

1 **Brief Communication**

2 **A bivalent HCV peptide vaccine elicits pan-genotypic neutralizing** 3 **antibodies in mice**

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16 **Author Contributions**

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

19 **Competing Interest Statement:** The authors declare that the research was conducted in the conflict of
20 interest.

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

29 **Introduction**

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

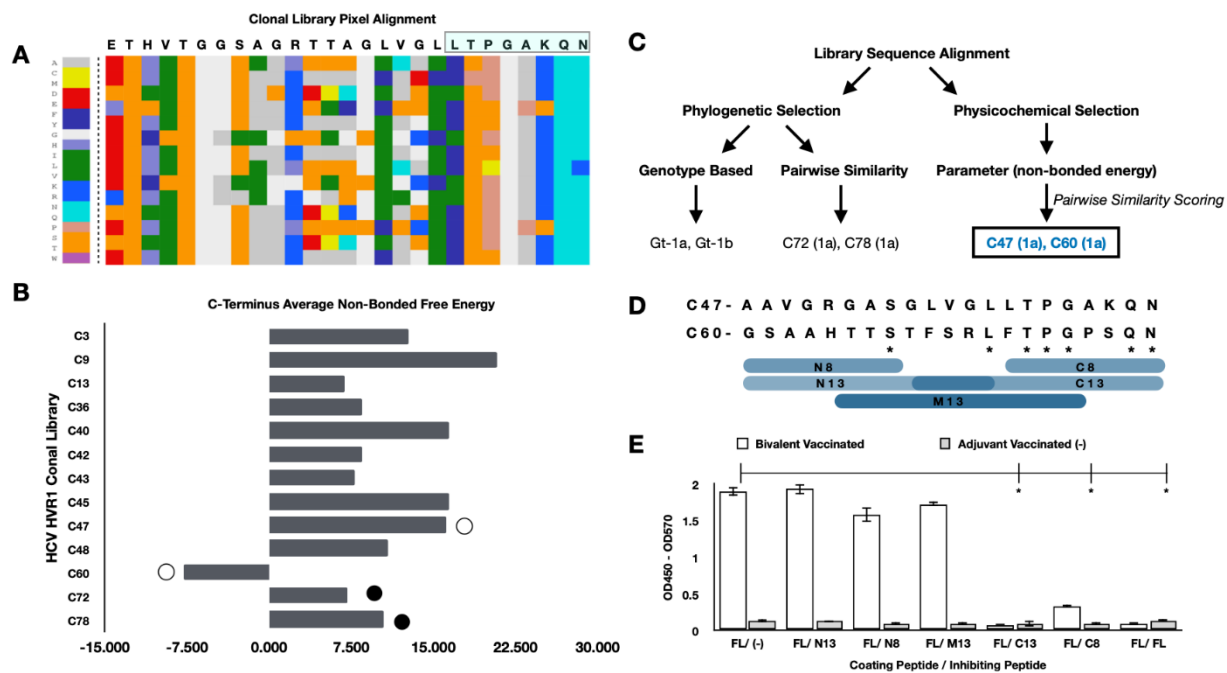
32 one strain are ineffective for another [1]. Understood as an emergent property involving host immune
33 responses, rather than solely an intrinsic feature of sequence diversity, antigenic variability is preserved
34 by the inability of the host adaptive-response to match the immunogenic breadth of the infecting pathogen
35 [2]. This dynamic is exemplified by HCV, with intrahost populations characterized by antigenic variants
36 positioned for divergent evolutionary trajectories depending on host immune responses, aggregating in
37 cycles of viral diversification and immune escape from AB targeting antigenically plastic neutralizing
38 epitopes [3].

