

Original Research

A Conserved Domain of HCV E2 Glycoprotein Interacts with Human CD81 and Induces Interferon-Gamma Secretion from Peripheral Blood Mononuclear Cells

Zhiyan Dai^{1,*}, Wei Zeng^{2,†}, Gang Li³, Xin Shu³¹Department of Gastroenterology and Hepatology, The Affiliated Hospital of Southwest Jiaotong University, The Third People's Hospital of Chengdu, Chongqing Medical University, 610031 Chengdu, Sichuan, China²Department of Critical Care Medicine, The Affiliated Hospital of Southwest Jiaotong University, The Third People's Hospital of Chengdu, Chongqing Medical University, 610031 Chengdu, Sichuan, China³Department of Infectious diseases, The Third Affiliated Hospital of Sun Yat-sen university, 510630 Guangzhou, Guangdong, China*Correspondence: wn9898@163.com (Zhiyan Dai)

†These authors contributed equally.

Academic Editor: Nikos Karamanos

Submitted: 2 December 2022 Revised: 15 April 2023 Accepted: 24 May 2023 Published: 18 October 2023

Abstract

Background: Hepatitis C virus (HCV) infection is a global health threat to the public, and vaccines against it are not yet available. The HCV envelope glycoprotein E2 is a key target for anti-HCV vaccines. The majority of previous studies have focused on the hypervariable region and the glycosylation sites of the HCV structural protein. This study aims to investigate a conserved domain of HCV E2 glycoprotein and explore its potential to induce an immune response against HCV. **Methods:** HCV E2 conserved domain (encompassing amino acids 505–702) was prepared in *Escherichia coli* (*E. coli*). Peripheral blood mononuclear cells (PBMCs) were isolated from patients with HCV or healthy controls. Interferon-gamma (IFN- γ) enzyme-linked immunosorbent spot assay was conducted to examine the HCV E2-specific immune response as reflected by IFN- γ -secreting cells/10⁶ PBMCs. **Results:** HCV E2 conserved domain was highly conserved among 25 HCV subtypes, and its recombinant soluble production in *E. coli* was recognized by anti-HCV E2 monoclonal antibodies. This study characterized *in vitro* direct interaction between bacterially expressed HCV E2 conserved domain and human CD81 (hCD81). Furthermore, the recombinant HCV E2 conserved domain markedly induced the production of IFN- γ by PBMCs from patients with HCV. Its stimulated specific immune response was significantly different from non-specific peptide controls or PBMCs isolated from healthy controls. **Conclusions:** HCV E2 conserved domain directly binds hCD81 and activates the production of IFN- γ in the PBMCs of patients with HCV. Therefore, the conserved domain of HCV E2 glycoprotein may be a new candidate for developing an HCV vaccine.

Keywords: hepatitis C virus; E2 glycoprotein; conserved domain; hCD81; protein–protein interaction; interferon-gamma; immune response

1. Introduction

Hepatitis C virus (HCV) infection remains a worldwide health threat to the public. Despite progress in therapeutic options, there is yet to be an effective and safe vaccine to prevent this virus. The HCV envelope E2 glycoprotein is one of three structural proteins (core, E1, E2) encompassing amino acids (aa) 384–746 of the viral polyprotein and carrying two hypervariable regions (HVR1 and HVR2) [1–3]. The high variation of HVR1 and HVR2 of HCV E2 glycoprotein and the use of error-prone RNA-dependent RNA polymerase for viral RNA replication generates a variety of HCV quasispecies, and they are considered a major viral escape mechanism. Several previous studies suggested that the epitopes aa 481–500 and aa 551–570 of HCV E2 glycoprotein may be critical for immunoreactivity. Furthermore, they added that most of the sites for binding to the human cell membrane protein CD81 (hCD81), including aa 420, 527, 529, 530, and 535, were found to

be highly conserved across various HCV genotypes [4–9]. Based on these previous findings, this study hypothesized that an HCV E2 fragment containing the highly conserved binding sites to hCD81 may circumvent this obstacle. To test this hypothesis, this study investigated the conserved domain and explored its potential as a target in developing a vaccine for HCV.

Extensive studies with soluble, truncated HCV E2 glycoprotein or virus-like particles containing E2 have shown its interaction with hCD81 and suggested that hCD81 was a receptor for HCV [10–14]. Moreover, a deglycosylated HCV E2 glycoprotein has been shown to bind hCD81 with similar binding efficiency compared with that of the core-glycosylated form. However, a highly glycosylated form of HCV E2 glycoprotein has shown a substantial reduction in binding affinity to CD81. Yurkova *et al.* [15] have reported the expression of glycosylated, soluble HCV E2 with a lack of its transmembrane domains in mammalian cells,



and the expression level was low. In addition, the purified product was found to be highly heterogeneous, even when a single cellular compartment was used for low protein isolation [15]. To increase the yield sufficient for a further binding assay and a functional study, it was decided to express recombinant HCV E2 glycoprotein using a prokaryotic expression system.

Hepatitis C virus E2-induced specific immune responses were demonstrated in patients with HCV as supported by cytotoxic T lymphocyte, lymphocyte proliferation, and IFN- α production. More importantly, a stronger HCV E2-specific immune response was associated with a better clinical outcome of interferon therapy and viral clearance [16–19]. However, little is known about whether a conserved fragment of HCV E2 could mediate specific T-cell responses. This study hypothesized that highly conserved and immuno-dominant domains within HCV E2 glycoprotein could elicit high cross-reactive immunity to different serotypes of HCV.

Based on these previous findings and to test this hypothesis, this study constructed a prokaryotic expression plasmid containing the coding region of the conserved domain of E2 protein (aa 505–702, including the positions at aa 527, 529, 530, and 535 for binding to hCD81). It was then examined whether the bacterially expressed conserved domain of HCV E2 glycoprotein could be functional by an assay binding to hCD81 and interferon- γ production using the enzyme-d immunosorbent spot (ELISPOT) assay. This study's findings may provide scientific evidence that the conserved domain of HCV E2 glycoprotein has the potential to serve as a new target for a vaccine against HCV infection.

2. Materials and Methods

2.1 Blood Samples

Blood samples were collected from 10 patients [6 men and 4 women, with a mean age of 42 years (range 22 \pm 62)] who were chronically infected with HCV subtype 1b in the Department of Infectious Disease in the Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, Guangdong, China). The diagnosis of chronic hepatitis C (CHC) was made, based on abnormally elevated serum aminotransferase for more than six months, the consistent detection of serum HCV RNA (Amplicor HCV Monitor, Roche, Basel, Switzerland), and histological examination. HCV antibodies [third-generation enzyme-linked immunosorbent assay (ELISA)] were present in each patient. All patients with HCV were negative for hepatitis B surface antigen and antibodies to human immunodeficiency virus 1 and 2. The patients were excluded if they presented other causes of liver diseases (e.g., autoimmunity, alcohol intake, drug toxicity, etc.) in accordance with the clinical and epidemiological information. A 25-year-old healthy man without any history of disease and with normal liver function who tested negative for the hepatitis virus and HIV infec-

tion was used as the healthy control. The study protocol for collecting blood samples from patients with chronic HCV infection and healthy control was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University.

