Salas, J. H. et al. (2022) An antigenically diverse, representative panel of envelope glycoproteins for HCV vaccine development. *Gastroenterology*, 162(2), pp. 562-574. (doi: 10.1053/j.gastro.2021.10.005).

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Gastroenterology

An antigenically diverse, representative panel of envelope glycoproteins for HCV vaccine development

--Manuscript Draft--

Manuscript Number:	GASTRO-D-21-00836R1
Full Title:	An antigenically diverse, representative panel of envelope glycoproteins for HCV vaccine development
Article Type:	Basic - Liver
Section/Category:	Basic
Corresponding Author:	Justin R. Bailey Johns Hopkins School of Medicine: Johns Hopkins University School of Medicine Baltimore, MD UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Johns Hopkins School of Medicine: Johns Hopkins University School of Medicine
Corresponding Author's Secondary Institution:	
First Author:	Jordan H. Salas, BS
First Author Secondary Information:	
Order of Authors:	Jordan H. Salas, BS
	Richard A. Urbanowicz, PhD
	Johnathan D. Guest, BS
	Nicole Frumento, BS
	Alexis Figueroa, BS
	Kaitlyn E. Clark, BS
	Zhenyong Keck, PhD
	Vanessa M. Cowton, PhD
	Sarah J. Cole, PhD
	Arvind H. Patel, PhD
	Thomas R. Fuerst, PhD
	Heidi E. Drummer, PhD
	Marian Major, PhD
	Alexander W. Tarr, PhD
	Jonathan K. Ball, PhD
	Mansun Law, PhD
	Brian G. Pierce, PhD
	Steven K. H. Foung, MD PhD
	Justin R. Bailey, MD PhD
Order of Authors Secondary Information:	
Abstract:	Background and Aims Development of a prophylactic hepatitis C virus (HCV) vaccine will require accurate and reproducible measurement of neutralizing breadth of vaccine-induced antibodies.

Currently available HCV panels may not adequately represent the genetic and antigenic diversity of circulating HCV strains, and the lack of standardization of these panels makes it difficult to compare neutralization results obtained in different studies. Here, we describe the selection and validation of a genetically and antigenically diverse reference panel of 15 HCV pseudoparticles (HCVpp) for neutralization assays.

Methods

We chose 75 envelope (E1E2) clones to maximize representation of natural polymorphisms observed in circulating HCV isolates, and 65 of these clones generated functional HCVpp. Neutralization sensitivity of these HCVpp varied widely. HCVpp clustered into 15 distinct groups based on patterns of relative sensitivity to seven broadly neutralizing monoclonal antibodies (bNAbs). We used these data to select a final panel of 15 antigenically representative HCVpp.

Results

Both the 65 and 15 HCVpp panels span four tiers of neutralization sensitivity, and neutralizing breadth measurements for seven bNAbs were nearly equivalent using either panel. Differences in neutralization sensitivity between HCVpp were independent of genetic distances between E1E2 clones.

Conclusions

Neutralizing breadth of HCV antibodies should be defined using viruses spanning multiple tiers of neutralization sensitivity, rather than panels selected solely for genetic diversity. We propose that this multi-tier reference panel could be adopted as a standard for the measurement of neutralizing antibody potency and breadth, facilitating meaningful comparisons of neutralization results from vaccine trials in different laboratories.

Order of Authors (with Contributor Roles):

Jordan H. Salas, BS (Conceptualization: Lead; Data curation: Lead; Formal analysis: Lead; Investigation: Lead; Methodology: Lead; Validation: Lead; Writing – original draft: Lead; Writing – review & editing: Lead)

Richard A. Urbanowicz, PhD (Conceptualization: Supporting; Data curation: Supporting; Formal analysis: Supporting; Investigation: Supporting; Methodology: Supporting; Resources: Supporting; Writing – review & editing: Supporting)

Johnathan D. Guest, BS (Conceptualization: Supporting; Data curation: Supporting; Formal analysis: Supporting; Methodology: Supporting; Writing – review & editing: Supporting)

Nicole Frumento, BS (Data curation: Supporting; Formal analysis: Supporting; Investigation: Supporting; Methodology: Supporting; Writing – review & editing: Supporting)

Alexis Figueroa, BS (Formal analysis: Supporting; Investigation: Supporting; Writing – review & editing: Supporting)

Kaitlyn E. Clark, BS (Formal analysis: Supporting; Investigation: Supporting; Writing – review & editing: Supporting)

Zhenyong Keck, PhD (Resources: Supporting; Writing – review & editing: Supporting)

Vanessa M. Cowton, PhD (Resources: Supporting; Writing – review & editing: Supporting)

Sarah J. Cole, PhD (Resources: Supporting; Writing – review & editing: Supporting)

Arvind H. Patel, PhD (Conceptualization: Supporting; Methodology: Supporting; Resources: Supporting; Writing – review & editing: Supporting)

Thomas R. Fuerst, PhD (Conceptualization: Supporting; Methodology: Supporting; Writing – review & editing: Supporting)

Heidi E. Drummer, PhD (Conceptualization: Supporting; Methodology: Supporting; Writing – review & editing: Supporting)

	Marian Major, PhD (Conceptualization: Supporting; Methodology: Supporting; Writing – review & editing: Supporting)
	Alexander W. Tarr, PhD (Conceptualization: Supporting; Formal analysis: Supporting; Investigation: Supporting; Methodology: Supporting; Resources: Supporting; Writing – review & editing: Supporting)
	Jonathan K. Ball, PhD (Conceptualization: Supporting; Methodology: Supporting; Resources: Supporting; Writing – review & editing: Supporting)
	Mansun Law, PhD (Conceptualization: Supporting; Methodology: Supporting; Resources: Supporting; Writing – review & editing: Supporting)
	Brian G. Pierce, PhD (Conceptualization: Supporting; Formal analysis: Supporting; Methodology: Supporting; Writing – review & editing: Supporting)
	Steven K. H. Foung, MD PhD (Conceptualization: Supporting; Methodology: Supporting; Resources: Supporting; Writing – review & editing: Supporting)
	Justin R. Bailey, MD PhD (Conceptualization: Lead; Data curation: Lead; Formal analysis: Lead; Funding acquisition: Lead; Investigation: Lead; Methodology: Lead; Project administration: Lead; Resources: Lead; Supervision: Lead; Validation: Lead; Writing – original draft: Lead; Writing – review & editing: Lead)

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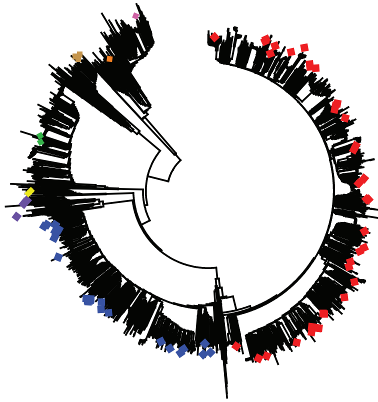
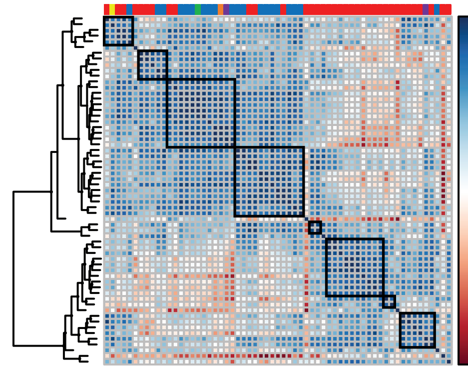
Selection of 75 genetically representative HCV Env genes

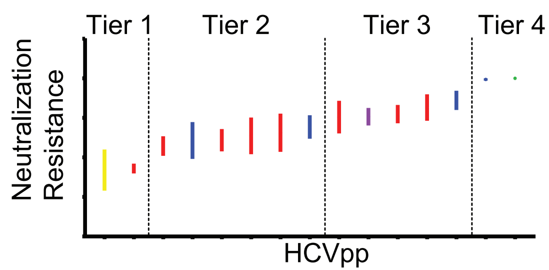
Generation of HCV pseudoparticles and phenotypic analysis using neutralizing mAbs

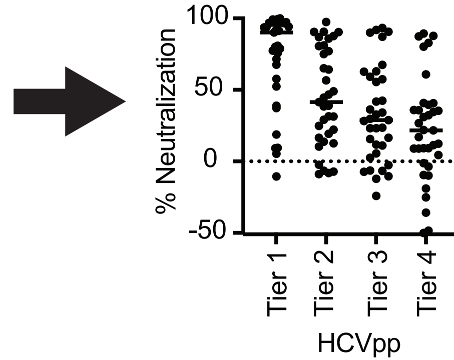




Selection of 15 representative pseudoparticles with 4 Tiers of neutralization sensitivity

Validation with genotype 1-6 immune plasma





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Title: An antigenically diverse, representative panel of envelope glycoproteins for HCV vaccine development

Authors: Jordan H. Salas[1], Richard A. Urbanowicz[2,3,4,#], Johnathan D. Guest[5,6], Nicole Frumento[1], Alexis Figueroa[1], Kaitlyn E. Clark[1], Zhenyong Keck[7], Vanessa M. Cowton[8], Sarah J. Cole[8], Arvind H. Patel[8], Thomas R. Fuerst[5,6], Heidi E. Drummer[9,10,11], Marian Major[12], Alexander W. Tarr[2,3,4], Jonathan K. Ball[2,3,4], Mansun Law[13], Brian G. Pierce[5,6], Steven K. H. Foung[7], and Justin R. Bailey[1]*

Affiliations

[1]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

[2]School of Life Sciences, Faculty of Medicine and Health Sciences, The University of Nottingham, Nottingham, UK

[3]Wolfson Centre for Global Virus Research, The University of Nottingham, Nottingham, UK

[4]NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust, Nottingham, UK

[#]Current address: Dept of Infection Biology and Microbiomes, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, UK

[5]University of Maryland Institute for Bioscience and Biotechnology Research, Rockville, MD, USA.

[6]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, USA.

[7]Department of Pathology, Stanford University School of Medicine, Stanford, CA, USA.

[8]Medical Research Council–University of Glasgow Centre for Virus Research, Glasgow, UK

[9]Viral Entry and Vaccines Group, Burnet Institute, Melbourne, VIC, Australia

[10]Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, VIC, Australia.

[11]Department of Microbiology, Monash University, Clayton, VIC, Australia.

[12]Division of Viral Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD, USA.

