Brief Communication

A bivalent HCV peptide vaccine elicits pan-genotypic neutralizing antibodies in mice

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Author Contributions

A.M., M.A., J.F., conceived the study. A.M., R.U., performed the research. A.M. prepared the manuscript. J.T., J.B., R.U., JF edited the manuscript.

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Abstract: Vaccine development for antigenically variable pathogens has faltered because extreme genetic diversity precludes induction of broadly neutralizing antibodies (nAB) with classical vaccines. Here, using the most variable epitope of any known human pathogen (HVR1 of HCV), we describe a novel approach capable of eliciting broadly neutralizing antibodies targeting highly variable epitopes. Our proof-of-concept vaccine elicited pan-genotypic nAB against HCV variants differing from the immunogen sequences by more than 70% at the amino acid level. These findings suggest broadly nAB to highly variable pathogens can be elicited by vaccines designed to target physicochemically conserved residues within hypervariable epitopes.

Introduction

Antigenic variability is characterized by the existence of multiple strains within a species, dispersed either within the host, between hosts, or spatiotemporally, for which protective immune responses induced by

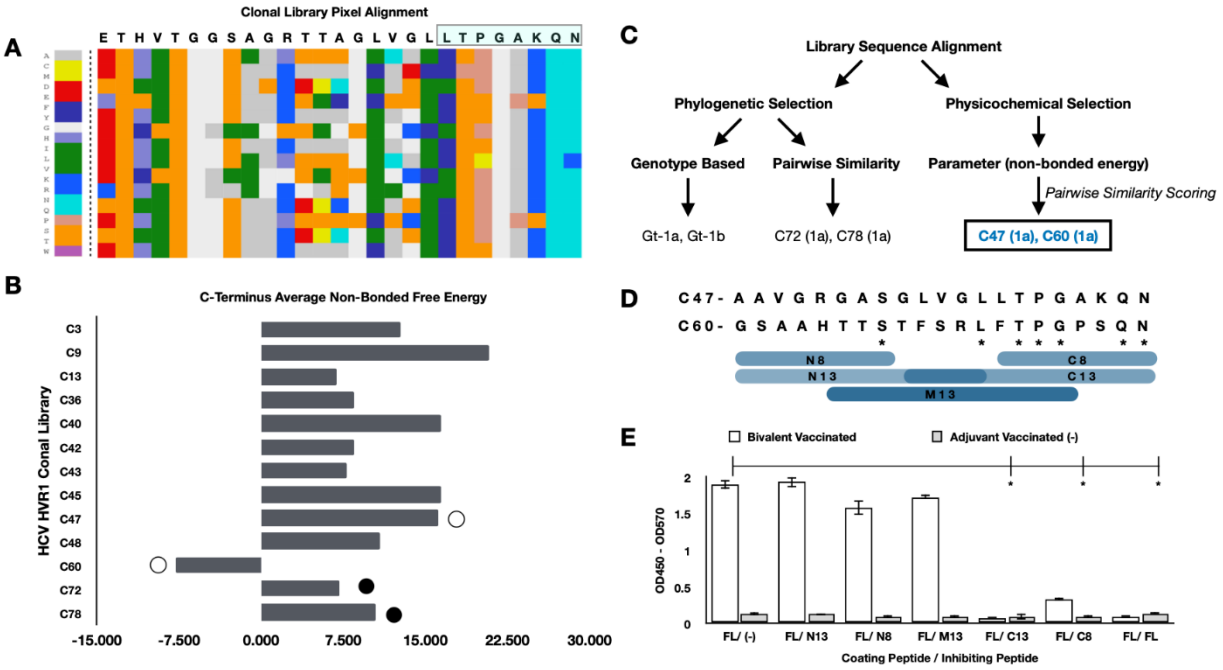
one strain are ineffective for another [1]. Understood as an emergent property involving host immune responses, rather than solely an intrinsic feature of sequence diversity, antigenic variability is preserved by the inability of the host adaptive-response to match the immunogenic breadth of the infecting pathogen [2]. This dynamic is exemplified by HCV, with intrahost populations characterized by antigenic variants positioned for divergent evolutionary trajectories depending on host immune responses, aggregating in cycles of viral diversification and immune escape from AB targeting antigenically plastic neutralizing epitopes [3].

