CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Clin Exp Vaccine Res 2023;12:47-59
https://doi.org/10.7774/cevr.2023.12.1.47
pISSN 2287-3651 • eISSN 2287-366X

Individual expression and processing of hepatitis C virus E1/E2 epitopes-based DNA vaccine candidate in healthy humans' peripheral blood mononuclear cells

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Received: March 25, 2022
Revised: May 26, 2022
Accepted: December 23, 2022

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No potential conflict of interest relevant to this article was reported.

We acknowledge the National Research Center (NRC) of Egypt for providing the facilities used to conduct the work and all needed in-kind support to perform the study. This work was financially supported by an in-house grant (number: 1010102) from the National Research Center and a GE-SEED grant (number: 24627) from the Science, Technology, and Innovation Funding authority both granted to Prof. Mahmoud Bahgat, as well as the PhD thesis grant for MSc Rola Nadeem.

Purpose: The development and study of hepatitis C virus (HCV) vaccine candidates' individualized responses are of great importance. Here we report on an HCV DNA vaccine candidate based on selected envelope (E1/E2) epitopes. Besides, we assessed its expression and processing in human peripheral blood mononuclear cells (PBMCs) and *in vivo* cellular response in mice.

Materials and Methods: HCV E1/E2 DNA construct (EC) was designed. The antigen expression of EC was assayed in PBMCs of five HCV-uninfected donors via a real-time quantitative polymerase chain reaction. Serum samples from 20 HCV antibody-positive patients were used to detect each individual PBMCs expressed antigens via enzyme-linked immunosorbent assay. Two groups, five Swiss albino mice each, were immunized with the EC or a control construct. The absolute count of lymph nodes' $CD4^+$ and $CD8^+$ T-lymphocytes was assessed.

Results: Donors' PBMCs showed different levels of EC expression, ranging between 0.83–2.61-fold in four donors, while donor-3 showed 34.53-fold expression. The antigens expressed in PBMCs were significantly reactive to the 20 HCV antibody repertoire (all $p=0.0001$). All showed comparable reactivity except for donor-3 showing the lowest reactivity level. The absolute count % of the $CD4^+$ T-cell significantly increased in four of the five EC-immunized mice compared to the control group ($p=0.03$). No significant difference in $CD8^+$ T-cells % was observed ($p=0.89$).

Conclusion: The inter-individual variation in antigen expression and processing dominance was evident, showing independence in individuals' antigen expression and reactivity levels to antibodies. The described vaccine candidate might result in a promising natural immune response with a possibility of $CD4^+$ T-cell early priming.

Keywords: Hepatitis C virus, DNA vaccines, Human peripheral blood mononuclear cells, Individuality, Envelope protein

Introduction

Hepatitis C virus (HCV) infection is a universal health threat. Prophylactic or therapeutic vaccine development is considered a milestone in the scientific community. The importance of vaccine development was emphasized despite the success of re-

cently developed direct-acting antivirals. This was due to the reported associated risks [1], viral drug resistance [2], and challenges of reinfection or relapses, as well as the related cost burden, especially in developing countries [3].

The positive single-stranded genome of HCV encodes for structural and non-structural proteins [4]. Among the structural proteins are the envelope E1 and E2, both are considered the main targets in HCV vaccine development approaches. E1 and E2 proteins represent key determinants for receptor binding, viral entry, and assembly. The E2 protein is known to be with the highest genetic diversity resulting in the host exhausting escape mutants, also harboring sequences that might interfere with the host immune response [4].

HCV vaccine design can mimic the immune response from reported events of spontaneous clearance occurring in 25%–30% of HCV patients in the first 6–12 months of infection [5]. The clearance was reported to be associated with early stimulation of humoral response and robust long-term cellular response of both $CD4^+$ and $CD8^+$ T-lymphocytes [5]. This was further supported by the reports that infection chronicity was associated with specific $CD4^+$ dysfunction followed by $CD8^+$ T-cells exhaustion [6]. Specific long-term $CD4^+$ T-cells, in particular, play an essential role in infection resolution [7]. It was reported to modulate the activation and production of B-lymphocytes, which was evident to correlate to the levels of neutralizing antibodies (nAb) in HIV co-infected patients, an infection known to destroy $CD4^+$ T-cells [8]. Also, $CD4^+$ T-cells modulate and prime $CD8^+$ T-cells, which play an important role in the recognition and damage of the infected cells [9]. In our study, the vaccine design and assessment target both, the possibility of the production of natural occurring nAbs as well as early cellular immune responses.

Recently, DNA vaccines showed a potential advantage over other traditional approaches. It is known to be stable, non-infectious, and easy to produce and formulate. Besides being cost-effective in terms of production and non-cold-chain transportation and distribution [10]. The best-fitting model for DNA vaccine *ex vivo* studies is known to be the peripheral blood mononuclear cells (PBMCs) [10]. PBMCs including the antigen-presenting cells that engulf DNA constructs are responsible for the humoral and cellular response efficiency of the DNA vaccination [11]. The cells also prime productive T-cell responses that are reported to be of the highest importance in anti-HCV immunity [12]. These reasons encourage interest in the study of an HCV DNA vaccine approach based on E1 and E2 proteins in the human PBMCs population.

E1 and E2 conserved and immunogenic sequences are required for efficient broad nAb (bnAb) production [13]. Past trials of HCV DNA vaccine approaches were based on the full, truncated, or selected E1 and E2 immunogenic regions. Despite showing promising results yet viral clearance and protection against infection relapses were not fully achieved. It was also reported that the vaccines based on the proteins' full length showed low immune responses, this was claimed to occur due to the inclusion of unfavorable regions that should be avoided. Among such regions are the hypervariable region 1 (HVR1) and the protein kinase receptor (PKR)-eIF2 alpha phosphorylation homology domain (PePHD).

