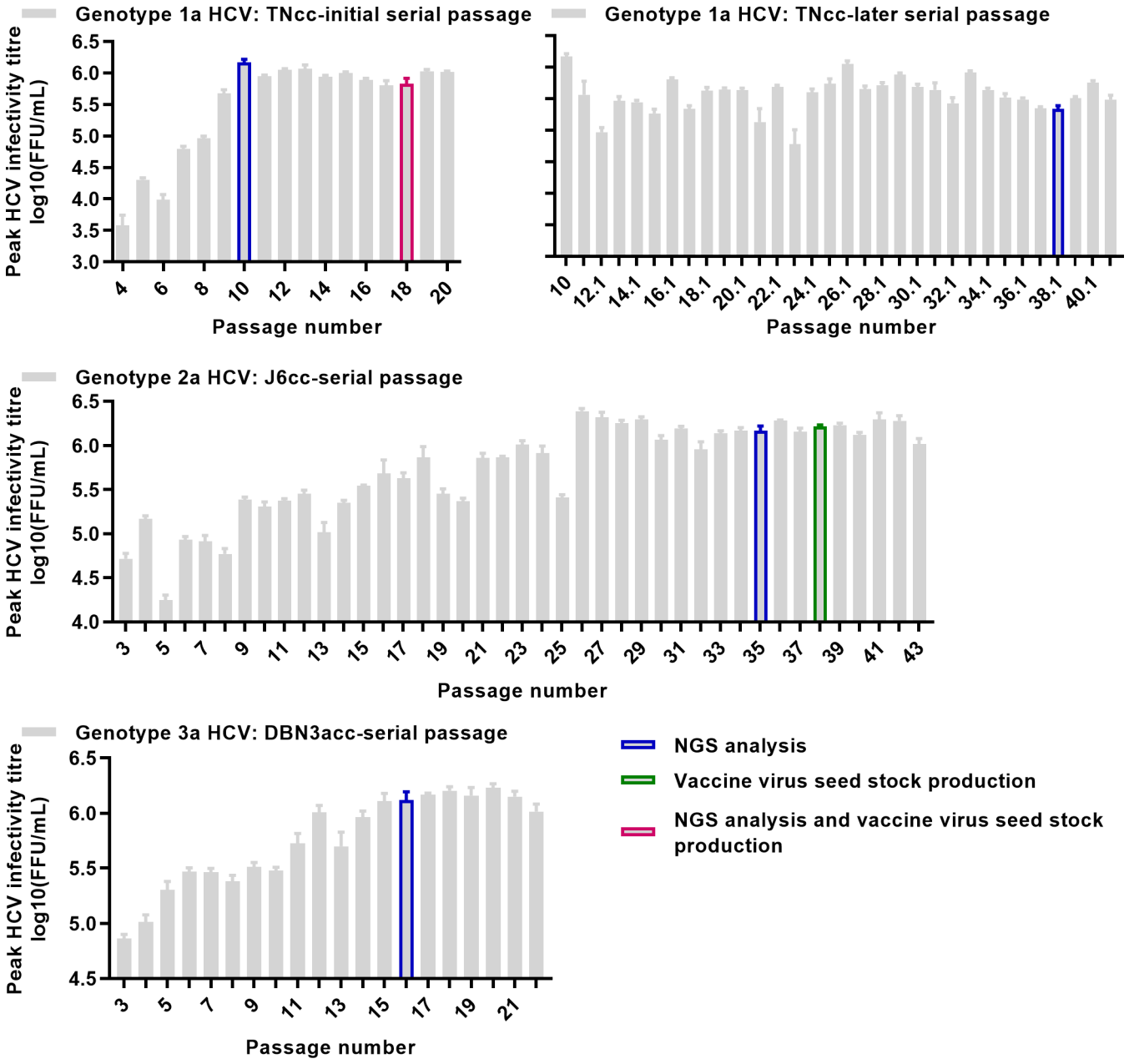


Figure 1 In vitro serial passage resulted in high-yield polyclonal genotype 1a, 2a and 3a HCV. Huh7.5 cells were inoculated with cell culture supernatant containing the specified viruses derived from the previous passage culture at the peak of infection, as determined by immunostaining. Every 2–3 days, passage cultures were split, and % HCV antigen positive cells and HCV infectivity titres were determined by immunostaining and infectivity titration, respectively. Peak supernatant HCV infectivity titres determined for the specified passages are means of three replicates with standard deviations (SD). For TNcc, the later passage line was inoculated with passage 10 virus from an initial passage line. Blue frames, passage subjected to NGS. Green frame, passage used for production of genotype 2a seed stock. Red frame, passage subjected to NGS and used for production of genotype 1a seed stock. The genotype 3a seed stock was based on the later developed DBNcc-HI recombinant. FFU, focus-forming unit; NGS, next-generation sequencing.

NGS results (online supplementary results, online supplemental figure 1, online supplemental tables 7–9).

Engineering of high-yield genotype 1a, 2a and 3a HCV recombinants

Based on genetic analysis of high-yield PP-viruses, we engineered high-yield (HI)-recombinants: TNcc-HI-18A and TNcc-HI-18B reflected the two main populations in the initial passage line (online supplemental figure 1 and online supplemental table 3). TNcc-HI reflecting TNcc-PP-38.1 in the later passage line, J6cc-HI reflecting J6cc-PP-35 and DBNcc-HI reflecting DBNcc-PP-16 harboured coding nucleotide changes with >80% frequency in NGS in combinations confirmed by subclonal analysis; as an exception, TNcc-HI also harboured G32S found at 48% frequency (unless otherwise indicated, all amino acid position numbers relate to the H77 reference polyprotein, Genbank accession number AF009606) (figure 2 and online supplemental tables 4–9).

Compared with the original recombinants, all HI-recombinants showed increased fitness in transfection and first passage

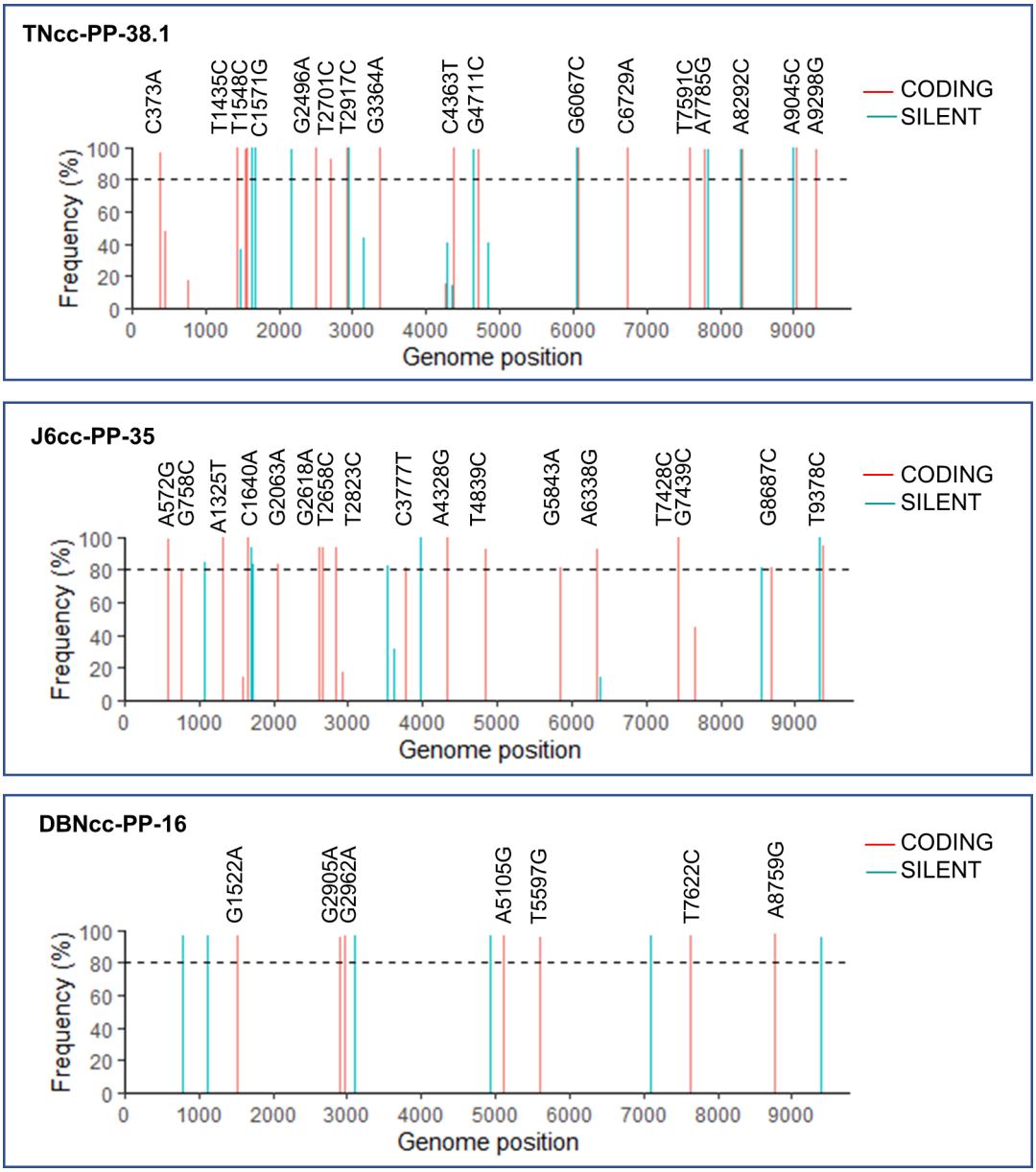


Figure 2 Nucleotide changes in serially passaged genotype 1a, 2a and 3a HCV. For TNcc-PP-38.1, J6cc-PP-35 and DBNcc-PP-16, coding and silent changes with allele frequency >10% determined by NGS of the complete ORF are shown. Coding changes with frequency of ≥80% are specified above bars. Genome positions relate to TNcc,[26] J6cc[27] and DBN3acc[28](GenBank accession numbers JX993348, JQ745650 and KX280714, respectively). For genome positions relating to the H77 (AF009606) reference genome and encoded amino acid changes, see online supplemental tables 4–6. NGS, next-generation sequencing; ORF, open reading frame.

infection kinetic experiments, with accelerated spread kinetics, monitored by determination of the % infected cells and of HCV infectivity titres and with increased peak infectivity titres. TNcc-HI-18A and TNcc-HI-18B peak infectivity titres were only approaching 5 log10 FFU/mL and thus fell short of the target of 6 log10 FFU/mL (online supplemental figure 2). In contrast, in transfection/infection experiments, TNcc-HI, J6cc-HI and DBNcc-HI yielded peak infectivity titres of 5.8/6.0, 6.1/6.8 and 6.4/7.0 log10 FFU/mL, respectively, while the respective original recombinants yielded 3.0/3.5, 3.5/3.4 and 5.3/5.3 log10 FFU/mL, respectively (figure 3). In infection experiments, infectivity titres of HI-viruses were comparable with those of PP-viruses (figure 3B). HI-recombinants were genetically stable following first viral passage (no acquisition of substitutions with >10% frequency with exception of TNcc-HI that acquired L179P and S1930Y with 15% and 39% frequency, respectively).

High-yield genotype 1a, 2a and 3a recombinants showed increased exposure of neutralising epitopes

Compared with the respective recombinants with in vivo derived envelope protein sequences without cell culture adaptive substitutions,[24 30] based on determined EC50 values, HI-recombinants showed 12-fold to 2472-fold increased sensitivity to neutralisation by human-derived mAb AR3A[17] and AR4A,[18] targeting conserved conformational epitopes in E2 and E1/E2 associated with protection, respectively, and by polyclonal IgG C211[19] derived from a patient chronically infected with genotype 1a (figure 4). In detail, TNcc-HI

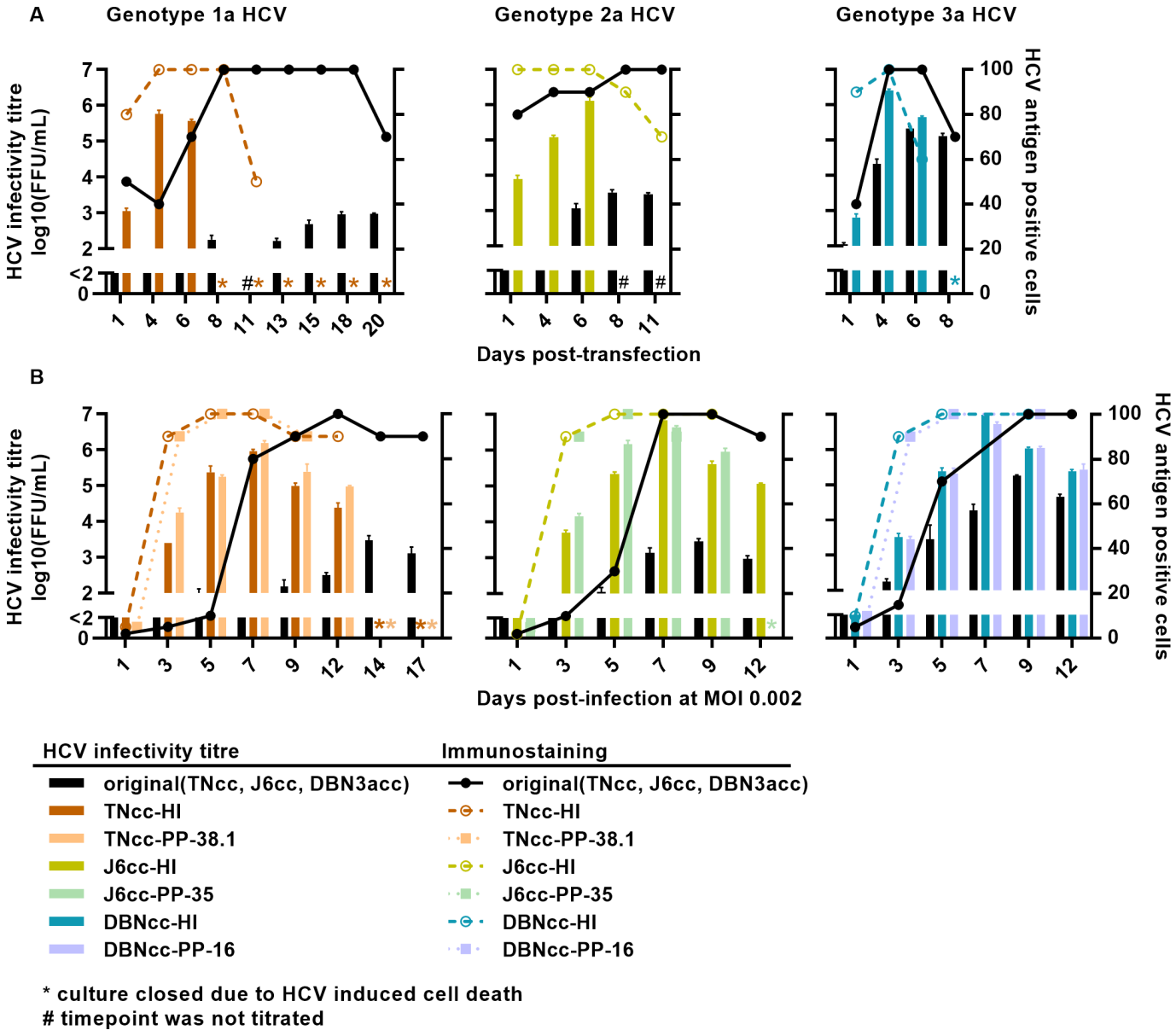
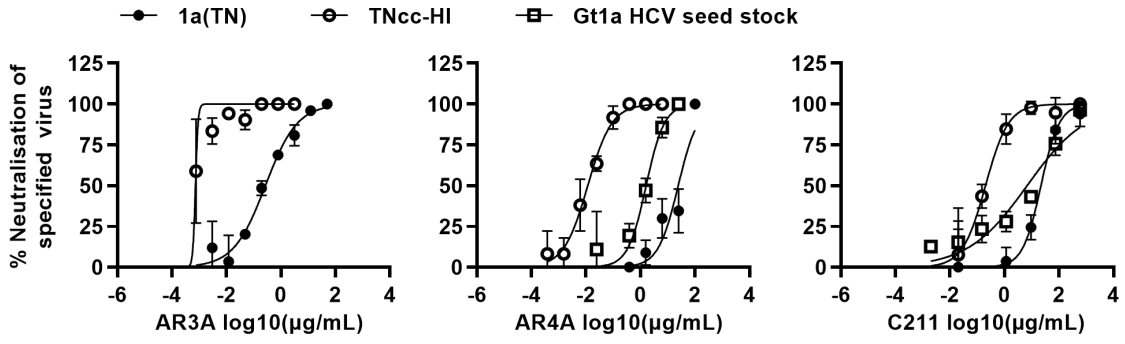


Figure 3 Engineered high-yield genotype 1a, 2a and 3a HCV recombinants had increased viral fitness in cell culture. (A) Specified recombinants were transfected into Huh7.5 cells using the same amount of HCV RNA in vitro transcripts for recombinants directly compared in each graph. (B) In first passage kinetic experiments, cells were infected at multiplicity of infection of 0.002 with original and HI-viruses using supernatants derived from the transfection experiment when peak infectivity titres were observed; polyclonal passage viruses were included for comparison. (A and B) Every 2–3 days, passage cultures were split, and % HCV antigen positive cells and HCV infectivity titres were determined by immunostaining and infectivity titration, respectively. HCV infectivity titres are means of three replicates with SD. FFU, focus-forming unit; MOI, multiplicity of infection.