2.2 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood samples using standard Ficoll density gradient centrifugation. Blood samples were mixed with Hank's balanced salt solution at an equal volume, followed by Ficoll-Hypaque density gradient centrifugation (GE HealthCare, Little Chalfont, Buckinghamshire, UK). After removing the supernatant, the resulting cells were washed twice and re-suspended in a complete cell culture medium [mg/mL streptomycin, and 50 mM 2-mercaptoethanol].

2.3 RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from the serum of patients with HCV or the PBMCs of the healthy control using the RNeasy Midi-Prep Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's manual. DNase I (Roche Diagnostics GmbH, Mannheim, Germany) was applied to remove potential genomic DNA contamination. Reverse transcription-polymerase chain reaction (RT-PCR) (MBI Fermentas, Vilnius, Lithuania) was conducted using the purified total RNA as a template, oligo(dT) as the RT primer, followed by PCR amplification using specific primers as listed in Table 1. The PCR conditions used in the RT-PCR were as follows: 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 1 min, for 30 cycles. Finally, the reaction was maintained at 72 °C for 10 min. The PCR amplified fragment encoding the conserved domain of HCV E2 [encompassing amino acids (aa) 505–702] and the large extracellular loop of human CD81 (hCD81) were used for the construction of their expression plasmids in *Escherichia coli* (*E. coli*).

2.4 Construction and Confirmation of Prokaryotic Expression Plasmids

The Novagen pET Expression System 22b, pET22b (+) vector, was used to construct prokaryotic expression plasmids for the preparation of the conserved domain of HCV E2 glycoprotein [encompassing amino acids (aa) 505–702, GenBank accession number QQ354893] and human CD81 (hCD81), respectively. The HCV E2 fragment encoding the conserved domain (encompassing aa 505–702) was amplified using the primers listed in Table 1 and ligated into the expression plasmid pET22b (+). The recombinant plasmids were purified, and the fragment was subjected to DNA sequencing.

Table 1. Primers used for the polymerase chain reaction amplification of hepatitis C virus E2 and human CD81.

Primer	Polarity	Primer sequence (5'–3')	Product	Position
P1	Forward	TCAGGATCCTCCAGTGTATTGCTTCAC	E2	aa505–702
P2	Reverse	TGCAAGCTTCAGGTATTGCACGTCCA		
P3	Forward	ATAGGATCCGATGGGAGTGGAGGGCTGCAC	hCD81	aa41–751
P4	Reverse	TCGAAGCTTTAGTACACGGAGCTGTCCGGATG		

Note: The forward primers (P1, P3) contain a start codon ATG incorporated into a *Bam*HI site (shown in italics) followed by nucleotides representing the 5' end. The reverse primer (P2) is followed by sequences encoding 6× histidine residues, TAA stop codon, and a *Hind*III site (shown in italics). The reverse primer (P4) is followed by TAA stop codon and a *Hind*III site (shown in italics), without sequences encoding 6× histidine residues. The nucleotide position was presented according to the numbering system for the prototype strain HCV-H (GenBank accession number AY460204) and human CD81 (GenBank accession number NM-004356).

2.5 Expression of Hepatitis C Virus E2 and hCD81 Fusion Proteins in *Escherichia coli* BL21

The constructed plasmids were transformed into *E. coli* BL21 (DE3), BL21 (DE3) pLysS, Rosetta-gami DE3, or Rosetta-gami pLysS cells (Novagen, San Diego, CA, USA) to express the conserved domain of HCV E2 or hCD81. The *E. coli* cells were grown in a lysogeny broth medium to reach an A_{600} of 0.4. The protein expression of the recombinant HCV E2 or hCD81 was induced by propylthio- β -galactoside to a final concentration of 1 mM. The *E. coli* cells were incubated for 4 h at a 15 °C–37 °C temperature.

Upon the completion of the protein expression, the *E. coli* cells were harvested, washed, and then resuspended in BugBuster (Novagen, San Diego, CA, USA). Cell suspensions were sonicated on ice for 4 min (cycles of 10 s on followed by 30 s off), and this step was repeated twice, followed by centrifugation at 10,000 g for 20 min at 4 °C. The resulting supernatant and pellet were collected.

The prokaryotic expression of the recombinant HCV E2 and hCD81 was examined using 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis on a Mini Protean III apparatus (Bio-Rad, Hercules, CA, USA). Protein gels were stained with Coomassie brilliant blue R-250 and destained with 5% acetic acid and 25% methanol.

2.6 Western Blot Analysis

Western blot (WB) analysis was conducted to examine the expression and purification of recombinant HCV E2 and hCD81. Total proteins were separated using 12% SDS-PAGE and electro-transferred onto the nitrocellulose membrane (Millipore, Billerica, MA, USA). The resulting membranes were blocked with 3% non-fat milk (KPL) in a phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T) for 2 h at room temperature (RT, 20 °C). The membranes were then incubated with a histidine (HIS)-tag monoclonal antibody (Novagen, San Diego, CA, USA) (dilution, 1:2000) or HCV E2-specific monoclonal antibody (Biosdesign, Denver, CO, USA) (dilution, 1:100) for 2 h. For the detection of hCD81, the primary monoclonal anti-

body against CD81 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used, and the dilution was 1:1000 in 1% non-fat milk in PBS-T. Subsequently, the membranes were incubated for 1 h with peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Sigma Chemical Co., St. Louis, Mo, USA) (dilution, 1:2000 in 1% non-fat milk in PBS-T) at RT. The bound antibodies were detected using tetramethylbenzidine antibodies for 10 min at RT.

2.7 Purification of Recombinant Hepatitis C Virus E2 by Affinity Chromatography

The HIS-tagged HCV E2 fusion protein was purified by affinity chromatography using the nickel-nitrilotriacetic acid (Ni-NTA) superflow resin (Novagen, San Diego, CA, USA) following the manufacturer's instructions. The *E. coli* cells with the expression of HIS-tagged HCV E2 fusion protein were suspended with a lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 6.8) complemented with 300 ug/mL lysozyme and 90 ug/mL phenylmethylsulfonyl fluoride. To facilitate cell lysis, the suspensions were kept on ice and sonicated for 4 min (cycles of 10 s on followed by 30 s off), and this step was repeated twice. After the completion of sonication, cells were centrifuged at 10,000 g for 20 min at 4 °C. The soluble fractions of cell lysate were collected and loaded onto the Ni-NTA pre-equilibrated column with the lysis buffer, followed by washing with a 5× wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 6.8). The HIS-tagged fusion protein was eluted with an elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 7.8).