The neutralizing anti-HVR1 antibodies were reported to act as a decoy for important receptor-binding nAbs, which leads to increased infectious events *in vivo* [14]. PePHD was found to inactivate the PKR, a viral replication suppressor innate mechanism [15]. A DNA vaccine approach, including PePHD region, resulted in increased immunogenicity and hinder the progression to chronicity yet did not stimulate sterilizing immunity [16]. Another approach, which included both HVR1 and PePHD regions, improved liver histology yet showed no major effects on viral clearance [17]. While another study compared vaccine candidates, including both HVR1 and PePHD regions, in the presence and absence of an adjuvant. The DNA vaccines resulted in a low immune response that was slightly induced in presence of the adjuvant [18]. Despite that each approach had its limitations, we do believe that one of the possible reasons was the lack of protein modification before vaccine construction. Thus, the selection of the immunogenic epitopes should consider the respective modifications and deletions to the virus proteins.

Inter-individual variability results in differences in the DNA uptake, expression, and antigen-processing patterns of introduced genes as well. This might be due to genetic or epigenetic variability, promotor activity, messenger RNA stability, or transcriptional factors [19]. Recent approaches tend to consider individual responses and interactions rather than studying the population mean. Personalized response studies aim at the evaluation of the vaccine fitness level among the population that can lead to the improvement tailored to the unfit groups accordingly [20].

In our study, we targeted the design of a DNA vaccine candidate based on selected immunogenic epitopes in the HCV E1 and E2 proteins. We also studied the differential expression and processing of the antigenic epitopes in PBMCs iso-

 https://doi.org/10.7774/cevr.2023.12.1.47

lated from HCV uninfected human donors. On the other hand, the designed vaccine candidate was tested for its capacity to stimulate early *in vivo* $CD4^+$ and $CD8^+$ T-lymphocytes in Swiss albino mice.

Materials and Methods

Design of the E1/E2 DNA vaccine candidate and *in vitro* confirmatory tests

The HCV E1 and E2 epitopes were selected according to previous reports. The epitopes were HCV-E1 313:327 domain and the E2-stretch 412: 538 that includes the 3 domains of 412:423, 436:446, and 523:538; numbering was according to the ED43 genotype 4a reference sequence (GenBank accession number: CAA72338.1). The sequence used in the construct was a consensus from 10 envelope protein sequences (accession numbers and steps are detailed in section A of Supplement 1). The separated equimolar expression of both E1 and E2 transcripts was maintained using the T2A cis-acting hydrolase element sequence (GSGEGRGSLLTCGDVEENPGP). The element was inserted at the 3` and 5`- ends of E1 and E2 sequences, respectively. The B-cell epitope prediction was done to test the recognition of the domains as separated antigens in case of T2A hydrolysis failure. The complete E1/T2A/E2 open-reading frame (ORF) sequence was submitted to the BepiPred-2.0: Sequential B-Cell Epitope Predictor Immune Epitope Database tool.

The E1/T2A/E2 sequence was synthesized and inserted in a mammalian expression construct (OriGene PS100026; OriGene, Rockville, MD, USA) under the control of a cytomegalovirus promotor (synthesized via Blue Heron Biotech LLC, Bothell, WA, USA) (envelope epitopes construct abbreviated as EC) while an empty construct (without the insert) was used as a control (control construct abbreviated as the CC). The design of the construct elements is detailed in Fig. 1. The plasmid was amplified and purified and its construction was confirmed via polymerase chain reaction (PCR) and sequencing (section B of Supplement 1).

in vitro experiments: Constructs transfection optimization and transcript amplification in 293T cells

The transfection and expression analysis of both EC and CC were optimized in the 293T mammalian cell line. Cells were seeded at a density of 1×10^6 in RPMI-1640 medium (Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% of fetal bovine serum (Lonza, Basel, Switzerland) and 1% antibiotic/antimycotic mixture (Lonza). After 24 hours the cells were transfected with the constructs using Xfect (Takara Bio, Kusatsu, Japan) nano-biopolymers complex with 7.5 μg DNA/well according to the manufacturer protocol. Transfected cells were examined for fluorescent green fluorescent protein (GFP)-reporter gene expression and harvested at 3-time points (24, 48, 72 hours post-transfection [hpt]).

RNA was extracted from the harvested cells using the RNeasy kit (QIAGEN, Düsseldorf, Germany). RNA extraction protocol included a DNase digestion step to get rid of the re-