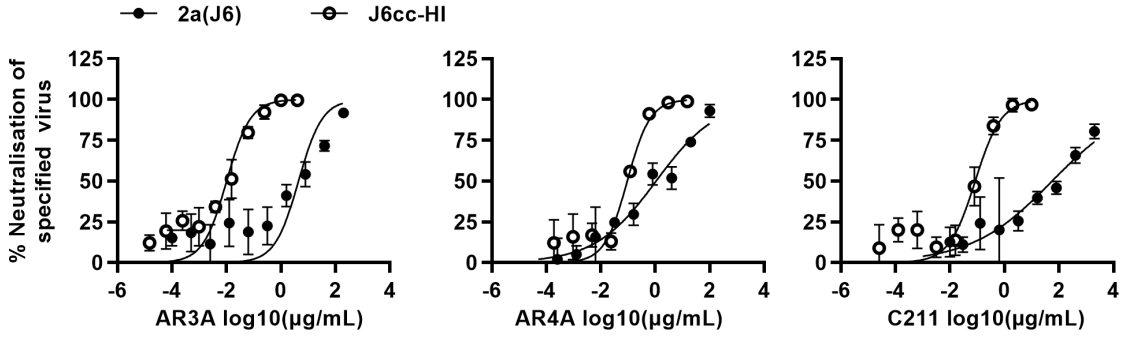
showed 300-fold, 2400-fold and 110-fold increased neutralisation sensitivity to AR3A, AR4A and C211, respectively, while a genotype 1a HCV seed stock derived from TN-PP-18 showed 3-fold, 15-fold and 3.2-fold increased sensitivity. J6cc-HI showed 440-fold, 12-fold and 633-fold increased neutralisation sensitivity. DBNcc-HI showed 1250-fold, 220-fold and 2472-fold, while DBN3acc showed 167-fold, 22-fold and 88-fold increased neutralisation sensitivity. For viruses with in vivo derived TN, J6 and DBN envelope protein sequences, determined half maximal effective concentrations (EC50) were in line with previous results.[20 40 41] Thus, HI-viruses showed greatly increased exposure of conserved conformational neutralising epitopes associated with protection against chronic HCV infection.

Generation of vaccine candidates based on inactivated high-yield genotype 1a, 2a and 3a recombinants

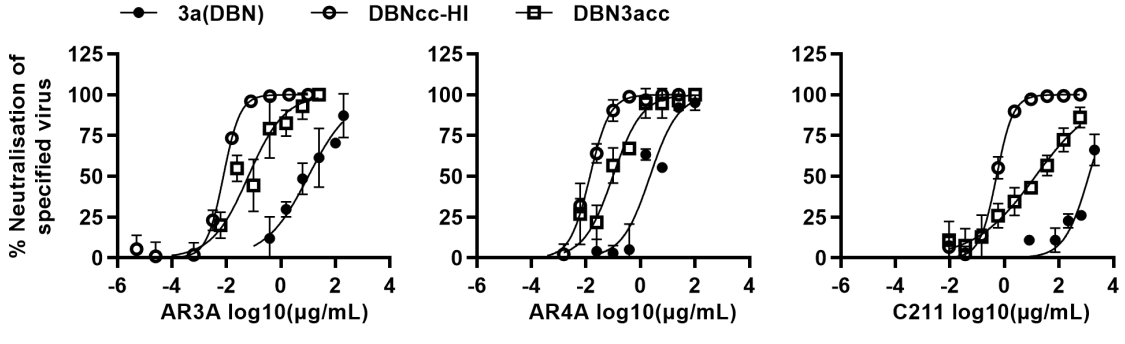
To produce viruses for vaccine experiments, HCV seed stocks were generated by inoculation of Huh7.5 cells with polyclonal virus preparation TNcc-PP-18, polyclonal virus preparation J6cc-PP-38 or a first viral passage DBNcc-HI virus, available on initiation of vaccine studies. Sequence confirmed genotype 1a, 2a and 3a HCV seed stocks with infectivity titres of 4.8, 6.2 and 6.4 log10 FFU/mL, respectively (online supplemental tables 3, 5 and 6), were used to inoculate Huh7.5 cells for HCV production in 10-layer cell factories, resulting in a total volume of 16 L HCV containing supernatant per virus (online supplemental figure 3). Supernatants were subjected to downstream processing, involving an initial filter clarification followed by two TFF steps,



	AR3A		AR4A		C211	
	EC50	fold	EC50	fold	EC50	fold
1a(TN)	0.3	na	24	na	22	na
TNcc-HI	0.001	300	0.01	2400	0.2	110
Gt1a HCV seed stock	0.1	3	1.6	15	6.8	3.2



	AR3A		AR4A		C211	
	EC50	fold	EC50	fold	EC50	fold
2a(J6)	4.4	na	1.1	na	57	na
J6cc-HI	0.01	440	0.09	12	0.09	633



	AR3A		AR4A		C211	
	EC50	fold	EC50	fold	EC50	fold
3a(DBN)	10	na	2.2	na	1236	na
DBNcc-HI	0.008	1250	0.01	220	0.5	2472
DBN3acc	0.06	167	0.1	22	14	8.8

Figure 4 Engineered high-yield genotype 1a, 2a and 3a HCV recombinants showed increased sensitivity to neutralisation by human-derived nAb. Recombinants with in vivo derived genotype(isolate) 1a(TN),[30] 2a(J6)[24] and 3a(DBN)[30] core-NS2 sequences, as well as TNcc-HI, J6cc-HI, DBNcc-HI, the genotype 1a HCV seed stock and DBN3acc[28] with envelope protein substitutions acquired during in vitro passage were subjected to neutralisation with human mAb AR3A and AR4A,[17 18] and polyclonal antibody C211.[19] Data points are means of three replicates with SD; curves were fitted, and EC50 were calculated with the formula $y=100/(1+10^{(\log10EC50-X)\times hillslope})$ using GraphPad prism. Fold increase in neutralisation sensitivity was calculated as [(EC50 of 1a(TN), 2a(J6) or 3a(DBN) virus with in vivo derived envelope protein sequence)/(EC50 of respective virus with in vitro derived envelope protein substitutions)]. Virus stock envelope protein sequences were confirmed by Sanger sequencing. EC50, half maximal effective concentration; gt, genotype; mAb, monoclonal antibody; nAb, neutralising antibody.

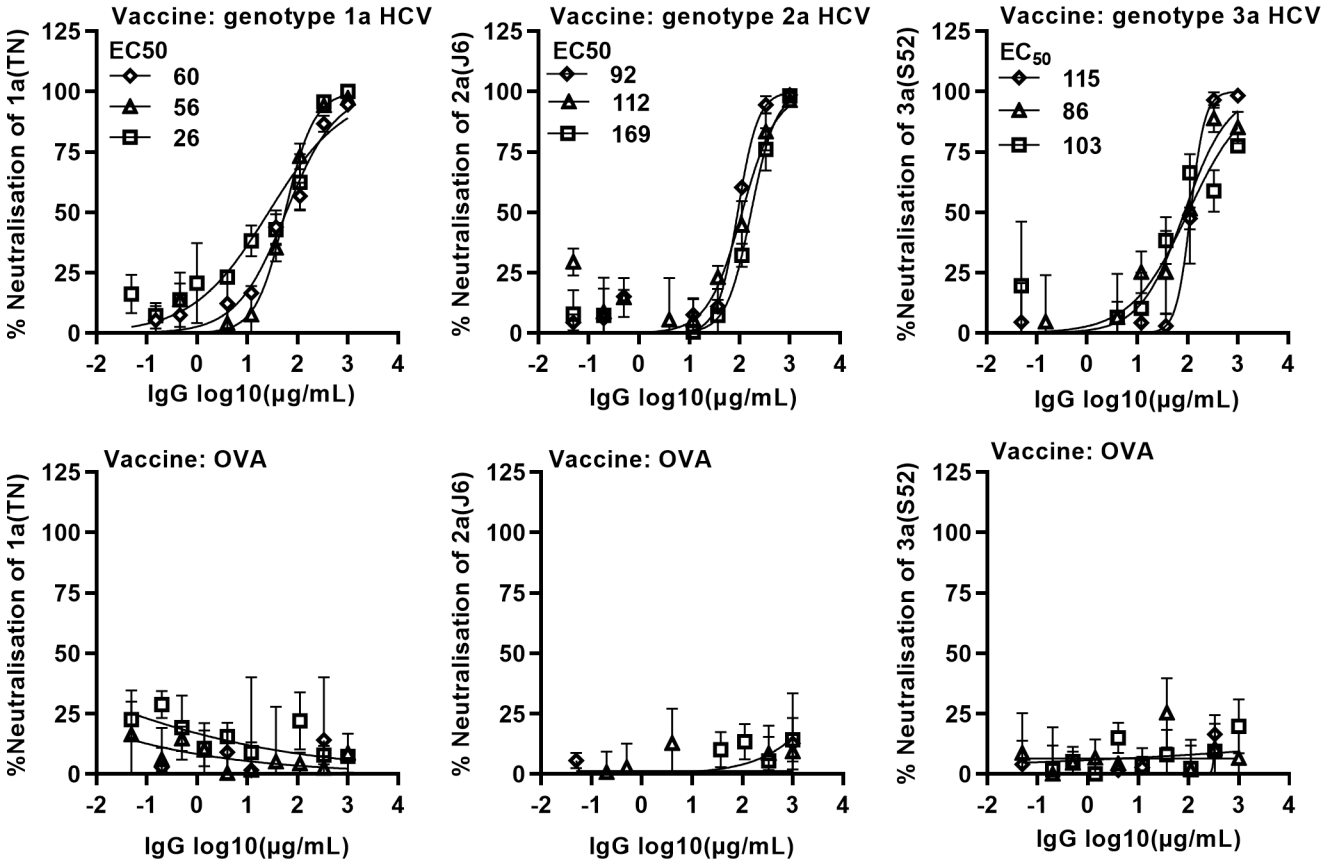


Figure 5 Immunisation with inactivated genotype 1a, 2a or 3a HCV elicited antibodies neutralising cell culture infectious HCV of the same genotype. Groups of three mice were immunised with inactivated genotype 1a, 2a and 3a HCV or OVA formulated with adjuvant AddaVax. Purified serum IgG from individual mice was used to neutralise recombinants containing in vivo derived genotype(isolate) 1a(TN),[30] 2a(J6)[24] and 3a(S52)[25] specific core-NS2. Data points are means of three replicates with SD; curves were fitted, and EC50 were calculated with the formula $y=100/(1+10^{(\log10EC50-X)\times hillslope})$ using GraphPad prism. Each concentration–response curve specified by unique symbols represents data from one animal. Virus stock envelope protein sequences were confirmed by Sanger sequencing. EC50, half maximal effective concentration; nAb, neutralising antibody; OVA, ovalbumin.

cushion ultracentrifugation, another TFF, gradient ultracentrifugation, chromatography and inactivation by UV irradiation (supplementary results in online supplemental file 1 and online supplemental figures 4 and 5).

Immunisation of mice with inactivated genotype 1a, 2a and 3a HCV vaccine candidates elicited broadly neutralising and envelope-protein binding antibodies

Processed inactivated genotype 1a, 2a or 3a HCVcc or control antigen OVA were formulated with the adjuvant AddaVax, an analogue of the adjuvant MF-59, which is licenced for human use, and used for immunisation of BALB/c mice.

Purified serum IgG from individual animals neutralised HCV with in vivo derived envelope protein sequences of the same genotype as used in the respective vaccine in concentration dependent manner, with mean EC50 of 47, 124 and 101 µg/mL for genotype 1a, 2a and 3a HCV vaccines, respectively (figure 5). Close to complete neutralisation was achieved at the highest applied IgG concentration of 1000 µg/mL. OVA immunised mice did not elicit HCV nAb.

Moreover, IgG pools neutralised genotype 1–5 HCV with in vivo derived envelope sequences and genotype 6 HCV with two vital cell culture adaptive substitutions in the envelope proteins with similar efficacy and in concentration dependent manner. Across neutralised viruses, mean EC50 of IgG from genotype 1a, 2a and 3a vaccinated animals were 67, 68 and 77 µg/mL, respectively, and close to complete neutralisation was observed at 1000 µg/mL (figure 6). Mouse IgG neutralisation capacity compared favourably with that of IgG from patients with CHC regarding efficacy and broadness (figure 7). Interestingly, these data confirmed that 5a(SA13) had relatively high neutralisation sensitivity, while 2a(J6) and 3a(S52) had relatively low neutralisation sensitivity.[42] Furthermore, neutralisation capacity of IgG from genotype 3a infected patients was lower than that from genotype 1a, 2a or 2b infected patients.

Finally, IgG pools from all HCV immunised animal groups efficiently bound TNcc-HI, J6cc-HI and DBNcc-HI E1/E2 complexes in concentration-dependent manner (figure 8, online supplemental figure 6). Such binding was not observed for pooled IgG from OVA immunised animals. Pools of immune-sera had endpoint titres of up to 32 000 (figure 8, online supplemental figure 7).