HCV vaccine candidates have therefore focused on the induction of neutralizing AB (nAB) targeting evolutionarily constrained epitopes [4]. However, conserved epitope masking by glycans, hypervariable domains, and host-lipoproteins, limits the protective efficacy of conserved epitope-targeting AB in vivo, and the failure of efforts to elicit broadly nAB with HVR1-deleted immunogens suggests the problem of antigenic diversity cannot be circumvented by simply amputating variable epitopes [5]. In contrast, though hypervariable neutralizing epitopes are sterically accessible, immunodominant, and their cognate AB are associated with resolution of acute infection, they elicit highly strain-specific AB [6]. Based on observations of antigenic convergence and physicochemical constraints within HVR1, we hypothesized that a multivalent vaccine designed to maximize physicochemical breadth, rather than sequence-based phylogenetic distance, would elicit broadly neutralizing antibodies to a hypervariable epitope [7]. We show that a bivalent HCV vaccine candidate based on this novel design approach elicited pangenotypic nAB to variants differing from the immunogen amino acid sequences by more than 70%.

Results

To formulate a polyvalent HVR1 vaccine maximally representing the antigenic, rather than sequence or phylogenetic, space, we developed a novel method combining physicochemical analysis of candidate sequences with pairwise redundancy reduction. This approach used the average non-bonded free energy of candidate HVR1 immunogens, calculated from primary sequences *in silico*, to identify sequence pairs with the broadest physicochemical coverage of HVR1 (Fig. 1A, B). In our case, this resulted in the selection of the physicochemically distinct clones 47 & 60 for the bivalent vaccine formulation (Fig. 1C). In contrast, traditional sequence based approaches, which are vulnerable to the Sequence-Metric Problem

(discrepancy between coding and structural similarity among aligned sequences), would have selected for the sequence divergent, yet physicochemically convergent, clones 78 & 72 (Fig. 1C) [10]. To evaluate if these immunogens could elicit antibodies to the C-terminal neutralizing epitope of HVR1, groups of 4, female Balb/C mice were immunized with either peptides C47, C60, or both in a bivalent preparation. Each vaccine elicited high-titre (1:100,000) immunogen-specific IgG (not shown). The epitopes targeted by bivalent vaccine elicited AB were mapped using competitive ELISA with a series of overlapping HVR1 peptide fragments (N8, N13, M13, C13, C8). Figure 1D shows that vaccine elicited AB exclusively target