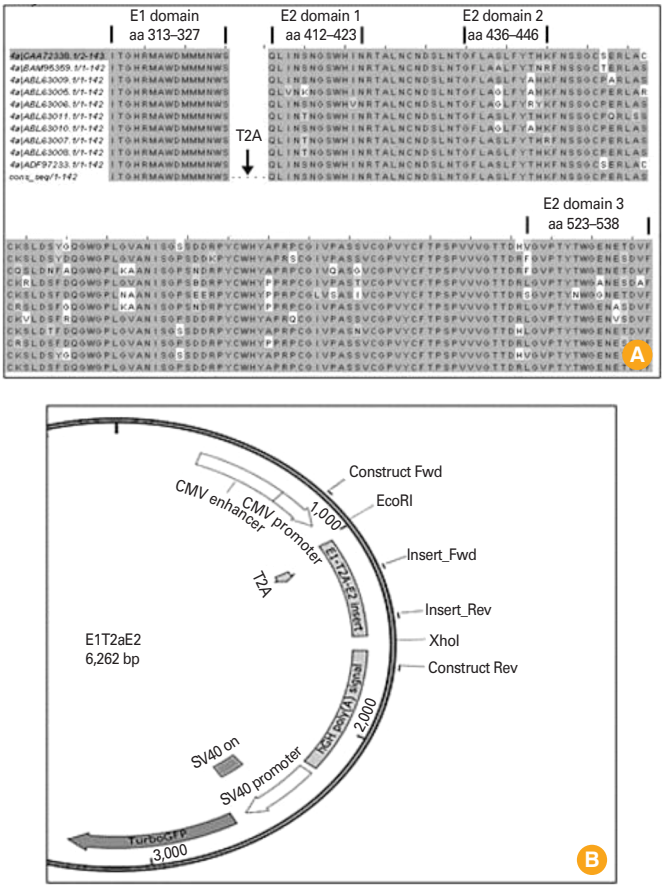

Fig. 1. Illustrative view for the epitopes multiple sequence alignment (MSA) of the selected hepatitis C virus (HCV) envelope and the mammalian expression construct design. (A) Amino acid (aa) MSA of HCV envelope protein sequences used for the consensus sequence development (consensus sequence is the last row). The gap between E1 and E2 is the position of the T2A hydrolysis element. (B) E1/T2A/E2 DNA mammalian expression construct map developed by SnapGene software (GSL Biotech LLC, Boston, MA, USA). E1/T2a/E2 insert is under the control of a cytomegalovirus promotor, downstream to it the unfused reporter gene of Turbo green fluorescent protein under control of an SV40 promoter. The position of the primers used for both the insert detection and correct orientation confirmation (insert forward and reverse) and the primers used in the insert sequencing analysis (construct forward and reverse) are labeled.

maining constructs' DNA before reverse transcription. And 250 ng of clean RNA from each treatment was reverse transcribed using an Omniscript RT kit (QIAGEN) and the expressed transcript was detected by PCR using *Taq* DNA polymerase (QIAGEN) and the insert specific primer set (forward: 5'-ACTGGGTTCTTGGCTAGCTT-3' and reverse: 5'-AGGGC TGGGAGTGAAACAAT-3').

Ex-vivo experiments: constructs expression in HCV-uninfected human PBMCs

Five female blood donors (designated hereafter as D1 through D5) of 29–42 years age range (mean age=35.5 years) were selected as HCV uninfected individuals. This was confirmed by both HCV-antibody rapid test (Acon Laboratories, San Diego, CA, USA) and target PCR. Blood was collected at midday in the same time range from the five donors. The collected blood was divided into two portions, the first was collected on lithium heparin for PBMCs separation and the other portion was clotted for autologous serum separation. PBMCs were separated using the Ficoll gradient separation protocol (Lymphoprep; ProteoGenix, Schiltigheim, France). Each donor's cells were seeded in duplicates for each treatment at a density of 1×10^6 in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% respective autologous serum and 1% antibiotic/antimycotic mixture (Lonza). After 24 hours the cells were transfected with the constructs using Xfect (Takara Bio) nano-biopolymers complex with 7.5 μg DNA/well according to the manufacturer protocol.

Cells were harvested 72 hpt, the harvest was divided into two portions. the first was subjected to RNA extraction (as described in the former *in vitro* PCR analysis methods section) for gene expression evaluation via quantitative PCR (qPCR). While the second portion was lysed for antigen detection via direct enzyme-linked immunosorbent assay (ELISA) assay (lysis buffer: 50 mM Tris base, 150 mM NaCl, 1% NP-40, 1 mM PMSF: pH=8).

Gene expression evaluation via semi-quantitative qPCR

Equal complementary DNA (cDNA) concentration of each treatment was subjected individually to a real-time semi-quantitative PCR reaction targeting both GAPDH reference gene (forward: 5'-GAGTCAACGGATTTGGTCGT-3' and reverse: 5'-TTGATTTTGGAGGGATCTCG-3') [21] and insert transcript (mentioned in the *in vitro* PCR confirmation method). Reactions were done in duplicates using Quantitect SYBR green master mix (QIAGEN). The cycle quantitation threshold (Ct) of both genes was recorded and the mean Ct for duplicates was calculated. The efficiency (E) of our reference gene amplification PCR method was typically 90%. It was calculated using the $E=10^{-1/m}-1$ equation, where m is the slope of the standard curve.

Detection of the expressed E1/E2 antigens via direct ELISA

Direct ELISA assay was used to evaluate the reactivity of each individual donor's expressed antigens. Sera collected from 20 HCV antibody-positive patients were used as the individual HCV antibody repertoire, while the five donors' sera were as negative HCV antibody control. Each donor's treatment with either EC (intervention treatment) or CC (baseline treatment) was tested individually against each individual serum (i.e., five individual donors' reactions against 25 individual serum samples). An equal concentration of 1 μg/mL from each individual treatment total protein was used to coat the ELISA plates. The detailed assay steps are described in section C of Supplement 1. Data points were statistically analyzed as detailed later and net values of intervention (subtracting each baseline read from its respective intervention read) were plotted.

In-vivo experiment: cell-mediated immune response analysis in immunized mice

Two groups of five female Swiss albino mice each, randomly selected as healthy mice at the age of 8 weeks, were immunized with either EC or CC prepared in phosphate buffer saline. Mice were immunized simultaneously with 20 μg of DNA/injection, 4 doses one week apart in a combined delivery route. Prime immunization was intraepidermal in the ear area and the 3 booster doses were as an intra-tibial intramuscular injection. Mice have been sacrificed 1-week post-last immunization. Mesenteric lymph nodes were collected from each mouse individually. Cells extracted from lymph nodes of each individual mouse were stained using FITC-conjugated anti-mouse $CD4^+$ and $CD8^+$ antibodies (BioLegend, San Diego, CA, USA). The absolute count of each T-lymphocyte type per 1,000 total cell count was recorded.

