Antibodies Targeting Novel Neutralizing Epitopes of Hepatitis C Virus Glycoprotein Preclude Genotype 2 Virus Infection

Kai Deng1☯, Ruyu Liu2☯, Huiying Rao1, Dong Jiang3, Jianghua Wang1, Xingwang Xie1, Lai Wei1*

1 Peking University People’s Hospital, Peking University Hepatology Institute, Beijing Key Laboratory of Hepatitis C and Immunotherapy for Liver Diseases, Beijing, China, 2 Liver Diseases Center, Beijing Ditan Hospital, Capital Medical University, Beijing, China, 3 Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing Key Laboratory of Emerging infectious Diseases, Beijing, China

☯ These authors contributed equally to this work.
* weelai@163.com

Abstract

Currently, there is no effective vaccine to prevent hepatitis C virus (HCV) infection, partly due to our insufficient understanding of the virus glycoprotein immunology. Most neutralizing antibodies (nAbs) were identified using glycoprotein immunogens, such as recombinant E1E2, HCV pseudoparticles or cell culture derived HCV. However, the fact that in the HCV acute infection phase, only a small proportion of patients are self-resolved accompanied with the emergence of nAbs, indicates the limited immunogenicity of glycoprotein itself to induce effective antibodies against a highly evolved virus. Secondly, in previous reports, the immunogen sequence was mostly the genotype of the 1a H77 strain. Rarely, other genotypes/subtypes have been studied, although theoretically one genotype/subtype immunogen is able to induce cross-genotype neutralizing antibodies. To overcome these drawbacks and find potential novel neutralizing epitopes, 57 overlapping peptides encompassing the full-length glycoprotein E1E2 of subtype 1b were synthesized to immunize BALB/c mice, and the neutralizing reactive of the induced antisera against HCVpp genotypes 1–6 was determined. We defined a domain comprising amino acids (aa) 192–221, 232–251, 262–281 and 292–331 of E1, and 421–543, 564–583, 594–618 and 634–673 of E2, as the neutralizing regions of HCV glycoprotein. Peptides PUH126 (aa 444–463) and PUH145 (aa 604–618)-induced antisera displayed the most potent broad neutralizing reactive. Two monoclonal antibodies recognizing the PUH126 and PUH145 epitopes efficiently precluded genotype 2 viral (HCVcc JFH and J6 strains) infection, but they did not neutralize other genotypes. Our study mapped a neutralizing epitope region of HCV glycoprotein using a novel immunization strategy, and identified two monoclonal antibodies effective in preventing genotype 2 virus infection.
Introduction

Hepatitis C virus (HCV) is one of the major causes of liver disease. An estimated 185 million people worldwide are infected with hepatitis C [1] and have a high risk of liver cirrhosis, hepatocellular cancer and death [2]. There is no prophylactic or therapeutic vaccine available for HCV, although rapid progress in hepatitis C treatment has been made due to the emergence of direct-acting antiviral (DAA) drugs. Once infected with HCV, most patients develop chronic hepatitis and only a small number of individuals clear the virus. Cellular immunity is thought to play a vital role in viral clearance [3–5]. Recently, accumulating evidence has highlighted the importance of humoral immunity in controlling infection [6,7]. Neutralizing antibodies (nAbs) were associated with the eradication of the virus both in the acute and chronic infection phases [7,8].

HCV glycoprotein, which mediates virus entry by interplay with host co-receptors, is the natural target of nAbs. Many nAbs with potent cross-genotype neutralizing reactive have been identified based on artificial glycoprotein immunogens, including recombinant E1E2, soluble E2, HCV pseudoparticles (HCVpp) and cell culture-derived HCV (HCVcc), mimicking the spare structure of the wild type virus glycoprotein [9–11]. Recently, the crystal structure of E2 was determined. The epitopes of these nAbs were mostly mapped to the "broadly neutralizing face", mainly within the N terminal of E2 and approximately comprising amino acids (aa) 412–453 and 502–535 [12–14]. The E2-CD81 interaction region was also thought to be within this domain. The fact that only a few infected patients are resolved during the acute phase in the presence of nAbs implies that the epitopes recognized by the most potent and effective nAbs may be relatively weakly immunogenic and not reactive in most patients with hepatitis C. In the HCV E1E2 steric structure, the epitopes may be buried by adjacent conformation and not accessible for nAbs. On the contrary, variable regions of E2 are immunodominant [15], but they only raise strain-specific protective immunity, which is unable to neutralize highly evolved HCV [16]. Thus, the strategy of solely adopting a glycoprotein immunogen may miss some neutralizing epitopes outside of the "broadly neutralizing face". It is of interest to determine whether there are other novel neutralizing epitopes using a different immunization approach.

Another factor deserving attention is that, in previous studies, the glycoprotein sequence was based on the H77 strain, which represented the most prevalent genotype 1a worldwide. Other genotypes/subtypes were rarely studied, although theoretically one genotype/subtype immunogen was capable of inducing a cross-genotype nAbs [9], and the sera of chronic hepatitis C patients of one subtype were reported to have broadly neutralizing potential [17].