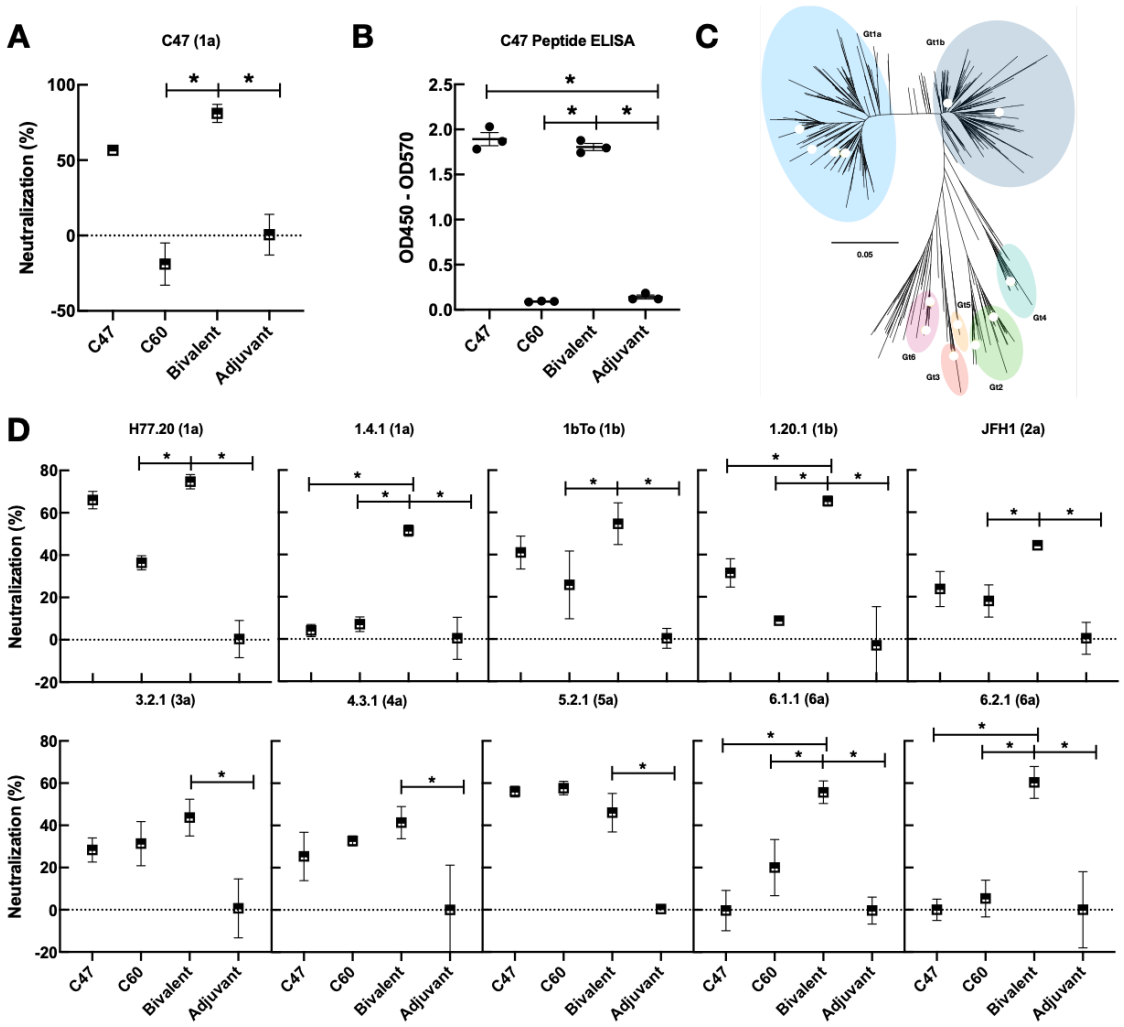
the HVR1 C-terminus (Fig. 1E).

Figure 1. Physicochemically Informed Multivalent Vaccine Design

(A) Coding diversity of a patient-derived clonal library is visualized via Pixel alignment of HVR1, with blue shading on consensus sequence highlighting the putative neutralizing epitope. (B) The average non-bonded free energy of the C-terminus of each HVR1 sequence was computed using the CRASP program [8]. (C) Physicochemical based immunogen selection from the patient library using average non-bonded free energy, followed by redundancy reduction via pairwise similarity scoring, yields immunogens C47 & C60. (D) Immunogens C47 & C60 were synthesized as 20-mer peptides for mouse immunization. (E) Short, overlapping peptides spanning C47 were used to map the epitope targeted by bivalent vaccine elicited AB via competitive

ELISA (1:1000 serum dilution). Averages of data from triplicates are shown. Error bars indicate standard deviation. Statistical analysis was done by one-tailed, unpaired t-test. *, $P < 0.001$.

We then examined if vaccine elicited AB were neutralizing. To evaluate homologous neutralization, HCVpp pseudotyped with C47 were incubated with antisera from each vaccine group (C47, C60, Bivalent, Adjuvant (-)) (Fig. 2A). We observed significant neutralizing activity in groups vaccinated either bivalently, or with C47 alone. In contrast, antisera from mice vaccinated with C60 were neither cross-reactive, nor neutralizing, underscoring the challenge of antigenic variability for vaccine elicited AB (Fig. 2A, B). To evaluate our primary hypothesis, that a bivalent HCV vaccine designed to maximize physicochemical breadth would induce bnAB, we employed a panel of 11 HCVpp representing the global phylogenetic space (Fig. 2C). We observed broad, cross-genotypic neutralization, with the infectivity of 11/11 HCVpp, derived from Gt 1-6, significantly inhibited by bivalent antisera (Fig. 2A, D). Further, antisera from bivalently vaccinated mice neutralized 8/11 HCVpp more potently than either, or both, of the monovalent



vaccines (Fig. 2D).