DISCUSSION

In this study, we developed high-yield genotype 1a, 2a and 3a HCV cell culture systems to facilitate development of whole virus inactivated vaccine candidates. Compared with the original viruses, high-yield viruses showed increased exposure of conserved conformational neutralising epitopes associated with protection against chronic HCV infection, as suggested by

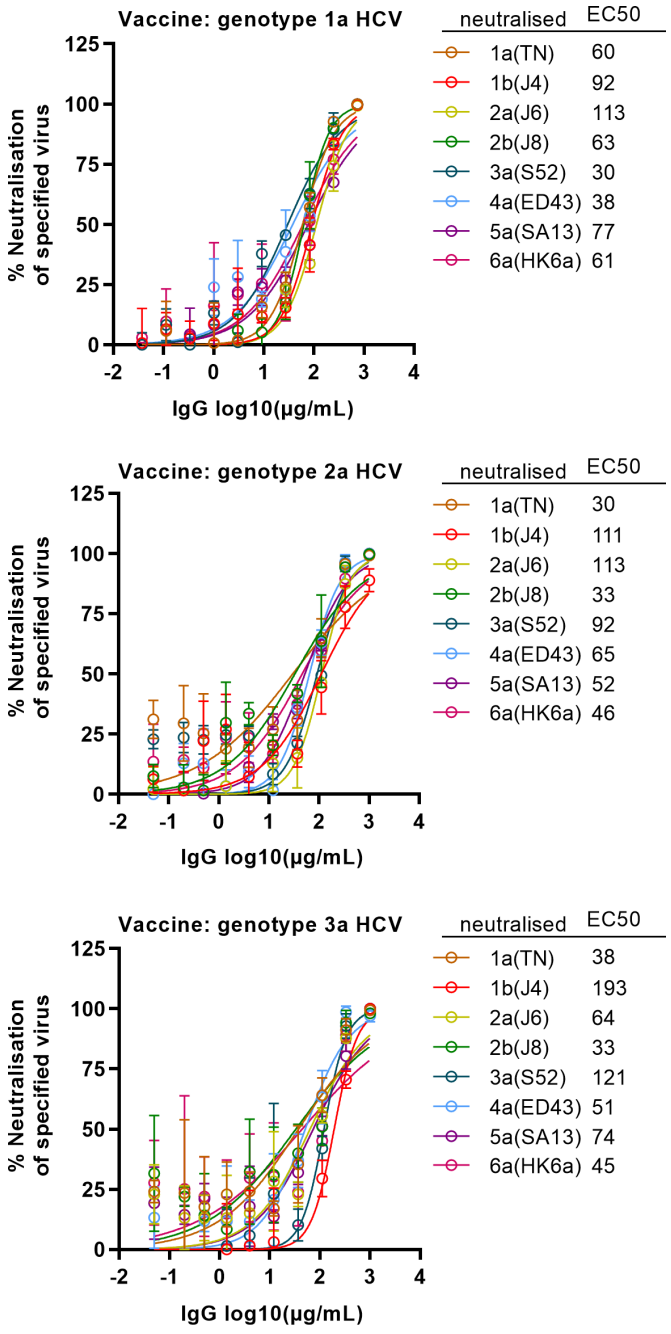


Figure 6 Immunisation with inactivated genotype 1a, 2a or 3a HCV elicited broadly nAb. Purified IgG from mice of each group, immunised with either genotype 1a, 2a or 3a HCV, was pooled using the same amount of IgG from each animal. IgG pools were used to neutralise recombinants containing in vivo derived genotype(isolate) 1a(TN), 1b(J4), 2a(J6), 2b(J8), 3a(S52), 4a(ED43) and 5a(SA13) core-NS2 sequences; 6a(HK6a) contained two vital cell culture adaptive substitutions in E1 and E2.[24 25 30] Data points are means of three replicates with SD; curves were fitted, and EC50 were calculated with the formula $y=100/(1+10^{(\log10EC50-X)\times hillslope})$ using GraphPad prism. Virus stock envelope protein sequences were confirmed by Sanger sequencing. EC50, half maximal effective concentration; nAb, neutralising antibody.

increased sensitivity to neutralisation by mAb AR3A and AR4A. In mouse immunogenicity studies, high-yield genotype 1a, 2a or 3a viruses, formulated with an analogue of the human MF-59 adjuvant, each had the capacity to induce efficient nAb broadly neutralising HCV of all major genotypes with recognised epidemiological importance.

For efficient production of whole virus inactivated vaccines, high-yield virus production is required. For example, SARS-CoV-2 used for vaccine production grow to infectivity titres of 6.5–7 log10 TCID50/mL.[43] For selection of high-yield variants of previously developed full-length recombinants of the most prevalent HCV genotypes,[26–28] we employed a serial passage approach, previously used for further adaption of a JFH1-based genotype 5a virus.[31] Applying this evolutionary approach until no further increase in viral infectivity titres is observed, and until a homogeneous viral population with no obvious evidence for ongoing selection of additional putative adaptive substitutions is recorded is expected to result in selection of highly fit and genetically stable virus populations.[31] Thus, the developed genetically stable, high-yield HCV recombinants can in the future be used to initiate virus vaccine antigen production with sequence confirmed early viral passage seed stocks.[44 45] Based on results from this study using full-length recombinants, as well as on results from the previous study using a JFH1-based recombinant,[31] the upper limit for HCV infectivity titres in monolayer Huh7.5 cell cultures is between 6 and 7 log10 FFU/mL, which might be due to limited availability of required host cell factors. Future studies should focus on investigation of the effect of acquired viral substitutions on the viral life cycle. Interestingly, several substitutions selected in this study were also selected during cell culture adaptation of other HCV recombinants, suggesting a general role for HCV cell culture adaptation (online supplemental table 10).

In future studies, it will be of special interest to investigate which of the selected envelope substitutions conferred increased exposure of the conserved conformational epitopes targeted by AR3A and AR4A. Several genetic changes in E2 such as deletion of HVR1 (aa 384-410),[40] abrogation of N-linked glycosylation,[19 46] but also specific substitutions in HVR1 (aa 400-404) and the E2 front layer (aa 414, 431 and 453; front layer: aa 411–461)[20] were described to increase neutralisation sensitivity, which was linked to an open E1/E2 state.[19 20] TN-PP-18 and TN-PP-38.1 acquired N410K in HVR1, while TN-PP-38.1 in addition acquired F403L in HVR1. J6cc-PP-35 acquired H434N in the E2 front layer and DBNcc-PP-16 acquired G395R in HVR1, while DBN3acc harboured S449A in the E2 front layer.[28] Furthermore, localising to E2 outside these specific regions previously associated with changes in neutralisation sensitivity, TN-PP-38.1 acquired V719I and J6cc-PP-35 acquired A573T, while DBN3acc harboured D474A, T528N and V629A.[28]

A positive correlation between viral fitness and neutralising epitope exposure was also observed in previous studies for HCV[31] and HIV.[47] For HCV, in vivo protection of conserved conformational neutralising epitopes might be associated with a fitness cost as closed E1/E2 states might decrease access of the main HCV entry receptor CD81 to its binding site, which is overlapping with AR3.[2]

For HCV, deletion of HVR1 led to a maximally open E1/E2 state associated with high neutralisation sensitivity.[19 40] For the full length genotype 1–3 HI-viruses developed in this study and the previously developed JFH1-based high-yield genotype 5a HCV,[31] the AR3A epitopes were as accessible as in HVR1-deleted viruses, while the AR4A epitopes were approximately 10-fold to 100-fold less exposed.[35 40] However, HVR1-deleted viruses typically show relatively low infectivity titres, hampering vaccine production.[48] Compared with the original viruses, HI-viruses showed ~300 to 1250-fold higher exposure of AR3A epitopes and ~12 to 2400-fold higher exposure of AR4A epitopes; the

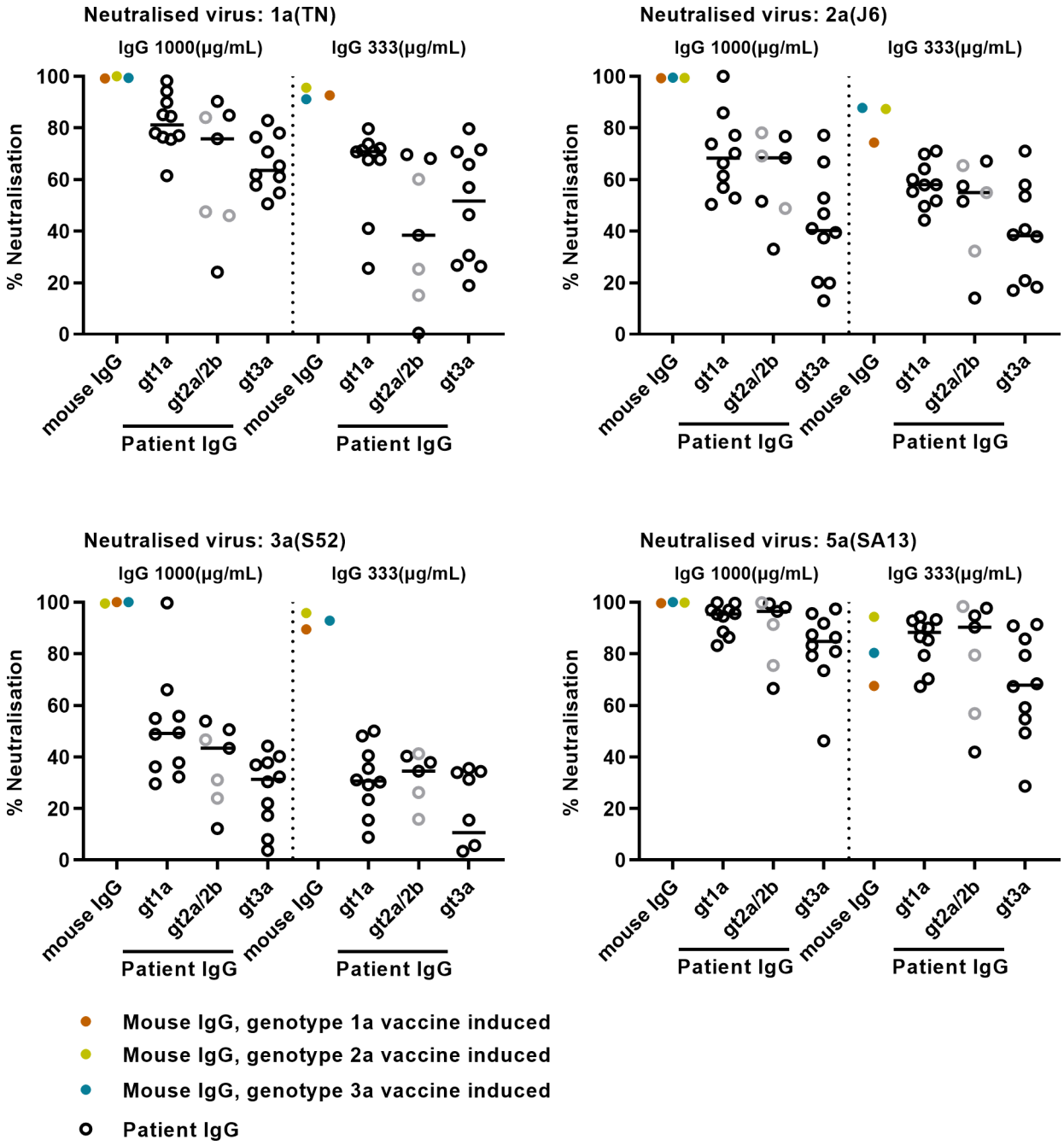


Figure 7 Vaccine-induced nAb responses compare favourably with those in patients with chronic HCV infection. Purified IgG from patients chronically infected with HCV of genotype 1a, 2a/2b or 3a at the specified concentrations was used to neutralise recombinants containing in vivo derived genotype(isolate) 1a(TN), 2a(J6), 3a(S52) and 5a(SA13) core-NS2 sequences.[24 25 30] Genotype 2a versus 2b patients are indicated by grey versus black open circles. Data points obtained in neutralisation assays with the same concentrations of purified mouse IgG shown in figure 6 are replotted for comparison. All data points are means of three replicates. Virus stock envelope protein sequences were confirmed by Sanger sequencing. gt, genotype; nAb, neutralising antibody.

high-yield genotype 5a HCV showed only ~10 fold higher exposure of AR3A and AR4A epitopes than the original already highly neutralisation sensitive genotype 5a HCV.[35]

Therefore, the genotype 1a, 2a and 3a HI-viruses developed in this study and the high-yield JFH1-based genotype 5a HCV[31] present interesting vaccine antigens as they might facilitate induction of antibodies targeting epitopes that are conserved between HCV variants and that are mediating protection in humans. A vaccine antigen exposing such conserved epitopes with the ability to induce broadly nAb might make a multivalent vaccine approach unnecessary.

Indeed, immunisation with genotype 1–3 PP-viruses and HI-viruses and with high-yield genotype 5a HCV[31] resulted in induction of broadly nAb. Fifty per cent neutralisation titres and ELISA endpoint titres of vaccine-induced antibodies were comparable with those reported for licenced antiviral vaccines and with those in chimpanzees protected from HCV challenge following vaccination with the E1/E2 heterodimer vaccine.[6 10]

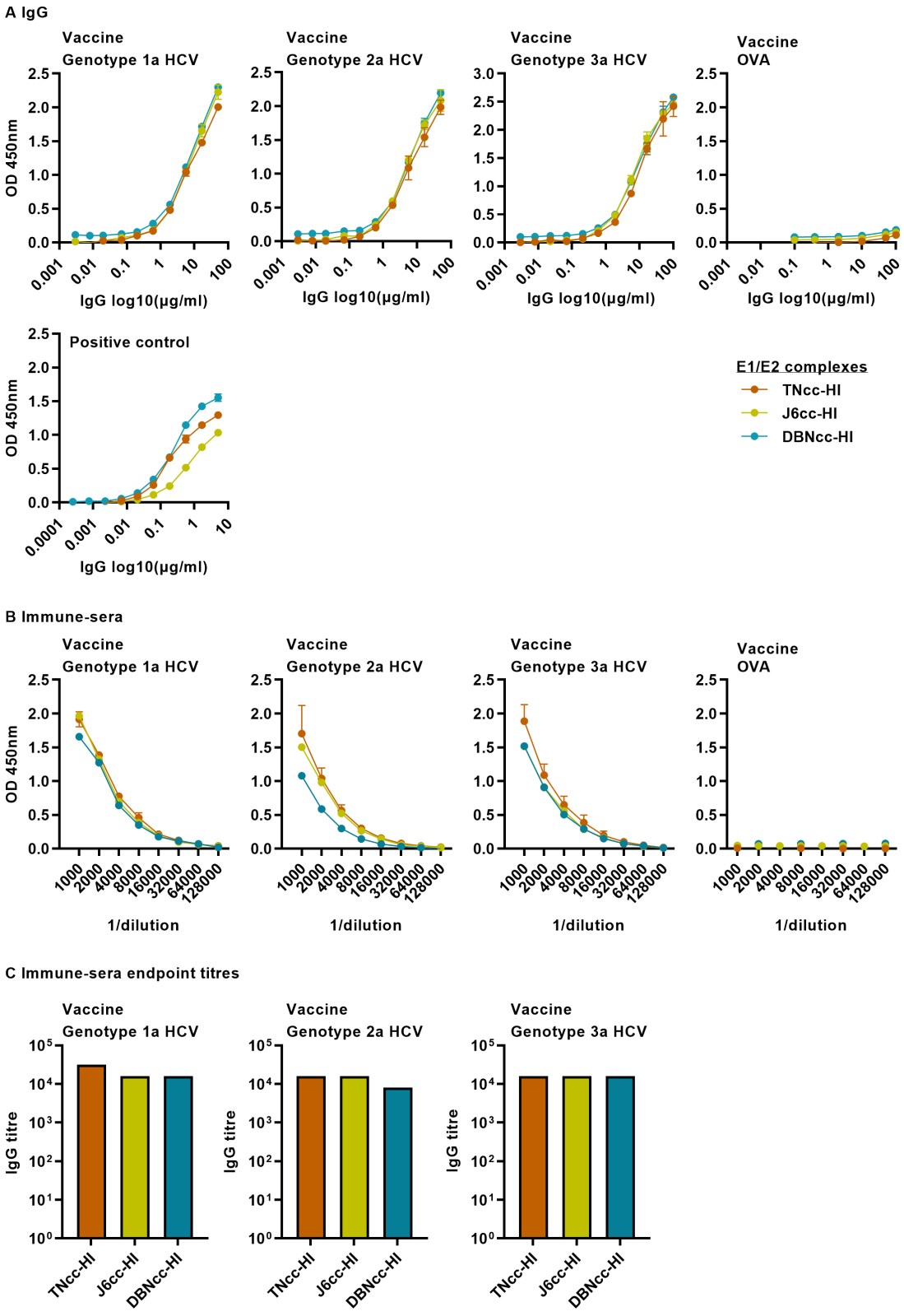


Figure 8 Immunisation with inactivated genotype 1a, 2a or 3a HCV elicited antibodies binding to HCV envelope proteins. Binding capacity of pooled (A) purified serum IgG or (B and C) immune-sera from mice immunised with inactivated genotype 1a, 2a or 3a HCV or OVA to E1/E2 complexes of the specified HI-recombinants was evaluated by ELISA. Values are optical density (OD) reads at 450 nm following subtraction of mean OD of eight negative controls. Data points are means of two replicates with SD. (A) Positive controls: instead of serum IgG, well-characterised primary antibodies were used: AP33[38] for binding to TNcc-HI and DBNcc-HI E1/E2 and H77.39[39] for binding to J6cc-HI E1/E2. Negative controls: no IgG or immune-sera were used, and TNcc-HI, J6cc-HI and DBNcc-HI E1/E2 were incubated with secondary antibody only; for negative controls, OD reads were ~0.05. (A and B) In the OVA graphs, data points reflecting binding to J6cc-HI E1/E2 and DBNcc-HI E1/E2 were nudged by 0.04 and 0.08 units in the y direction, respectively. (C) Immune-sera endpoint titres were determined as the highest serum dilution yielding an OD >2 fold mean OD of negative controls. OVA, ovalbumin.