2.8 Analysis of the Interaction between Histidine-E2 and Non-Histidine-hCD81

Freshly prepared soluble fractions of *E. coli* cell lysate containing the recombinant conserved domain of HIS-HCV E2 (5 mL) or hCD81 (5 mL) were mixed and incubated at 30 °C for 90 min. The mixture with potential HIS-E2-hCD81 complex was loaded onto the Ni-NTA affinity column pre-equilibrated with a lysis buffer (QIAexpressionist, Qiagen, Hilden, Germany) and was allowed to bind to the Ni-NTA resin for at least 1 h. After washing three times with a 5×

wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 6.8), the bound fusion proteins were eluted with an elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 7.8). The final eluted samples were then separated on SDS-PAGE and further examined with WB analysis using anti-hCD81 and anti-HIS primary antibodies.

2.9 Interferon-Gamma Enzyme-Linked Immunosorbent Spot Assay

The Human IFN- γ ELISPOT Set was purchased from BD PharMingen (San Diego, CA, USA) and used to examine the *ex vivo* cellular immune response to the recombinant HCV E2. The analysis was performed following the manufacturer's instructions but with a modification [20]. PBMCs were prepared from the whole blood samples of patients with CHC or the healthy control. The cells were seeded at a density of 2×10^5 PBMCs per well in a 96-well plate for the testing samples or the negative control, and 2×10^3 PBMCs per well were used for the stimulant phytohemagglutinin as a positive control. For the examination of stimulation, PBMCs were treated with purified HCV E2 protein at 1 μ g/mL. All experiments were carried out in duplicates, and plate images were captured.

2.10 Statistical Analysis

Statistical analysis was conducted using the SPSS software (version 23.0, IBM, Armonk, New York, USA), and differences in mean values were determined using the Student's *t*-test. Furthermore, $p < 0.05$ was considered statistically significant.

3. Results

3.1 Expression of the Soluble Recombinant Conserved Domain of Hepatitis C Virus E2 and hCD81 in *Escherichia coli*

This study generated an expression vector harboring the conserved domain of HCV E2 glycoprotein using the pET22b prokaryotic expression system. A 6 \times HIS-tag was introduced at the C-terminus of the protein to facilitate purification. To achieve a high yield sufficient for further analysis and to obtain the target protein found in soluble form, this study chose to express recombinant HCV E2 in *E. coli* cytoplasm.

To optimize conditions for the target protein expression, this study tested different strains of host cells [BL21 (DE3), BL21(DE3) pLysS, and Rosetta-gami strains] and a wide temperature range (+20, +25, +30, and +37 $^{\circ}$ C) (the data is not shown). By comparison, it was decided to use BL21(DE3) pLysS as host cells to express the target protein at 37 $^{\circ}$ C. As shown in Fig. 1, the conserved domain of HCV E2 glycoprotein was highly expressed in the *E. coli* strain BL21(DE3) pLysS (Fig. 1). The results suggested that the prokaryotic expression system gave the best yield of the soluble recombinant HCV E2 protein (Fig. 1A,B1,C1,D1,E1).

With the optimized prokaryotic expression system, the vector containing the hCD81 coding region was transformed into *E. coli* strain BL21(DE3) pLysS. As shown in Fig. 1B2,C2,D2,E2, the target protein hCD81 was highly expressed in BL21(DE3) pLysS.

3.2 Similarity Analysis of Amino Acid Sequences of the Immunodominant Domain within Hepatitis C Virus E2 Glycoprotein among Different Strains and Serotypes of Hepatitis C Virus

To evaluate the similarity of amino acid sequences of the acquired conserved domain of HCV E2 glycoprotein among the main HCV serotypes and strains across different geographic regions, amino acid sequences (aa 500–700) were aligned with those of 15 representative strains of different HCV subtypes using the DNAssist software (version 2.0, Softonic, Barcelona, Spain). As schematically presented in Fig. 2 (Ref. [21]), the positions of amino acid residues 527, 529, 530, 535, 544, and 551, known as critical sites for binding to hCD81, shared 100% similarity and were identical among all the strains from HCV serotypes as expected.

3.3 Interaction of the Conserved Domain of Hepatitis C Virus E2 Glycoprotein with hCD81

This study assayed the binding of the recombinant conserved domain of HCV E2 glycoprotein to hCD81 as described in Materials and Methods. Freshly prepared HIS-E2 protein was incubated with non-HIS-hCD81, and a complex was expectedly formed. As illustrated in Fig. 3A, SDS-PAGE analysis of the final eluted protein samples showed two dominant bands of a bound complex on the Ni-NTA column. Western blot analysis was further carried out using anti-HIS tag monoclonal antibody to detect HIS-E2 (Fig. 3B) and anti-hCD81 monoclonal antibody (Fig. 3C). The Western blot images indicated that the bound complex resulted from the direct interaction between the conserved domain of HCV E2 glycoprotein and hCD81 (Fig. 3B,C).

3.4 The Conserved Domain of Hepatitis C Virus E2 Glycoprotein Stimulated the Secretion of Interferon Gamma by Peripheral Blood Mononuclear Cells from Chronic Hepatitis C Patients *In Vitro*

Finally, this study evaluated whether the recombinant conserved domain of HCV E2 glycoprotein could induce specific T-cell responses as reflected by the secretion of IFN- γ by PBMCs from CHC patients using the IFN- γ ELISPOT assay. PBMCs were isolated from 10 anti-HCV IgG-positive patients or the healthy control. As shown in Fig. 4A–F, the IFN- γ ELISPOT assays revealed HCV E2-specific IFN- γ production in PBMCs of patients with HCV but not in those of the healthy control, suggesting T-cell immune responses against HCV upon stimulation with the conserved domain of HCV E2 glycoprotein. Furthermore, quantitative analysis was carried out to compare the number of IFN- γ secreting cells (ISC)/ 10^6 PBMCs that