To address the issues mentioned above, we employed a different immunization strategy. First, we synthesized overlapping peptides encompassing the full-length glycoprotein E1E2 (not including the transmembrane domain of E2) instead of glycoprotein as the immunogen. Secondly, the immunogen sequence was mostly according to subtype 1b strain H77, which was prevalent globally and was the dominant subtype in China. Our study revealed that peptides of subtype 1b did induce nAbs, and the neutralizing epitopes of HCV glycoprotein were more broadly distributed than expected. Furthermore, we identified two monoclonal antibodies (mAbs), 2O18 and 2C21, recognizing epitopes aa 454–463 and aa 611–618 of E2, respectively, which efficiently blocked genotype 2 virus (HCVcc, JFH and J6 strains) infection in vitro.

Taken together, our study reveals the neutralizing domain of HCV glycoprotein from a new angle and also identifies two monoclonal antibodies that recognize novel glycoprotein epitopes blocking genotype 2 virus infection. These results facilitate future vaccine design and development.
Materials and Methods

Ethics Statements

All immunization procedures in BALB/c mice were conducted by Abmart Inc. (Shanghai, China; http://www.ab-mart.com) according to national guidelines (the Regulations for the Administration of Affairs Concerning Experimental Animals, China) and were approved by the Ethics Committee of Peking University People's Hospital.

Peptide Synthesis

A peptide library consisting of 57 peptides (Tables 1 and 2) averaging 20 amino acid residues long and overlapping by 10 residues encompassing the complete sequence of HCV glycoprotein E1E2 (not including the transmembrane domain of E2, aa 718–746) of a subtype 1b “reference strain” was synthesized by Invitrogen Corp. (Shanghai, China). The “reference strain” was a consensus sequence generated by alignment of 43 chronic hepatitis C patient viral sequences belonging to subtype 1b (S1 File).

Animal Immunization and Antibody Generation

Fifty μg of peptide was used to inoculate BALB/c mice (n = 3 for each peptide) with complete adjuvant to elicit polyclonal antibodies (antisera), and repeated at day 7, 14 and 21 with

Table 1. Amino acid sequences of peptides PUHI 1–25 used to raise antibodies.

| Peptide (PUHI) | aa Position | Sequence         |
|----------------|-------------|------------------|
| 1              | 192–211     | YEVRNVSGVYHTNDCSNSS |
| 2              | 202–221     | HVTNDCSNSSIYVEAADMIM |
| 3              | 212–231     | IVYEAADIMHTPGCVPCVR |
| 4              | 222–241     | HTPGCVPCVRENNSRCWVA |
| 5              | 232–251     | ENNSSRCWVALPTLAARNA |
| 6              | 242–261     | LPTLAARNASVPTTTIRRH |
| 7              | 252–271     | SVPTTTIRRHVDDLVGAAAF |
| 8              | 262–281     | VDLVGAAAFCSAMYVGDLC |
| 9              | 272–291     | CSAMYVGDLCGSVLQSQLF |
| 10             | 282–301     | GSVFLVSQFHTFSPPRHTETV |
| 11             | 292–311     | TFSPRHRVTQDCNCSIYPG |
| 12             | 302–321     | QDCNCSIYPGHSVGHRMAWD |
| 13             | 312–331     | HSVGHRMADMNNMNSPTTA |
| 14             | 322–341     | MMMNWPTTALVSQQLRIP |
| 15             | 332–353     | LVVSQRLIPOAVDVMVAGAHW |
| 16             | 384–403     | GTYVTGQAHTTRGAFSL |
| 17             | 394–413     | HTTRGAFSLFTPQPSQKL |
| 18             | 404–418     | TPGPSQIKQLVLNTNG |
| 19             | 409–423     | QKIQLVLNTNGSWHIN |
| 20             | 414–428     | VNTNGSWHINRTALN |
| 21             | 419–433     | SWHINRTALNCDSL |
| 22             | 424–438     | RTALNCDSLNTGFL |
| 23             | 429–443     | CNDSLNTGFLAALFY |
| 24             | 434–448     | NTGFLAALFYTHKFN |
| 25             | 439–453     | AALFYTHKFNSSGCP |

The amino acid positions are reported relative to the subtype 1a H77 strain.

doi:10.1371/journal.pone.0138756.t001
incomplete adjuvant. The antisera were collected 4 weeks post-immunization. The antibody concentration in the sera was validated by ELISA (>1:200). The production of the monoclonal antibody was conducted according to standard hybridoma technology. Ascites from inoculated BALB/c mice were collected at 4–6 weeks post-immunization, and monoclonal antibodies were purified from ascites using a protein A column. After the experiment, all mice were euthanized by CO2 asphyxiation.

**Cell Lines and Antibodies**

HEK 293T (ATCC CRL-1573) and human Huh7.5 hepatoma cells were maintained and propagated as described previously [18]. Mouse anti-HCV NS3 was purchased from Abcam (Cat. 13830) and goat anti-mouse Alexa Fluor 488 was purchased from Invitrogen (Cat. A-11001).