However, compared with IgG from chronically infected patients, antibodies elicited by the E1/E2 heterodimer vaccine[8–10] and different vaccine candidates based on soluble E2 protein,[46 49–51] nAb induced in this and the previous study[35] showed increased capacity to neutralise different HCV variants. Finally, 50% neutralisation titres were comparable with those reported for a genotype 2a inactivated vaccine candidate in mice and non-human primates when experimental adjuvants not suitable for human use were applied.[21 29] In the study of non-human primates, application of the licenced adjuvant aluminium hydroxide did not result in induction of efficient nAb.[29] AddaVax/MF-59 appears to be more immunogenic than aluminium hydroxide.[35 52] Furthermore, for other viruses, increased neutralising epitope exposure was suggested to result in increased immunogenicity.[53 54] In future studies, it would be interesting to investigate, whether increased exposure of conserved neutralising epitopes increases immunogenicity and whether different HCV genotypes/serotypes differ in immunogenicity.[42 55] However, this would require development of high-yield viruses without E2 substitutions mediating epitope exposure, which might not be possible, as it is likely that increased fitness was at least partly mediated by such substitutions. Soluble E2 or E1/E2 heterodimer vaccine platforms might be more amenable for such studies; however, they might not reflect the native envelope protein conformation on the whole virus particle. In such studies, deletion of the three variable E2 regions was reported to result in a certain increase in immunogenicity, whereas deletion of HVR1 and/or modification of glycosylation sites had no or a minor effect on immunogenicity.[46 49–51 56] Deletion of HVR1 of the already highly neutralisation sensitive high-yield genotype 5a HCV, facilitated by subsequent culture adaptation, did not result in increased immunogenicity.[35] In addition, future studies requiring larger amounts of HI-virus vaccine induced nAb and most likely derived mAb could investigate which epitopes are targeted by these antibodies.

Further preclinical and clinical development requires optimisation of vaccine production and processing conditions to ensure compatibility with vaccine manufacturing.[36 44] Moreover, further research should define the most powerful of the developed vaccine candidates, based on performance in an optimised bioprocess and detailed immunogenicity studies of resulting antigens, as well as dose finding studies. In initial upstream bioprocess studies employing a scalable bioreactor[44 57] and virus seed stocks generated from early viral passages following transfection, genotype 2a and 3a HI-viruses and high-yield genotype 5a HCV[35] yielded considerably higher infectivity titres than the genotype 1a HI-virus, signifying an advantage for these three candidates in the production process. No immunocompetent HCV in vivo challenge model is available. While future studies might employ specialised small animal models such as the human liver chimeric uPA-SCID mouse model to study certain aspects of vaccine-induced protection[48] and larger animals to confirm vaccine safety and immunogenicity,[9] promising vaccine candidates likely need to proceed to clinical trials involving controlled human infection models[58] to evaluate their true protective potential. Finally, in the quest for an HCV vaccine, it will be important to facilitate cross-comparison of vaccine candidates by application of standardised assays.[42 55]

In conclusion, we developed high-yield genotype 1a, 2a and 3a HCV constituting a basis for inactivated vaccine candidates that could be used for further preclinical and clinical development.

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SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Plasmids encoding cell culture infectious HCV recombinants

Original cell culture infectious genotype 1a strain TN (TNcc), genotype 2a strain J6 (J6cc) and genotype 3a strain DBN (DBN3acc) recombinants were developed previously.[26–28] High-yield HI-recombinants were engineered based on generated subclones harboring the desired mutations using In-Fusion technology. In brief, 50ng of gel purified PCR amplicons generated using the subclones with the sequences of interest as templates were incubated with 2µl of 5x In-Fusion HD enzyme (Takara Bio) and H_2O added to a total volume of 10µl. The reaction was incubated for 15min at 50°C and 4µl of the mix was transformed in 50µl Stellar Competent Cells (Takara) according to the manufacturers´ protocol.

Previously developed cell culture infectious HCV recombinants with genotype(isolate) 1a(TN), 1b(J4), 2a(J6), 2b(J8), 3a(S52), 3a(DBN), 4a(ED43), 5a(SA13), 6a(HK6a)[24,25,30] specific core-NS2 and remaining sequences of genotype 2a isolate JFH1 were used in *in vitro* neutralization assays. Except for 6a(HK6a) containing two vital cell culture adaptive substitutions in the envelope proteins, these viruses reflected the consensus envelope protein sequences determined *in vivo*.

For all plasmid preparations used in experiments the complete HCV sequence was verified by Sanger sequencing (Macrogen).

Maintenance of Huh7.5 and HEK283 cells

The human hepatoma cell line Huh7.5[25] and the human embryonic kidney cell line HEK293 were cultured with Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (Sigma), penicillin 100U/ml and streptomycin 100µg/ml (P/S) (Gibco/Invitrogen) at 37°C and 5% CO_2; cultures were split every 2–3 days using trypsin (ThermoFisher Scientific) as described.[25]

Transfection of HCV *in vitro* RNA transcripts in Huh7.5 cells

In vitro HCV RNA transcripts were generated and transfections were carried out as previously described.[25] Briefly, plasmids were linearized with Xbal (New England Biolabs) following the sequence encoding the 3´end of the HCV genome, and RNA *in vitro* transcription reactions were carried out using T7 RNA polymerase (Promega). RNA concentrations were measured using the Qubit RNA BR Assay Kit (Thermo Fisher Scientific); for recombinants directly compared in the same graph, equal amounts of RNA transcripts were used. Prior to the day of transfection, 400,000

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cells were seeded per well of a 6 well plate (Nunc); 7.6µg of TNcc or TNcc-HI, 7.5µg of J6cc or J6cc-HI, and 5µg of DBN3acc or DBNcc-HI RNA transcripts were combined with 5µl Lipofectamine2000 (Invitrogen) in a total of 500µl Opti-MEM (Gibco/Invitrogen) and cells were incubated with the resulting transfection complexes in a total of 2ml Opti-MEM for 4-6h prior to washing with PBS and addition of serum containing medium (DMEM+10%FBS+P/S). Transfected cultures were split every 2-3 days. When cells were split replicate cell cultures were plated in chamber slides for immunostaining to monitor the % of infected cells. In addition, supernatant was collected and stored at -80°C for determination of HCV infectivity titers.

Infection of Huh7.5 cells with HCV

400,000cells/well in 6 well plates plated the previous day, were inoculated at the specified multiplicity of infection (MOI) using supernatants derived from the transfection experiment at the peak of infection, as determined by immunostaining and infectivity titration. Infected cultures were split every 2-3 days. When cells were split, replicate cell cultures were plated in chamber slides for immunostaining to monitor the % of infected cells. In addition, supernatant was collected and stored at -80°C for determination of HCV infectivity titers.

Serial passage of HCV in Huh7.5 cells

For serial passage of cell culture infectious HCV recombinants, 10^6 cells were seeded into T25 flasks (ThermoFisher Scientific) and the following day inoculated with up to 1ml cell culture supernatant derived from the previous passage at the peak of infection as determined by immunostaining. The first passage culture was inoculated with up to 1ml of supernatant derived from a prior transfection culture at the peak of infection. When cells were split, replicate cell cultures were plated in chamber slides for immunostaining of HCV antigen to monitor the % of infected cells and supernatant was collected and stored at -80°C for determination of HCV infectivity titers.

Generation of virus stocks

For generation of HCV stocks for neutralization assays and of seed stocks for vaccine virus production, $6x10^6$ cells were plated in T175 flasks and the next day inoculated with cell culture supernatant derived from the peak of infection. When cells were split, replicate cell cultures were plated in chamber slides for immunostaining to monitor the % of infected cells. In addition, supernatant was collected and stored at -80°C for determination of HCV infectivity titers.

Supernatants derived on days where a high % of HCV infected cells was recorded by immunostaining, and/or a high infectivity titer was recorded, were pooled and used as virus stocks. For virus stocks used in neuralization assays, the envelope protein sequence was confirmed by Sanger sequencing. For virus seed stocks for vaccine virus production, the complete HCV open reading frame (ORF) was analyzed by next generation sequencing (NGS).

Immunostaining of HCV antigens in cell culture

Immunostaining of HCV antigens was done on replicate cultures derived when HCV infected cells were split using primary monoclonal anti-HCV core antibody C7-50 ((EnzoLifeSciences diluted 1:5000 in BSK (PBS supplemented with 0.2% skim milk (Easis) and 1% Bovine Serum Albumin, (Sigma)) and monoclonal anti-HCV NS5A antibody 9E10[24] diluted 1:5000 in BSK.[25] After at least 1h of incubation and subsequent washing, secondary antibody Alexa Flour 488 goat anti mouse IgG (H+L) (Invitrogen) diluted 1:500 in BSK was added and cell nuclei were counterstained with Hoechst reagent (Invitrogen).[25,34] The % of infected cells was scored from 0% to 100%, using intervals of 10%, by fluorescence microscopy with a Zeiss Axio Vert.A1 microscope.

Determination of HCV infectivity titers

HCV infectivity titers were determined in cell culture supernatant as focus-forming units (FFU)/ml as previously described.[25] In brief, 6000 cells were seeded per well of 96 well plates (Nunc) and infected the following day with serially diluted cell culture supernatants, testing each dilution in triplicate. After 48h of incubation, cells were fixed using methanol and stained for HCV antigens using primary anti-HCV core antibody C7-50 (EnzoLifeSciences) diluted 1:1000 in BSK and anti-HCV NS5A antibody 9E10 diluted 1:3000 in BSK, followed by secondary antibody ECL sheep anti-mouse IgG diluted 1:500 in BSK.[34] The HCV antigen positive cells were visualized and FFU were automatically counted using an Immunospot series 5 UV analyzer (CTL Europe GmbH) as described.[25]

Sequence analysis of cell culture derived HCV

HCV RNA was extracted from cell culture supernatants using Trizol LS (Life technology) and the RNeasy MinElute kit (QIAGEN) as described.[33] RNA was eluted and used for reverse transcription with Maxima H Minus Reverse Transcriptase (ThermoScientific) and genotype specific reverse primers binding to the HCV 3´UTR variable region as described[33,32] (Supplementary Table 1).

Polymerase chain reaction (PCR)-based amplification of the complete open reading frame (ORF) was carried out using Q5 Hot start High-Fidelity DNA polymerase and genotype specific primers as described[33,32] (Supplementary Table 2). Alternatively, for virus stocks used for neutralization assays, a PCR amplicon spanning the envelope proteins was generated (Supplementary Table 2). The sequence of the resulting PCR amplicons was either determined by Sanger sequencing or by NGS. NGS was carried out as described.[33] In brief, PCR amplicons were loaded on a gel with SYBR safe DNA gel stain (Invitrogen), visualized with blue light and gel extracted with the Large Fragment DNA recovery kit (Zymo Research). Size selection was done with magnetic beads and adaptors with unique barcodes with the use of TruSeq Nano DNA library kit (Illumina). Finally, paired-end sequencing was performed on the Miseq platform. Viruses from serial passage experiments, first passage kinetic experiments and virus seed stocks used for vaccine production, were subjected to NGS of the complete ORF. Virus stocks for neutralization assays were subjected to Sanger sequencing of genome regions encoding E1 and E2.

Subclonal analysis

For serially passaged viruses, PCR amplicons spanning the complete ORF were subcloned using the TOPO-XL Cloning kit (Invitrogen) following the manufacturers´ instructions. The HCV sequence of the resulting plasmids was determined by Sanger sequencing. TNcc-PP-10, TNcc-PP-18, TNcc-PP-38.1, J6cc-PP-35 and DBNcc-PP-16 were analyzed like this. For TNcc-PP-10, TNcc-PP-18 and TNcc-PP-38.1, phylogenetic analysis was carried out using MAFFT for aligning sequences and subsequently PhyML for building the phylogeny visualized by FigTree v1.4.3.

HCV production for vaccine generation

HCV production for immunization studies was done using previously developed protocols.[35] In brief, $18x10^6$ cells were plated in T500 triple layer cell culture flasks (ThermoFisher Scientific). The next day, cells were infected at MOI 0.003 with genotype 1a, 2a and 3a seed stocks. On day 1 post infection, around $1.8x10^8$ cells were plated in 10-layer cell-factories (ThermoFisher Scientific). When 80% of cells were estimated to be infected by monitoring of a replicate T25 cell culture, cells in the cell-factory were washed with pre-warmed PBS and DMEM was replaced by Adenovirus Expression Medium (AEM) supplemented with P/S.[34] From the 10-layer cell-factories, supernatant was harvested every 2–3 days. Each factory yielded 5 harvests of 800ml each. For each HCV recombinant,

4 cell-factories were done, collecting a total volume of ~16l of virus containing supernatant. Harvested supernatant was stored at -80°C until further processing.

Processing of HCV for vaccine generation

HCV was processed for vaccine development using previously established protocols.[35] In brief, initial clarification of ~16l virus containing supernatant harvested from cell-factories was carried out through a 5µm capsule filter, followed by a 0.65µm Sartopure® PP3 filter (Sartorius). Afterwards, two tangential flow filtration (TFF) steps were carried out using a fiber with a surface area (SA) of 2600cm^2 and a molecular weight cut off (MWCO) of 500kDa and subsequently a fiber with a SA of 790cm^2 and a MWCO of 500kDa (MINIKROS 65cm 500KD MPES, MINIKROS 20cm 500KD MPES, Repligen), concentrating ~16l to ~350ml and subsequently to ~35ml. For a subsequent 3-cushion ultracentrifugation (UC) step, 3 cushions containing 1ml of 60%, 28% and 10% Optiprep Density Gradient Medium (Sigma), respectively, were layered on top of each other in 6 UC tubes. To each tube, ~6ml of virus sample were added and the total volume was adjusted to 11ml with PBS. After centrifugation with a Beckman SW-41 rotor at 40,000 revolutions per minute (rpm) at 4°C for 2h, fraction 1 (8ml), fraction 2 (1.2ml) and fraction 3 (2ml) were collected from the top of each tube. Fraction 2 was pooled from 6 tubes and adjusted to a total volume of 20ml with PBS prior to TFF with a fiber with a SA of 20cm^2 and a MWCO of 500kDa (MICROKROS 20CM 500K MPES 0.5MM, Repligen) resulting in concentration to ~2ml. For a subsequent equilibrium density gradient UC step one day prior to the experiment a semi-continuous 40% to 10% iodixanol gradient was prepared by layering 2.5ml of 40%, 30%, 20% and 10% Optiprep Density Gradient Medium on top of each other followed by equilibration at 4°C. The ~2ml sample resulting from the previous step was added on top of the gradient and ultracentrifugated at 40,000rpm with a Beckman SW-41 rotor for 6h at 4°C. Eighteen fractions of ~ 550µl were collected from the bottom of the tube and each fraction was weighed for determination of buoyant density. The three fractions with a density closest to 1.1g/ml were pooled, obtaining a final volume of ~1.5ml. For a subsequent size exclusion chromatography step 1.54g of Sephadex G-100 (Sigma Aldrich) was mixed with 35ml sterile water 3 days prior to the experiment and added to a chromatography PD-10 column (PD-10 reservoirs, GE Healthcare Life Science). The ~1.5ml of sample resulting from the previous step were then added to the column, and 12 fractions of ~1ml were eluted with NaCl (9 mg/ml). Based on absorbance determined with a NanoDrop (Thermo Scientific) at an OD of 230nm, 5 fractions were pooled, obtaining a final volume of close to 5ml. Finally, the resulting preparation was subjected to UV-

irradiation with a UVG-54 Handheld UV lamp (240nm UV, 6 watt) (Analytik Jena) for 25min with frequent agitation using a 6 well plate with 1.25ml of sample per well. To confirm inactivation, cells seeded one day prior to the experiment were inoculated with 100µl of UV inactivated sample, and cell cultures were followed for 3 weeks by splitting and immunostaining every 2-3 days.