[13]Department of Immunology and Microbiology, The Scripps Research Institute, La Jolla, CA, USA.

Running title: A diverse hepatitis C virus neutralization panel

***Corresponding author:**
Justin R. Bailey
Department of Medicine
Johns Hopkins University School of Medicine
855 N. Wolfe St
Rangos Building Suite 520
Baltimore, MD 21205
Phone: (410) 614-6087 Fax: (410) 614-9775 email: jbailey7@jhmi.edu

Conflict of Interest Statement: The authors declare no conflicts of interest.

Author Contributions: JHS, RAU, JDG, AHP, TRF, HED, MM, AWT, JKB, ML, BGP, SKHF, and JRB conceived and designed the study. RAU, AHP, ZK, VMC, SJC, AWT, JKB, ML, SKHF, and JRB provided reagents. JHS, RAU, AWT, NF, AF, and KEC performed experiments. JHS, RAU, AWT, JDG, NF, AF, BGP, and JRB performed primary data analysis. All authors edited multiple drafts of the manuscript and approved the final draft.

Data sharing: All data are included in the manuscript or supplemental materials. E1E2 clones will be made available through Addgene.

Funding: JRB is supported by NIH grants AI127469 and AI088791. ML is supported by the NIH grants AI079031 and AI123861. SF is supported by NIH grant AI123862. TRF, BGP, and SF are supported by NIH grant AI132213, and TRF and BGP are supported by NIH grant AI144083. JB, AT, and RU are supported by Medical Research Council [grant number MR/R010307/1]. AHP, VMC, and SJC are supported by the UK Medical Research Council grant MC_UU12014/2. JDG was supported by a graduate training grant (NIH T32 AI125186).

Abstract

Background and Aims: Development of a prophylactic hepatitis C virus (HCV) vaccine will require accurate and reproducible measurement of neutralizing breadth of vaccine-induced antibodies. Currently available HCV panels may not adequately represent the genetic and antigenic diversity of circulating HCV strains, and the lack of standardization of these panels makes it difficult to compare neutralization results obtained in different studies. Here, we describe the selection and validation of a genetically and antigenically diverse reference panel of 15 HCV pseudoparticles (HCVpp) for neutralization assays.

Methods: We chose 75 envelope (E1E2) clones to maximize representation of natural polymorphisms observed in circulating HCV isolates, and 65 of these clones generated functional HCVpp. Neutralization sensitivity of these HCVpp varied widely. HCVpp clustered into 15 distinct groups based on patterns of relative sensitivity to seven broadly neutralizing monoclonal antibodies (bNAbs). We used these data to select a final panel of 15 antigenically representative HCVpp.

Results: Both the 65 and 15 HCVpp panels span four tiers of neutralization sensitivity, and neutralizing breadth measurements for seven bNAbs were nearly equivalent using either panel. Differences in neutralization sensitivity between HCVpp were independent of genetic distances between E1E2 clones.

Conclusions: Neutralizing breadth of HCV antibodies should be defined using viruses spanning multiple tiers of neutralization sensitivity, rather than panels selected solely for genetic diversity. We propose that this multi-tier reference panel could be adopted as a standard for the measurement of neutralizing antibody potency and breadth, facilitating meaningful comparisons of neutralization results from vaccine trials in different laboratories.

94 **Keywords:** hepatitis C virus, broadly neutralizing antibodies, neutralizing breadth, vaccine

Introduction

Direct-acting antiviral (DAA) therapies for hepatitis C virus (HCV) infection represent a major advancement toward reducing the global burden of liver disease[1]. However, due to limited uptake of treatment and high incidence of new infections, few countries are on target to achieve the elimination of HCV as a public health problem by 2030, a goal set by the World Health Organization (WHO)[2]. Therefore, an effective prophylactic vaccine is needed to advance efforts toward HCV elimination. HCV prevention with a prophylactic vaccine is also important to prevent HCV-induced hepatocellular carcinoma (HCC), as the risk of HCC persists in some patients even after successful treatment with DAAs[3, 4].

The extreme genetic diversity of HCV is a barrier to vaccine development[5, 6], but some broadly neutralizing antibodies (bNAbs) recognize relatively conserved envelope glycoprotein (E1E2) domains and block infection by genetically diverse HCV isolates[7-14]. Early development of bNAbs is associated with natural control of HCV infection in humans, and bNAbs can prevent HCV infection in animal models[7, 11, 15-22]. Thus, an effective HCV vaccine will certainly need to induce bNAbs or a broadly neutralizing polyclonal antibody response. Therefore, accurate and standardized measurement of neutralizing breadth of antibodies is critical to guide vaccine development.

Antibody neutralizing breadth is typically measured using panels of HCV pseudoparticles (HCVpp) or replication-competent cell culture viruses (HCVcc). HCVpp are lentiviral particles with HCV E1E2 proteins on their surface, which enable the measurement of single rounds of viral entry into hepatoma cells[23-25]. Despite structural differences between HCVcc and HCVpp[26, 27], multiple studies have demonstrated concordance between neutralization results of identical

E1E2 clones expressed in either HCVpp or HCVcc[28-31], suggesting that either approach can be used to measure antibody neutralizing activity in vitro.

HCVpp and HCVcc panels used until now to measure neutralizing breadth have notable limitations. Our previous panel of 81 patient-derived clones was tested against a limited set of CD81 binding site mAbs[29]. Most other panels are relatively small and do not represent the polymorphisms present in naturally circulating HCV isolates[32-34]. In addition, these panels were generally assembled with an emphasis on genetic rather than antigenic diversity, and they have not been evaluated with a standard set of neutralizing antibodies or immune sera to define the range of neutralization sensitivity of isolates in each panel. Some panels may contain only neutralization sensitive or neutralization resistant isolates. Lack of standardization makes it difficult to compare neutralization results obtained in different studies.

Here, we describe the selection and validation of a genetically and antigenically diverse reference panel of 15 HCVpp for use in neutralization assays. These HCVpp were selected to maximize the representation of natural polymorphisms observed in HCV sequences that are available in international sequence databases, with a focus on genotype 1 since infections with this genotype are most prevalent worldwide. In addition, we selected antigenically diverse HCVpp that demonstrated a wide range of neutralization sensitivity to diverse broadly neutralizing monoclonal antibodies and human plasma. We propose that this reference panel could be adopted as a standard for the measurement of antibody neutralizing potency and breadth to enable comparisons of neutralization results from different laboratories.

Materials and Methods

Cell Lines.

A single source of both wild type human embryonic kidney 293T (HEK293T) and Huh7 human hepatoma cell lines[29] were used. As indicated, CD81 knockout HEK293T cells[35] (Dr. Joe Grove, University of Glasgow, Glasgow, Scotland), were used for production of some HCVpp. Cells were grown in Dulbecco's modified essential medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 0.1 mM nonessential amino acids (Invitrogen).

Antibodies.

HCV MAbs CBH-7[9], HC84.26[8], HC33.4, and HC33.1[10], and negative control MAb R04 (to cytomegalovirus) were produced by Steven Foung. MAbs AR3A[7] and AR4A[11] were produced by Mansun Law. MAb hAP33 (a chimeric mouse AP33-human Fc antibody)[14, 36] was produced by Arvind Patel, and mAb HCV1[37] was a kind gift of Yang Wang, MassBiologics, Boston, MA. MAbs HEPC74, HEPC98, HEPC108, HEPC111, HEPC112, HEPC146 were a kind gift of James E. Crowe, Jr., Vanderbilt University Medical Center, Nashville, TN[13, 38].

Plasma.

Genotype 1-3-infected plasma samples were obtained from the Baltimore Before and After Acute Study of Hepatitis[39]. Plasma samples representing genotype 4-6 HCV infections were obtained from the University of Nottingham Trent HCV Cohort study[40]. All subjects provided written informed consent for blood donation, and protocols were approved by the Institutional Review Board of Johns Hopkins University School of Medicine or the Northern & Yorkshire Multicentre Research Ethics Committee (ref. MREC/98/3/55).

Sequence analysis.

Genotype 1-7 HCV E1E2 amino acid sequences were downloaded from NCBI (www.ncbi.nlm.nih.gov), euHCVdb[41] and LANL HCV[42] databases. Redundant or incomplete sequences were removed. This set of sequences and E1E2 sequences from two previously published HCVpp panels[28, 29] (166 sequences) were aligned using MAFFT[43] (version 7.3). Pairwise sequence distance matrices were calculated from the multiple sequence alignment (MSA), which were input to R (www.r-project.org) to perform hierarchical phylogenetic clustering. For each candidate panel of size N, the hierarchical clustering cutoff was specified to generate N clusters, and candidate sequence panels were identified by selecting one exemplar sequence for each cluster. Polymorphism coverage for each candidate panel was calculated using the MSA and an in-house Perl script. Genbank accession numbers for the 75 E1E2 clones used for HCVpp production are included in Supplemental Table 1. E1E2 expression plasmids for the final panel of 15 HCVpp are available from Addgene (www.addgene.org).

HCVpp production.

HIV Gag-packaged HCVpp were generated by lipofectamine-mediated transfection of HCV E1E2 plasmid, pNL4-3.Luc.R-E- plasmid containing the env-defective HIV proviral genome (NIH AIDS Reagent Program), and pAdVantage (Promega) plasmid into HEK293T cells as previously described[44]. MLV Gag-packaged HCVpp were produced by polyethylenimine (Polyscience) mediated transfection of HCV E1E2 plasmid, luciferase-encoding reporter plasmid (pTG126), and phCMV MLV Gag/Pol packaging construct (phCMV-5349) plasmid into HEK293T cells as described[44]. Mock pseudoparticles (mockpp) generated without E1E2 plasmid were used as a negative control for each transfection. CD81 knockout (CD81ko) HEK293T cells were used for production of HCVpp used for testing of genotype 2 and 3 sera in Figure 5 and for production of HCVpp used for E2 quantitation. As previously shown[35], HCVpp

produced in CD81ko HEK293T cells consistently showed greater entry of hepatoma cells, but neutralization results obtained using HCVpp produced in wild type or CD81ko HEK293T cells were highly correlated (Supplemental Figure 2).

HCVpp entry.

15,000 Huh7 cells per well were plated in 96-well solid white flat bottom microplates and incubated overnight. Then, 50 μl of HCVpp were added to the Huh7 cells in triplicate and plates were incubated at 37°C for 5 hours. HCVpp were removed and replaced with phenol-free media and cells incubated for 72 hours at 37°C. HCVpp entry was determined by measurement of luciferase activity of cell lysate in relative light units (RLU). Sixty genotype 1a and 1b HIV Gag-packaged HCVpp showed greater specific entry than MLV-Gag HCVpp (Supplemental Figure 3), so the HIV-Gag production protocol was used to produce HCVpp for neutralization testing.