Statistical analysis

All statistical analyses were done using the calculators available on the Socscistatistics website (https://www.socscistatistics.com/). The recorded optical density reads of the ELISA assay followed two statistical test types that apply for a single-case study, to preserve the individuality of the data points

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during the analysis. The data were grouped as follows: (1) the intervention data (Ag+ coat: Ab+ serum): represents the reaction of each donor's EC transfected cell lysate with each of the 20 HCV-antibody positive serum (n=20 per donor). (2) Baseline data (Ag- coat: Ab+ serum): represents the reaction of each donor's CC transfected cell lysate with each of the 20 HCV-antibody positive serum (n=20 per donor). (3) Negative control data (Ag+ or Ag- coat: Ab- serum): represents the reaction of donor's cell lysate transfected with either EC (n=5) or CC (n=5) with each of the five HCV-antibody negative serum (total n=10 per donor).

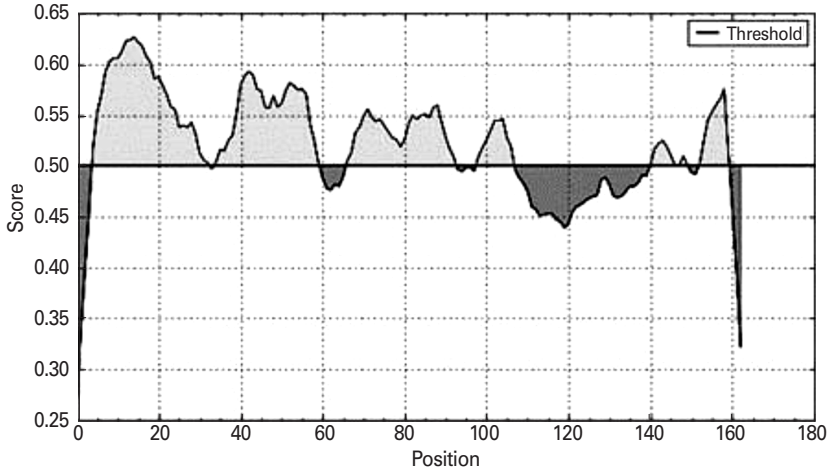
To compare the intervention and baseline/negative groups for each donor, the non-parametric independent Mann-Whitney U test was used. The test parameters were two-tailed $\alpha=0.05$, number of intervention data points=20, number of baselines and negative data points=30, $U_{critical}=200$. All cases where $U \leq U_{critical\ no.}$, p-value ≤ 0.05 reflect significant dominance of intervention over baseline/control group.

The non-parametric dependent Wilcoxon signed ranks test was applied to compare the five donors' reactivity to the 20 HCV antibody repertoires. The test parameters were two-tailed $\alpha=0.05$, the number of intervention data points per donor=20, critical value=52. In this test, we applied an interpretational twist, where we rather accept than reject the null hypothesis, which will refer to that there was no significant difference between the two groups of data and that both donors reacted the same. In all cases where $|W| \geq W_{critical\ value.}$, p-value ≥ 0.05 reflects the significant similarity in reactivity of the two compared donors.

The *in vivo* experiment statistical analysis of the two groups' mean cell counts comparison was calculated using the unpaired two-sample Student t-test (p-value ≤ 0.05). A sample size of 5 for each group was accepted, this is in reference to a previous study showing that for n=5 the type I error rate reaches a nominal level of 5% [22].

Ethics approval

The study protocol was approved by Medical Research Ethics Committee at the National Research Center (registration number: 16-159). The Animals were both purchased from,



No.	Start	End	Peptide	Length
1	5	33	E1/cleavage peptide	29
2	35	60	E2 domain 1 stretch	26
3	67	93	E2 domain 2 stretch	27
4	99	107	Inter-domain stretch	9
5	141	147	Inter-domain stretch	7
6	149	150	E2 domain 3 residues	2
7	153	160	E2 domain 3 stretch	8

Fig. 2. B-cell binding domains prediction of the whole hepatitis C virus (HCV) envelope open-reading frame (ORF) using the BepiPred-2.0 (DTU Health Tech, Lyngby, Denmark): sequential B-cell epitope predictor Immune Epitope Database tool. The x-axes depict the residue positions in the sequence of the unseparated ORF, while the y-axes depict for each residue correspondent score. The table includes the detected B-cell antigenic determinants (numbering refers to positions in the insert sequence).

and maintained at, the animal facility of the National Research Centre (NRC) in Egypt. Animals were fed on a standard diet and maintained at an ambient temperature according to the animal welfare protocols of the NRC animal house. Anesthesia procedures complied with the guidelines of the National Institutes of Health in the USA. Before injection, the animal was anesthetized using diethyl ether vapor discontinuous sniffs till numbness to reduce awareness of pain. Animals were checked for normal vital signs (breath, movement, feeding) and leg movement for 30 minutes post-injection procedure. Before dissection and sacrifice, the animals were anesthetized to complete sedation using xylazine (10 mg/kg body weight). All animals and tissues were placed in labeled biohazard bags and handed to the waste management unit at the NRC.

Results

The four E1/E2 peptides recognized as separate B-cell binding antigenic determinants

The unseparated E1/T2A/E2 ORF prediction supports the detection of the four domains as a separated B-cell domain antigenic determinant even in the minor cases of hydrolysis failure (Fig. 2).