### Table 2. Amino acid sequences of peptides PUHI 26–57 used to raise antibodies.

| Peptide (PUHI) | aa Position | Sequence                      |
|----------------|-------------|-------------------------------|
| 26             | 444–463     | THKFNSSGCPERMASCRRPID         |
| 27             | 454–473     | ERMASCRRPIDKFQGWPIT           |
| 28             | 464–483     | KFAQGWGPTYAEPPSSDOR           |
| 29             | 474–493     | YAEPPSSDORPYCWHYAPRP          |
| 30             | 484–503     | PYCWHYAPRPCCGIVPASQVC         |
| 31             | 494–513     | CGIVPASQCGPVVCFTPS            |
| 32             | 504–523     | GPPVCFTPSPPPVVGTDDRGF         |
| 33             | 514–528     | VVVGTDDRGFGVPTYN              |
| 34             | 519–533     | TDRFGVPTYNWGENCE              |
| 35             | 524–538     | VPTYNWNGENTDVLL               |
| 36             | 529–543     | WGENETDVLLLNNTR              |
| 37             | 534–548     | TDVVLLNTRPPQGN               |
| 38             | 539–553     | LNNTRPPQGNWFGCT              |
| 39             | 544–563     | PPQGNWFGCTWMNSTGFTKT          |
| 40             | 554–573     | WMNSTGFTKTCGGPCCNIGG          |
| 41             | 564–583     | CGGPPCNIGGVGNNTLTCPT          |
| 42             | 574–593     | VGNNTLTCTPDCFRKHPEAT           |
| 43             | 584–603     | DCFRKHPEATYTGKCGSPW           |
| 44             | 594–613     | YTKGCGSPWLTPCRLVDPY           |
| 45             | 604–618     | TPRLVDPYPRLWY                |
| 46             | 609–623     | VDYPYPRLWHYPCTVN             |
| 47             | 614–628     | RLWHYPCTVNFITFK               |
| 48             | 619–633     | PCTVNFITFKVRMY               |
| 49             | 624–643     | FTIFKVRFMYGGVHEHLNAA          |
| 50             | 634–653     | GGGVHEHLNACNWTGGERCD         |
| 51             | 644–663     | CNWTGGERCDLEDRRSELS          |
| 52             | 654–673     | LEDRRSELSPLLSTTEWO            |
| 53             | 664–683     | PLLLSTTEWOILPCSFTTLP          |
| 54             | 674–693     | ILPCSFTTLPALSTGLIHLH         |
| 55             | 684–703     | ALSTGLIHLHONIVQGYYL          |
| 56             | 694–713     | QNIVDVOYLYGVGSAVVSFA          |
| 57             | 704–717     | GVGSAVISFSAIKWE              |

The amino acid positions are reported relative to the subtype 1a H77 strain.

doi:10.1371/journal.pone.0138756.t002
Normal mouse IgG was from Santa Cruz (SC-2025). The neutralizing antibodies CBH-5 and CBH-7 served as the positive controls [19].

**HCV Pseudoparticle (HCVpp) Production and Concentration**

293T cells were co-transfected with expression plasmids encoding the HCV envelope glycoproteins, HIV gag/pol (pLP1), HIV rev (pLP2), and pcDNA3 encoding luciferase protein. HCV envelope expression plasmids included genotype 1a strain H77 (provided by F. L. Cosset, INSERM U758, Lyon, France), genotype 1b strain Con1 (provided by C. Rice, Rockefeller University, New York, NY), and genotypes 2a (clone UKN2A1.2), 3a (clone UKN3A1.28C), 4 (clone UKN4.21.16), 5 (UKN5.14.4) and 6 (UKN6.5.340) (provided by J. K. Ball, The University of Nottingham, United Kingdom). At 48 and 72 hours post-co-transfection, the virus-containing supernatants were harvested, filtered through 0.45 μm membranes, concentrated with a 100K Centrifugal Device (Pall, USA) and stored in aliquots at -80°C until use.

**Cell Culture Derived HCV (HCVcc) Production**

Cell culture supernatant was collected from 10 μg full-length HCV RNA transfected Huh7.5 cells and was used to infect Huh7.5 cells grown in 100 mm dishes at a multiplicity of infection (MOI) of 0.01. The infected cells were passaged at 3-day intervals. At day 14 post-infection, viral supernatants were obtained and clarified by centrifugation and stored in aliquots at -80°C. The FFU of the HCVcc stock was measured using the end-point dilution method as described previously [11]. The HCVcc strains included J4 (1b), JFH (2a), J6 (2a), S52 (3a), ED43 (4a), SA13 (5a) and HK6a (6a) [20].

**Neutralization Assays**

For HCVpp neutralization assays, 8×10³ Huh7.5 cells were seeded into 96-well plates one day before infection. Ten μL HCVpp stock was incubated with antiserum, monoclonal antibodies or normal mouse serum/IgG (control group) at various concentrations, plus 4 μg/ml polybrene at 37°C for 1 hour. The mixtures (100 μL in total) were then added to each well. After incubation at 37°C for 6 hours, the mixtures were replaced with complete culture medium and incubated for 72 hours. HCV infection was evaluated by measuring luciferase activity (Promega, Cat. E1501). The value of %Neutralization was calculated as (1 - luciferase value of experimental group/luciferase value of control group) x 100%. The IC₅₀ of the antibody (required to neutralize 50% of virus) was determined based on a neutralization curve generated from a series of 2-fold dilutions tested in triplicate.