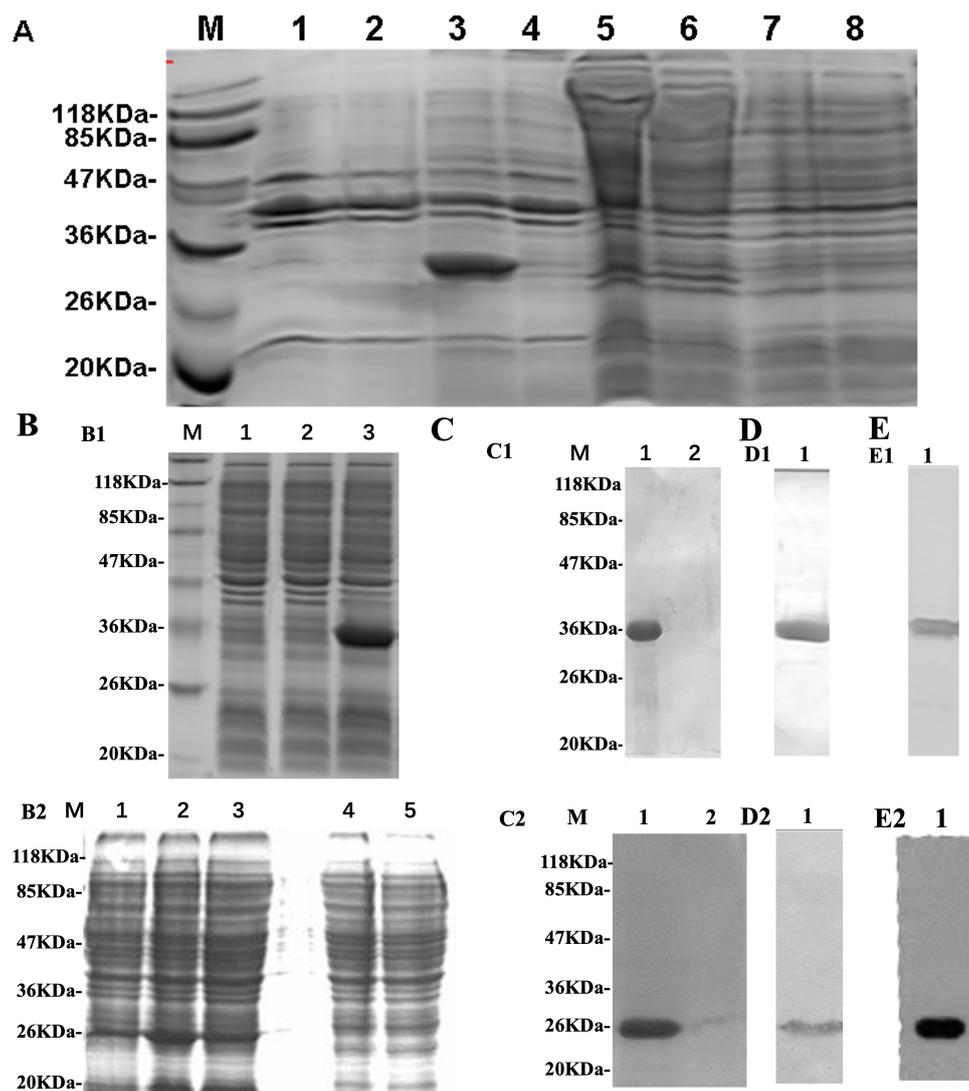


Fig. 1. Analysis of hepatitis C virus E2 and hCD81 protein expression and purification. Prokaryotic expression plasmids were transformed into *Escherichia coli* strain BL21, and hepatitis C virus E2 or hCD81 protein (the large extracellular loop) expression was induced by propylthio- β -galactoside and purified by nickel-nitrilotriacetic acid affinity chromatography. (A) 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis analysis of cell extract from the supernatant (Lanes 1–4) and precipitate samples (Lanes 5–8). Lane M: protein marker; Lane 1: pET-22b (+) empty vector control; Lane 2: phenobarbital without propylthio- β -galactoside induction; Lane 3: phenobarbital with propylthio- β -galactoside induction for 4 h; Lane 4: untreated cells; Lane 5: pET-22b (+) empty vector control; Lane 6: phenobarbital without propylthio- β -galactoside induction; Lane 7: phenobarbital with propylthio- β -galactoside induction for 4 h; Lane 8: untreated cells. (B) 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis analysis of fusion protein. B1, 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis analysis of hepatitis C virus E2 protein. Lane M: protein marker; Lane 1: pET-22b (+) empty vector control; Lane 2: fusion protein E2 expression without induction with propylthio- β -galactoside; Lane 3: induction of fusion protein E2 expression by propylthio- β -galactoside; B2, 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis analysis of hCD81 protein. Lane M: protein marker; Lanes 1–3: induction of fusion protein hCD81 in different concentrations by propylthio- β -galactoside; Lanes 4–5: fusion protein hCD81 expression without propylthio- β -galactoside induction. (C) 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis analysis of fusion protein purification. C1, Hepatitis C virus E2 protein purification. Lane M: protein marker; Lane 1: his-tagged purified E2 protein in the elution fraction; Lane 2: non-his-tagged protein as negative control; C2, hCD81 protein purification. Lane M: protein marker; Lanes 1: his-tagged purified hCD81 protein in the elution fraction; Lane 2: non-his-tagged protein as negative control. (D) Western blot analysis of fusion protein expression. D1, Western blot analysis of hepatitis C virus E2 protein expression. Lane 1: hepatitis C virus E2 protein detected by an anti-histidine monoclonal antibody; D2, Western blot analysis of hCD81 protein expression. Lane 1: hCD81 protein detected by an anti-histidine monoclonal antibody. (E) Western blot analysis of fusion protein expression. E1, Western blot analysis of hepatitis C virus E2 protein expression. Lane 1: hepatitis C virus E2 protein detected by anti-hepatitis C virus E2 monoclonal antibody; E2, Western blot analysis of hCD81 protein expression. Lane 1: hCD81 protein detected by anti-hCD81 monoclonal antibody.