In vitro experiments: constructs transfection and expression in 293T cells

The cultured 293T cell line transfected with the EC showed a gradual increase in the GFP-reporter intensity through the 24, 48, and 72 hpt treatments (Fig. 3B–D, respectively). Expression was further supported by the gradual increase in the

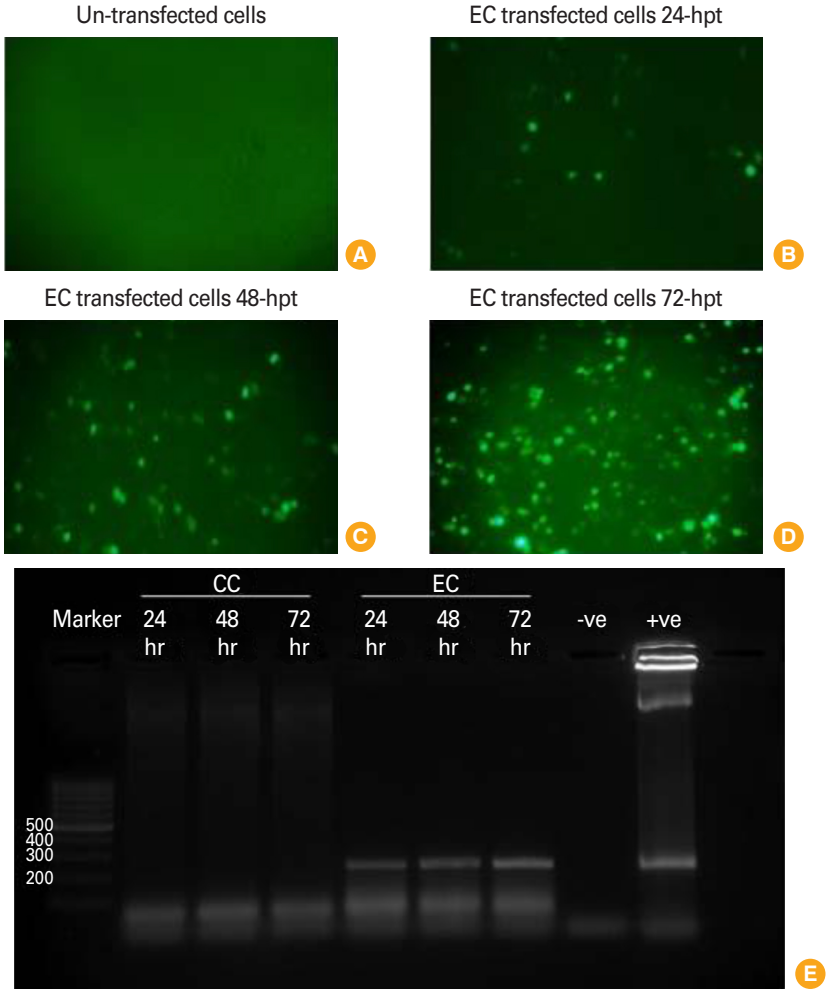


Fig. 3. Constructs expression optimization in 293T cell line. (A) Un-transfected 293T control cells. (B–D) Showing a gradual increase in the expression of the green fluorescent protein-reporter gene through image (A). (E) While image shows the amplification of the 240 bp E1/E2-insert transcript from 293T cells post-transfection with both EC and CC. There is gradual amplification intensity in EC reactions 24-, 48-, and 72-hpt in comparison to clean reactions of the CC reactions. EC, E1/E2 construct; CC, control construct; htp, hours post-transfection.

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intensity of the specific 240 bp amplification product, compared to the clean CC transfection reactions (Fig. 3E).

Ex-vivo experiments: individual transcript expression levels by semi-qPCR

The CC treatment resulted in no Ct values; it was not applicable to use the 2^-ddCt equation to calculate the relative fold change in gene expression referenced to a control value. This was expected as the EC construct expresses an exogenous peptide that has no normal expression level in naïve HCV uninfected PBMCs control or CC expression. The normalized expression level of each donor's reaction was calculated as an

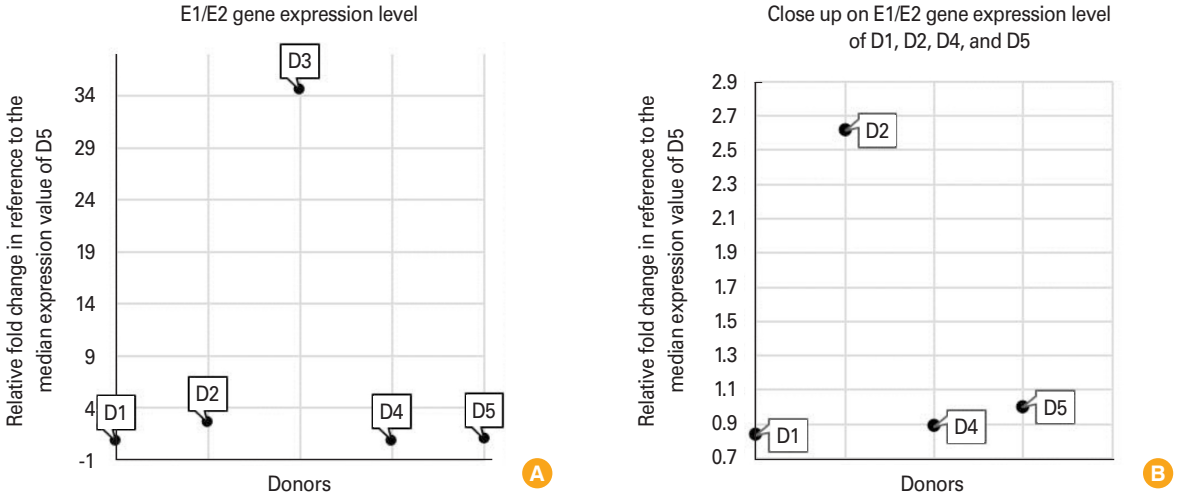
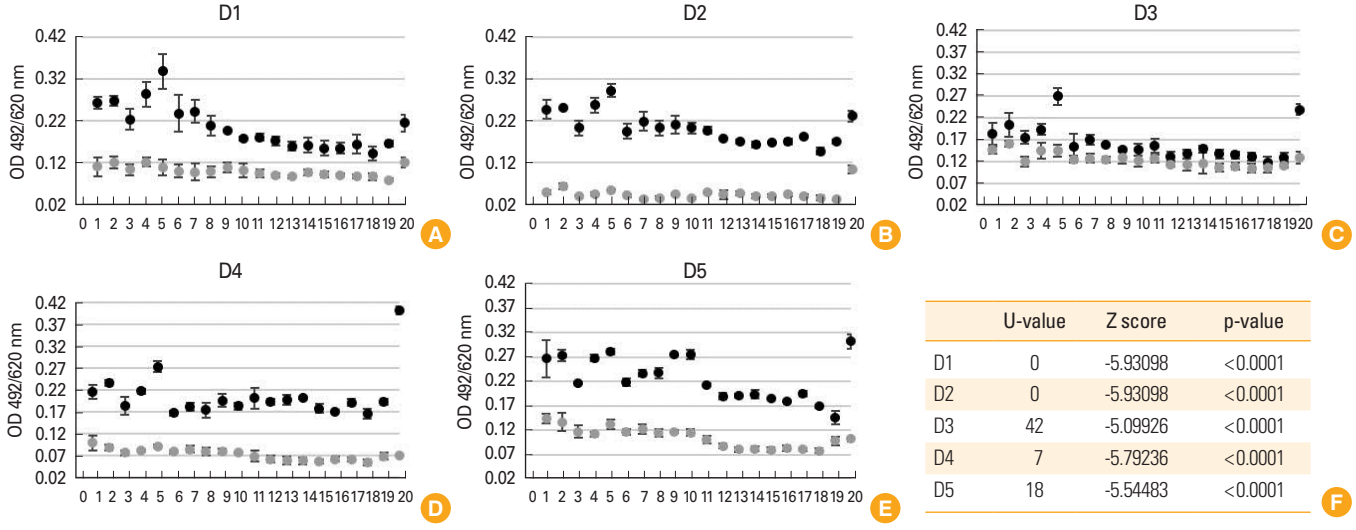