For the HCVcc neutralization assays, 6×10³ Huh7.5 cells were seeded into 96-well plates one day before infection. A sample of 100 FFU HCVcc was incubated with monoclonal antibodies or normal mouse IgG (control group) at 37°C for 1 hour. The mixture was then incubated with Huh7.5 cells for 4 hours. Seventy-two hours post-infection, HCV infection was evaluated by counting HCV NS3-positive foci in an indirect immunofluorescence assay [21]. Each test was performed in triplicate. %Neutralization was calculated as (1 - foci of experimental group/foci of control group) x 100%.

**Epitope Mapping**

To map the precise epitopes of mAbs 2O18 and 2C21, three overlapping peptides covering aa 444–463 and aa 604–618, respectively, were synthesized. The plates were coated with 5 μg/ml of peptide and blocked with 4% PBST. The two mAbs were incubated and binding was detected in an ELISA format as described previously [22]. Irrelevant rabies virus peptide
(VNLHDFRSDEIE) served as the negative control. Peptide PUH126 (aa 444–463) and PUH145 (aa 604–618) served as the positive controls.

**Alanine Replacement Mutagenesis**

To identify residues crucial for mAbs binding, alanine mutagenesis of the epitope residues was analyzed in a GNL capture ELISA assay [23]. The OD value of antibody binding to epitope with replacing <50% of wild type residues was defined as positive. Each experiment was performed in triplicate.

**Data Analysis and Software**

Statistical comparison of the neutralization between experimental and control groups was performed using the \( \chi^2 \) test (GraphPad Prism 5). The neutralizing entropy of 47 peptides inducing antisera against HCVpp was analyzed with Genesis software (http://genome.tugraz.at/genesisclient/genesisclient_description.shtml) [24].

**Results**

**Neutralizing Epitope Domain of HCV Glycoprotein E1E2**

A series of 57 overlapping peptides covering the full-length glycoprotein E1E2 (subtype 1b) was used as an immunogen to produce antibodies (Tables 1 and 2). The peptides were an average of 20-mers, with an overlap of 10 amino acids. Forty-seven of 57 peptides induced antisera containing high-titer polyclonal antibodies (>1:200 in ELISA). However, the remaining 10 peptides, including peptide PUHI 9 (aa 272–291), 10 (aa 282–301), 15 (aa 332–353), 18 (aa 404–418), 19 (aa 409–423), 24 (aa 434–448), 48 (aa 619–633), 55 (aa 684–703), 56 (aa 694–713) and 57 (aa 704–717), did not raise sufficient antibodies in BALB/c mice sera, although we repeated the immunization procedure 3 times (three mice for each peptide immunogen).

To determine the neutralizing reactive of the antibodies induced by 47 peptides, the antisera were 50-fold diluted and tested in HCVpp neutralizing assays (genotypes 1–6). The relative neutralization of antisera was determined (Tables 3 and 4) and analyzed with Genesis software, as described previously [24]. The neutralizing values of the antisera were converted to different kinds and degrees of colors (Fig 1A). The green color indicated neutralizing reactive antisera, and the red color indicated antisera without neutralizing reactive or promoting virus entry.

Our results revealed a huge difference in the neutralizing reactive of antisera induced by various peptides. Antisera induced by PUH126 (aa 444–463) had the most potent neutralizing reactive against genotype 1a HCV pp (74.8%). The same antisera also showed different neutralizing activities against various genotypes of HCVpp; e.g., the neutralization of antisera induced by PUH12 (aa 202–221) against genotypes 1a, 3, 5 and 6 HCVpp was 30.0%, 59.8%, 54.0% and 16.1%, respectively, but did not neutralize genotypes 1b, 2 and 4 HCVpp entry.

There is not a precise value of relative neutralization to define antisera as “neutralizing antisera”. In this study, we did not adopt rigorous standards (such as neutralization >50%) because during the neutralizing mAbs screening course, we found that the same peptide immunogen simultaneously induced neutralizing and non-neutralizing antibodies, and the latter somewhat promoted virus infection, which may offset the effect of neutralizing antibodies in antisera (S1 Fig). In our study, the difference between antisera with neutralizing reactive ≥30% and the normal mice serum group (5.6%) was statistically significant \( (P< 0.01, \chi^2 \) test). In addition, the sera with neutralizing reactive ≥30% prevented virus entry in a dose-dependent manner (S2 Fig). Thus, we defined the antisera with relative neutralization ≥30% as “neutralizing antisera”.

Antibodies Targeting Novel Epitopes Preclude HCV Infection
Twenty-two peptides induced antisera that neutralized ≥30% against 3 genotypes/subtypes in the HCVpp neutralizing assay (Tables 3 and 4). Therefore, four regions of HCV E1 (aa 192–221, aa 232–251, aa 262–281 and aa 292–331) were defined as the broadly neutralizing domains. Four regions of HCV E2 (aa 421–543, aa 564–583, aa 594–618 and aa 634–673) were also defined as broadly neutralizing domains (Fig 1B).