Immunization of mice

To evaluate immunogenicity of the developed vaccine candidates, 6-8 week-old female BALB/c mice were acquired from Taconic Farms, Denmark, and were housed in certified animal facilities at the University of Copenhagen. Animals were resting for at least one week following arrival to the animal facility. Animals were subcutaneously immunized four times every 3 weeks with processed and inactivated HCV or as a control with 100µg EndoFit OVA (Invitrogen) formulated with adjuvant AddaVax 50%/50% (v/v). Genotype 1a, 2a and 3a HCV vaccines contained an equivalent of 6.8, 8.6, and 8.6 log10 FFU, respectively, determined prior to inactivation. Each experimental group including the control group consisted of 3 animals. Thus, a total of 12 animals were used. Animals were randomly assigned to the different groups and treatments were administered in random order. The size of the groups was determined based on availability of vaccine antigen. Two weeks after the last immunization mice were sacrificed and serum was obtained. IgG was purified from serum of each animal and was evaluated in *in vitro* neutralization assays as the primary outcome measure; in addition, IgG pools derived from each group were evaluated in *in vitro* neutralization assays as the primary outcome measure and in HCV E1/E2 complex ELISA assays as a secondary outcome measure. No animals were excluded from the study. All data obtained from all animals were reported. During the experiments, persons handling animals and samples were blinded to the identity of the vaccine antigen. Experiments were conducted in accordance with national Danish guidelines regarding animal experiments (Amendment # 1306 of November 23, 2007), approved by the Danish Animal Experiments Inspectorate, Ministry of Justice, permission numbers 2015-15-0201-00623.

Patient samples

Sera or plasma from patients with chronic hepatitis C (CHC) were collected between May 2011 and August 2021 in biobanks attached to the Danish Database for Hepatitis B and C and the HCV Tandem cohort at the Department of Infectious Diseases, Copenhagen University Hospital, Hvidovre. Patients were ≥18 years, had no previous history of treatment for CHC, no co-infection with human immunodeficiency virus or hepatitis B virus, and no recent intravenous drug use. The HCV Tandem

cohort was approved by the Regional Ethical Committee (H-21004361), and the Danish Data Protection Agency (2012-58-0004); written informed consent was provided by all patients.

Purification, concentration and quantification of IgG from mouse serum and patient serum or plasma

IgG from individual mouse serum or individual patient serum / plasma samples was purified with the Amicon® Pro Affinity Concentration Kit Protein G with 50kDa Amicon® Ultra-0.5 Device (Merck Millipore), in accordance to the manufacturers´ instructions. Briefly, for mouse samples 200µl and for patient samples 600µl of Protein G resin was added to the column, followed by a wash step. Next, serum or plasma was added and incubated for 1h at room temperature on a shaker; 300-500µl of mouse serum or 600µl of patient serum or plasma was processed. Following the second wash, elution and neutralization buffer were added to obtain IgG. Concentration of IgG was done with the Vivaspin 500, 30,000 MWCO (GE Lifescience) kit according to the manufacturers´ instructions. Briefly, IgG resulting from the previous step was diluted in PBS to a total volume of 500µl and centrifugated at 14,000g until a volume of ~80µl was obtained. Concentration of the resulting mouse IgG preparations was determined with the IgG (TOTAL) mouse uncoated ELISA Kit (ThermoFisher) according to the manufacturers´ instructions. Briefly, 96 well plates were coated with capture antibody and incubated overnight, followed by incubation with blocking buffer. After several washes, concentrated and serially diluted IgG was added in duplicates together with the provided standards. Then, the detection antibody was added and incubated for two hours on a shaker. Next, substrate was added followed by stop solution and absorbance at an optical density (OD) of 490nm was measured (FLUOstar OPTIMA, BMG Labtech). The generated standard curve was used to calculate the IgG concentration. Concentration of the resulting patient IgG preparations was determined with the Cedex Bio Analyzer (Roche) according to the manufacturers´ instructions.

In vitro neutralization assay

Neutralization with human derived monoclonal antibodies AR3A and AR4A,[17,18]and polyclonal IgG C211[19] was done as described in.[25] In brief, these antibodies were serially diluted in cell growth medium (DMEM+10%FBS+P/S), added to the virus and incubated in a total of 100µl for 1h at 37°C. Each antibody concentration was tested in triplicate. Virus only controls were prepared by mixing virus with cell growth medium. Virus-antibody mixes, virus-medium mixes or medium only were then added to Huh7.5 cells, which had been seeded at 6000cells/well in 96 well poly-D-Lysine coated plates (Nunc) the day prior to the experiment. Following 3.5h incubation, cells were washed with

PBS and 100µl cell growth medium was added per well. After 48h of incubation at 37°C, plates were fixed with methanol and stained for HCV core and NS5A antigen as described for infectivity titrations.

Neutralization with purified mouse serum IgG or patient serum / plasma IgG was done using previously established assays.[37] In brief, these IgG were serially diluted in cell growth medium in a total of 3µl and added to virus diluted in 7µl to a total of 10µl and incubated for 1.5h at 37°C. Each antibody concentration was tested in triplicate. Virus only controls were prepared by mixing virus with cell growth medium. Subsequently, 30µl of cell growth medium were added to virus-IgG or virus-medium mixes and the resulting samples were added to Huh7.5 cells, which had been seeded at 6000cells/well in 96 well poly-D-Lysine coated plates (Nunc) the day prior to the experiment. Following 4.5h incubation at 37°C, cells were washed with PBS and 100µl of cell growth medium were added per well. As a positive neutralization control, C211 antibody and a well-defined genotype 5a virus[31] were mixed and added to each 96-well plate. After 48h of incubation at 37°C, plates were fixed with methanol and BSK was added at 300µl/well. Following 1h incubation, BSK was removed and 100µg/ml Fab Fragment Goat anti-mouse IgG (Jackson ImmunoResearch) diluted in PBS was added and incubated for 1h at room temperature. Next, plates were washed and 50µl of monoclonal primary antibody 9E10 diluted 1:5000 in BSK was added per well. The remaining staining steps were done as described above for infectivity titrations.

For all neutralization assays, the percentage of neutralization was calculated relating the number of FFU in experimental wells to the mean number of FFU in virus only wells. For all neutralization assays, half maximal effective concentration (EC50) was calculated based on concentration-response curves, using top and bottom constrains of 0 and 100% and the formula $y=100/(1+10^{(\log 10EC50-X)\times hillslope})$ using GraphPad Prism, as described.[34] Fold-changes in neutralization sensitivity given in Figure 4 were calculated as [(EC50 of original virus)/(EC50 of virus with envelope substitutions)]; these calculated values are not meant to reflect data accuracy.

HCV E1/E2 complex ELISA

Expression plasmids harboring E1 and E2 envelope protein sequences of TNcc-HI, J6cc-HI and DBNcc-HI were constructed using the In-Fusion HD cloning kit (Takara Bio) and 50ng of PCR amplicons harboring E1 and E2 sequences of the TNcc-HI, J6cc-HI or DBNcc-HI plasmid and an HCV pseudo-particle expression plasmid. The E1 and E2 sequence in the resulting plasmids was sequence confirmed (Macrogen). Plasmids were transfected in HEK293 cells, plated at

$8x10^5$cells/well in 6-well plates 1 day prior to the experiment, using 5µl Lipofectamine2000 (Invitrogen) and 5µg plasmid in a total volume of 2ml of Opti-MEM (Invitrogen). Following 6h of incubation, Opti-MEM was replaced with DMEM+10%FBS+P/S. In a replicate culture, after 48h of incubation at 37°C, transfection efficiency was determined by immunostaining for E1/E2 protein using the monoclonal antibody AR4A[17] diluted 1:5000 in BSK for 1h at room temperature, followed by application of the anti-human Alexa Fluor 488 coupled secondary antibody, as described above. Following confirmation of expression, cell growth medium was discarded, and total protein was collected in lysate buffer (1% Triton X-100, 50nM Tris-HCl, 150nM NaCl, pH8.0). Next, the cell lysate was treated with Benzonase endonuclease (Sigma) and 2nM of $MgCl_2$ and finally, the generated protein was measured with BCA protein assay kit (Pierce™) according to the manufacturers´ instructions.

For ELISA assays, 96-well plates (Nunc) were coated with *Galanthus nivalis* lectin (Medicago) and the next day the plate was washed with PBS and blocked with 200µl of PBS-5% non-fat milk (Easis). The next day, the plates were washed with PBS containing 0.1% Tween (PBST) and 50µl of E1/E2 complexes (200µg/ml) were added to the plates and incubated at 4°C overnight. After several washes with PBST, purified mouse serum IgG or immune-sera were serially diluted in PBST-1% non-fat milk, dilutions were added to plates in duplicates, and plates were incubated for 2h at room temperature, followed by a washing step with PBST. Binding of antibodies to E1/E2 complexes was detected by secondary antibody ECL sheep anti-mouse IgG horseradish-peroxidase linked whole antibody (GE Healthcare) diluted 1:1000 in PBST-1% non-fat milk and incubated for 1h at room temperature, followed by a washing step with PBST. TMB substrate (3,3′, 5,5′-tetramethylbenzidine, Thermo Scientific) was then added for 10min followed by ELISA Stop Solution (Invitrogen). Absorbance was determined at 450nm with the use of an ELISA plate reader (BIO-TEK Instruments, Inc.). As positive controls, mouse anti-E2 antibody AP33[39] was used to bind TNcc-HI E1/E2 and DBNcc-HI E1/E2 complexes and H77.39[40] was used to bind J6cc-HI E1/E2 complexes. As negative control, only secondary antibody, ECL sheep anti-mouse IgG horseradish-peroxidase linked whole antibody (GE Healthcare) was added to the E1/E2 complexes omitting prior addition of mouse serum IgG, immune-sera or control antibodies.

Supplementary Results

Phylogenetic analysis of subclones of polyclonal passaged genotype 1a HCV from an initial passage line

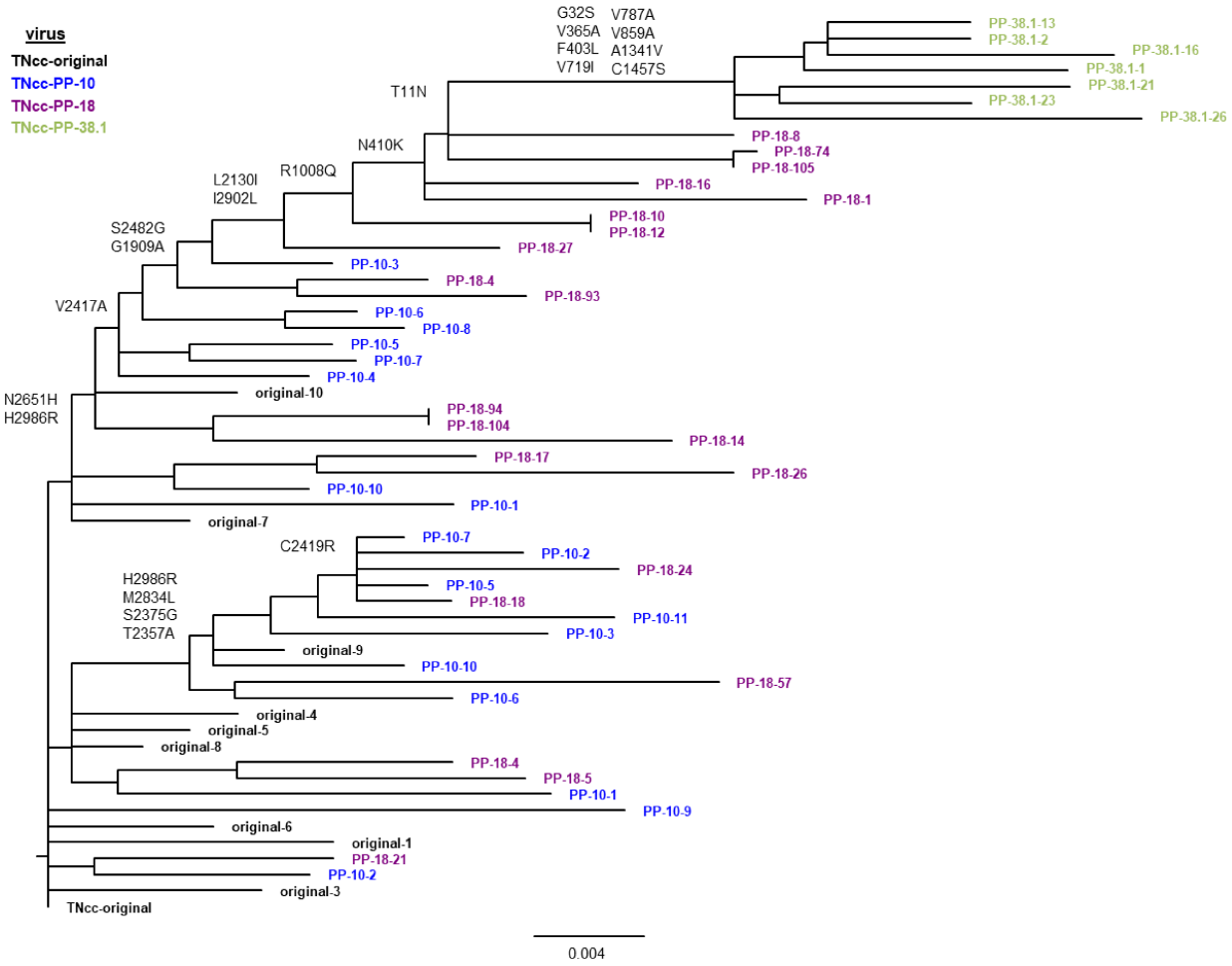
For the original TNcc, TNcc-PP-10, TNcc-PP-18 and TNcc-PP-38.1, generated subclones were subjected to phylogenetic analysis using PhyML; subclones for the original TNcc were generated and reported previously.[33] Results of this analysis are shown in Supplementary Figure 1. In comparison to the TNcc plasmid sequence, in passage 10 and 18 viruses, this analysis revealed two main quasispecies populations, designated A and B. The A population was mainly characterized by signature substitutions N2651H and H2986R. The B population was mainly characterized by signature substitutions T2357A, S2375G, M2834L and H2986R. In the passage 10 subclones, the A and B population had similar frequency, with 8/18 subclones belonging to A and 7/18 subclones to B, respectively. In the passage 18 subclones, the A population appeared to prevail over the B population with 15/21 and 3/21 subclones belonging to the A and B population, respectively. TNcc-PP-38.1 was characterized by population A signature substitutions and acquisition of additional substitutions corresponding to the substitutions found with >80% frequency in TNcc-PP-38.1 (Supplementary Table 4). Thus, subclonal analysis described in this section reflected results obtained by NGS analysis of polyclonal cell culture virus (Supplementary Table 3, 4 and 7).