Figure 2. Bivalent Vaccination Elicits Broad, Pan-Genotypic Neutralizing Antibody Response

(A) Neutralization activity of antisera (1:50 dilution) against HCVpp pseudotyped with homologous C47. (B) An ELISA plate was coated with peptide C47. Sera from immunized mouse groups were added at 1:1000 dilution. The binding of antibody was detected with anti-mouse secondary antibody. (C) HCV reference sequence database (gpE2) were supplemented with HCVpp panel sequences (white circles) for neighbour joining in Molecular Evolutionary Genetics Analysis version 7 (MEGA7). (D) Neutralization of heterologous HCVpp pseudotyped with 10 strains (H77.20, 1.4.1, 1bTo, 1.20.1, JFH1, 3.2.1, 4.3.1, 5.2.1, 6.1.1, 6.2.1). Neutralization activity was quantified by measuring luciferase activity (relative light units), and normalized to adjuvant immunized sera. For (A), (B), and (C), averages of data from triplicates are shown. Error bars indicate standard error of the mean. Statistical analysis was done by unpaired t-test followed by FDR (Q=0.05) adjustment for multiple comparison (*, FDR-adjusted $P<0.05$).

Discussion

Using a novel approach to vaccine design for antigenically variable pathogens, based on the hypothesis that emergent physicochemical features better predict cross-reactivity than sequence homology, we described a bivalent vaccine candidate capable of eliciting pan-genotypic nAB targeting the antigenically convergent, sterically accessible C-terminus of the immunodominant HVR1. Intriguingly, and in contrast to previous reports, the observed cross-reactivity of nAB elicited by HVR1 vaccination was not genotypically defined [11]. Furthermore, despite competitive ELISA mapping the 8 C-terminal AA of HVR1 as the epitope targeted by nAB, no resistance conferring residues within this region could be identified. These results are inconsistent with an entirely sequence-defined conception of antigenicity, and, coupled with previous reports of antigenic convergence and global physicochemical constraint in HVR1, imply that the immunological space is *distinct from and smaller* than the sequence space [7,12].

We also observed, for the first time, that broad, cross-genotype neutralizing antibodies to HVR1 could be elicited by vaccination with only *two mono-genotypic* immunogens. The broad-reactivity of these AB, in contrast to the restricted specificity of the combined AB elicited by monovalent vaccination, indicates that our vaccine approach altered, rather than merely expanded, the epitope specificity of stimulated clonal lineages, as the breadth of neutralization following bivalent vaccination exceeded the summed

neutralization breadth of its monovalent constituents. Collectively, these findings suggest functional constraints within hypervariable epitopes can be targeted by polyvalent vaccines designed with physicochemically informed immunogen selection.

Materials and Methods

To design the vaccine candidate, a set of degenerate primers specific for the N-terminus of Core (nt 328-342) and C-terminus of E2 (2566-2580) were used to generate an HCV structural polyprotein coding clonal library from 72 patient-derived amplicons [13]. Clones were functionally characterized by co-transfection with pNL4. 3 HIV-1 into HEK 293T cells, as described, with infectious clones then sequenced for subsequent immunogen selection [14]. The HVR1 from each clone was used to construct maximum likelihood trees (PhyML) to identify the two most sequence-distal clones. HVR1 sequences were then transformed into sequence-length matched values representing average-non bonded energy, reported as an aggregate value across each peptide, using the program CRASP [8]. Physiochemically distinct peptide-pairs were then resolved via pairwise redundancy reduction, intended to identify the maximally divergent set among candidate pairs, to yield C47/C60.