Fig 2 shows the peptides inducing antisera, with the top 5 most potent neutralizing reactive against genotypes/subtypes 1–6 HCVpp. Their epitopes were mostly within aa 421–543 of E2, followed by regions aa 594–618 and aa 564–583. Aa 421–543 represented the main domain, accounting for inducing the most potent neutralizing antisera against various genotypes of the virus. The neutralization of PUHII26 (aa 444–463)-induced antiserum against genotypes/subtypes 1a, 1b, 3, 4, 5 and 6 HCVpp was 74.8%, 56.4%, 69.5%, 73.5%, 70.5% and 76.1%, respectively. The neutralization of PUHI45 (aa 604–618)-induced antiserum against genotypes/subtypes 1a, 1b, 2, 3, 5, and 6 HCVpp at 25.0%, 34.9%, 65.0%, 52.7%, 52.9% and 68.0%, respectively. These two peptide immunogens induced the most potent broadly neutralizing antisera (Fig 2).

Development of Monoclonal Antibodies Recognizing Neutralizing Epitopes

Because the peptides PUHII26 (aa 444–463) and PUHI45 (aa 604–618) induced broadly neutralizing antisera, mAb isolates targeting the epitopes were produced using hybridoma techniques and tested in a HCVpp neutralizing assay (S1 Fig). Two mAbs, 2O18 and 2C21, recognizing aa 444–463 and aa 604–618, respectively, were identified as having neutralizing reactive (Fig 3). The neutralizing reactive of 2O18 against different genotypes of HCVpp

Table 3. Average neutralization reactive of PUH11–22 induced antisera against genotypes/subtypes 1–6 HCVpp.

| PUH11 | % Neutralization against genotypes/subtypes 1–6 HCVpp |
|-------|--------------------------------------------------------|
| 1     | 1a 15.3 1b -50.6 2 35.9 4 22.2 5 44.0 6 28.9 |
| 2     | 30.0 -1.1 -43.8 59.8 -111.5 54.0 16.1 |
| 3     | 47.5 28.8 2.9 21.1 -745.1 13.3 -2.4 |
| 4     | 18.0 13.6 -37.9 44.7 12.3 70.1 24.4 |
| 5     | 36.9 22.5 12.4 35.5 11.3 66.2 68.5 |
| 6     | 43.2 5.1 -5.1 -54.6 28.7 75.3 9.9 |
| 7     | 10.6 -21.2 -8.3 2.0 13.4 6.6 -71.5 |
| 8     | 18.3 2.1 -1.3 35.8 -104.6 60.5 73.3 |
| 11    | 20.7 48.2 35.0 28.3 -9.6 21.5 36.7 |
| 12    | 23.9 -7.2 23.7 -7.5 21.8 -39.5 9.4 |
| 13    | 12.8 35.1 32.3 34.0 -133.9 -155.4 49.1 |
| 14    | 18.1 -58.1 -29.6 23.0 68.5 20.8 29.2 |
| 16    | 30.3 45.9 -26.4 -22.8 -5.6 -221.8 25.6 |
| 17    | -42.9 29.2 20.3 9.0 -11.9 22.9 -202.6 |
| 20    | 6.9 -1.0 24.6 37.9 -4.1 -14.4 -156.7 |
| 21    | -14.3 31.2 -65.9 -27.3 1.2 -6.1 -4.6 |
| 22    | 33.3 -21.2 54.8 -18.5 -99.8 6.9 -2.9 |

%Neutralization is calculated as (1-luciferase value of experimental group/luciferase value of control group) x100%. Peptides inducing antisera with neutralization values ≥30% are in bold type.

doi:10.1371/journal.pone.0138756.t003

Antibodies Targeting Novel Epitopes Preclude HCV Infection
varied, with the most potent neutralization against the con1 strain of genotype 1b (IC\textsubscript{50} 35.8 μg/ml), and moderate neutralization against the genotype 6 strain (IC\textsubscript{50} 167.7 μg/ml). Similarly, 2C21 displayed the most potent neutralizing reactive against genotype 6 virus (IC\textsubscript{50} 34.1 μg/ml), but barely neutralized the genotype 1a H77 strain.

To test 2O18 and 2C21 dose-dependent neutralizing reactive against various genotypes of HCVcc, they were serially 10-fold diluted from 100 μg/ml to 0.01 μg/ml and assayed in the HCVcc neutralizing assay. Both demonstrated potent neutralizing characteristics against genotype 2 JFH and J6 strains (Fig 4). However, they could not completely block other genotypes of HCVcc, including J4 (1b), S52 (3a), ED43 (4a), SA13 (5a) and HK6a (6a), even at 100 μg/ml.
Epitope Mapping and Determination of Residues Crucial for Antibody Binding

To map the precise epitopes of 2O18 and 2C21, three overlapping peptides, encompassing aa 444–463 and aa 604–618, were synthesized and their relative binding to antibodies was determined in by ELISA (Fig 5). The results demonstrated that 2O18 and 2C21 recognized epitopes aa 454–463 and aa 611–618, respectively.
To determine the residues crucial for mAbs binding, we conducted alanine replacement mutagenesis (Fig 6). The results demonstrated that residues R460, P461, I462 and D463 were crucial for 2O18 binding, while L615 and W616 were responsible for 2C1 binding.

Conservation Analysis of 2O18 and 2C21 Epitopes

To analyze the conservation of 2O18 and 2C21 epitopes, a total of 536 HCV sequences comprising genotypes/subtypes 1–6 were manually retrieved from the NCBI database (Table 5, S2 File). All the sequences were submitted to Clusta Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) for alignment, and the conserved epitope residues are marked in Fig 7. Residues M456, C459 and I462 of the 2O18 epitope were conserved, while R614 and L615 of the 2C21 epitope were conserved. Thus, the conserved residue crucial for 2O18 binding was I462, and L615 for 2C21.