Downstream processing of HCV for vaccine experiments

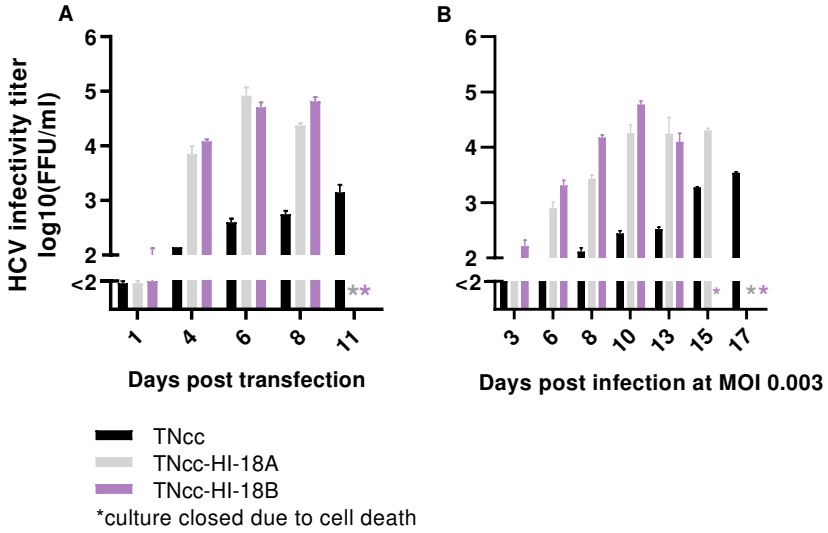
While the general protocol for HCV downstream processing is described in Materials and Methods / Supplementary Materials and Methods, this section describes the results obtained during downstream processing of genotype 1a, 2a and 3a vaccine viruses. Downstream processing was initiated with a first filter clarification step using filters with a pore size of 5µm and 0.65µm, followed by two TFF steps with two hollow fibers with different surface areas, which resulted in a volume reduction from ~16l to ~35ml. The resulting material had HCV infectivity titers of 7.5, 9.2 and 8.8 log10 FFU/ml for genotype 1a, 2a and 3a HCV, respectively (Supplementary Figure 4). The resulting material was distributed into six different ultracentrifugation vials which were subjected to 3-cushion ultracentrifugation resulting in three fractions. Fraction 2 from each of the six vials (~1.2ml each) were pooled amounting to a total volume of ~7.5ml with HCV infectivity titers of 7.8, 9.5 and 9.1log10 FFU/ml for genotype 1a, 2a and 3a HCV, respectively (Supplementary Figure 4). This was followed by TFF using a small hollow fiber reducing the volume to ~2ml with infectivity titers of 8.5, 11 and 9.6log10 FFU/ml for genotype 1a, 2a and 3a HCV, respectively (Supplementary Figure

4). These samples were subjected to gradient ultracentrifugation; 18 fractions (~550µl each) were collected and weighed to determine their buoyant densities. The three fractions with densities closest to 1.1g/ml were pooled amounting to a total volume of ~1.5ml. These pools yielded infectivity titers of 7.8, 9.9 and 9.6 log10 FFU/ml for genotype 1a, 2a and 3a HCV, respectively (Supplementary Figure 4). These pools were then subjected to Sephadex chromatography, where 12 fractions (~1ml each) were collected. Five of these fractions were pooled based on absorbance at OD of 230nm amounting to a total volume of ~4.8ml with infectivity titers of 7.4, 9.2 and 9.1 log10 FFU/ml for genotype 1a, 2a and 3a HCV, respectively (Supplementary Figure 4). Thus, recoveries in downstream processing were 9%, 17% and 30% for genotype 1a, 2a and 3a HCV, respectively. The pools resulting from chromatography were UV inactivated to yield the final vaccine antigens. To confirm virus inactivation, replicate cell cultures were inoculated and maintained for three weeks; cells were split every 2-3 days, when replicate cultures for immunostaining for HCV antigen were plated, to confirm inactivation based on absence of HCV antigen positive cells (Supplementary Figure 5).

Supplementary Figures



Supplementary Figure 1. Phylogenetic analysis of subclones of full ORF amplicons of polyclonal passaged genotype 1a HCV**.** Phylogenetic analysis and ancestral reconstruction of subclones of RT-PCR amplicons spanning the entire ORF of the original TNcc used as starting material for serial passage experiments, TNcc-PP-10, TNcc-PP-18 and TNcc-PP-38.1 (Figure 1 and 2 and Supplementary Table 3, 4 and 7). Respective subclones are designated PP-10-X, PP-18-X and PP-38.1-X, with "X" being the subclone number.



Supplementary Figure 2. HCV genotype 1a recombinants engineered based on an initial passage line had increased viral fitness in cell culture. (A) Original TNcc and newly engineered TNcc-HI-18A and TNcc-HI-18B HCV recombinants were transfected into Huh7.5 cells using the same amount of HCV RNA *in vitro* transcripts. (B) First passage kinetic experiments were inoculated at MOI 0.003 with TNcc and HI-viruses using supernatants derived from the transfection experiment when peak infectivity titers were observed. Cultures were split every 2-3 days. On these days HCV infectivity titers given in log10 FFU/ml were determined with an infectivity titration assay as means of three replicates with standard deviation (SD). *, cell culture was closed due to HCV induced cell death.

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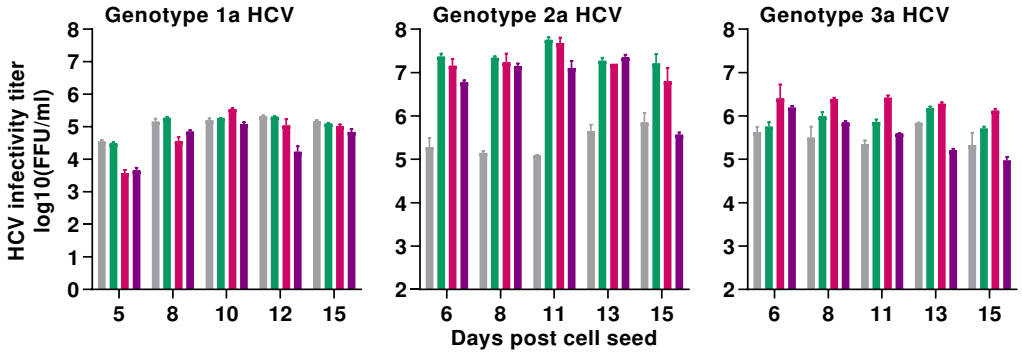

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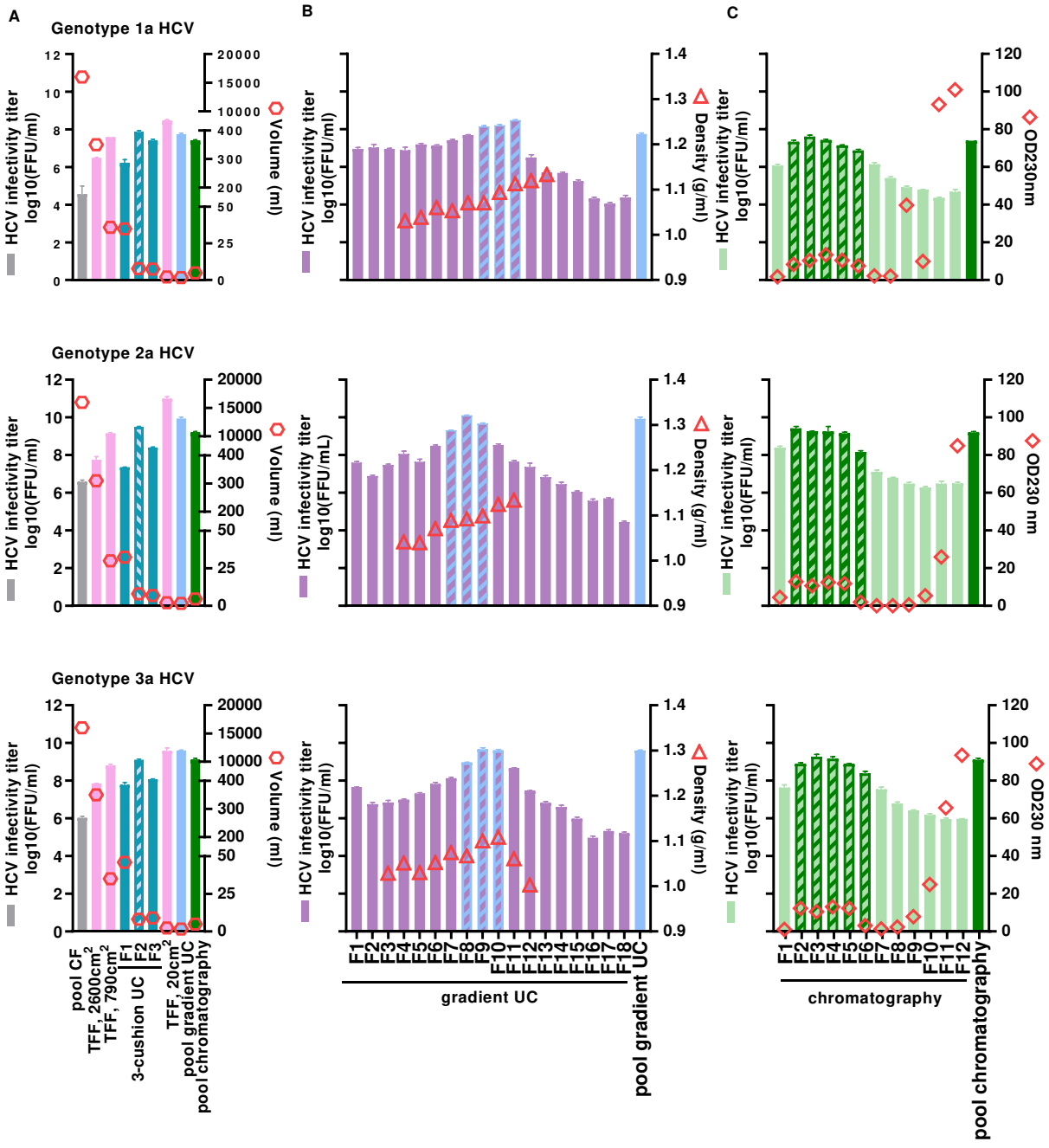
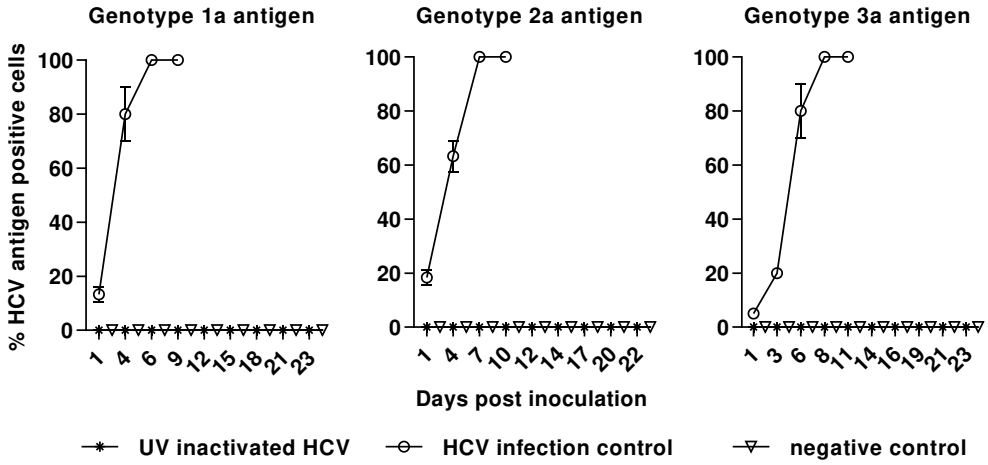
348 Supplementary Figure 3. Production of genotype 1a, 2a, and 3a vaccine viruses. Huh7.5 cells were
349 infected with genotype 1a, 2a and 3a HCV seed stocks at MOI 0.003 and transferred to 10-layer cell-
350 factories. For each genotype four cell-factories were processed as indicated by different colors. Per
351 cell-factory, five harvests of 800ml supernatant were collected at the indicated days post cell seed
352 into the cell-factory. HCV infectivity titers in harvests were determined as log10 FFU/ml and are
353 means of three replicates with SD. Thus, per virus 16l HCV containing supernatant were produced
354 and pooled for further processing.

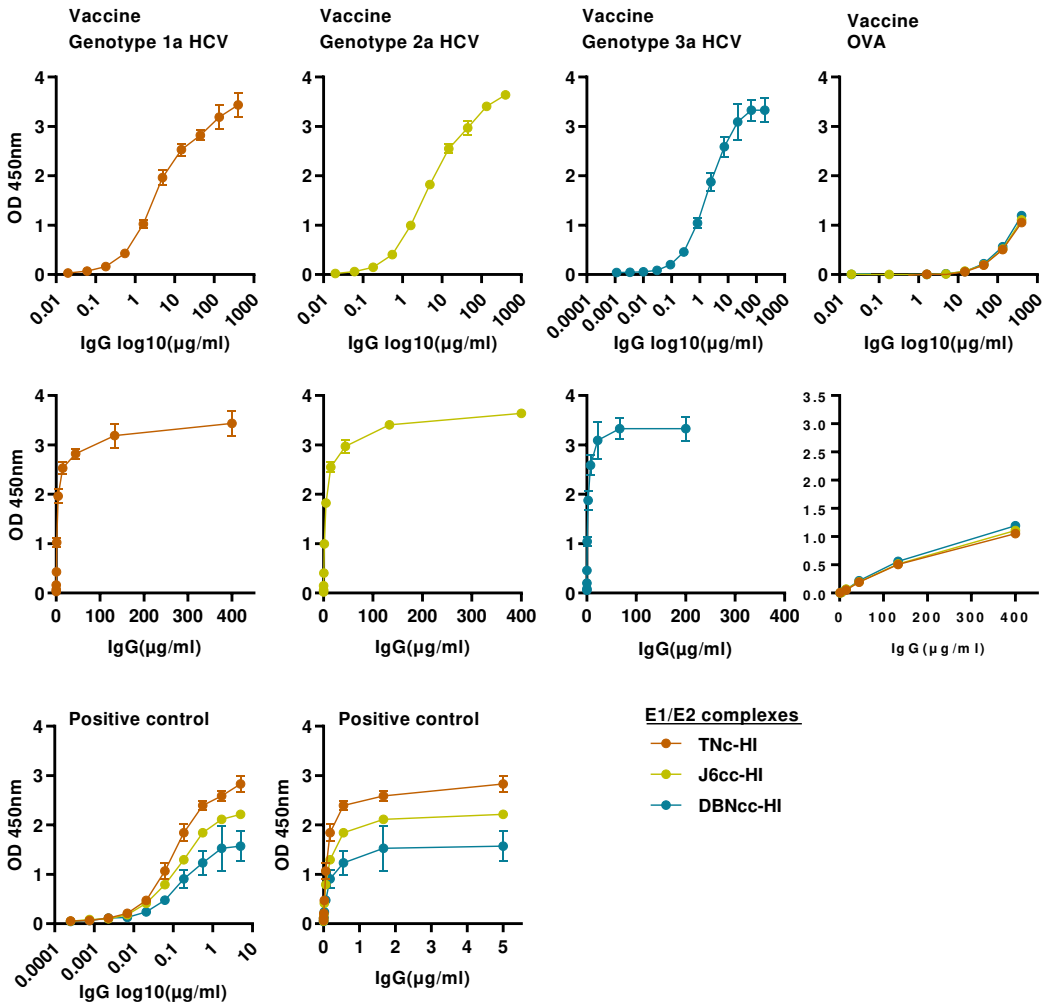


Supplementary Figure 4. Downstream processing of cell culture produced genotype 1a, 2a and 3a HCV. For each of genotype 1a, 2a and 3a HCV, ~16l of pooled virus containing cell culture supernatant produced in cell-factories were concentrated for vaccine production. (A) HCV infectivity titers (left y-axis) and volumes (right y-axis) of material from the different processing steps: pool CF,

pooled supernatant from cell-factories; TFF, 2600cm^2 and TFF, 790cm^2, TFF with hollow fibers with specified surface area; 3-cushion UC F1, F2 and F3, fraction 1, 2 and 3 from 3-cushion ultracentrifugation with the striped bar indicating F2 subjected to further processing; TFF, 20cm^2, TFF with hollow fiber with specified surface area; pool gradient UC, pool of 3 fractions selected from fraction 7-11 shown in B; pool chromatography, pool of fractions selected from fraction 2-6 shown in C. (B) HCV infectivity titers (left y-axis) and buoyant densities (right y-axis) of 18 fractions collected following equilibrium gradient ultracentrifugation (gradient UC). Striped bars indicate the fractions that were pooled for further processing (pool gradient UC, same samples also shown in A). (C) HCV infectivity titers (left y-axis) and OD230nm determinations (right y-axis) for 12 fractions collected during Sephadex chromatography. Striped bars indicate the fractions that were pooled prior to UV inactivation (pool chromatography, samples also shown in A). (A-C) HCV infectivity titers are given as log10 FFU/ml and as means of three replicates with SD. For buoyant density and OD determination, a single measurement was done.