Fig. 4. Differential semi-quantitative expression level in individual blood donors. E1/E2 expression of the EC transfected peripheral blood mononuclear cells of each donor normalized to GAPDH reference gene 72 hpt. (A) The relative fold change of the normalized absolute gene expression (calculated by 2^-dCt equation) level of each donor's reaction was related to the median gene expression of D5. The five donors showed different comparative fold changes in gene expression, while D3 showed a 34.5 relative folds increase. (B) Close-up on values of D1, D2, D4, and D5 showing the relatively close relative fold change to D5 except for D2 that showed a slightly higher value. This chart excludes D3 for a better visual comparison, showing differential expression between the four donors. EC, E1/E2 construct; htp, hours post-transfection; D1 to D5, donor 1 to donor 5.



	U-value	Z score	p-value
D1	0	-5.93098	<0.0001
D2	0	-5.93098	<0.0001
D3	42	-5.09926	<0.0001
D4	7	-5.79236	<0.0001
D5	18	-5.54483	<0.0001

F

Fig. 5. Reactivity of each individual's E1/E2 expressed antigens to hepatitis C virus positive antibody repertoire compared to the cell lysate transfected with the control construct. (A–E) Shows reactivity of the antibody repertoire of the 20 infected patient's sera (along x-axis) with the antigens in the lysate of transfected cells with EC (black dots) or CC (grey dots) represented as optical density [OD]±standard deviation. (F) Mann-Whitney U value of each donor showing a statically significant intervention: baseline dominance in aal donors except with high U value of D3 that represents less overall dominance. EC, E1/E2 construct; CC, control construct; D1 to D5, donor 1 to donor 5.

absolute value using the 2^-dCt equation, then in order to calculate a relative fold change the individual samples' values were compared to the sample of median value (donor number D5). This will refer to the D5 expression level of the value=1, referencing other donor's values to it.

D1 and D4 resulted in 0.83 and 0.88 relative fold values, respectively (Fig. 4B). D2 resulted in a 2.61 relative fold increase (Fig. 4B) while D3 showed an extreme 34.53 relative fold increase (Fig. 4A).

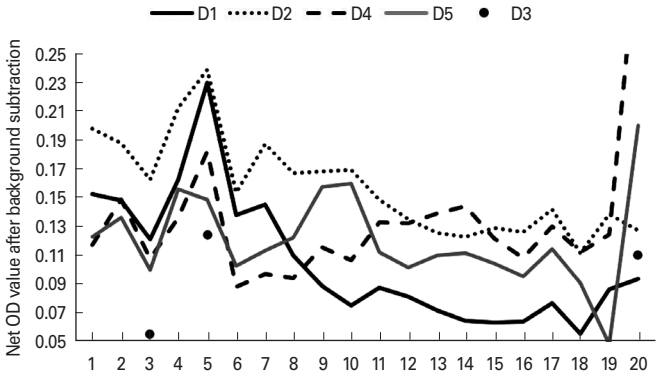


Fig. 6. Net values of donor's EC antigen reactivity to hepatitis C virus (HCV) antibody repertoires subtracting the background of donor's CC reaction value. The y-axis represents the net optical density (OD) value of each donor reaction to each HCV patient's sera (numbered along x-axis). The graph is showing that D3 has the lowest reactivity except in 3 out of the 20 (number 3, 5, and 20) of the antibody repertoires. This graph can be considered as the visual representation of the Wilcoxon test numerical values (shown in Table 1). EC, E1/E2 construct; CC, control construct; D1 to D5, donor 1 to donor 5.

Individual HCV antigen detection by the HCV antibody repertoires

The outcomes of the Mann-Whitney test resulted in a U value less than the $U_{critical}$ of 200 (all $p<0.0001$) for the five donors (Fig. 5A–E). This reflected a significant difference in the EC antigen interaction with the HCV antibody repertoires than that of the baseline/negative control. By comparing the donors' respective U values (Fig. 5F), D1, D2, and D4 showed a close overall interaction level with a U value of 0, 0, and 7, respectively. D5 showed lower yet comparable dominance levels with U values of 18. D3 showed the lowest dominance of intervention over the baseline/negative treatments with a U value of 42. The U value of D3 is far from the critical value of 200 yet higher than that of the other donors' values. Despite that, the significance of the D3 U value, plotting the net values showed that three out of the 20 reactions (of patients 3, 5, and 20) resulted in a comparable reactivity to other donors even though the other seventeen reactions were not considered as reactive (Fig. 6).