Discussion

Development of an effective vaccine against HCV, such as a neutralizing antibody, is an urgent need because of the inaccessibility and unaffordability of DAAs in many developing countries.
Fig 3. Dose-dependent neutralization of genotypes/subtypes 1–6 HCVpp with mAbs 2O18 and 2C21. 2O18 and 2C21 were 2-fold diluted, from 128 μg/ml to 1 μg/ml, mixed with HCVpp and incubated for 1 hour and then added to Huh7.5 cells. Seventy-two hours post-infection, viral infection was evaluated by determining luciferase activity. HCVpp without antibody served as the positive control. One-half inhibitory concentrations for virus neutralization values (IC50) against each HCVpp were calculated using GraphPad Prism software. NA, not applicable. The experiment was performed in triplicate and the error bars represented the standard error of the means (SEM).

doi:10.1371/journal.pone.0138756.g003
Neutralizing antibody plays an important role in controlling HCV infection and is associated with viral clearance [6,7]. Many nAbs were identified using glycoprotein immunogens, with the immunogen sequence according to the subtype 1a H77 strain. These epitopes were mostly mapped to a “neutralizing face”, mainly within the aa 412–535 region [12,25]. In this study, we adopted a different immunization strategy, using overlapping peptides instead of glycoproteins, and the 1b strain sequence instead of H77, to map the neutralizing domain of HCV glycoprotein. Our study revealed that the chief neutralizing
Fig 5. mAbs 2O18 and 2C21 epitope mapping. Three overlapping peptides, encompassing aa 444–463 and aa 604–618, were synthesized. The binding of mAbs to each peptide fragment was determined by ELISA. +, with binding activity; -, without binding activity. Peptides aa 444–463 and 604–618 served as the positive controls for 2O18 and 2C21, respectively, while the rabies virus peptide served as the negative control. Blank, without peptide.

doi:10.1371/journal.pone.0138756.g005
Fig 6. Alanine-replacement mutagenesis of the 2O18 and 2C21 epitopes. The amino acid residues mutated to alanine are shown. The relative binding of mAbs to each mutant was determined by ELISA and expressed as 490 nm OD value. WT (PUHI26 and PUHI45) and rabies virus peptide served as the positive and negative controls, respectively.

doi:10.1371/journal.pone.0138756.g006

Table 5. Sequences retrieved from the NCBI database. In total, 536 protein sequences comprising HCV genotypes/subtypes 1–6 were retrieved from the NCBI database for conservation analysis.

| Genotypes/subtypes | Number |
|--------------------|--------|
| 1a                  | 85     |
| 1b                  | 272    |
| 1c                  | 2      |
| 2a                  | 20     |
| 2b                  | 82     |
| 2c                  | 5      |
| 3a                  | 37     |
| 3b                  | 1      |
| 4                   | 10     |
| 5                   | 3      |
| 6                   | 19     |
| Total               | 536    |

doi:10.1371/journal.pone.0138756.t005
domain of the glycoprotein was located at aa 421–543 of E2, which induced the most potent broadly neutralizing antisera, consistent with previously defined “neutralizing face”. The second neutralizing regions were aa 594–618 and aa 624–653 of E2. Others have also reported neutralizing epitopes at aa 611–616, aa 644–651 and aa 292–331, although E2 was generally considered to be the immunodominant glycoprotein. Of note, there were some peptides within previously reported neutralizing epitopes, such as PUH1 19 (409–423) within epitope aa 412–423, that did not yield detectable antibody, although we repeated the immunization procedure. This may be due to the weak immunogenicity of the epitope. Indeed, epitope aa 412–423, recognized by nAb AP33, was detected in less than 2.5% of serum samples collected from acute and chronic hepatitis C patients [29]. The use of a particular carrier in combination with a peptide immunogen may improve the production of the antibody [30]. Of note, 2O18 and 2C21 antibodies appeared to promote infections of most tested HCVpp at low doses (<16 µg/ml for 2O18, <8 µg/ml for 2C21), the antibody Fc receptor was probably account for that [31].

Based on the identified neutralizing epitopes, we isolated two nAbs, 2O18 and 2C21. Both displayed cross-genotype neutralizing reactive in HCVpp neutralizing assays, and potently blocked HCVcc genotype 2 JFH and J6 strain infection. Epitope mapping revealed that some of the residues crucial for antibody binding were highly conserved, indicating that the interplay between antibodies and virus epitopes was relatively stable. Because genotype 2 is prevalent in the Asian-Pacific region and accounts for a large proportion of HCV infection, it is advisable to further test the efficacy of 2O18 and 2C21 in more HCVcc genotype 2 strains, and in vivo.

2O18 and 2C21 did not neutralize HCVcc of genotypes other than genotype 2, but displayed broad neutralizing reactive in HCVpp. The reason for this difference is elusive and needs further elucidation. The strains used in HCVpp and HCVcc were not exactly the same. Recently there were similar observations for other neutralizing antibodies [32].

In summary, our study reveals the neutralizing region of HCV glycoprotein by use of the peptide immunization strategy. We developed two monoclonal antibodies with potent neutralizing reactive against genotype 2 viruses. These findings have important implications for HCV vaccine design and development.