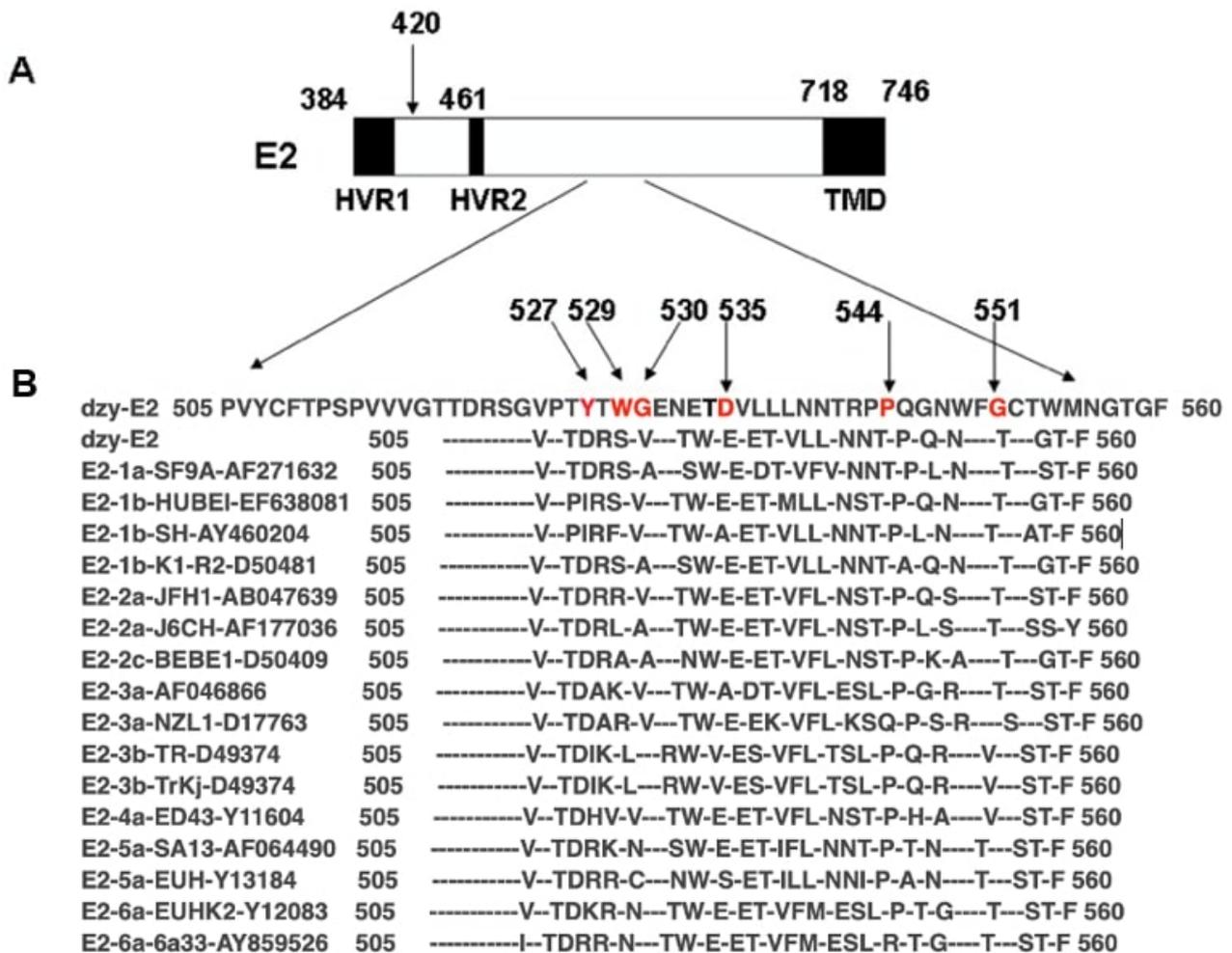


Fig. 2. Alignment of amino acid sequences (aa 505–560) in hepatitis C virus envelope glycoprotein E2 among different hepatitis C virus genotypes. (A) Schematic representation of hepatitis C virus envelope glycoprotein E2. The numbers corresponded to the amino acid positions in the hepatitis C virus polyprotein of the human prototype strain H of the hepatitis C virus as a reference strain (GenBank accession no. AY460204). Hypervariable regions 1 (HVR1) and 2 (HVR2) and the transmembrane domain (TMD) were denoted. (B) Alignment of amino acid sequences (aa 505–560) in dzy-E2 (GenBank accession no. QQ354893) and hepatitis C virus E2 among 15 representative hepatitis C virus strains, including hepatitis C virus genotype 1a (GenBank accession no. AF271632); hepatitis C virus genotype 1b (EF638081, AY460204, D50481); hepatitis C virus genotype 2a (AB047639, AF177036); 2c (D50409); hepatitis C virus genotype 3a (AF046866, D17763); hepatitis C virus genotype 3b (D49374); 4a (Y11604); hepatitis C virus genotype 5a (AF064490, Y13184); and hepatitis C virus genotype 6a (Y12083, AY859526). As indicated by prefixes, the 15 subtypes represented hepatitis C virus isolates of each hepatitis C virus genotype. Amino acids identical to those in the consensus sequence were indicated by a hyphen. The arrows indicated the positions of the amino acid at 420 (A), and at 527, 529, 530, 535, 544, and 551 in the conserved domain (B), which were previously reported to be potentially involved in binding to hCD81 [21].

reflected the HCV E2-induced specific immune response against HCV between the three tested groups (unstimulated control, healthy control; HCV positive control), and the differences were statistically significant ($p < 0.001$) (Fig. 4F). The Pearson correlation coefficient analysis suggested no significant correlation between the HCV E2-induced immune response and HCV RNA or ALT levels in Patient 1 (Fig. 4G).

4. Discussion

Previous studies on the expression of the recombinant HCV E2 glycoprotein in bacteria resulted in an insoluble product at a low yield [15,22–24]. This study constructed a prokaryotic expression vector carrying the conserved domain of HCV E2 glycoprotein, and the target protein was highly expressed in *E. coli* in a soluble form. The major novel findings were summarized as follows: (1) the conserved domain of HCV E2 glycoprotein prepared in the *E. coli* strain BL21 was recognized by an anti-HCV E2 mono-