Neutralization.

Neutralization assays were performed as described previously[44]. MAbs were serially five-fold diluted, starting at a concentration of 100 μg/mL (leaving the last well as PBS only), and incubated with HCVpp for one hour at 37°C before addition to Huh7 target cells in duplicate. HCVpp entry was measured as above. The percentage of neutralization was calculated as $[1 – (RLU_{mAb} / RLU_{PBS})] \times 100$ with the PBS RLU values averaged across three plates. R04 and polyclonal human IgG (Thermo Fisher) were used as negative controls. Log_{10} fifty percent inhibitory concentrations ($log_{10}IC_{50}$) were calculated from neutralization curves fit by nonlinear regression [log(inhibitor) vs. normalized response, variable slope] in Prism v8 (GraphPad Software). Mab-HCVpp tests that did not reach 50% inhibition were assigned an IC_{50} of 100 μg/mL. IC_{50} values for 7 mAbs generated with the final panel of 15 HCVpp are shown in

Supplemental Table 2. Plasma samples were tested for neutralization at a 1:20 dilution. Pooled plasma from HCV-negative donors also at 1:20 dilution was used as a negative control. Percentage neutralization of each HCVpp was calculated as $[1 - (RLU_{immune\ plasma} / RLU_{control\ plasma})] \times 100$.

Hierarchical clustering.

$Log_{10}IC_{50}$ values for each of 7 mAbs (with HCV1 and AP33 values averaged together) for each HCVpp were compared pairwise for all HCVpp using Pearson correlation, as described in Results. Rho (r) values were used as input for hierarchical clustering as implemented in the "pvclust" package for R (http://cran.r-project.org/web/packages/pvclust/index.html). This clustering, depicted as a tree, was also used to order a matrix of correlation values produced using the "corrplot" package for R (http://cran.r-project.org/web/packages/corrplot/index.html).

E2 quantitation.

HCVpp were concentrated 30-fold using a 100 kDa molecular weight cutoff (ThermoFisher) and run on 4–12% denaturing, reducing BIS-TRIS gels (ThermoFisher). Proteins were visualized with mAbs HC33.1 and anti-HIV-1 p24 (Abcam, cat: 9044), HRP-conjugated secondary antibody, and SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher), and imaged on a BioRad ChemiDoc XRS+. For ELISA, HCVpp were diluted 1:10 in TBS with 0.5% bovine serum albumin, 1.0% sodium dodecyl sulfate (SDS), and 50nM dithiothreitol (DTT) and boiled at 100°C, serially diluted 1:2 12 times, and then incubated overnight in GNA-lectin-coated microwells. Wells were washed and incubated with HC33.1 or IgG (negative control), followed by anti-human IgG HRP-conjugated secondary antibody, and then TMB substrate.

Results

Selection of HCV E1E2 clones for HCVpp production.

We downloaded all available genotype 1-7 E1E2 sequences from NCBI GenBank, LANL[42], and euHCVdb[41] databases, including 2587 (77%) genotype 1 and 830 (23%) genotype 2-7 sequences. We focused our initial polymorphism analysis on genotype 1 sequences, since genotype 1 infection is most prevalent worldwide, and best represented in sequence databases. Using the aligned set of 2587 genotype 1 E1E2 amino acid sequences, we developed a script to identify all possible amino acid polymorphisms appearing at each position in E1E2. To focus on common variations, and ignore universally conserved positions, we only tabulated polymorphisms appearing in 2-80% of database isolates. Since the functional status of the majority of sequence database E1E2 isolates is unknown, we assessed representation of these database sequence polymorphisms by a known-functional set of 166 genotype 1a and 1b E1E2 clones[28, 29]. The entire set of 166 functional clones contained 93.8% of amino acid polymorphisms present in 2-80% of genotype 1 database sequences. Interestingly, we found that we could identify a subset of 60 functional E1E2 clones that contained as many database polymorphisms (93.8%) as the full 166 functional E1E2 panel (Fig. 1A). Since the inclusion of additional clones added no additional polymorphism coverage, we limited subsequent genotype 1 phenotyping to these 60 E1E2 clones. HCVpp produced with three of these clones (1a18, UKNP1.21.4, and UKNP1.21.5) were poorly functional in initial tests (Supplemental Figure 3), so replacement clones genetically similar to each were added to the phenotyping set (UKNP1.2.5, UKNP1.21.2, and UKNP1.21.3, respectively). In addition, we selected twelve genotype 2-6 E1E2 clones from among the limited set of functional clones that had been previously described, choosing clones previously shown to be either highly neutralization

sensitive or highly resistant[29]. The 75 E1E2 clones selected for phenotypic analysis were widely distributed across clades in a phylogenetic tree of 3583 genotype 1-7 sequences from all subtypes (Figure 1B).

Wide variation in hepatoma cell entry of HCVpp.

We measured HCVpp entry into Huh7 hepatoma cells to determine the relative function of each of the 75 E1E2 clones (Figure 2A). Mock pseudoparticles lacking E1E2 (mockpp) were produced and tested in parallel with HCVpp to quantitate nonspecific entry. Specific entry of each HCVpp was calculated as a ratio of HCVpp entry relative to mockpp entry. Genotype 2-6 E1E2 clones that did not produce functional HCVpp using the HIV-Gag production system were then produced using an MLV-Gag production system. HCVpp demonstrated a wide range of entry (0.38-2532 fold greater than mockpp). Of 75 HCVpp, 63 (84%) demonstrated entry greater than the pre-selected threshold of 10-fold above mockpp entry using the HIV-Gag production system, while two HCVpp (clones UKNP2.2.1 and UKNP3.2.1) exceeded this threshold only when generated using the MLV-Gag HCVpp production system. Of 75 HCVpp, 10 (13%) failed to exceed the 10-fold above mockpp threshold when produced using either the HIV-Gag or the MLV-Gag production systems. HCVpp expressing E1E2 from genotypes 2-6 were distributed across the ranking of genotype 1 HCVpp entry. The 65 HCVpp that exceeded the 10-fold above mockpp entry threshold (genotype 1 n=56, genotype 2-6 n=9) carried 96.1% of genotype 1 polymorphisms and 84.8% of genotype 2-6 polymorphisms present in 2-80% of database sequences. These 65 HCVpp were used for subsequent neutralization testing.

Wide variation in neutralization sensitivity of HCVpp.

We measured neutralization of each of the 65 HCVpp by serial dilutions of seven well-characterized neutralizing mAbs, which were selected because they bind to a range of neutralizing epitopes across the E2 glycoprotein or E1E2 heterodimer, with a range of neutralizing breadth previously documented using other HCVpp or HCVcc panels[7-10, 14, 37, 45]. These seven mAbs recognize five distinct antigenic sites, including Domain B/AR3 (mAb AR3A), Domain C (mAb CBH-7), Domain D (mAb HC84.26), AR4 (mAb AR4A), and Domain E/AS412 (HC33.4, HCV1, and hAP33). All mAbs were isolated from HCV infected humans, except HCV1, which was generated by immunizing a transgenic mouse expressing human antibody genes[37], and hAP33, which was generated by immunization of a wild type mouse[14], and then subsequently produced as a mouse-human chimera (i.e. its variable heavy and variable light chains grafted onto a human IgG1 Fc backbone)[36]. The 65 HCVpp displayed a wide range of neutralization sensitivity to the panel of seven mAbs. We ranked each HCVpp from lowest to highest mean $\log_{10}IC_{50}$ across seven mAbs (mean $\log_{10}IC_{50}$ -0.80 to >2 μg/mL) (Figure 2B). Given high similarity between epitopes and neutralization profiles of mAbs HCV1 and AP33 (Supplemental Figure 4), $\log_{10}IC_{50}$ values for these two mAbs were averaged, giving each mAb half the weight of the other five mAbs in this analysis. Based upon the normal distribution of these mean $\log_{10}IC_{50}$ values, we separated the HCVpp into four tiers: HCVpp with mean $\log_{10}IC_{50}$ values more than one standard deviation below the overall mean ($\log_{10}IC_{50}<0.22$ μg/mL; Tier 1; 8 HCVpp), within one standard deviation below the mean ($\log_{10}IC_{50}$ 0.22-0.83 μg/mL; Tier 2; 24 HCVpp), within one standard deviation above the mean ($\log_{10}IC_{50}$ 0.83-1.45 μg/mL; Tier 3; 24 HCVpp), and greater than one standard deviation above the mean ($\log_{10}IC_{50}>1.45$ μg/mL; Tier 4, 9 HCVpp).

In agreement with prior studies[16, 29], some HCVpp generated using E1E2 clones from the same subtype differed dramatically in their sensitivity to neutralization, while many HCVpp generated using E1E2 clones from different subtypes or even different genotypes demonstrated very similar neutralization sensitivity. As expected based on prior studies[34], HCVpp 1a154 (strain H77) was relatively sensitive to neutralization, falling in Tier 2. Within or across subtypes, the genetic distance between E1E2 clones did not correlate with the difference in neutralization sensitivity of HCVpp produced from those clones (Supplemental Figure 5A). There was also no correlation between the magnitude of hepatoma cell entry of each HCVpp and the relative neutralization sensitivity of that HCVpp (Supplemental Figure 5B). It is noteworthy that some of the most neutralization sensitive and neutralization resistant HCVpp were genotype 2-6 strains, although this was not surprising since these E1E2 clones were selected in part based on prior testing showing them to be either highly neutralization sensitive or resistant[29].

Hierarchical clustering based on patterns of relative neutralization sensitivity reveals antigenic relationships among HCVpp.