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

51 **Results**

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

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



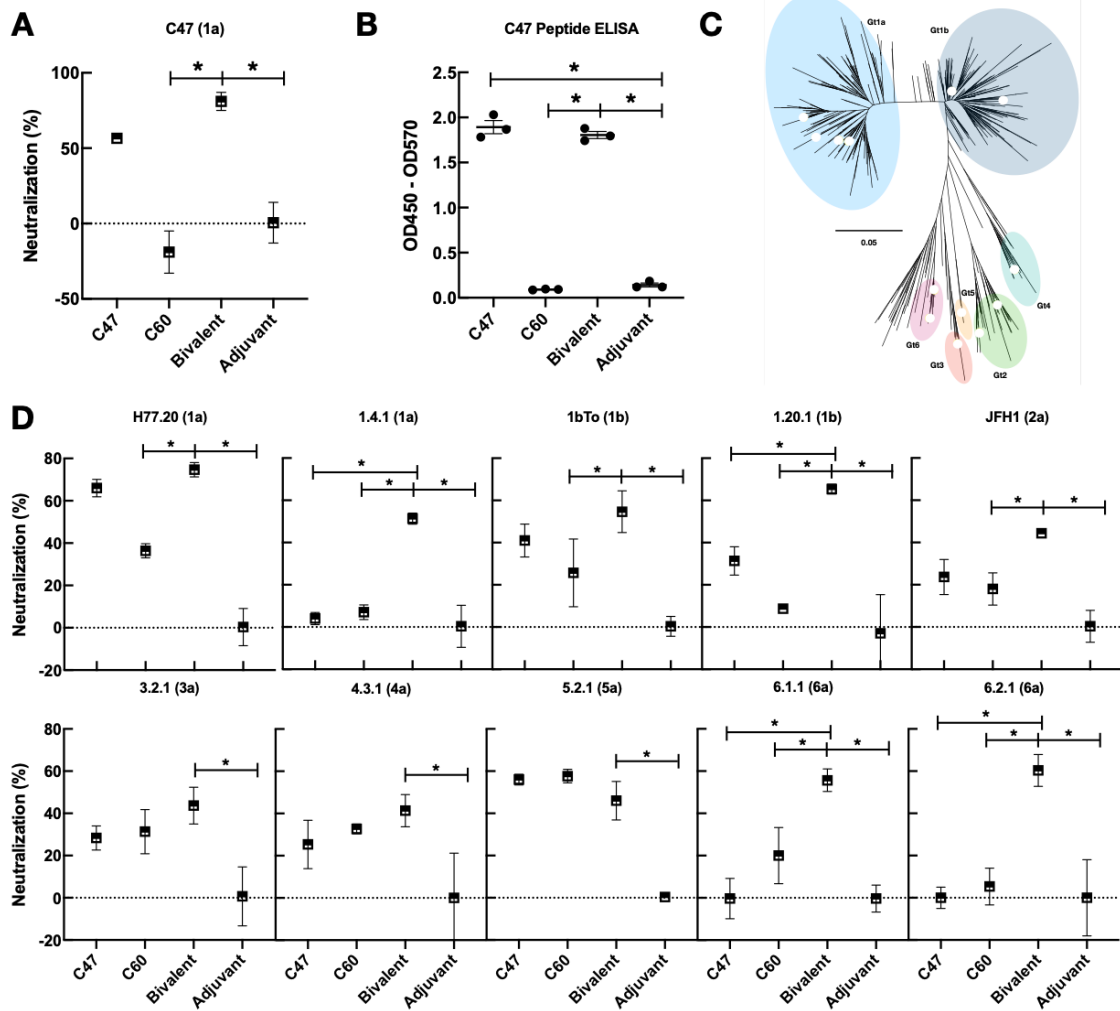
66 the HVR1 C-terminus (Fig. 1E).

67 **Figure 1. Physicochemically Informed Multivalent Vaccine Design**

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

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

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



86 vaccines (Fig. 2D).

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

88 (A) Neutralization activity of antisera (1:50 dilution) against HCVpp pseudotyped with homologous C47. (B) An ELISA plate was
89 coated with peptide C47. Sera from immunized mouse groups were added at 1:1000 dilution. The binding of antibody was
90 detected with anti-mouse secondary antibody. (C) HCV reference sequence database (gpE2) were supplemented with HCVpp
91 panel sequences (white circles) for neighbour joining in Molecular Evolutionary Genetics Analysis version 7 (MEGA7). (D)
92 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,
93 6.2.1). Neutralization activity was quantified by measuring luciferase activity (relative light units), and normalized to adjuvant
94 immunized sera. For (A), (B), and (C), averages of data from triplicates are shown. Error bars indicate standard error of the
95 mean. Statistical analysis was done by unpaired t-test followed by FDR (Q=0.05) adjustment for multiple comparison (*, FDR-
96 adjusted P<0.05).

97

98 **Discussion**

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

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

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

117

118 **Materials and Methods**

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

129 For immunizations, C47 and C60 were synthesized into peptides using Fmoc chemistry, N-terminally
130 conjugated to keyhole limpet hemocyanin via maleimide linkage, and mixed at 1:1 ratio with Freund's
131 complete or incomplete adjuvant (primary/booster). Mice were subcutaneously injected (35 µg peptide +
132 35 µL adjuvant) at days 0, 28, and 38, with terminal bleed via cardiac puncture at day 48 (4 female, 4-6
133 week old Balb/c per group - protocol approved by University Health Network (UHN) Animal Care
134 Committee (ACC)). Mock immunization used adjuvant with sterile PBS.

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

140 37 °C. Thereafter, media from Huh7 cells seeded one day prior in 96-wells was discarded, and replaced
141 with the pseudoparticle/sera mixture (100uL), in triplicates. Plates were then incubated (37°C, 5% CO₂)
142 for 72 hours before lysis with BrightGlo luciferase detection reagent (Promega). Neutralization was
143 determined by the triplicate averaged reduction in relative light units as normalized to adjuvant vaccinated
144 sera.

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

156

157 **Data Availability**

158 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
159 are available in GenBank (accession numbers: NC_038882, KU285161, KU285213, KF268446,
160 KU285200, KU285218, KU285223, KU285226, KU285227, KU285228). HVR1 sequences of 1bTO, I.1,
161 I.2, are available upon request. Sequences used for NPJ constructing are available from
162 (<https://hcv.lanl.gov/content/sequence/NEWALIGN/align.html>) with parameters “2008/ E2/ protein”, or
163 from corresponding author upon request.

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