Supporting Information

S1 Fig. Neutralizing reactive of monoclonal antibody isolates against epitopes aa444-463 and aa604-618. 20 mice ascites monoclonal antibodies against epitope aa 444–463 (11365 and 11366 groups), and 30 monoclonal antibodies against epitope aa 604–618 (11367 and 11368 groups), were 1:50 diluted and tested in HCVpp neutralizing assay (genotype 1a, 1b and 2a). Some of the antibodies (clones 2–6 and 14 in 11365 group, and clones 7–14, CBH-5 in 11368 group) were not tested in genotype 2a HCVpp neutralizing assay. Clone 2C21 in 11368 group was only tested in genotype 1b HCVpp neutralizing assay. Antibodies CBH-5 (13µg/ml) and
CBH-7 (130μg/ml) were served as positive controls, respectively. (TIFF)

S2 Fig. Dose-dependent neutralization of HCVpp (genotype 1b). The sera were 2-fold diluted (started 1:50), and assayed by HCVpp neutralization. All experiments were performed in triplicate and the error bars represented the standard error of the neutralization means (SEM). (TIFF)

S1 File. Genotype 1b HCV sequences alignment (Bioedit 7.09). HCV genotype 1b reference sequence (aa192-717). (ZIP)

S2 File. HCV sequences conservation analysis (Bioedit 7.09). (ZIP)

Author Contributions
Conceived and designed the experiments: LW KD RL. Performed the experiments: KD RL. Analyzed the data: KD RL HR DJ JW XX. Contributed reagents/materials/analysis tools: LW HR DJ JW. Wrote the paper: LW KD RL. Offered Huh7.5 cell line: RL.

References
1. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST (2013) Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. Hepatology 57: 1333–1342. doi:10.1002/hep.26141 PMID: 23172780
2. Lauer GM, Walker BD (2001) Hepatitis C virus infection. N Engl J Med 345: 41–52. PMID: 11439948
3. Claassen MA, Janssen HL, Boonstra A (2013) Role of T cell immunity in hepatitis C virus infections. Curr Opin Virol 3: 461–467. doi: 10.1016/j.coiviro.2013.05.006 PMID: 23735335
4. Rehermann B (2009) Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. J Clin Invest 119: 1745–1754. doi: 10.1172/JCI39133 PMID: 19587449
5. Walker CM (2010) Adaptive immunity to the hepatitis C virus. Adv Virus Res 78: 43–86. doi: 10.1016/B978-0-12-385032-4.00002-1 PMID: 21040831
6. Dowd KA, Netski DM, Wang XH, Cox AL, Ray SC (2009) Selection pressure from neutralizing antibodies drives sequence evolution during acute infection with hepatitis C virus. Gastroenterology 136: 2377–2386. doi: 10.1053/j.gastro.2009.02.080 PMID: 19303013
7. Osburn WO, Snider AE, Wells BL, Latanich R, Bailey JR, Thomas DL, et al. (2014) Clearance of hepatitis C infection is associated with the early appearance of broad neutralizing antibody responses. Hepatology 59: 2140–2151. doi: 10.1002/hep.27013 PMID: 24245349
8. Ishii K, Rosa D, Watanabe Y, Katayama T, Harada H, Wyatt C, et al. (1998) High titers of antibodies inhibiting the binding of envelope to human cells correlate with natural resolution of chronic hepatitis C. Hepatology 28: 1117–1120. PMID: 9755251
9. Wong JA, Bhat R, Hockman D, Logan M, Chen C, Levin A, et al. (2014) Recombinant hepatitis C virus envelope glycoprotein vaccine elicits antibodies targeting multiple epitopes on the envelope glycoproteins associated with broad cross-neutralization. J Virol 88: 14278–14288. doi: 10.1128/JVI.01911-14 PMID: 25275133
10. Deng Y, Guan J, Wen B, Zhu N, Chen H, Song J, et al. (2013) Induction of broadly neutralising HCV antibodies in mice by integration-deficient lentiviral vector-based pseudotyped particles. PLoS One 8: e62684. doi: 10.1371/journal.pone.0062684 PMID: 23628946
11. Akazawa D, Mortiyama M, Yokokawa H, Omi N, Watanabe N, Date T, et al. (2013) Neutralizing antibodies induced by cell culture-derived hepatitis C virus protect against infection in mice. Gastroenterology 145: 447–455 e441–e444. doi: 10.1053/j.gastro.2013.05.007 PMID: 23673355
12. Cashman SB, Marsden BD, Dustin LB (2014) The Humoral Immune Response to HCV: Understanding is Key to Vaccine Development. Front Immunol 5: 550. doi: 10.3389/fimmu.2014.00550 PMID: 25426115
13. Khan AG, Whidby J, Miller MT, Scarborough H, Zatorski AV, Cygan A, et al. (2014) Structure of the core ectodomain of the hepatitis C virus envelope glycoprotein 2. *Nature* 509: 381–384. doi: 10.1038/nature13117 PMID: 24553139

14. Liu R, Rao H, Wang J, Xie X, Jiang D, Pan X, et al. (2013) Determination of the human antibody response to the neutralization epitopes encompassing amino acids 313–327 and 432–443 of hepatitis C virus E1E2 glycoproteins. *PLoS One* 8: e66872. doi: 10.1371/journal.pone.0066872 PMID: 23826163