While HCVpp can be ranked by overall neutralization sensitivity to a set of mAbs, they also have unique patterns of relative sensitivity to individual mAbs targeting different neutralizing epitopes. For example, while two HCVpp might have equivalent mean IC_{50} values for a set of mAbs, one HCVpp might be most sensitive to mAb AR3A and most resistant to mAb AR4A, while another is most sensitive to AR4A and most resistant to AR3A. We defined the neutralization profile for each HCVpp as a set of 6 $\log_{10}IC_{50}$ values ($\log_{10}IC_{50}$s for AR3A, CBH-7, HC84.26, AR4A, HC33.4, and the average $\log_{10}IC_{50}$ of HCV1 and hAP33) . We performed hierarchical clustering of HCVpp based on pairwise Pearson correlations between their

neutralization profiles (Figure 3). Four HCVpp were fully resistant to all reference mAbs (IC_{50}>100 μg/mL), so they were excluded from this analysis. HCVpp were assigned to the same antigenic group if they clustered with approximately unbiased (AU) values >70, with mean $r>0.81$ for the group, corresponding to $p<0.05$. Based on clustering analysis, 61 HCVpp fell in 15 distinct antigenic groups, with each group containing from one to twelve HCVpp. Notably, clustering was not driven by genotype or subtype. Six groups contained HCVpp from multiple subtypes, and nine groups contained only genotype 1a HCVpp, which was expected by chance given the large number of genotype 1a HCVpp included in the analysis. Of genotype 1a HCVpp, 16 (44%) fell in multi-subtype groups, while 20 (56%) fell in 1a-only groups ($p=0.48$ by Fischer's exact test). In some cases, neutralization profiles of genotype 2-6 HCVpp were highly correlated with profiles of one or more genotype 1 HCVpp [e.g. UKNP4.1.1 (4a) and UKNP1.20.4 (1b), $r=0.94$, $p=0.006$]. Overall, this analysis showed that a large number of genetically diverse HCVpp could be clustered based on neutralization profiles into a relatively small number of antigenic groups, which were not dictated by genotype or subtype.

Identification of an antigenically and genetically diverse and representative subset of 15 HCVpp.

For ease of use and to limit redundancy, we selected a representative subset of the larger panel of 65 HCVpp based on five criteria. First, we selected HCVpp that would preserve the same distribution of overall neutralization sensitivity across Tiers 1-4 that we observed with the full panel of 65 HCVpp. Second, we selected HCVpp that would best preserve the $\log_{10}IC_{50}$ mean, range, and standard deviation observed with the 65 HCVpp panel for each of the 7 reference mAbs. Third, we selected HCVpp to maximize the representation of the 15 hierarchical

antigenic clusters. Fourth, we included HCVpp from multiple genotypes and subtypes to maintain genetic diversity. Fifth, we selected HCVpp that demonstrated robust hepatoma cell entry after production using the HIV-Gag HCVpp protocol (to maximize ease of use and reproducibility). By satisfying these five criteria, we identified 15 HCVpp that were robustly functional and antigenically representative of the larger panel of 65 HCVpp (Figure 4). Notably, the prototype reference strain H77 (HCVpp 1a154) was included in Tier 2 of this final panel.

Overall neutralization sensitivity of the 65 and 15 HCVpp panels was very similar (mean (range) IC_{50} 37.80 (0.41 to >100) μg/mL vs. 35.94 (0.63 to >100) μg/mL). Like the larger panel, the panel of 15 HCVpp could be distributed across four tiers of overall neutralization resistance, with 2 HCVpp (13.5%) in Tier 1, 6 HCVpp (40%) in Tier 2, 5 HCVpp (33%) in Tier 3, and 2 HCVpp (13.5%) in Tier 4 (Figure 4). In addition, neutralizing breadth (% of the panel neutralized) for each mAb at IC_{50} thresholds of 100, 10, or 1 μg/mL was very similar when quantitated using either the panel of 65 or the panel of 15 HCVpp (Table 1). Interestingly, these 15 HCVpp were highly antigenically representative of the larger panel of 65 HCVpp despite expressing only 79.6% of genotype 1 polymorphisms and 60.3% of genotype 2-6 polymorphisms present in 2-80% of database sequences, indicating that many E1E2 amino acid differences across strains and genotypes are irrelevant for bNAb sensitivity.

To better understand the differences in hepatoma cell entry and neutralization sensitivity between these 15 HCVpp, we used Western blot and ELISA (Supplemental Figure 1A-B), to measure incorporation of E2 into each HCVpp. We found that incorporation of E2 varied greatly across HCVpp, and the level of E2 incorporation correlated significantly with the level of hepatoma cell entry of each HCVpp (Supplemental Figure 1C). However, the level of E2 incorporation did not correlate with HCVpp neutralization sensitivity (Supplemental Figure 1D),

indicating that differences in sensitivity between HCVpp are not dictated by these differences in E2 incorporation.

Panel validation using human immune plasma samples and additional neutralizing mAbs.

We selected thirty-five plasma samples from HCV-infected humans for neutralization breadth testing using the 15 HCVpp panel. The samples were obtained from individuals infected with genotype 1 (n=9), 2 (n=5), 3 (n=6), 4 (n=5), 5 (n=5), or 6 (n=5) virus. The majority of samples were obtained from individuals with chronic infection, and samples were not matched for duration of infection. As expected, these samples displayed a range of neutralizing breadth when tested at a 1:20 plasma dilution (0%-100% of the panel neutralized) (Figure 5A and Supplemental Table 3). Interestingly, some plasma samples with moderate or poor neutralizing breadth enhanced rather than inhibited entry of some HCVpp, which was demonstrated by greater entry of HCVpp incubated with immune plasma relative to entry of HCVpp incubated with HCV-negative control plasma, as has been previously described (Supplemental Table 3)[46-48]. For unclear reasons, genotype 4-6-infected plasma displayed greater neutralizing breadth than genotype 1-3-infected plasma (median 87% vs 27% of the panel neutralized). However, across all genotype 1-6 plasma samples, Tier 1 HCVpp were significantly more sensitive to plasma neutralization than Tiers 2, 3, and 4 ($p<0.001$ for each comparison). Tier 2 HCVpp were significantly more sensitive than Tiers 3 and 4 ($p<0.05$, $p<0.0001$, respectively). Tier 3 HCVpp were significantly more sensitive than Tier 4 ($p<0.05$). (Figure 5B). Notably, this trend of increasing neutralization resistance from Tier 1-4 was also consistent when genotype 1-6-infected plasma samples were segregated by genotype, although not all comparisons between

Tiers were statistically significant, likely due to smaller numbers of samples in each group (Figure 5C).

Seven additional neutralizing mAbs (HEPC74, HEPC98, HEPC108, HEPC111, HEPC112, HEPC146, and HC33.1) were also tested using the 15 HCVpp panel because they bind to unique epitopes relative to the seven reference mAbs used to select the panel. Four of these mAbs (HEPC98, HEPC108, HEPC112, HEPC146) target distinct antigenic sites relative to the initial mAb reference panel [29, 38]. As expected, the mAbs showed a wide range of neutralizing breadth when tested at 20 µg/mL concentration (62% or 5% of the panel neutralized by HEPC74 or HEPC112, respectively) (Supplemental Figure 6A). We expected that neutralization of the panel by HEPC74 would be similar to neutralization we had observed with AR3A, given structural analyses demonstrating that the two mAbs bind to highly similar epitopes. This was confirmed, as % neutralization of each HCVpp by HEPC74 was highly correlated with values obtained with AR3A ($r=0.67$, $p=0.008$) but not with unrelated mAb HC33.4 ($r=0.33$, $p=0.23$) (Supplemental Figure 6B).

Taken together, these data confirmed that immune plasma samples and novel mAbs displayed a wide range of neutralizing breadth across the HCVpp panel. Regardless of the infecting genotype of the immune plasma source, Tier 1 HCVpp were most sensitive to neutralization, followed by Tier2, Tier 3, and then Tier 4.

Panel validation by independent neutralization testing in a second research laboratory.

To ensure reproducibility of neutralization measured using this panel, the panel of 15 HCVpp was reproduced by plasmid transfection at the University of Nottingham, and neutralization testing was repeated with the original seven reference mAbs (HCV1, hAP33,

AR3A, CBH-7, HC84.26, AR4A, and HC33.4). Neutralization results ($log_{10}IC_{50}$ values) for each HCVpp and mAb combination obtained at the University of Nottingham were highly correlated with those previously obtained at Johns Hopkins ($r = 0.92$, $p<0.0001$) (Figure 6A). Mean IC_{50} for each HCVpp across seven mAbs ranged from 0.63 to >100 μg/mL in Johns Hopkins testing, and from 0.74 to 91.38 μg/mL in University of Nottingham testing. None of the HCVpp switched neutralization sensitivity tiers in the repeat testing (Figure 6B).

Discussion

Development of a prophylactic HCV vaccine will require accurate and reproducible measurement of neutralizing breadth and potency of vaccine-induced antibodies. By analyzing hepatoma cell entry, neutralization sensitivity, and neutralization profiles of HCVpp generated with diverse E1E2 clones, we identified a robustly functional and reproducible panel of 15 HCVpp that gave neutralizing potency and breadth measurements for 7 bNAbs that were remarkably similar to results obtained with a more genetically diverse 65 virus panel.

We observed a wide range of neutralization sensitivity across a large number of subtype 1a and 1b HCVpp and a smaller number of genotype 2-6 HCVpp. In agreement with prior studies[16, 28, 29, 34, 49], the genetic distance between E1E2 clones did not correlate with differences in neutralization sensitivity between HCVpp generated from those clones (Supplemental Figure 5). We also observed very close correlations between neutralization profiles of some HCVpp with very divergent E1E2 sequences, including some close correlations between neutralization profiles of HCVpp from different genotypes. These findings are supported by a recent study by Bankwitz, et al., which also found that neutralization profiling could be used to select a relatively small number of isolates to represent a larger HCV panel[49]. Neutralizing breadth of four of the reference mAbs used in this study was previously measured using a widely adopted panel of genotype 1-6 HCVcc [strains H77 (gt1a), J6 (gt2a), S52 (gt3a), ED43 (gt4a), SA13 (gt5a), HK6a (gt6a)][8, 10, 11, 32], so it is useful to compare those prior results to the results obtained with this new HCVpp panel. Both panels include some sensitive isolates, but the neutralization breadth of each mAb (defined here as a percentage of isolates in the panel neutralized by ≤ 10 μg/mL of mAb) was lower using the HCVpp panel than it was in prior studies using the HCVcc panel

(Supplemental Table 4), indicating that the HCVpp panel incorporates more antigenic diversity than the HCVcc panel, despite including fewer HCV genotypes. Taken together, these data confirm that the neutralizing breadth of antibodies should be defined by neutralization of antigenically diverse isolates representing multiple tiers of neutralization sensitivity, rather than by neutralization of isolates from multiple genotypes.