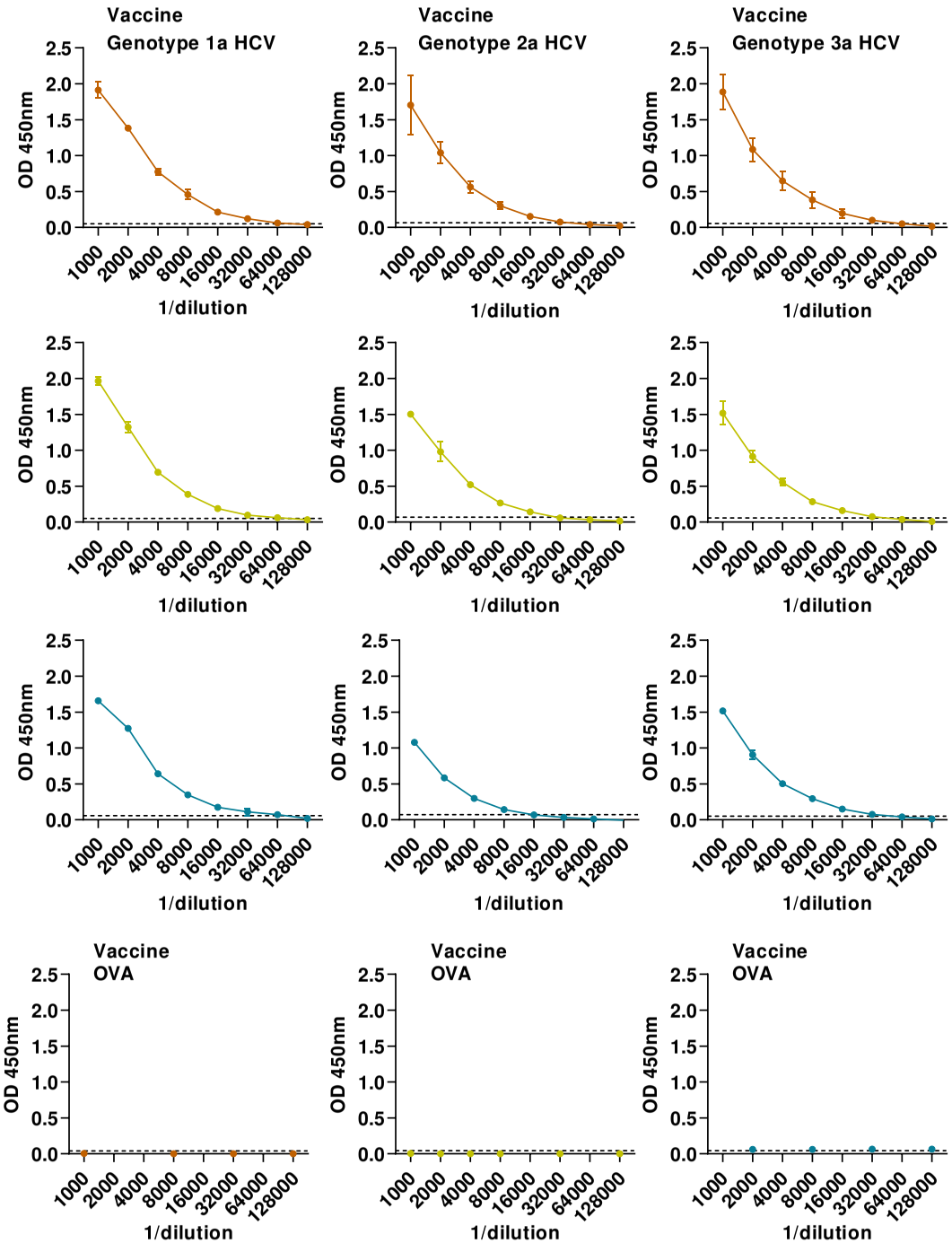


Supplementary Figure 5. Absence of HCV antigen positive cells in cell cultures inoculated with UV inactivated HCV. Three Huh7.5 cell cultures plated the previous day at 80000 cells per well in a 24-well dish, were inoculated with 100µl of UV irradiated HCV of the specified genotype. Positive HCV infection control cultures were inoculated with a genotype 5a HCV[31] at an MOI that resulted in robust infection. Negative control cultures were not inoculated. Cultures inoculated with irradiated HCV and negative control cultures were followed for 3 weeks by splitting every 2-3 days and determination of the percentage of HCV antigen positive cells by immunostaining for HCV core and NS5A. HCV infection control cultures were followed until peak of infection. Percentages of HCV antigen positive cells are means of immunostainings from three replicate cell cultures with SD. For the negative control datapoints are nudged by 0.5 units in the x-axis direction.



Supplementary Figure 6. Immunization with inactivated genotype 1a, 2a or 3a HCV elicited IgG binding to HCV envelope proteins approaching a binding plateau. Binding capacity of pooled purified serum IgG from mice immunized with inactivated genotype 1a, 2a or 3a HCV or OVA to E1/E2 complexes of the homologous HI-recombinants was evaluated by ELISA. Values are OD reads at 450nm following subtraction of mean OD of 8 negative controls. Datapoints are means of two replicates with SD. Positive controls: Instead of serum IgG, well-characterized primary antibodies were used: AP33[38] for binding to TNcc-HI and DBNcc-HI E1/E2 and H77.39[39] for binding to J6cc-HI E1/E2. Negative controls: No IgG was used and TNcc-HI, J6cc-HI and DBNcc-HI E1/E2 were incubated with secondary antibody only; for negative controls, OD reads were ~0.05. Data are shown

either with logarithmic or linear x axis. Compared to Figure 8, higher IgG concentrations were used to evaluate if a binding plateau could be approached.



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Alzua GP, *et al*. *Gut* 2022;0:1–13. doi: 10.1136/gutjnl-2021-326323

Supplementary Figure 7. Immune-sera binding to HCV envelope proteins and determination of endpoint titers. Binding capacity of immune-sera from mice immunized with inactivated genotype 1a, 2a or 3a HCV or OVA to E1/E2 complexes of the specified HI-recombinants was evaluated by ELISA. Values are OD reads at 450nm following subtraction of mean OD of 8 negative controls. Datapoints are means of two replicates with SD. Negative controls: No immune-sera were used and TNcc-HI, J6cc-HI and DBNcc-HI E1/E2 were incubated with secondary antibody only; for negative controls, OD reads were ~0.05. Broken-lines: 2-fold mean OD of negative controls, used as cut-off for determination of endpoint titers.

405 **Supplementary Tables**

406 Supplementary Table 1. Primers for reverse transcription for generation of full-length HCV ORF
407 amplicons.

3' cDNA primers	Target virus	Primer sequence (5'-3')
1a-9405-RT	1a(TNcc, TNcc-HI)	TAAGAGGCCGGAGTGTTTAC
2a-9481-RT	2a(J6cc, J6cc-HI)	CTATGGAGTGTACCTAGTGTGTGC
3a-9435-RT	3a(DBNcc, DBNcc-HI)	AAAAGAATGGAGTGTTAT

408

409 Supplementary Table 2. Primers for PCR for generation of full-length HCV ORF amplicons and
410 amplicons spanning E1/E2.

Primers	Target virus	Primer sequence (5'-3')
1a-209-F	1a(TNcc, TNcc-HI)	TGCCTGATAGGGTGCTTGCG
1a-9402-R	1a(TNcc, TNcc-HI)	AGGCCGGAGTGTTTACCCCA
1a-3285R	1a(TNcc, TNcc-HI)	TGGTCTCCATCTGGGAAAAG
2a-303-F	2a(J6cc, J6cc-HI)	CTTGCGAGTGCCCCGGGAGG
2a-9467-R	2a(J6cc, J6cc-HI)	TGGAGTGTACCTAGTGTGTGCCGCTC
2a-3774-R	2a(J6cc, J6cc-HI)	GGGATGACATCAGCGTTCCGCGTGAC
3a-293-F	3a(DBNcc, DBNcc-HI)	GATAGGGTGCTTGCGAGTGCC
3a-9432-R	3a(DBNcc, DBNcc-HI)	AGAATGGAGTGTTATCCTACCAGCTCA
3a-3694R	3a(DBNcc, DBNcc-HI)	CTGGCCACCCAACRAGRTCCT

411

412 Supplementary Table 3. Coding nucleotide changes identified in polyclonal passaged genotype 1a
413 HCV from an initial passage line.

HCV protein[a]	Nucleotide change[b]			Allele frequency (%)[c]			AA change TNcc reference[d]	Engineered construct[e]	
	TNcc position	TNcc reference	change	TNcc-PP-10	TNcc-PP-18	Gt 1a HCV seed stock		TNcc-HI-18A	TNcc-HI-18B
core	373	C	A	16	26	41	T11N		
	430	T	C	7	12	11	I30T		
E2	1571	C	G	18	27	42	N410K		
	2464	G	A	11	29	16	S708N		
NS2	2822	G	A	5	16	24	M827I		
	2895	A	G	17	30	15	R852G		
	2935	A	C	1	14	21	N865T		
	3364	G	A	18	28	44	R1008Q		
NS3	3804	C	G	20	33	15	P1155A		
	3978	A	T	2	10	23	N1213Y		
	4848	G	A	5	10	10	A1503T		
NS4B	5812	G	A	15	5	12	G1824D		
	6067	G	C	25	36	59	G1909A		
NS5A	6729	C	A	4	24	40	L2130I		
	7296	C	T	5	15	23	P2319S		
	7410	A	G	24	34	17	T2357A		x
	7464	A	G	29	45	27	S2375G		x
	7588	A	G	9	20	23	D2416G		
	7591	T	C	32	34	46	V2417A		
	7596	T	C	25	43	29	C2419R		x
NS5B	7785	A	G	18	29	43	S2482G	x	
	8292	A	C	61	60	80	N2651H	x	
	8841	A	T	29	38	20	M2834L		x
	8901	A	C	5	11	11	I2854L		
	8985	A	C	9	18	24	I2882L		
	9045	A	C	7	27	43	I2902L	x	
	9298	A	G	72	96	98	H2986R	x	x

414

415 [a], HCV protein, in which specified change was located.

416 [b], Nucleotide change specified by nucleotide position and identity relating to the TNcc genome
417 (Genbank accession number JX993348) as a reference.[26] Coding changes with allele frequency of at
418 least 10% in one of the analyzed samples are listed.

419 [c], Allele frequency of the identified nucleotide change. Polyclonal passage 10 (TNcc-PP-10) and 18
420 (TNcc-PP-18) viruses from the initial passage line (Figure 1), and the seed stock used for vaccine
421 production (Gt 1a HCV seed stock) were analyzed.

422 [d], Amino acid change specified by amino acid position and identity relating to the TNcc polyprotein;
423 amino acid position numbers are identical to position numbers in relation to the reference H77
424 polyprotein (Genbank accession number AF009606).
425 [e], x indicates that the respective nucleotide change was engineered for generation of TNcc-HI-18A
426 and TNcc-HI-18B, respectively.

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Supplementary Table 4. Coding nucleotide changes identified in polyclonal passaged genotype 1a HCV from a later passage line**.**

HCV protein[a]	Nucleotide change[b]			Allele frequency (%)[c]	AA change TNcc reference[d]	Engineered construct[e]
	TNcc position	TNcc reference	change			
				TNcc-PP-38.1		TNcc-HI
core	373	C	A	97	T11N	x
	435	G	A	48	G32S	x
	756	C	A	17	L139I	
E1	1435	T	C	99	V365A	x
E2	1548	T	C	99	F403L	x
	1571	C	G	100	N410K	x
	2496	G	A	100	V719I	x
p7	2701	T	C	92	V787A	x
NS2	2917	T	C	100	V859A	x
	3364	G	A	100	R1008Q	x
NS3	4267	C	T	15	A1309V	
	4363	C	T	100	A1341V	x
	4711	G	C	99	C1457S	x
NS4B	6067	G	C	100	G1909A	x
NS5A	6729	C	A	100	L2130I	x
	7591	T	C	100	V2417A	x
NS5B	7785	A	G	99	S2482G	x
	8292	A	C	99	N2651H	x
	9045	A	C	100	I2902L	x
	9298	A	G	99	H2986R	x

[a], HCV protein in which specified change was located.

[b], Nucleotide change specified by nucleotide position and identity relating to the TNcc genome (Genbank accession number JX993348) as a reference.[26] Coding changes with allele frequency of at least 10% in one of the analyzed samples are listed.

[c], Allele frequency of the identified nucleotide change. Polyclonal passage 38.1 (TNcc-PP-38.1) virus from the later passage line was analyzed (Figure 1).

[d], Amino acid change specified by amino acid position and identity relating to the TNcc polyprotein; amino acid position numbers are identical to position numbers in relation to the reference H77 polyprotein (Genbank accession number AF009606).

[e], x indicates that the respective nucleotide change was engineered for generation of TNcc-HI.

Supplementary Table 5. Coding nucleotide changes identified in polyclonal passaged genotype 2a HCV.

HCV Protein[a]	Nucleotide change[b] J6cc position	J6cc reference	change	Allele frequency (%)[c] J6cc-PP-35	Gt 2a HCV seed stock	AA change J6cc reference[d]	AA position H77 reference[e]	Engineered construct[f] J6cc-HI
core	572	A	G	99	99	K78E	78	x
	758	G	C	81	81	V140L	140	x
E1	1325	A	T	100	100	T329S	329	x
E2	1583	A	G	14	14	N415D	415	
	1640	C	A	100	100	H434N	434	x
	2063	G	A	83	83	A575T	573	x
p7	2618	G	A	94	94	A760T	756	x
	2658	T	C	94	94	V773A	769	x
NS2	2823	T	C	93	93	V828A	824	x
	2913	A	C	17	17	E858A	854	
NS3	3777	C	T	82	82	A1146V	1142	x
	4328	A	G	100	100	I1330V	1326	x
	4839	T	C	93	93	L1500P	1496	x
NS4B	5843	G	A	82	82	V1835I	1831	x
NS5A	6338	A	G	93	93	T2000A	1996	x
	7428	T	C	100	92	L2363P	2363	x
	7439	G	C	100	100	A2367P	2367	x
	7661	T	C	45	45	C2441R	2419	
NS5B	8687	G	C	81	81	E2783Q	2761	x
	9378	T	C	95	45	L3013S	2991	x

[a], HCV protein in which specified change was located.

[b], Nucleotide change specified by nucleotide position and identity relating to the J6cc genome (Genbank accession number JQ745650) as a reference.[27] Coding changes with allele frequency of at least 10% in one of the analyzed samples are listed.

[c], Allele frequency of the identified nucleotide change. Polyclonal passage 35 (J6cc-PP-35) virus (Figure 1) and the seed stock used for vaccine production (Gt 2a HCV seed stock) were analyzed.

[d], Amino acid change specified by amino acid position and identity relating to the J6cc polyprotein (Genbank accession number JQ745650).

[e], Amino acid position relating to the H77 reference polyprotein (Genbank accession number AF009606).

[f], x indicates that the respective nucleotide change was engineered for generation of J6cc-HI.

455 Supplementary Table 6. Coding nucleotide changes identified in polyclonal passaged genotype 3a
456 HCV.

HCV protein[a]	Nucleotide change[b]			Allele frequency (%)[c]		AA change DBN3acc reference[d]	AA position H77 reference[e]	Engineered construct[f]
	DBN3acc position	DBN3acc reference	change					
				DBNcc-PP-16	Gt 3a HCV seed stock			DBNcc-HI
E2	1522	G	A	97	100	G395R	395	x
NS2	2905	G	A	96	100	A856T	849	x
	2962	G	A	97	100	G875R	868	x
NS3	5105	A	G	97	100	N1589S	1582	x
NS4B	5597	T	G	96	100	I1753S	1746	x
NS5A	7622	T	C	97	100	V2428A	2417	x
NS5B	8759	A	G	97	100	D2807G	2796	x

457

458 [a], HCV protein in which specified change was located.

459 [b], Nucleotide change specified by nucleotide position and identity relating to the DBN3acc genome
460 (Genbank accession number KX280714) as a reference.[28] Coding changes with allele frequency of at
461 least 10% in one of the analyzed samples are listed.

462 [c], Allele frequency of the identified nucleotide change. Polyclonal passage 16 (DBNcc-PP-16) virus
463 (Figure 1) and the seed stock used for vaccine production (Gt 3a HCV seed stock) were analyzed.

464 [d], Amino acid change specified by amino acid position and identity relating to the DBN3acc
465 polyprotein (Genbank accession number KX280714).

466 [e], Amino acid position relating to the H77 reference polyprotein (Genbank accession number
467 AF009606).

468 [f], x indicates that the respective nucleotide change was engineered for generation of DBNcc-HI.