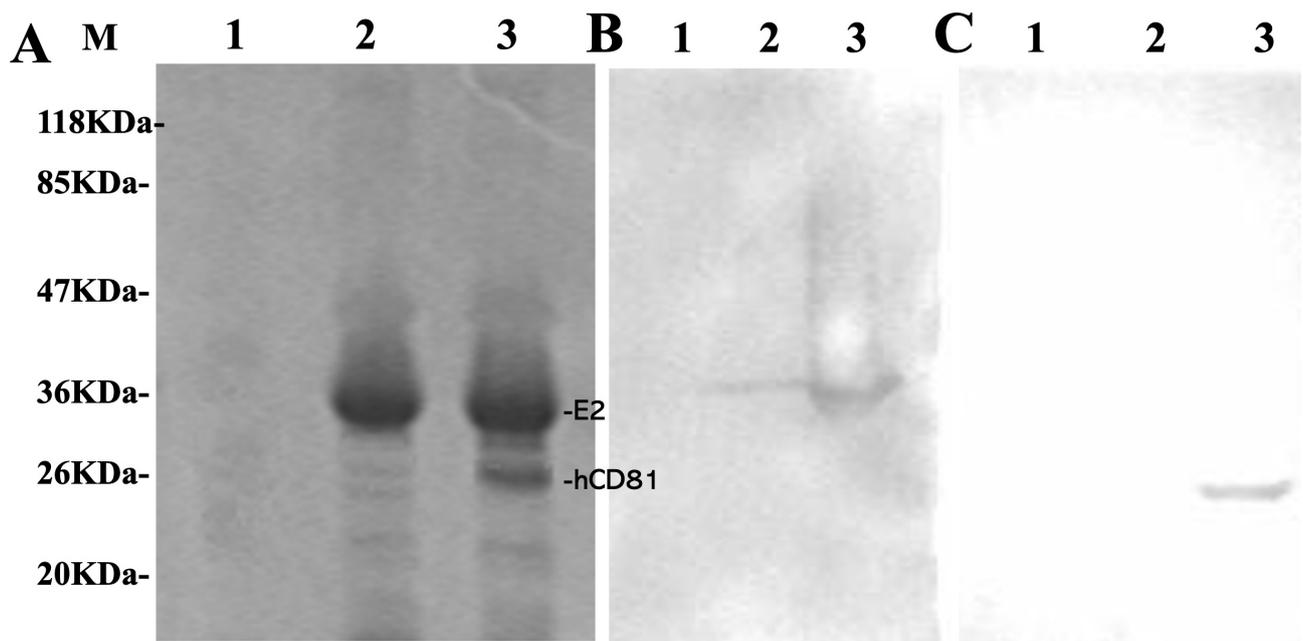


Fig. 3. Analysis of the interaction between histidine-hepatitis C virus E2 and non-histidine-hCD81. Freshly prepared soluble fractions of *Escherichia coli* cell lysate containing histidine-E2, non-histidine-hCD81, or a mixture of both were loaded onto the nickel-nitrilotriacetic acid affinity chromatography column. After unbound proteins were washed thoroughly, bound proteins were eluted and examined by (A) Sodium dodecyl-sulfate polyacrylamide gel electrophoresis and Western blot analysis using (B) an anti-histidine tag antibody or (C) an anti-hCD81 monoclonal antibody. Lane M: protein marker; Lane 1: non-histidine-hCD81; Lane 2: histidine-HCV E2; Lane 3: a mixture of histidine-hepatitis C virus E2 and non-histidine-hCD81.

clonal antibody; (2) the bacterially expressed conserved domain of HCV E2 glycoprotein showed a direct interaction with hCD81 in the binding assay; (3) the recombinant conserved domain of HCV E2 glycoprotein markedly induced IFN- γ production by PBMCs from patients with HCV, suggesting its specific T-cell immune response against HCV. These results suggested the direct binding of the conserved domain of HCV E2 glycoprotein to hCD81, thereby leading to IFN- γ production in the immune response against HCV.

One of the strengths of this study was the intention to investigate the function of cross-serotype conserved domain with HCV E2 glycoprotein. Evaluation of the similarity of amino acid sequences showed that the bacterially expressed conserved domain of HCV E2 glycoprotein contained amino acid residues 527, 529, 530, 535, 544, and 551, which were well documented as important sites for binding to hCD81. The conserved domain shared 100% similarity with that among all the strains from HCV serotypes, which was expected. It was found that the high variable E2 sequence also has several conservative regions that would help maintain the function stabilization of E2 and support vaccine design for effective cross-serotype protection against HCV.

To test whether the bacterially expressed conserved domain of HCV E2 glycoprotein as prepared in this study is functional, a modified method like the “pull-down” assay [25,26] was applied to determine its interaction with

hCD81. The results indicated that the bacterially expressed conserved domain of HCV E2 glycoprotein was functional. This study showed that the conserved domain of HCV E2 glycoprotein directly interacted with the HCV receptor, hCD81, *in vitro*. In the binding assay, it was found that the eluted samples were recognized by HIS-tag McAb or E2 McAb. The antigenic and hCD81-binding properties of the conserved domain of HCV E2 glycoprotein indicated similar functions compared with its native glycosylated counterpart. The results of the binding assay also demonstrated that the non-glycosylated recombinant conserved domain of HCV E2 glycoprotein may retain the functionality of the native glycosylated counterpart. Considering that the soluble conserved domain of HCV E2 glycoprotein can be prepared in adequate amounts, it can be used as a surrogate model of a native counterpart. It was also confirmed that the binding domains of HCV E2 to hCD81 existed in a respective conserved region. These findings may help design hCD81-based mimics that inhibit the binding of HCV-E2 to CD81.

The conserved domain of HCV E2 stimulated the production of IFN- γ using the cell culture system based on PBMCs from patients with HCV [27]. The results indicated that PBMCs from anti-HCV IgG-positive donors and the healthy control were tested with the ELISPOT assay. Given that T-cell-mediated immunity, such as the release of cytokines and protective antibodies, is crucial in the development of vaccines, including the hepatitis C vaccine,