The neutralization panel described here has some limitations. First, we characterized relatively few genotype 2-6 E1E2 clones, and no genotype 7 or 8 clones. However, genotype 2-6 HCVpp were distributed among genotype 1 HCVpp in both the neutralization sensitivity and neutralization profile analyses, confirming that neutralization phenotypes do not segregate by genotype or subtype[46]. In addition, neutralization sensitivities of Tier 1-4 HCVpp were consistent regardless of the infecting genotype of immune plasma tested. Thus, this reference panel is likely to be antigenically representative of genotype 2-8 as well as genotype 1 strains. Nevertheless, it will be important to continue to phenotype additional genotype 2-8 isolates. Second, this panel was generated with HCVpp rather than HCVcc to facilitate widespread use. HCVpp are generally more neutralization sensitive than HCVcc, likely because HCVcc incorporate apolipoprotein E and HCVpp do not[26, 30]. Therefore, it will be helpful to incorporate some or all of these E1E2 clones into chimeric HCVcc, to confirm that relative neutralization sensitivity is consistent across HCVpp and HCVcc, as has been observed in prior studies[28-31].

It is interesting that most genotype 4-6-infected plasma samples tested with this panel displayed greater neutralizing breadth than genotype 1-3-infected plasma. This observation might be the result of different durations of infection at the time of plasma sampling, since longer duration of infection has been associated with greater neutralizing breadth[16, 50]. Genotype

1-3 samples were obtained from the BBAASH acute infection cohort[39], with subjects infected a median (range) of 389 (228-963) days at the time of sampling, while genotype 4-6 samples were obtained from the University of Nottingham Trent HCV cohort[40], from individuals with chronic infection of unknown duration. Further studies with time-matched samples from multiple genotypes will be needed to clarify this observation.

While this panel can be used immediately to compare neutralizing breadth of antibodies induced by candidate vaccines, further work is needed to determine the level of serum neutralizing breadth and neutralizing potency that is necessary for protection against HCV infection. These data might be obtained by using this panel to test serum from animal challenge models of HCV infection or by measuring plasma neutralizing breadth in vaccine trials performed in at-risk human populations. As this panel is adopted in new laboratories, quality control of HCVpp will be important. HCVpp should be used in experiments only if they demonstrate entry at least ten-fold above background entry by mockpp. In addition, one or more of the reference bNAbs from this study could be included in experiments as a positive control to confirm that neutralization breadth (Table 1) and IC_{50} values (Supplemental Table 2) are similar to those obtained here. Controls for plasma or serum neutralization experiments should also be carefully considered, since we and others have observed enhancement, rather than inhibition of infection by some plasma samples. This enhancement of HCVpp entry may be due to lipoproteins in plasma that increase entry when neutralizing antibodies levels are low or absent[47, 48]. To control for these effects, neutralization by immune plasma or serum should be calculated relative to control wells containing pre-immune plasma or serum, or immunoglobulins should be purified from plasma prior to testing.

In conclusion, we have rationally selected and validated a genetically and antigenically diverse panel of 15 HCVpp for use in neutralization assays. This study also demonstrated that neutralizing breadth of HCV antibodies should be defined using viruses spanning multiple tiers of neutralization sensitivity, rather than panels selected solely for genetic diversity. We propose that this panel could be adopted as a standard for measurement of antibody neutralizing potency and breadth, advancing HCV vaccine development by facilitating comparisons of neutralization results from laboratories around the world.

Acknowledgements

We would like to thank Dr. Stuart Ray and Dr. Michael Houghton for helpful discussions. JRB is supported by NIH grants AI127469, AI088791, and AI158193. ML is supported by the NIH grants AI079031 and AI123861 and AI158193. SF is supported by NIH grant AI123862. TRF, BGP, and SF are supported by NIH grant AI132213, and TRF and BGP are supported by NIH grant AI144083. JB, AT, and RU are supported by Medical Research Council [grant number MR/R010307/1]. AHP, VMC, and SJC are supported by the UK Medical Research Council grant MC_UU12014/2. JDG was supported by a graduate training grant (NIH T32 AI125186).

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Figure Legends

Figure 1. Selection of genetically diverse and representative E1E2 strains for HCVpp phenotyping. A. Percent representation by candidate panels of polymorphisms observed with 2-80% frequency in a set of 2587 nonredundant gt1 E1E2 sequences. Polymorphism coverage by a set of 166 functional gt1 E1E2 clones is shown as a black dotted line (93.8%). Polymorphism coverage by previously published panels of 19 gt1 HCVpp ("JHU 2014"), 113 gt1 HCVpp ("JHU 2017")[28], and 58 gt1 HCVpp ("UoN 2016")[29], are indicated. Candidate gt1 E1E2 panels of various sizes, selected by hierarchical phylogenetic clustering ("Cluster-based"), are shown as black points, and coverage by an optimized panel of 60 gt1 E1E2 clones ("OptGt1") is shown as a blue point circled in red (93.8%). **B.** Neighbor-joining phylogenetic tree of 3583 E1E2 amino acid sequences representing all subtypes of gt 1-7. Tree was generated in Mega v7.0.21 using the Jones-Taylor-Thornton model with gamma distribution. Branches are drawn to scale, and positions containing gaps and missing data were eliminated. Symbols indicate 75 gt 1-6 clones selected for HCVpp production.

Figure 2. Wide variation in hepatoma cell entry and neutralization sensitivity of HCVpp. A. Hepatoma cell entry of 75 HCVpp expressed as fold increase in relative light units (RLU) compared to the background entry of mockpp. In some cases, HCVpp were first generated using HIV Gag (circles) and then MLV Gag (open diamonds). Data represent 1-5 independent experiments for each HCVpp, with each experiment performed with 2-6 replicates. A threshold of 10-fold above mockpp entry is indicated with a dotted line. Asterisks indicate HCVpp that were freeze-thawed prior to testing. **B.** Neutralization of 65 HCVpp by 7 bNAbs targeting diverse epitopes across E1E2. Mab/HCVpp tests that did not reach 50% inhibition were assigned

an IC_{50} of 100 μg/mL. HCVpp are arranged from most to least neutralization sensitive based on mean $log_{10}IC_{50}$ measured for 7 mAbs and divided into four tiers of sensitivity based on the normal distribution of the data. Each mAb-HCVpp combination was tested with nine serial mAb dilutions in duplicate. Points indicate means and whiskers SEM. Prototype reference strain H77 is highlighted in red.

Figure 3. Hierarchical clustering based on patterns of relative neutralization sensitivity reveals antigenic relationships among HCVpp. A heat map was generated showing all pairwise correlations among neutralization profiles of 61 HCVpp (neutralization profile = $log_{10}IC_{50}$ values for 7 mAbs). HCVpp are arrayed in the same order along the x and y axes, with the genotype of each HCVpp indicated on the x-axis. Circles at each intersection are scaled by the magnitude of the Pearson correlation (r) between neutralization profiles. Hierarchical clustering analysis using these pairwise correlations is depicted as a tree. Circles at tree nodes indicate approximately unbiased (AU) test values >70, indicating strength of support for a particular cluster. HCVpp were assigned to the same antigenic group (black boxes) if they clustered with approximately unbiased (AU) values >70, with mean $r>0.81$ for correlations among the group, corresponding to $p<0.05$. Arrows indicate HCVpp selected for the final 15 HCVpp panel.

Figure 4. Identification of an antigenically and genetically diverse and representative subset of 15 HCVpp. HCVpp selected as representative of the larger HCVpp panel based on the distribution of neutralization sensitivity across Tiers 1-4, preservation of the $log_{10}IC_{50}$ mean, range, and standard deviation for each of the 7 reference mAbs, representation of hierarchical

antigenic clusters, inclusion of multiple genotypes and subtypes, and robust hepatoma cell entry. Points indicate means and whiskers SEM. Prototype reference strain H77 is highlighted in red.

Figure 5. Panel validation using human immune plasma samples. A. % neutralization of the 15 HCVpp panel (Tiers 1-4) by plasma samples at 1:20 dilution from 35 persons infected with genotype (gt) 1-6 HCV, measured in duplicate. **B-C.** Plasma neutralization is arranged according to HCVpp Tier. Each point indicates mean neutralization of all HCVpp in the indicated Tier by a single plasma sample from panel A. All plasma samples (gt1-6) were analyzed together in **B**, or separated by infecting HCV genotype in **C**. Horizontal lines are medians. Groups were compared by one-way ANOVA if data were normally distributed (gt1, 2, 3, 5, 6 graphs) or by Friedman test if data were not normally distributed (gt1-6, gt4 graphs). All tests were adjusted for multiple comparisons using the Benjamani, Krieger, and Yekutieli method, with adjusted $p<0.05$ considered significant. * <0.05, ** <0.005, *** <0.001, **** <0.0001.

Figure 6. Panel validation by independent neutralization testing in a second research laboratory. Repeat production of the HCVpp panel and neutralization testing with 7 reference mAbs tested with serial dilutions in duplicate. **A.** Correlation between $\log_{10}IC_{50}$ values for each HCVpp-mAb combination obtained at Johns Hopkins or the University of Nottingham. R and p values from Pearson correlation. **B.** For each HCVpp, $\log_{10}IC_{50}$ results for 7 mAbs obtained at Johns Hopkins are on the left (black) and repeat results obtained at the University of Nottingham are on the right (gray). Points indicate means and whiskers SEM. Prototype reference strain H77 is highlighted in red.

Supplemental Figure 1. Quantitation of E2 incorporated into HCVpp. A. Western blot of a denaturing, reducing gel run with concentrated HCVpp, probed with an anti-E2 mAb (HC33.1) targeting a linear epitope that is intact in all E2 variants and an anti-HIV p24 mAb. **B.** ELISA with serial 2-fold dilutions of concentrated, denatured HCVpp bound to GNA-lectin-coated wells, probed with anti-E2 (HC33.1). Values are means of duplicate wells, and error bars are SEM. **C.** Positive correlation of the amount of E2 incorporated into each HCVpp (area under the curve calculated in B) with entry of that HCVpp into Huh7 hepatoma cells (expressed as fold entry above background entry of mock HCVpp with no E1E2). **D.** No correlation of the amount of E2 incorporated into each HCVpp (area under the curve calculated in B) with neutralization sensitivity of that HCVpp (mean $\log_{10}IC_{50}$ for each HCVpp from Figure 2). R and p values were calculated using the Pearson method, with $p<0.05$ considered significant.