469 Supplementary Table 7. Subclonal analysis of polyclonal passaged genotype 1a HCV from a later
470 passage line.

HCV protein[a]	**core**				**E1**		**E2**						
Nucleotide position[b]	373	435	463	756	1188	1435	1497	1512	1548	1571	1842	2232	2496
TNcc reference	C	G	G	C	T	T	T	A	T	C	A	A	G
Nucleotide change	A	A	C	A	C	C	C	G	C	G	G	G	A
Allele frequency (%)[c]	97	48	nd	17	nd	99	6	9	99	100	nd	nd	100
# Subclones[d]													
1	x	x				x			x	x			x
2	x	x				x			x	x			x
3	x	x			x	x			x	x			x
4	x	x				x			x	x	x		x
5	x			x		x		x	x	x		x	x
6	x	x				x		x	x	x			x
7	x	x	x	x		x	x		x	x			x
AA change TNcc reference[e]	T11N	G32S	G41A	L139I	S283P	V365A	Y386H	S391G	F403L	N410K	S501G	M631V	V719I
Engineered construct[f]	x	x				x			x	x			x

HCV protein[a]	**p7**		**NS2**			**NS3**							
Nucleotide position[b]	2650	2701	2917	2959	3364	3876	3907	4071	4138	4267	4363	4711	5130
TNcc reference	T	T	T	T	G	T	T	T	C	C	C	G	G
Nucleotide change	C	C	C	C	A	A	G	C	G	T	T	C	A
Allele frequency (%)[c]	1	92	100	8	100	nd	nd	nd	nd	15	100	99	nd
# Subclones[d]													
1		x	x		x	x					x	x	
2		x	x	x	x						x	x	
3	x	x	x		x		x	x			x	x	
4		x	x		x						x	x	
5		x	x		x						x	x	x
6		x	x		x						x	x	
7		x	x		x				x		x	x	
AA change TNcc reference[e]	V770A	V787A	V859A	V873A	R1008Q	L1179I	V1189G	Y1244H	A1266G	A1309V	A1341V	C1457S	A1597T
Engineered construct[f]		x	x		x						x	x	

HCV protein[a]	**NS4B**		**NS5A**								
Nucleotide position[b]	5575	6067	6315	6729	7288	7386	7404	7407	7422	7500	7591
TNcc reference	T	G	A	C	T	A	T	T	A	T	T
Nucleotide change	C	C	G	A	A	T	C	C	T	C	C
Allele frequency (%)[c]	1	100	nd	100	nd	nd	1	6	0.4	nd	100
# Subclones[d]											
1		x		x			x				x
2		x		x		x					x
3		x	x	x							x
4		x		x						x	x
5		x		x	x						x
6		x		x				x			x
7	x	x		x					x		x
AA change TNcc reference[e]	V1745A	G1909A	K1992E	L2130I	L2316H	T2349S	S2355P	S2356P	T2361S	S2387P	V2417A
Engineered construct[f]		x		x							x

HCV protein[a]	**NS5B**					
Nucleotide position[b]	7785	7993	8292	8779	9045	9298
TNcc reference	A	T	A	C	A	A
Nucleotide change	G	C	C	T	C	G
Allele frequency (%)[c]	99	nd	99	nd	100	99
# Subclones[d]						
1	x	x	x		x	x
2	x		x		x	x
3	x		x		x	x
4	x		x		x	x
5	x		x		x	x
6	x		x		x	x
7	x		x	x	x	x
AA change TNcc reference[e]	S2482G	V2551A	N2651H	A2813V	I2902L	H2986R
Engineered construct[f]	x		x		x	x

[a], HCV protein, in which specified change was located.

[b], Nucleotide position, identity and change relating to the TNcc genome (Genbank accession number JX993348).[26] Coding changes occurring in at least one subclone are listed.

[c], Allele frequency of the identified nucleotide change in TNcc-PP-38.1 as determined by NGS (Supplementary Table 4). nd, not detected in NGS.

[d], Number of subclone. x, indicates presence of the respective change. Grey shadings indicate which subclone fragments were used for construction of the engineered construct.

479 [e], Amino acid change specified by amino acid position, identity and change relating to the TNcc
480 polyprotein (Genbank accession number JX993348).
481 [f], x, indicates that the respective nucleotide change was engineered for generation of TNcc-HI.

482 Supplementary Table 8. Subclonal analysis of polyclonal passaged genotype 2a HCV.

HCV protein[a]	**core**										**E1**		
Nucleotide position[b]	382	383	405	527	572	758	830	845	872	908	1030	1118	1199
J6cc reference	C	A	T	C	A	G	T	T	T	T	G	A	G
Nucleotide change	A	C	C	del	C	C	C	A	G	A	C	G	T
Allele frequency (%)[c]	nd	nd	nd	nd	99	80	nd	nd	nd	nd	nd	nd	7
# Subclones[d]													
2					x	x							
10					x	x							x
11			x		x	x						x	
13					x			x					
14					x	x							
18					x	x							
19					x	x				x			
20					x	x							
22	x	x		x	x	x			x				x
26					x	x					x		
27					x	x	x						
AA change J6cc reference[e]	N14K	T15P	V22A		K78E	V140L	F164L	L169I	L178V	S190T	E230D	T260A	A287S
Engineered construct[f]					x	x							

HCV protein[a]	**E1**				**E2**									
Nucleotide position[b]	1318	1325	1418	1439	1536	1583	1590	1640	1940	2063	2126	2174	2228	2454
J6cc reference	G	A	G	G	T	A	A	C	A	G	A	C	A	T
Nucleotide change	A	T	del	C	C	G	G	A	G	A	G	A	G	A
Allele frequency (%)[c]	nd	100	nd	nd	4	12	4	99,8	8	82	2	nd	nd	3
# Subclones[d]														
2		x						x		x				
10		x						x		x				
11		x		x				x		x				
13		x						x		x				
14	x	x						x		x				
18		x			x			x		x				
19		x				x		x		x				
20		x						x		x		x		
22		x					x	x	x					x
26		x						x		x			x	
27		x	x			x		x		x	x			
AA change J6cc reference[e]	W326*	T329S	na	A367P	L399P	N415D	N417I	N434H	N532D	A575T	T596A	L612M	I630V	F705Y
Engineered construct[f]		x						x		x				

483

HCV protein[a]	**p7**					**NS2**								
Nucleotide position[b]	2616	2618	2658	2685	2735	2823	2879	2904	2913	2984	3011	3160	3212	3295
J6cc reference	A	G	T	T	T	T	T	C	A	G	G	G	A	G
Nucleotide change	G	A	C	ins	C	C	G	T	C	del	A	C	G	C
Allele frequency (%)[c]	nd	93	93	nd	nd	92	nd	nd	15	nd	nd	nd	nd	nd
# Subclones[d]														
2		x	x			x						x		
10		x	x			x			x					
11		x	x			x								
13		x	x		x	x	x			x				
14	x	x	x			x								
18		x	x	x		x								x
19		x	x			x			x				x	
20		x	x			x								
22								x			x			
26		x	x			x								
27		x	x			x			x					
AA change J6cc reference[e]	H759R	A760T	V773A	na	S799P	V828A	L846V	T855I	E858A	na	D891N	M940I	T958A	K985N
Engineered construct[f]		x	x			x								

HCV protein[a]	**NS3**												**NS4A**
Nucleotide position[b]	3702	3705	3777	3870	4173	4328	4694	4839	5154	5157	5157	5208	5432
J6cc reference	C	A	C	G	T	A	T	T	C	C	C	T	G
Nucleotide change	T	G	T	A	C	G	C	C	T	del	ins	C	A
Allele frequency (%)[c]	nd	nd	81	nd	nd	100	nd	93	nd	nd	nd	nd	0.2
# Subclones[d]													
2			x			x		x	x				x
10			x	x		x		x					
11	x		x			x		x				x	
13			x		x	x		x					
14			x			x		x					
18			x			x		x		x			
19		x	x			x		x			x		
20			x			x		x					
22			x			x		x					
26			x			x		x					
27			x			x	x	x					
AA change J6cc reference[e]	T1121I	K1122R	A1146V	R1177K	I1278T	I1330V	F1452L	L1500P	P1605L	na	na	V1623A	V1698I
Engineered construct[f]			x			x		x					

484

HCV protein[a]	NS4B								NS5A				
Nucleotide position[b]	5562	5563	5571	5787	5796	5843	6078	6210	6338	6509-6694	6563	6581	6596
J6cc reference	A	G	C	T	G	G	G	T	A		G	A	A
Nucleotide change	G	C	G	ins	A	A	A	C	G	del	T	T	G
Allele frequency (%)[c]	nd	0.1	nd	nd	nd	81	nd	nd	92	nd	nd	9	nd
# Subclones[d]													
2						x			x				
10						x	x		x				
11					x	x			x				
13						x			x		x		
14						x			x				
18				x		x			x				
19			x			x			x			x	
20	x	x				x			x			x	x
22						x			x	x			
26						x			x				
27						x		x	x				
AA change J6cc reference[e]	Q1741R	Q1741H	S1744C	na	W1819*	V1835I	G1913E	I1957T	T2000A	na	V2075L	N2081Y	I2086V
Engineered construct[f]						x			x				

HCV protein[a]	NS5A												
Nucleotide position[b]	6639	6861	6951	7026	7059	7107	7122	7187	7204	7335	7358	7428	7436
J6cc reference	A	T	G	A	A	T	A	C	G	C	A	T	G
Nucleotide change	G	C	T	C	G	C	G	T	T	G	G	C	T
Allele frequency (%)[c]	2	nd	nd	nd	nd	nd	nd	1	nd	nd	nd	92	nd
# Subclones[d]													
2					x						x	x	
10		x										x	
11								x				x	
13		x		x								x	
14			x									x	
18							x					x	
19												x	
20	x											x	
22									x			x	x
26												x	
27						x				x		x	
AA change J6cc reference[e]	H2100R	V2174A	S2204I	D2229A	D2240G	L2256P	E2261G	P2283S	W2289C	T2332R	I2340V	L2363P	G2366W
Engineered construct[f]												x	

485

HCV protein[a]	NS5A							NS5B							
Nucleotide position[b]	7439	7523	7586	7625	7640	7649	7661	7908	8209	8559	8639	8687	9017	9308	9378
J6cc reference	G	C	C	T	G	G	T	G	A	A	G	G	G	T	T
Nucleotide change	C	ins	del	C	A	C	C	A	del	G	A	C	ins	C	C
Allele frequency (%)[c]	100	nd	nd	nd	6	nd	39	nd	nd	nd	nd	81	nd	nd	95
# Subclones[d]															
2	x		x				x	x				x			x
10	x						x					x	x		x
11	x		x				x				x	x			x
13	x				x							x			x
14	x						x					x			x
18	x		x		x							x			x
19	x						x					x			x
20	x	x					x			x		del			x
22	x					x						x		x	x
26	x								x			x			x
27	x			x								x			x
AA change J6cc reference[e]	A2367P	na	na	W2429R	E2434K	D2437H	C2441R	R2523K	na	K2740R	E2767K	E2783Q	na	S2990P	L3013S
Engineered construct[f]	x											x			x

[a], HCV protein, in which specified change was located.

[b], Nucleotide position, identity and change relating to the J6cc genome (Genbank accession number JQ745650).[27] Coding changes as well as insertions (ins) or deletions (del) occurring in at least one subclone are listed.

[c], Allele frequency of the identified nucleotide change in J6cc-PP-35 as determined by NGS (Supplementary Table 5). nd, not detected in NGS.

[d], Number of subclone. x, indicates presence of the respective change. Grey shadings indicate which subclone fragments were used for construction of the engineered construct.

[e], Amino acid change specified by amino acid position, identity and change relating to the J6cc polyprotein (Genbank accession number JQ745650). na, not applicable. *, stop codon.

[f], x, indicates that the respective nucleotide change was engineered for generation of J6cc-HI.

498 Supplementary Table 9. Subclonal analysis of polyclonal passaged genotype 3a HCV.

HCV protein[a]	core				E1	E2				p7
Nucleotide position[b]	386	529	618	755	1453	1501	1522	1544	2068	2731
DBN3acc reference	T	C	G	T	G	A	G	T	G	A
Nucleotide change	C	A	A	C	T	G	A	C	A	G
Allele frequency (%)[c]	nd	nd	nd	nd	nd	7	97	nd	0.6	nd
# Subclones[d]										
1							x		x	
2				x			x			
3			x				x			
4	x	x			x		x	x		
5						x	x			
6							x			
7				x			x			
8							x			
9							x			
10							x			x
AA change DBN3acc reference[e]	I16T	P64T	W93*	L139P	A372S	I388V	G395R	L402S	E577K	T798A
Engineered construct[f]							x			

HCV protein[a]	NS2				NS3					NS4A		
Nucleotide position[b]	2905	2941	2962	3152	4093	4588	4771	5105	5158	5549	5589	5597
DBN3acc reference	G	G	G	G	G	A	T	A	C	A	A	T
Nucleotide change	A	del	A	A	C	G	ins	G	T	G	T	G
Allele frequency (%)[c]	96	nd	97	0.7	nd	0.4	nd	97	nd	0.6	0.4	96
# Subclones[d]												
1	x		x					x	x			x
2						x	x	x				x
3	x		x					x		x		x
4	x	x	x					x				x
5	x		x					x			x	x
6	x		x	x	x			x				x
7						x	x	x				x
8	x		x					x				x
9	x		x					x				x
10	x		x					x				x
AA change DBN3acc reference[e]	A856T	na	G875R	G938E	V1252L	M1417V	na	N1589S	P1607S	E1737G	Q1750H	I1753S
Engineered construct[f]	x		x					x				x

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HCV protein[a]	**NS4B**			**NS5A**					**NS5B**				
Nucleotide position[b]	5726	5872	6427	6800	7048	7409	7465	7622	8552	8571	8612	8759	8824
DBN3acc reference	C	G	G	T	A	A	A	T	G	T	A	A	G
Nucleotide change	T	C	A	T	G	T	T	C	C	del	C	G	A
Allele frequency (%)[c]	nd	nd	nd	ins	nd	nd	nd	97	nd	nd	nd	97	3
# Subclones[d]													
1								x		x	x	x	
2				x			x	x				x	
3								x				x	
4		x						x				x	
5			x					x	x			x	
6					x			x				x	x
7				x			x	x				x	
8	x							x				x	
9						x		x				x	
10								x				x	
AA change DBN3acc reference[e]	S1796F	A1845P	G2030S	na	N2237D	K2357I	T2376S	V2428A	G2738A	na	D2758A	D2807G	E2829K
Engineered construct[f]								x				x	

[a], HCV protein, in which specified change was located.

[b], Nucleotide position, identity and change relating to the DBN3acc genomes (Genbank accession number KX280714).[28] Coding changes as well as insertions (ins) or deletions (del) occurring in at least one subclone are listed.

[c], Allele frequency of the identified nucleotide change in DBNcc-PP-16 as determined by NGS (Supplementary Table 6). nd, not detected in NGS.

[d], Number of subclone. x, indicates presence of the respective change. Grey shadings indicate which subclone fragments were used for construction of the engineered construct.

[e], Amino acid change specified by amino acid position, identity and change relating to the DBN3acc polyprotein (Genbank accession number KX280714). na, not applicable. *, stop codon

[f], x indicates that the respective nucleotide change was engineered for generation of DBNcc-HI.

512 Supplementary Table 10. Substitutions acquired during serial passage of HCV in this study and
513 previously reported cell culture infectious HCV recombinants.

AA position H77 reference[a]	Substitutions in this study[b]			Substitutions in previous studies[c]						References[d]
	TNcc-P38	J6cc-P35	DBNcc-P16	H77cc	HCV1cc	H77-JFH1	J4-JFH1	J6-JFH1	JFH1	
32	G-S								G-S	Aligeti et al., *J Virol.* 2015;22:11523-11533
78		K-E						K-E		Bungyoku et al., *J Gen Virol.* 2009;7:1681-1691
787	V-A					V-A				Scheel et al., *Proc Natl Acad Sci USA.* 2008;3:997-1002
1326		I-V			I-V					Li et al., *J Virol.* 2015;1:811-823
1496		L-P					Q-L			Gottwein et al, *Hepatology.* 2009;49:364-377
1909	G-A			G-S						Li et al., *J Virol.* 2015;1:811-823
2417	V-A		V-A	V-A						Li et al., J Virol. 2015;1:811-823

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515 [a], Amino acid position, relating to the H77 reference polyprotein (Genbank accession number
516 AF009606).

517 [b], Amino acid substitutions identified in the TNcc-P38.1, J6cc-P35 and DBNcc-P16 polyproteins that
518 were subsequently engineered.

519 [c], Amino acid substitutions reported in previously developed cell culture infectious HCV
520 recombinants.

521 [d], literature references