For immunizations, C47 and C60 were synthesized into peptides using Fmoc chemistry, N-terminally conjugated to keyhole limpet hemocyanin via maleimide linkage, and mixed at 1:1 ratio with Freunds complete or incomplete adjuvant (primary/booster). Mice were subcutaneously injected (35 µg peptide + 35 µL adjuvant) at days 0, 28, and 38, with terminal bleed via cardiac puncture at day 48 (4 female, 4-6 week old Balb/c per group - protocol approved by University Health Network (UHN) Animal Care Committee (ACC)). Mock immunization used adjuvant with sterile PBS.

To evaluate neutralization breadth, clones from a previously described multi-genotype HCVpp panel were selected to maximize both genotypic coverage and neutralization sensitivity [10]. Selected variants were situated within the global sequence space using a neighbour joining tree constructed with an E2 reference set in MEGA7 [15]. Neutralization assays were performed as previously described [14]. In brief, infectious HCVpp (100ul) containing media was mixed with sera diluted 1:50, and incubated 1 hour at

37 °C. Thereafter, media from Huh7 cells seeded one day prior in 96-wells was discarded, and replaced with the pseudoparticle/sera mixture (100uL), in triplicates. Plates were then incubated (37°C, 5% CO2) for 72 hours before lysis with BrightGlo luciferase detection reagent (Promega). Neutralization was determined by the triplicate averaged reduction in relative light units as normalized to adjuvant vaccinated sera.

For competitive ELISA epitope mapping, PBST diluted sera (1:1000) were incubated at room temperature with peptides (1ug/well) corresponding to either full length HVR1, or a series of overlapping peptides spanning full length HVR1, for 1 hour at room temperature. Peptide-sera mixes were then added, in triplicate, to 96-wells pre-coated with full length HVR1 (1ug), and incubated 1 hour. Post-incubation, plates were washed 5 times in PBST, and HRP conjugated donkey anti-mouse IgG was diluted 1:10,000, added across wells, and incubated for 1 hour to detect primary AB. After a final three washes, TMB substrate was added to each well, dark-incubated for 15 minutes, then reaction terminated with Stop-Solution (0.16M sulfuric acid). Absorbance was read at 450nm, in triplicate, with measurements corresponding to the visual colour change in each well. Statistical analysis was done by unpaired t-test followed by a Benjamini-Hochberg false discovery rate (FDR) adjustment for multiple comparisons (Q=0.05) using Prism8 [16].

Data Availability

Sequences used to pseudotype HCVpp H77.20, 1.4.1, 1.20.1, JFH1, 3.2.1, 4.3.1, 5.2.1, 6.1.1, and 6.2.1 are available in GenBank (accession numbers: NC_038882, KU285161, KU285213, KF268446, KU285200, KU285218, KU285223, KU285226, KU285227, KU285228). HVR1 sequences of 1bTO, I.1, I.2, are available upon request. Sequences used for NPJ constructing are available from (https://hcv.lanl.gov/content/sequence/NEWALIGN/align.html) with parameters “2008/ E2/ protein”, or from corresponding author upon request.

References

Domingo, Esteban, et al. “Viral Quasispecies Evolution.” *Microbiology and Molecular Biology Reviews*, vol. 76, no. 2, June 2012, pp. 159–216. *mmbr.asm.org*, doi:10.1128/MMBR.05023-11

Servín-Blanco, R., et al. “Antigenic Variability: Obstacles on the Road to Vaccines against Traditionally Difficult Targets.” *Human Vaccines & Immunotherapeutics*, vol. 12, no. 10, June 2016, pp. 2640–48. *PubMed Central*, doi:10.1080/21645515.2016.1191718

Di Lello, Federico A., et al. “Inter and Intrapatient Evolution of Hepatitis C Virus.” *Annals of Hepatology*, vol. 14, no. 4, July 2015, pp. 442–49. *ScienceDirect*, doi:10.1016/S1665-2681(19)31164-0