Supplemental Figure 2. A. Hepatoma cell (Huh7) entry of 15 HCVpp produced by transfection of either wild type (WT) HEK293T cells or CD81 knockout (CD81ko) HEK293T cells, expressed as fold increase in RLU above background entry of mock HCVpp without E1E2 transfected and tested in parallel. **B.** Correlation between percent neutralization values obtained using mAb HEPC74 at 20 μg/mL concentration and 15 HCVpp produced in WT HEK293T cells or CD81ko HEK293T cells. R and p values were calculated using Pearson correlation.

Supplemental Figure 3. Initial testing to compare specific entry of HIV-Gag HCVpp to MLV-Gag HCVpp. Sixty genotype 1a or 1b E1E2 were co-transfected with either *env*-deficient HIV-1 with a luciferase reporter gene (HIV-Gag HCVpp) or with a luciferase-encoding reporter plasmid and an MLV Gag/Pol packaging construct (MLV-Gag HCVpp). Mockpp (lacking E1E2)

were produced in parallel, and entry was calculated as a ratio of HCVpp entry relative to mockpp entry. The pre-selected threshold of 10-fold greater than mockpp entry is indicated with a dotted line.

Supplemental Figure 4. Correlation between IC_{50} values across 65 HCVpp for bNAbs HCV1 and hAP33. Given the similarity of epitopes targeted by these bNAbs, we measured the correlation between $\log_{10}IC_{50}$ values for these mAbs across the HCVpp panel. Due to this high correlation in neutralization profiles, $\log_{10}IC_{50}$ values for these two mAbs for each HCVpp were averaged (given half the weight of the other 5 bNAbs) when ranking HCVpp by neutralization sensitivity and for hierarchical clustering of HCVpp (Figures 2-4, 6). R and p calculated by the Pearson method.

Supplemental Figure 5. Genetic distance and magnitude of hepatoma cell entry do not predict neutralization sensitivity of HCVpp. A. For each of 64 HCVpp, E1E2 amino acid p distance and absolute difference in mean $\log_{10}IC_{50}$ were calculated relative to reference strain 1a154 (H77, genotype 1a) HCVpp. The expected genetic distances were observed between H77 and genotype 1a (red), genotype 1b (blue), and genotype 2-6 (black) E1E2 clones, but this distance was not correlated with the difference in neutralization sensitivity of HCVpp generated with these clones relative to H77 HCVpp sensitivity. **B.** No correlation between the magnitude of hepatoma cell entry (Figure 2A) and mean $\log_{10}IC_{50}$ (Figure 2B) of 65 HCVpp. R and p values calculated using the Spearman method.

Supplemental Figure 6. A. % neutralization of the 15 HCVpp panel by 7 additional neutralizing mAbs at 20 μg/mL concentration, measured in duplicate. **B.** Correlation between % neutralization values obtained with HEPC74 and related mAb AR3A, or HEPC74 and unrelated mAb HC33.4, tested at 20 μg/mL concentration. Each point represents neutralization of a single HCVpp measured in duplicate. R and p values determined using the Spearman method.

Table 1

Table 1. Neutralizing breadth of reference mAbs measured using 65 or 15 HCVpp panels.

		Neutralizing breadth[1] (%)		
mAbs	# HCVpp	100 µg/mL	10 µg/mL	1 µg/mL
hAP33	65	91	82	46
	15	93	80	47
HCV1	65	89	80	28
	15	87	87	40
AR4A	65	91	80	20
	15	87	80	33
HC84.26	65	74	57	34
	15	73	67	33
AR3A	65	74	54	18
	15	60	53	13
HC33.4	65	77	48	20
	15	73	40	27
CBH-7	65	31	12	3
	15	33	13	13

[1]Percent of isolates in 65 or 15 HCVpp panels neutralized with IC_{50}<100, 10, or 1 µg/mL.

Figure 1

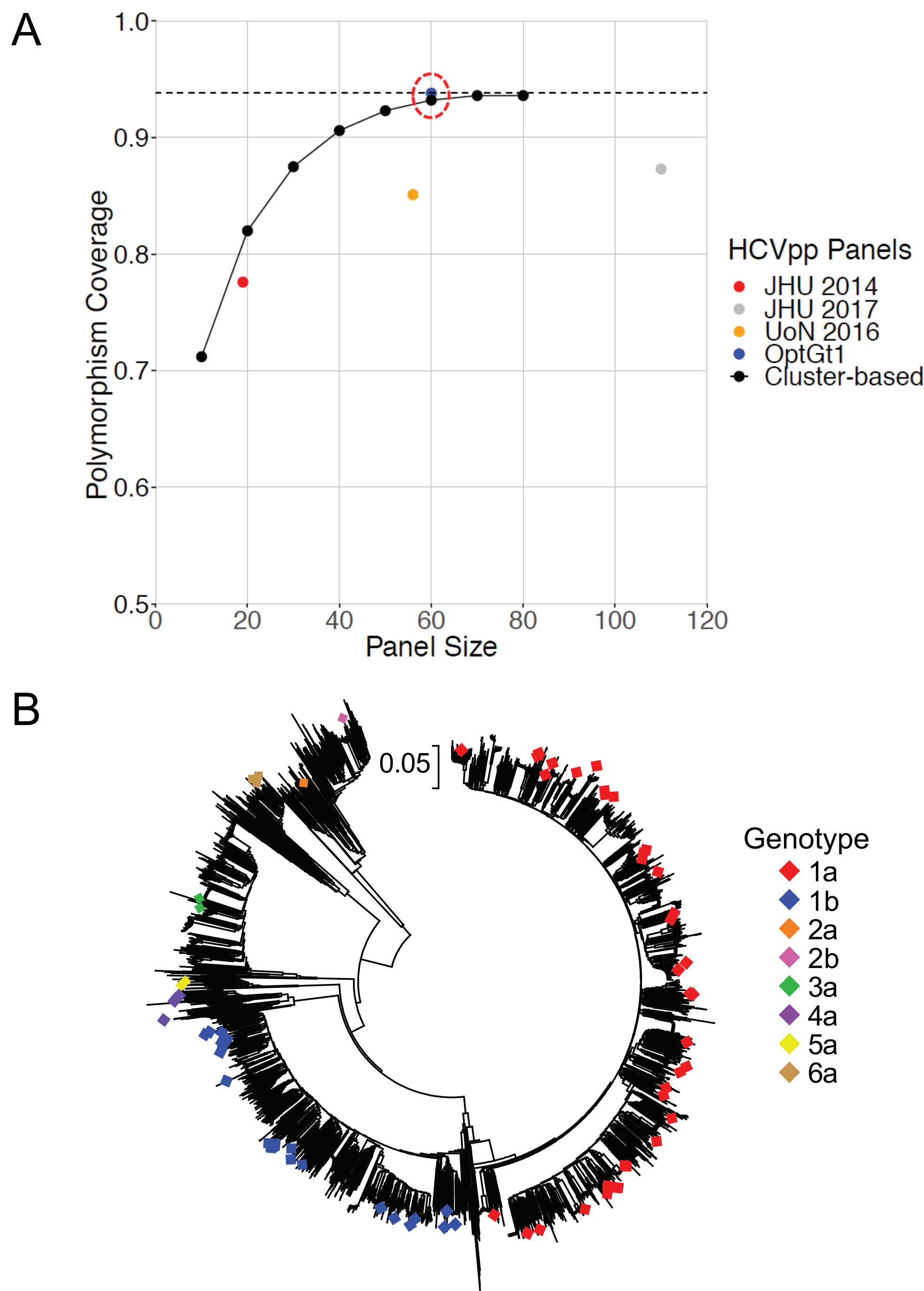

Figure 1. Selection of genetically diverse and representative E1E2 strains for HCVpp phenotyping. A. Percent representation by candidate panels of polymorphisms observed with 2-80% frequency in a set of 2587 nonredundant genotype 1 E1E2 sequences. Polymorphism coverage by the full set of 166 functional genotype 1 E1E2 clones that were considered for inclusion is shown as a black dotted line (93.8%). Polymorphism coverage by previously published panels of 19 gt1 HCVpp ("JHU 2014") and 113 gt1 HCVpp ("JHU 2017")[27], and 58 gt1 HCVpp ("UoN 2016")[28], are shown as red, gray, and orange points, respectively. Candidate genotype 1 E1E2 panels of various sizes, selected by hierarchical phylogenetic clustering ("Cluster-based"), are shown as black points, and coverage by an optimized diverse panel of 60 genotype 1 E1E2 clones ("OptGt1") is shown as a blue point circled in red (93.8%). **B.** Neighbor-joining phylogenetic tree of 3583 E1E2 amino acid sequences representing all subtypes of genotypes 1-7. Tree was generated in Mega v7.0.21 using the Jones-Taylor-Thornton model with gamma distribution. Branches are drawn to scale, and positions containing gaps and missing data were eliminated. Symbols indicate 75 genotype 1-6 clones selected for HCVpp production and phenotypic analysis.