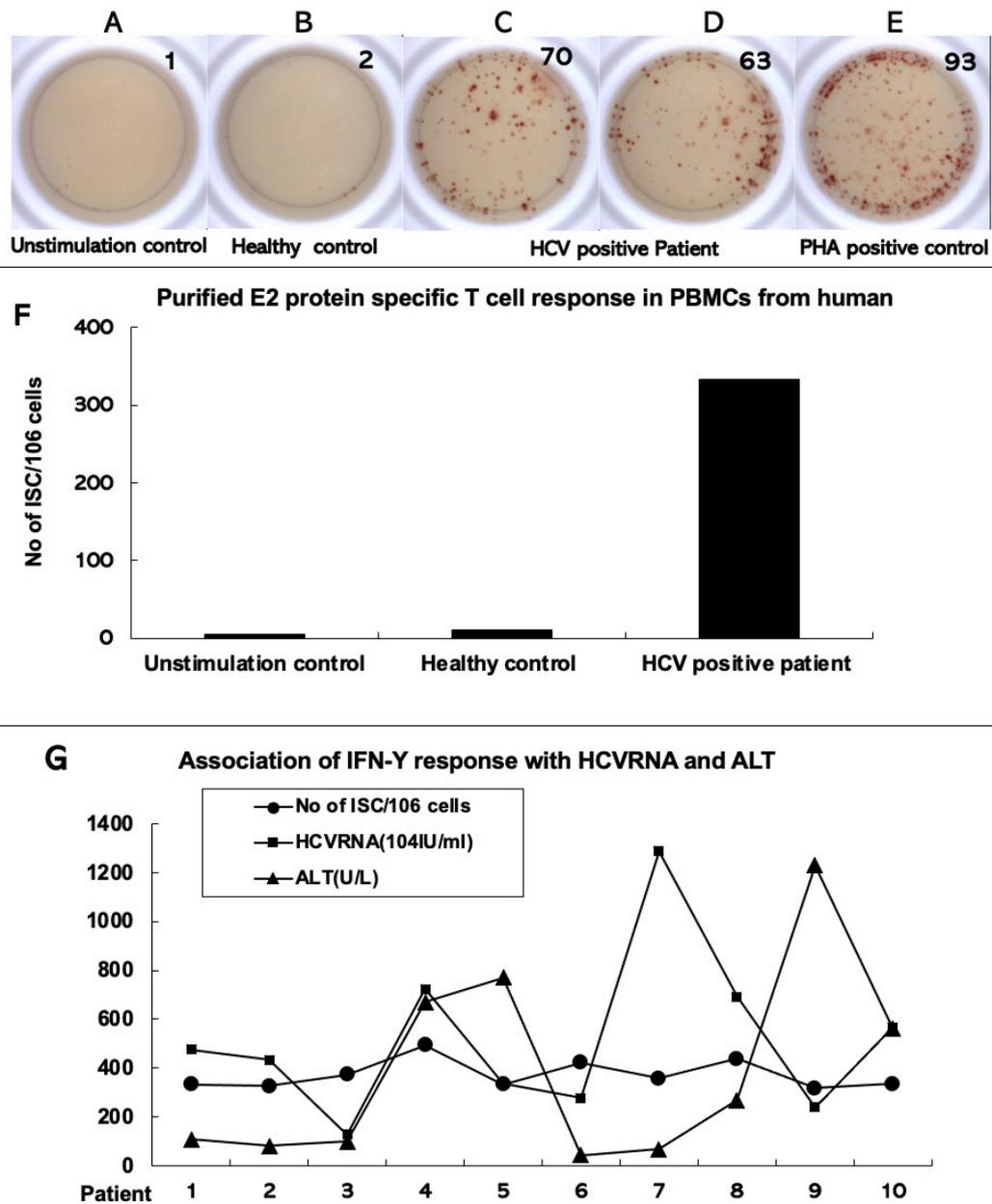


Fig. 4. Interferon gamma enzyme-linked immunosorbent spot assays to test hepatitis C virus E2 protein-stimulated T-cell response in peripheral blood mononuclear cells of patients with chronic hepatitis C. Peripheral blood mononuclear cells were isolated from the whole blood samples of patients with chronic hepatitis C ($n = 10$). They were stimulated *in vitro* with purified hepatitis C virus E2 protein for IFN- γ enzyme-linked immunosorbent spot assay. Peripheral blood mononuclear cells were seeded in a 96-well plate at a density of 2×10^5 per well for the testing samples or the negative control, and 2×10^3 peripheral blood mononuclear cells per well were used for the positive control. All experiments were conducted in duplicate. Plate images were captured and representative images were illustrated. (A) Representative image of enzyme-linked immunosorbent spot wells containing peripheral blood mononuclear cells from patients with chronic hepatitis C treated with a non-specific peptide control. (B) Representative image of enzyme-linked immunosorbent spot wells containing peripheral blood mononuclear cells from the healthy control. (C,D) Representative images of enzyme-linked immunosorbent spot wells containing peripheral blood mononuclear cells from patients with chronic hepatitis C treated with hepatitis C virus E2 as stimulant. (E) Representative image of enzyme-linked immunosorbent spot wells containing peripheral blood mononuclear cells from patients with chronic hepatitis C treated with stimulant phytohemagglutinin as the positive control. (F) Quantitative analysis of images of enzyme-linked immunosorbent spot wells ((A), non-specific peptide as non-stimulant control; (B), healthy control; (C,D), peripheral blood mononuclear cells from patients with chronic hepatitis C treated with hepatitis C virus E2). The differences between the three tested groups were significant ($p < 0.001$). (G) Association of immune response with hepatitis C virus RNA and ALT levels. Hepatitis C virus E2 IFN- γ response for anti-hepatitis C virus-positive donor was reflected by the number of IFN- γ -secreting cells (ISC)/ 10^6 peripheral blood mononuclear cells.

the findings in this study suggested HCV-specific T-cell immune responses. Therefore, this study hypothesized that HCV E2 glycoprotein-containing is highly conserved and that immuno-dominant domains could elicit high immunity against HCV infection. With the promising findings in this PBMCs-based *in vitro* culture system that the recombinant HCV E2 conserved domain elicited T-cell responses and induced the production of IFN- γ against HCV, it is worth performing further studies, including a conformation study using any well-characterized conformational antibody. Moreover, it is suggested to perform an immunization study on an animal model to determine the immunogenicity of the recombinant HCV E2 conserved domain, T-cell response, and specific neutralizing antibody response against HCV.

5. Conclusions

In conclusion, this study has shown that the conserved domain of HCV E2 glycoprotein directly interacts with hCD81, through which it activates IFN- γ production in an immune response to HCV infection. Therefore, the findings of this study may help explain the pathogenicity of HCV. Furthermore, these new findings may mean that the conserved domain of HCV E2 glycoprotein as prepared in the prokaryotic expression system could be a promising new candidate for the development of a vaccine to prevent HCV infection.

Abbreviations

HCV, Hepatitis C virus; HVR1 and HVR2, hyper-variable regions; hCD81, human cell membrane protein CD81; VLP, virus-like particles; IFN- γ , interferon-gamma; ELISA, third-generation enzyme-linked immunosorbent assay; HBsAg, hepatitis B surface antigen; HIV-1 and HIV-2, human immunodeficiency virus 1 and 2; PBMCs, peripheral blood mononuclear cells; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; WB, Western blot; PBS, phosphate-buffered saline; RT, room temperature; Ni-NTA, nickel-nitrilotriacetic acid; CHC, chronic hepatitis C; ISC, IFN- γ secreting cells; HCV-H, human prototype strain H of HCV; TMD, transmembrane domain; ELISPOT, enzyme-linked immunosorbent spot.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

ZYD and GL designed the research study. ZYD and WZ performed the research. XS analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study had been approved by the Ethics Committee of the Sun Yat-sen University. All methods were carried out in accordance with relevant guidelines and regulations (SYSU, 2022017882). All the patients/participants provided their written informed consent to participate in this study.