Quadeer, Ahmed A., et al. “Identifying Immunologically-Vulnerable Regions of the HCV E2 Glycoprotein and Broadly Neutralizing Antibodies That Target Them.” *Nature Communications*, vol. 10, no. 1, May 2019, pp. 1–11. *www.nature.com*, doi:10.1038/s41467-019-09819-1

Law, John L. M., et al. “Role of the E2 Hypervariable Region (HVR1) in the Immunogenicity of a Recombinant Hepatitis C Virus Vaccine.” *Journal of Virology*, vol. 92, no. 11, May 2018. *PubMed Central*, doi:10.1128/JVI.02141-17

Keck, Zhen-yong, et al. “Antibody Response to Hypervariable Region 1 Interferes with Broadly Neutralizing Antibodies to Hepatitis C Virus.” *Journal of Virology*, vol. 90, no. 6, Jan. 2016, pp. 3112–22. *PubMed*, doi:10.1128/JVI.02458-15

Campo, David S., et al. “Hepatitis C Virus Antigenic Convergence.” *Scientific Reports*, vol. 2, Feb. 2012. *PubMed Central*, doi:10.1038/srep00267

Afonnikov, Dmitry A. et al. Kolchanov. “CRASP: A Program for Analysis of Coordinated Substitutions in Multiple Alignments of Protein Sequences.” *Nucleic Acids Research*, vol. 32, July 2004, pp. W64–68. *PubMed Central*, doi:10.1093/nar/gkh451.

Atchley, William R., et al. “Solving the Protein Sequence Metric Problem.” *Proceedings of the National Academy of Sciences*, vol. 102, no. 18, May 2005, pp. 6395–400. *www.pnas.org*, doi:10.1073/pnas.0408677102

Urbanowicz, Richard A., et al. “A Diverse Panel of Hepatitis C Virus Glycoproteins for Use in Vaccine Research Reveals Extremes of Monoclonal Antibody Neutralization Resistance.” *Journal of Virology*, vol. 90, no. 7, Dec. 2015, pp. 3288–301. *PubMed*, doi:10.1128/JVI.02700-15

Johnson, Janelle., et al. “A Recombinant Hepatitis C Virus Genotype 1a E1/E2 Envelope Glycoprotein Vaccine Elicits Antibodies That Differentially Neutralize Closely Related 2a Strains Through Interactions of the N-Terminal Hypervariable Region 1 of E2 With Scavenger Receptor B1.” Journal of virology, Oct. 29, 2019. https://doi.org/10.1128/JVI.00810-19.

Penin, François, et al. “Conservation of the Conformation and Positive Charges of Hepatitis C Virus E2 Envelope Glycoprotein Hypervariable Region 1 Points to a Role in Cell Attachment.” *Journal of Virology* 75, no. 12, Jun. 2001, pp. 5703–10. https://doi.org/10.1128/JVI.75.12.5703-5710.2001.

Donlin, Maureen J., et al. “Pretreatment Sequence Diversity Differences in the Full-Length Hepatitis C Virus Open Reading Frame Correlate with Early Response to Therapy.” *Journal of Virology,* vol. 81, no. 15, Aug. 2007, pp. 8211–24. https://doi.org/10.1128/JVI.00487-07.

13. Bailey, Justin R., et al. “Standardized Method for the Study of Antibody Neutralization of HCV Pseudoparticles (HCVpp).” *Methods in Molecular Biology (Clifton, N.J.)*, vol. 1911, 2019, pp. 441–50. *PubMed Central*, doi:10.1007/978-1-4939-8976-8_30

14. Kumar, Sudhir., et al. “MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets.” *Molecular Biology and Evolution* 33, no. 7 (2016): 1870–74. https://doi.org/10.1093/molbev/msw054.

Yu, Xuesong, et al. “Statistical Approaches to Analyzing HIV-1 Neutralizing Antibody Assay Data.” *Statistics in Biopharmaceutical Research* 4, no. 1, Jan. 2012, p. 1–13. *PubMed Central*, doi.org/10.1080/19466315.2011.633860