Figure 2

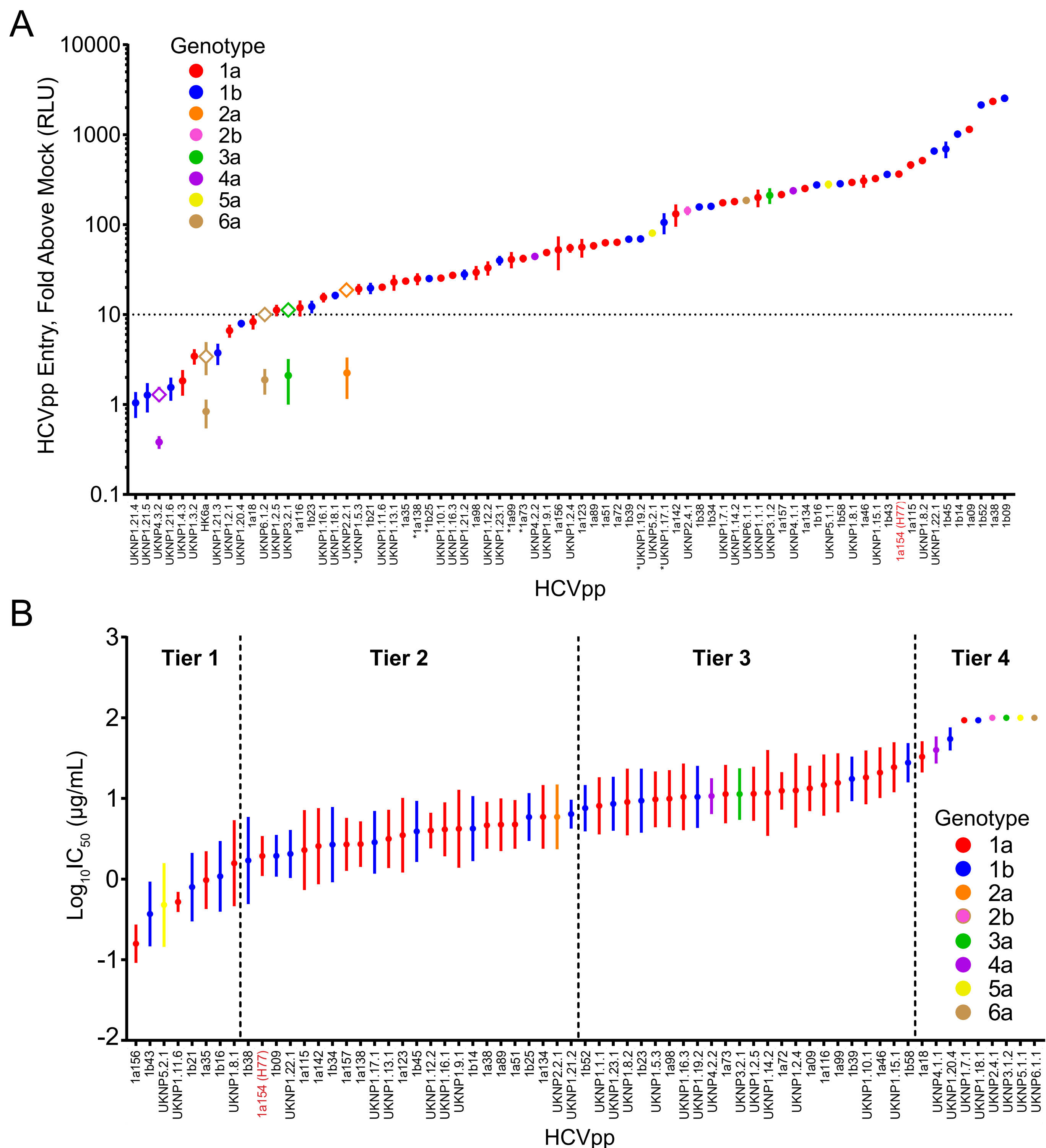

Figure 2. Wide variation in hepatoma cell entry and neutralization sensitivity of HCVpp. A. Hepatoma cell entry of 75 HCVpp expressed as fold increase in relative light units (RLU) compared to the background entry of mockpp (pseudoparticles without E1E2). In some cases, HCVpp were first generated using HIV Gag (circles) and then MLV Gag (open diamonds). Data represent 1-5 independent experiments for each HCVpp, with each experiment performed with 2-6 replicates. A pre-selected threshold of 10-fold above mockpp entry is indicated with a dotted line. Asterisks indicate HCVpp that were freeze-thawed prior to testing. **B.** Neutralization of 65 HCVpp by 7 bNAbs targeting diverse epitopes across E1E2. Mab/HCVpp tests that did not reach 50% inhibition were assigned an IC_{50} of 100 μg/mL. HCVpp are arranged from most to least neutralization sensitive based on mean $log_{10}IC_{50}$ measured for 7 mAbs and divided into four tiers of sensitivity based on the normal distribution of the data. Each mAb-HCVpp combination was tested with nine serial mAb dilutions in duplicate. Points indicate means and whiskers SEM. Label for prototype reference strain H77 is highlighted in red.

Figure 3

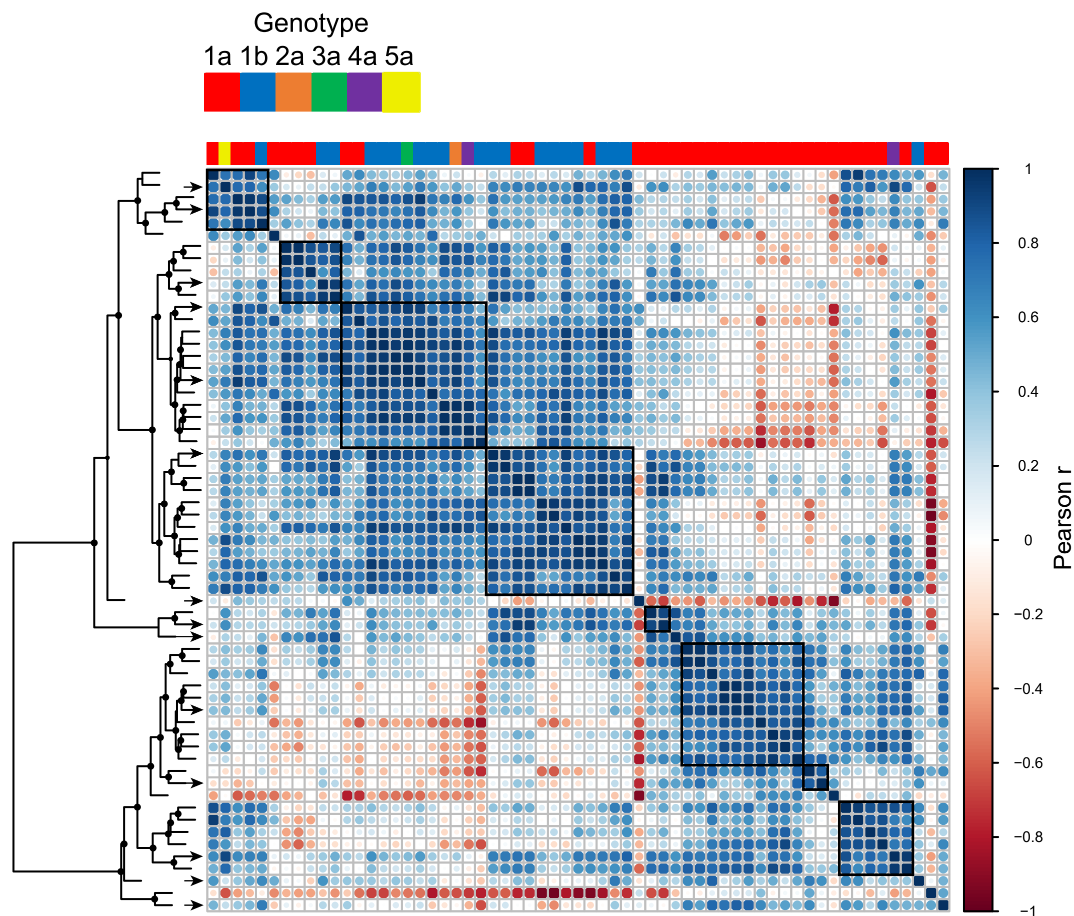

Figure 3. Hierarchical clustering based on patterns of relative neutralization sensitivity reveals antigenic relationships among HCVpp. A heat map was generated showing all pairwise correlations among neutralization profiles of 61 HCVpp (neutralization profile = $\log_{10}IC_{50}$ values for 7 mAbs, with IC_{50}s for mAbs HCV1 and hAP33 averaged to a single value given high similarity of these mAbs). HCVpp are arrayed in the same order along the x and y axes, with the genotype of each HCVpp indicated on the x-axis. Four HCVpp were excluded because they were fully resistant to all reference mAbs. Circles at each intersection are scaled by the magnitude of the Pearson correlation (r) between neutralization profiles of each HCVpp pair (darker blue=higher r, darker red=more negative r). Hierarchical clustering analysis using these pairwise correlations is depicted as a tree. Circles at tree nodes indicate approximately unbiased (AU) test values >70, which indicate strength of support for a particular cluster. HCVpp were assigned to the same antigenic group (black boxes) if they clustered with approximately unbiased (AU) values >70, with mean r>0.81 for correlations among the group, corresponding to p<0.05. Arrows indicate representative HCVpp selected for the final 15 HCVpp panel.

Figure 4

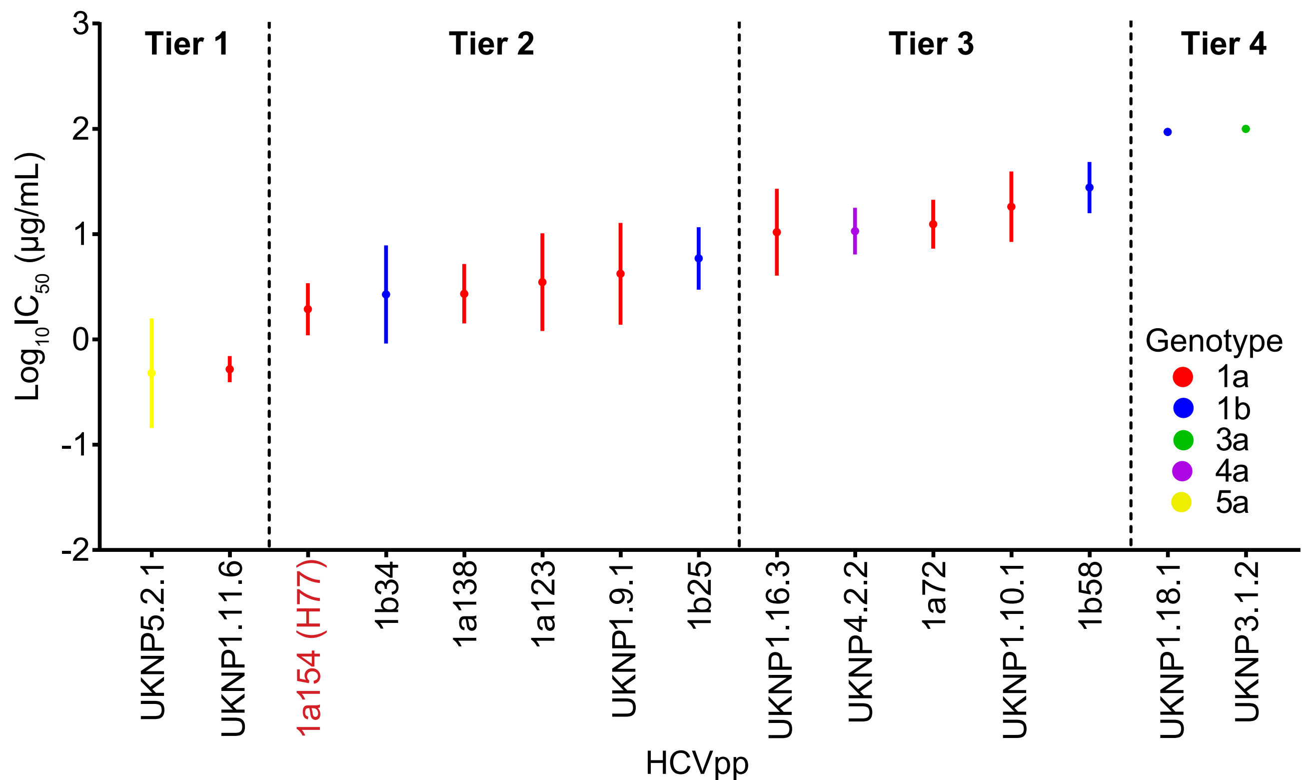

Figure 4. Identification of an antigenically and genetically diverse and representative subset of 15 HCVpp. HCVpp selected as representative of the larger HCVpp panel based on the distribution of neutralization sensitivity across Tiers 1-4, preservation of the $\log_{10}IC_{50}$ mean, range, and standard deviation for each of the 7 reference mAbs, representation of hierarchical antigenic clusters, inclusion of multiple genotypes and subtypes, and robust hepatoma cell entry. Points indicate means and whiskers SEM. Label for prototype reference strain H77 is highlighted in red.