Acknowledgment

This work was supported by the Research Unit and Vaccine Research Institute of the Third Affiliated Hospital of Sun Yat-sen University, and Gene Therapy Program of the University of Pennsylvania.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Farci P, Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome [Science 1989;244:359-362]. *Journal of Hepatology*. 2002; 36: 582–585.
- [2] Khan AG, Whidby J, Miller MT, Scarborough H, Zatorski AV, Cygan A, *et al.* Structure of the core ectodomain of the hepatitis C virus envelope glycoprotein 2. *Nature*. 2014; 509: 381–384.
- [3] Lindenbach BD, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, *et al.* Complete replication of hepatitis C virus in cell culture. *Science (New York, N.Y.)*. 2005; 309: 623–626.
- [4] Ciczora Y, Callens N, Montpellier C, Bartosch B, Cosset FL, De Beeck AO, *et al.* Contribution of the charged residues of hepatitis C virus glycoprotein E2 transmembrane domain to the functions of the E1E2 heterodimer. *The Journal of General Virology*. 2005; 86: 2793–2798.
- [5] Flint M, Dubuisson J, Maidens C, Harrop R, Guile GR, Borrow P, *et al.* Functional characterization of intracellular and secreted forms of a truncated hepatitis C virus E2 glycoprotein. *Journal of Virology*. 2000; 74: 702–709.
- [6] Boo I, teWierik K, Douam F, Lavillette D, Pombourios P, Drummer HE. Distinct roles in folding, CD81 receptor binding and viral entry for conserved histidine residues of hepatitis C virus glycoprotein E1 and E2. *The Biochemical Journal*. 2012; 443: 85–94.
- [7] Afzal S, Idrees M, Akram M, Awan Z, Khubaib B, Aftab M, *et al.* Mutations in the E2-PePHD region of hepatitis C virus genotype-3a and correlation with response to interferon and ribavirin combination therapy in Pakistani patients. *Virology Journal*. 2010; 7: 377.
- [8] Keck ZY, Saha A, Xia J, Wang Y, Lau P, Krey T, *et al.* Mapping a region of hepatitis C virus E2 that is responsible for escape from neutralizing antibodies and a core CD81-binding region that does not tolerate neutralization escape mutations. *Journal of Virology*. 2011; 85: 10451–10463.
- [9] Drummer HE, Boo I, Maerz AL, Pombourios P. A conserved Gly436-Trp-Leu-Ala-Gly-Leu-Phe-Tyr motif in hepatitis C virus glycoprotein E2 is a determinant of CD81 binding and viral entry. *Journal of Virology*. 2006; 80: 7844–7853.
- [10] Nattermann J, Zimmermann H, Iwan A, von Lilienfeld-Toal M, Leifeld L, Nischalke HD, *et al.* Hepatitis C virus E2 and CD81

interaction may be associated with altered trafficking of dendritic cells in chronic hepatitis C. *Hepatology* (Baltimore, Md.). 2006; 44: 945–954.

- [11] Ding Q, von Schaewen M, Hrebikova G, Heller B, Sandmann L, Plaas M, *et al.* Mice Expressing Minimally Humanized CD81 and Occludin Genes Support Hepatitis C Virus Uptake *In Vivo*. *Journal of Virology*. 2017; 91: e01799–16.
- [12] Cowton VM, Angus AGN, Cole SJ, Markopoulou CK, Owsianka A, Dunlop JI, *et al.* Role of Conserved E2 Residue W420 in Receptor Binding and Hepatitis C Virus Infection. *Journal of Virology*. 2016; 90: 7456–7468.
- [13] Owsianka AM, Timms JM, Tarr AW, Brown RJP, Hickling TP, Szejek A, *et al.* Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. *Journal of Virology*. 2006; 80: 8695–8704.
- [14] Farquhar MJ, Hu K, Harris HJ, Davis C, Brimacombe CL, Fletcher SJ, *et al.* Hepatitis C virus induces CD81 and claudin-1 endocytosis. *Journal of Virology*. 2012; 86: 4305–4316.
- [15] Yurkova MS, Patel AH, Fedorov AN. Characterisation of bacterially expressed structural protein E2 of hepatitis C virus. *Protein Expression and Purification*. 2004; 37: 119–125.
- [16] Li P, Wan Q, Feng Y, Liu M, Wu J, Chen X, *et al.* Engineering of N-glycosylation of hepatitis C virus envelope protein E2 enhances T cell responses for DNA immunization. *Vaccine*. 2007; 25: 1544–1551.
- [17] Zucchelli S, Capone S, Fattori E, Folgori A, Di Marco A, Casimiro D, *et al.* Enhancing B- and T-cell immune response to a hepatitis C virus E2 DNA vaccine by intramuscular electrical gene transfer. *Journal of Virology*. 2000; 74: 11598–11607.
- [18] Ward S, Lauer G, Isba R, Walker B, Klenerman P. Cellular immune responses against hepatitis C virus: the evidence base 2002. *Clinical and Experimental Immunology*. 2002; 128: 195–203.
- [19] Youn JW, Park SH, Cho JH, Sung YC. Optimal induction of T-cell responses against hepatitis C virus E2 by antigen engineering in DNA immunization. *Journal of Virology*. 2003; 77: 11596–11602.
- [20] Wang L, Figueredo J, Calcedo R, Lin J, Wilson JM. Cross-presentation of adeno-associated virus serotype 2 capsids activates cytotoxic T cells but does not render hepatocytes effective cytolytic targets. *Human Gene Therapy*. 2007; 18: 185–194.
- [21] Patel AH, Wood J, Penin F, Dubuisson J, McKeating JA. Construction and characterization of chimeric hepatitis C virus E2 glycoproteins: analysis of regions critical for glycoprotein aggregation and CD81 binding. *The Journal of General Virology*. 2000; 81: 2873–2883.
- [22] Xiang ZH, Cai WJ, Zhao P, Kong LB, Ye LB, Wu ZH. Purification and application of bacterially expressed chimeric protein E1E2 of hepatitis C virus. *Protein Expression and Purification*. 2006; 49: 95–101.
- [23] Li SW, Zhang J, Li YM, Ou SH, Huang GY, He ZQ, *et al.* A bacterially expressed particulate hepatitis E vaccine: antigenicity, immunogenicity and protectivity on primates. *Vaccine*. 2005; 23: 2893–2901.
- [24] Liu J, Kong Y, Zhu L, Wang Y, Li G. High-level expression of the C-terminal hydrophobic region of HCV E2 protein ectodomain in *E. coli*. *Virus Genes*. 2002; 25: 5–13.
- [25] Jia S, Peng J, Gao B, Chen Z, Zhou Y, Fu Q, *et al.* Relative quantification of protein-protein interactions using a dual luciferase reporter pull-down assay system. *PLoS ONE*. 2011; 6: e26414.
- [26] Rodina E, Vorobieva N, Kurilova S, Mikulovich J, Vainonen J, Aro EM, *et al.* Identification of new protein complexes of *Escherichia coli* inorganic pyrophosphatase using pull-down assay. *Biochimie*. 2011; 93: 1576–1583.
- [27] Tapia-Calle G, Born PA, Koutsoumpli G, Gonzalez-Rodriguez MI, Hinrichs WLJ, Huckriede ALW. A PBMC-Based System to Assess Human T Cell Responses to Influenza Vaccine Candidates *In Vitro*. *Vaccines*. 2019; 7: 181.