Comparison between donors' EC antigens' reactivity to the individual HCV antibody repertoires

The outcomes of the Wilcoxon signed ranks test reflected the comparison between every two donors regarding their respective matched pair of interaction with the same HCV antibody repertoire. As illustrated in Table 1, the significance of the matched pairs showed a resemblance in the EC antigens reactivity of D1, D2, and D4 (bold values in Table 1). Where the W values of D1-D2=92, D1-D4=89, and D2-D4=89

Table 1. Wilcoxon (W) matched-pair signed-rank test results of the five donors (D1:D5)

	D1	D2	D3	D4	D5
D1		**W=92** **Z-value: -0.4853** **p-value: 0.62414**	W=5 Z-value: -3.7333 p-value: 0.0002	**W=89** **Z-value: -0.5973** **p-value: 0.5485**	W=44 Z-value: -2.2773 p-value: 0.0226
D2			W=1 Z-value: -3.8826 p-value: 0.0001	**W=89** **Z-value: -0.5973** **p-value: 0.5485**	W=17 Z-value: -3.2853 p-value: 0.001
D3				W=0 Z-value: -3.9199 p-value: 0.00008	W=0 Z-value: -3.9199 p-value: 0.00008
D4					W=45 Z-value: -2.24 p-value: 0.0251
D5					

It shows the comparison in the donor's expressed antigens' reactivity to the hepatitis C virus patient's sera antibody repertoire. The critical value for $W_{n=20}$ ($p<0.05$, two-tailed) is 52. Bold values of insignificant p-values indicate the resemblance between each two compared donors.

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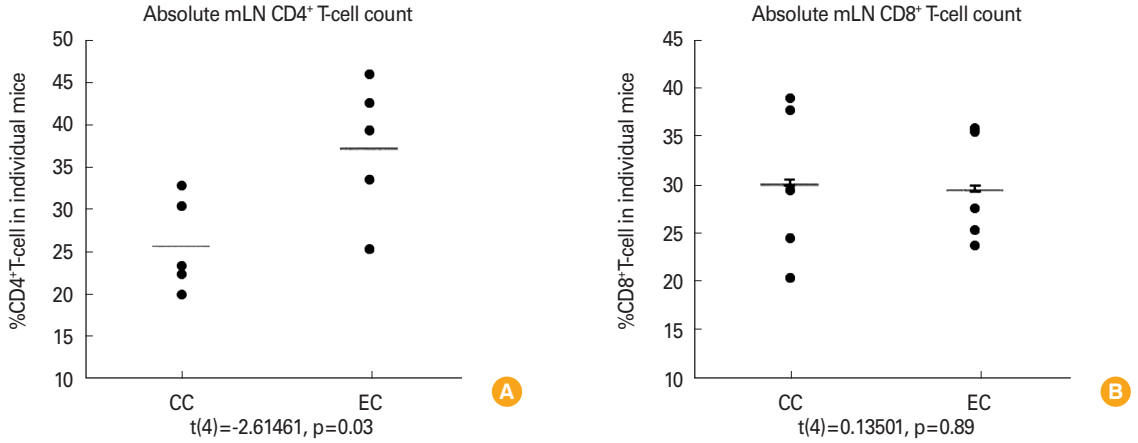

Fig. 7. Absolute CD4+ and CD8+ T-cell count per 1,000 cells in the mesenteric lymph node of immunized mice groups with EC and CC. Data is represented as individual values (black dots) with group mean horizontal line value±standard deviation. (A) Data shows a significant increase in CD4+ T-cell count in the mice group immunized with the EC group compared to the CC group. (B) Data shows no significant increase in CD8+ T-cell count in the mice group immunized with the EC group compared to the CC group. EC, E1/E2 construct; CC, control construct.

(p=0.62, 0.54, and 0.54, respectively) were higher than the $W_{critical}$ of 52. D5 showed a less resemblance to D1, D2, and D4, with W values less than the $W_{critical}$ of 52. Where their comparison resulted in W values of D5-D1=44, D5-D2=17, and D5-D4=45 (p=0.022, 0.001, and 0.025, respectively). D3 showed the least resemblance to the other four donors' interaction to the HCV antibody repertoire, where W values ranged 0–5 (all p<0.0002).

In vivo experiments: the absolute count of CD4+ and CD8+ T-lymphocytes of the immunized mice's mesenteric lymph nodes' cell population

The mean of the absolute cell count (n=5) of CD4+ T-lymphocytes in the mesenteric lymph nodes' cells of the EC immunized mice were significantly higher than that of the CC immunized group (t[4]=-2.61, p=0.03) (Fig. 7A). On the other hand, the CD8+ T-lymphocytes mean of the absolute cell count (n=5) comparison of the two groups showed an insignificant change (t[4]=0.135, p=0.89) (Fig. 7B).

Discussion

With the aim of developing an HCV DNA vaccine candidate that targets both humoral and cellular immune responses, we designed and assessed an E1- and E2-selected epitopes-based DNA construct according to previously reported epitopes immunogenicity. The selected E1 epitope (313–327) is one of the conserved bnAb that was found to be prevalent in infected human sera [23] and recognized by various monoclonal antibodies (mAbs). It also showed efficient results in the HCV pseudo-particle neutralization that was efficient in HCV infection prevention [23]. The selected E2 stretch included three different epitopes. The first E2 epitope (412–423) was reported to induce strong bnAbs [24] that are resistant to immune escape [25]. While the second E2 epitope (436–446) generates both specific and synergistic bnAbs that stimulate the E1 epitope (selected herein) neutralization activity [15]. Lastly, the third E2 epitope (523–538) was recognized by several bnAbs known to be resistant to the escape mutants [26]. The regions between the three epitopes also include conserved residues for the CD81 receptor binding to HCV envelope protein [27].