15. Puig M, Major ME, Mihalik K, Feinestone SM (2004) Immunization of chimpanzees with an envelope protein-based vaccine enhances specific humoral and cellular immune responses that delay hepatitis C virus infection. *Vaccine* 22: 991–1000. PMID: 15161076

16. Farci P, Shimoda A, Wong D, Cabezon T, De Gioannis D, Strazzera A, et al. (1996) Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci U S A* 93: 15394–15399. PMID: 8986822

17. Pedersen J, Carlsen TH, Prentoe J, Ramirez S, Jensen TB, Forns X, et al. (2013) Neutralization resistance of hepatitis C virus can be overcome by recombinant human monoclonal antibodies. *Hepatology* 58: 1587–1597. doi: 10.1002/hep.26524 PMID: 23729237

18. Keck Z, Wang W, Wang Y, Lau P, Carlsen TH, Prentoe J, et al. (2013) Cooperativity in virus neutralization by human monoclonal antibodies to two adjacent regions located at the amino terminus of hepatitis C virus E2 glycoprotein. *J Virol* 87: 37–51. doi: 10.1128/JVI.01941-12 PMID: 23097455

19. Owsianka AM, Tarr AW, Keck ZY, Li TK, Witteveldt J, Adair R, et al. (2008) Broadly neutralizing human monoclonal antibodies to the hepatitis C virus E2 glycoprotein. *J Gen Virol* 89: 653–659. doi: 10.1099/vir.0.83386-0 PMID: 18272755

20. Mathiesen CK, Jensen TB, Prentoe J, Krarup H, Nicosia A, Law M, et al. (2014) Production and characterization of high-titer serum-free cell culture grown hepatitis C virus particles of genotype 1–6. *Virology* 458–459: 190–208. doi: 10.1016/j.virol.2014.03.021 PMID: 24928051

21. Meunier JC, Russell RS, Goossens V, Priem S, Walter H, Depla E, et al. (2008) Isolation and characterization of broadly neutralizing human monoclonal antibodies to the e1 glycoprotein of hepatitis C virus. *J Virol* 82: 966–973. PMID: 17977972

22. Ndongo N, Berthillon P, Pradat P, Vieux C, Bordes I, Berby F, et al. (2010) Association of anti-E1E2 antibodies with spontaneous recovery or sustained viral response to therapy in patients infected with hepatitis C virus. *Hepatology* 52: 1531–1542. doi: 10.1002/hep.23862 PMID: 20890942

23. Ruwona TB, Giang E, Nieusma T, Law M (2014) Fine mapping of murine antibody responses to immunization with a novel soluble form of hepatitis C virus envelope glycoprotein complex. *J Virol* 88: 10459–10471. doi: 10.1128/JVI.01584-14 PMID: 24965471

24. Sturm A, Quackenbush J, Trajanoski Z (2002) Genetics: cluster analysis of microarray data. *Bioinformatics* 18: 207–208. PMID: 11836235

25. Edwards VC, Tarr AW, Urbanowicz RA, Ball JK (2012) The role of neutralizing antibodies in hepatitis C virus infection. *J Virol* 95: 1–19. doi: 10.1128/JVI.00395-10 PMID: 22049091

26. Clayton RF, Owsianka A, Aitken J, Graham S, Bhella D, Patel AH (2002) Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles. *J Virol* 76: 7672–7682. PMID: 12097581

27. Owsianka A, Clayton RF, Loomis-Price LD, McKeating JA, Patel AH (2001) Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. *J Gen Virol* 82: 1877–1883. PMID: 11457993

28. Keck ZY, Xia J, Wang Y, Wang W, Krey T, Prentoe J, et al. (2012) Human monoclonal antibodies to a novel cluster of conformational epitopes on HCV E2 with resistance to neutralization escape in a genotype 2a isolate. *PLoS Pathog* 8: e1002653. doi: 10.1371/journal.ppat.1002653 PMID: 22511875

29. Tarr AW, Owsianka AM, Jayaraj D, Brown RJ, Hickling TP, Irving WL, et al. (2007) Determination of the human antibody response to the epitope defined by the hepatitis C virus-neutralizing monoclonal antibody AP33. *J Gen Virol* 88: 2991–3001. PMID: 17947521

30. Torresi J, Fischer A, Grollo L, Zeng W, Drummer H, Jackson DC (2007) Induction of neutralizing antibody responses to hepatitis C virus with synthetic peptide constructs incorporating both antibody and T-helper epitopes. *Immunol Cell Biol* 85: 169–173. PMID: 17242693

31. Meyer K, Ait-Goughoulte M, Keck ZY, Foung S, Ray R (2008) Antibody-dependent enhancement of hepatitis C virus infection. *J Virol* 82: 2140–2149. PMID: 18094180

32. Carlsen TH, Pedersen J, Prentoe JC, Giang E, Keck ZY, Mikkelsen LS, et al. (2014) Breadth of neutralization and synergy of clinically relevant human monoclonal antibodies against HCV genotypes 1a, 1b, 2a, 2b, 2c, and 3a. *Hepatology* 60: 1551–1562. doi: 10.1002/hep.27298 PMID: 25043937