Figure 5

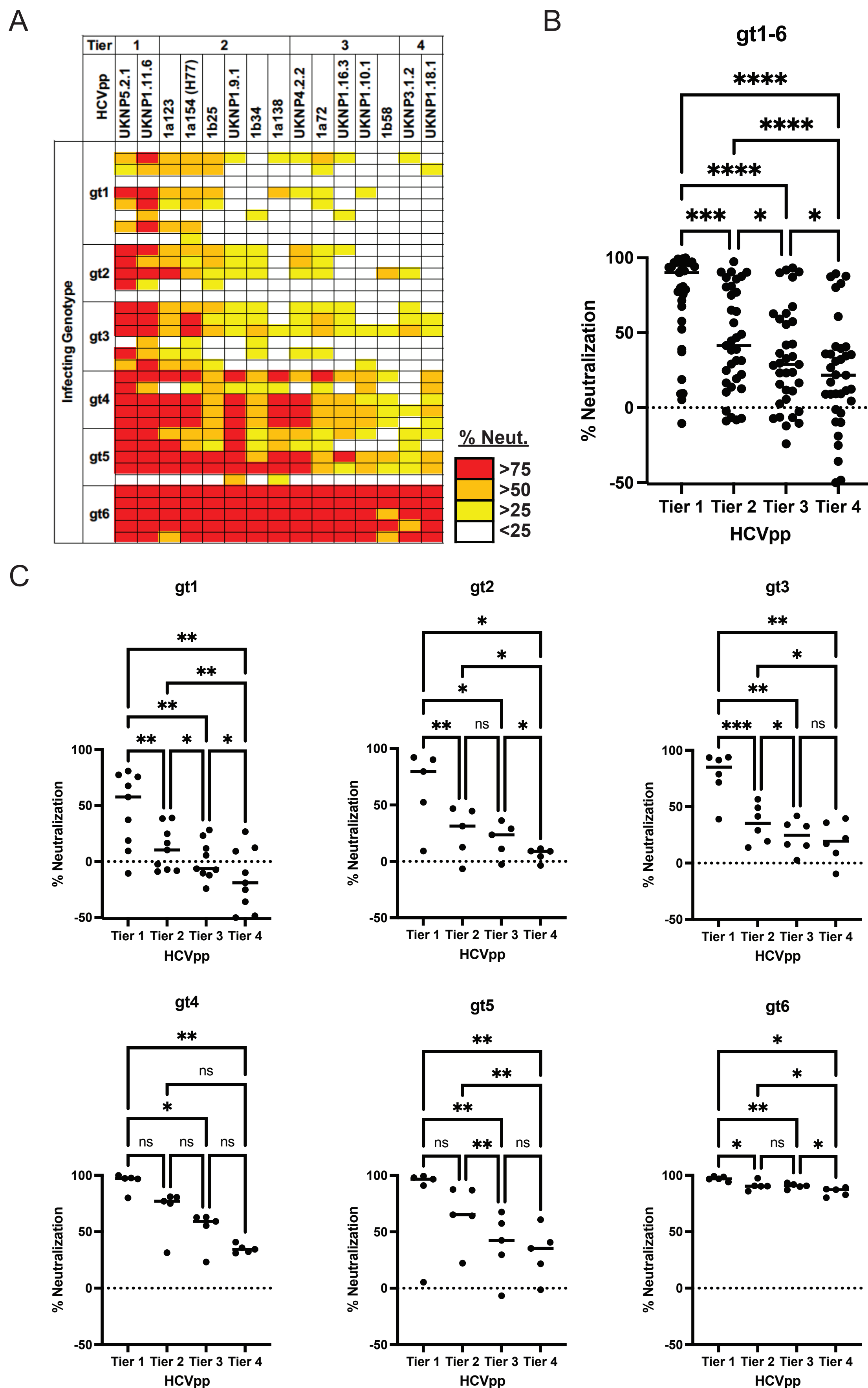

Figure 5. Panel validation using human immune plasma samples. A. % neutralization of the 15 HCVpp panel (Tiers 1-4) by plasma samples at 1:20 dilution from 35 persons infected with genotype (gt) 1-6 HCV, measured in duplicate. **B-C.** Plasma neutralization arranged according to HCVpp Tier. Each point indicates mean neutralization of all HCVpp in the indicated Tier by a single plasma sample from panel A. All plasma samples (gt1-6) were analyzed together in **B**, or separated by infecting HCV genotype in **C**. Horizontal lines are medians. Groups were compared by one-way ANOVA if data were normally distributed (gt1, 2, 3, 5, 6 graphs) or by Friedman test if data were not normally distributed (gt1-6, gt4 graphs). All tests were adjusted for multiple comparisons using the Benjamani, Krieger, and Yekutieli method, with adjusted p<0.05 considered significant. * <0.05, ** <0.005, *** <0.001, **** <0.0001.

Figure 6

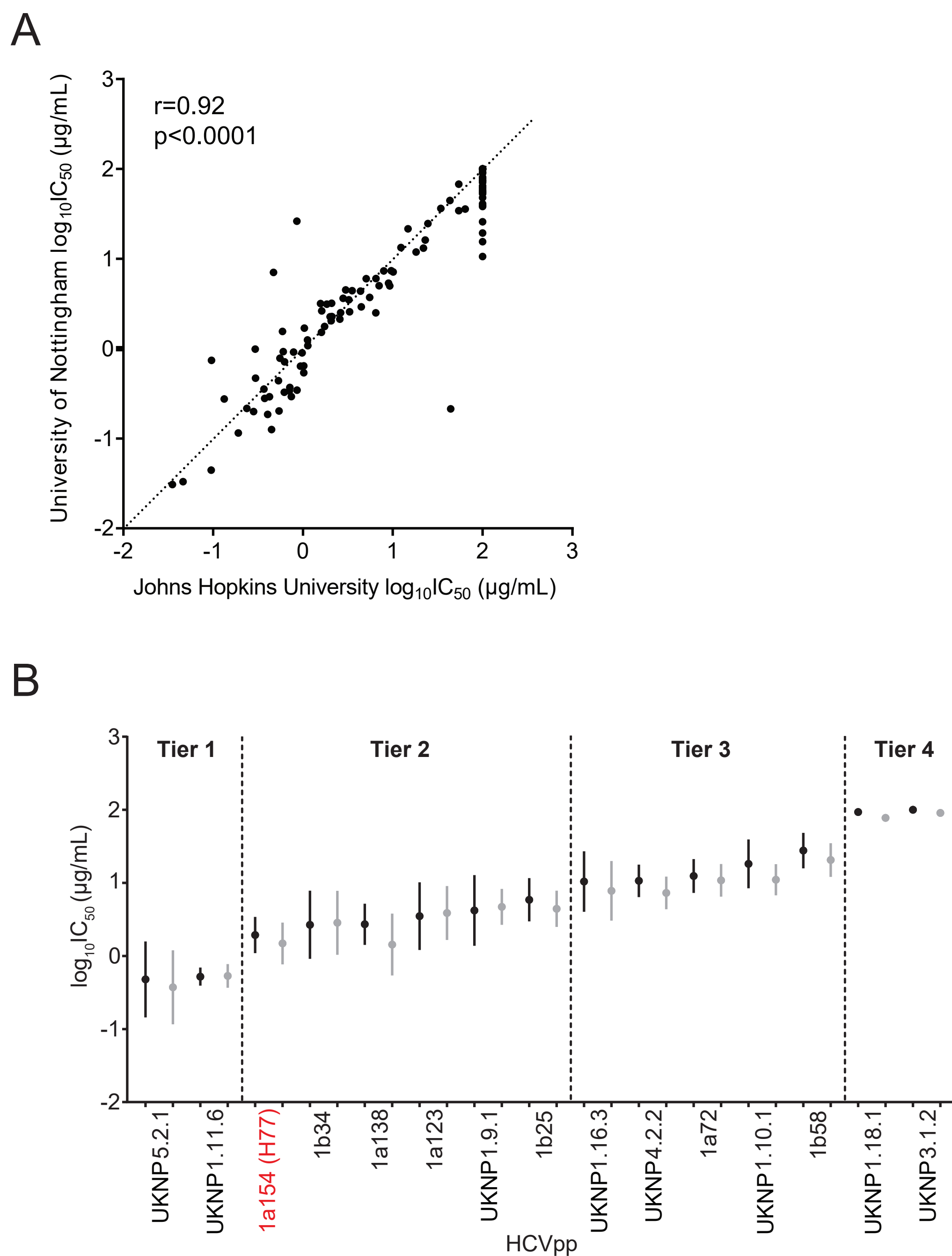

Figure 6. Panel validation by independent neutralization testing in a second research laboratory. Repeat production of the HCVpp panel and neutralization testing with 7 reference mAbs tested with serial dilutions in duplicate. **A.** Correlation between $\log_{10}IC_{50}$ values for each HCVpp-mAb combination obtained at Johns Hopkins or the University of Nottingham. R and p values from Pearson correlation. **B.** For each HCVpp, $\log_{10}IC_{50}$ results for 7 mAbs obtained at Johns Hopkins are on the left (black) and repeat results obtained at the University of Nottingham are on the right (gray). Points indicate means and whiskers SEM. Label for prototype reference strain H77 is highlighted in red.

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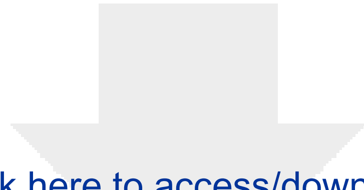

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Supplemental Material (large tables & data sets)

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