The EC transcript expression level in the five individual donors' PBMCs was evaluated using the semi-quantitative PCR assay. All five donors' cells' expression level was compared to the median value (D5) of the five data points. While the D5 expression level was considered the ground value of 1-fold, D1 and D4 expression were slightly different yet comparably alike to D5. D2 showed a slightly higher expression of 2.61-fold increase while D3 expression reached an extreme of 34.5-fold increase than the D5 median value. All experimental factors that might affect the reliability of the results were unified among the five donors' tests. There were several reports about the variability of gene expression among individuals that were evident in the results of our study. While three out of the five donors (60%) showed low variability, the other two donors were completely unrelated. This might be due to a difference in DNA uptake or the transcription regulation [28]. DNA uptake was reported to be variable among individuals that might lead to differences in transcriptional levels

and potency of the overall induced response [29].

The antigenicity of the EC expressed epitopes were assessed through their reactivity to individual antibody repertoires from 20 HCV patients' sera. All patients were at a chronic active state of the infection, thus expected to include a diverse antibody repertoire covering possible antigenic representation. The observed difference in reactivity can be interpreted on two levels. First, is the diversity shown by the 20 repertoire's reaction to the same processed epitopes by each individual donor's PBMCs (20 antibody repertoires versus a single donor's processed antigens). The variable level of reactivity was evident and can be compared using the Mann-Whitney U analysis values. D1 and D2 showed the highest overall comparable reactivity to the repertoires while D4 and D5 were slightly lower. Contrary to the expectations, D3 reactivity was the lowest and nearly similar to the control background. Fig. 5 shows an obvious individual difference in the antibody detection levels of the same donor's processed epitopes that might reflect antibody diversity and/or titer.

Second, is the diversity in the processing of the same construct antigen by each individual donor (comparable donors' antigen detection versus a single antibody repertoire). This was observed by tracking the different reactivity of the five-donor's antigens to the same individual antibody repertoire (Fig. 5). We performed a matched pair Wilcoxon signed ranked test (W) statistical analysis to compare the individual reactivity of the donors against each individual HCV repertoire and not in an overall pattern. Once again, the calculated W value shown in Table 1 was evident not only in the variability between the five donors but also in the near-ground level reaction of D3. We can rank the five donors' reactivity into three different levels: the highest to include the comparable D1, D2, and D4, the moderate to include D5, and the lowest to include D3. All such conclusions are only based on considering the uptake, expression, and processing of the vaccine candidate and do not represent the possible immune response.

The individual difference in both, the antigen processing and the antigenic dominance in antibody repertoires can be understood under the immune dominance theories. It is known that upon DNA uptake, the construct is imported to the nucleus for transcription. Gene transcripts are then exported to the cytosol and directed to the endoplasmic reticulum for translation. Translated peptides then undergo post-translational modifications and folding. In antigen-presenting cells, the major histocompatibility complex (MHC) II molecules aided by specific molecules bind different stretches of the antigens in an MHC II groove [30]. This binding, upon mediated dissociation, if it showed dissociation resistance will be captured as an MHC II molecule optimum antigenic determinant. Captured determinants are then proteolyzed by cellular proteases keeping the dominant optimum epitope bound to MHC II molecule ready to be presented for T-cell activation [31].

Some epitopes are capable of optimum binding to MHC II over others, gaining dominance regardless of their relevance to infection clearance. This is obvious in the diversity of HCV chronic patients' repertoire yet failed to clear the infection. The other subdominant epitopes, known as cryptic epitopes, are less represented but can elicit the T-cell activation in absence of the dominant epitopes [32]. It was reported that epitope immune dominance is not absolute, where an epitope might exchange its dominance. This exchange is controlled by some factors, including the simultaneous presence of other epitopes, such epitopes might be of a different pathogenic source but with a similar or a higher fitness competing to bind to MHC II molecules [31]. Another factor can be the availability of the epitope to bind at its optimum to the MHC II groove, being dominant only upon modifications [33]. Those factors, and others, result in the different presentation of the same pathogenic antigen according to the overall state that controls either its dominant or its cryptic presentation. This understanding can give an explanation for the difference among donors in processing the same modified E1/E2 antigen and the difference among patients in processing the pathogenic full-length envelope proteins. Antigen dominance and abundance might be affected by the donor's or the patients' MHC II optimum binding during exposure and there is a possibility for an exchange in processing dominance in the same individual of the five donors in a different condition [31].

Among the five donors, D3 showed a questionable activity, where the extreme transcript level was expected to reflect the antigenic abundance which was not the case. D3 showed the least comparable reactivity to antibody repertoires that reflected a deficiency in antigen expression or proper processing (Fig. 5). But, checking the comparable net reactivity values of D3 of patients 5 and 20 showed a reactivity comparable to the other donors (Fig. 6). HCV antibody repertoires of patients 5 and 20 showed the highest reactivity among all five donors, reflecting their diversity and/or high titer level in developed antibodies. Despite that the low overall reactivity of

 https://doi.org/10.7774/cevr.2023.12.1.47

D3 might reflect an impaired antigen processing/presentation that is reactive to the natural repertoires, its reactivity to patients 5 and 20 does not support that notion. This might direct our interpretations to the possibility that D3 showed a different immune dominance or processing of cryptic epitopes that are not presented in the other antibody repertoires than 5 and 20. This raises the question of whether this pattern would change if D3 reacted to HCV's spontaneously cleared antibody repertoire that was reported to include different antigenic dominance than that of chronic cases.

On the other side, D3 low reactivity might also reflect a deficiency that can be explained by a number of assumptions. First, is the possible formation of short defective ribosomal products that resulted in a pre-mature unfolded protein [34]. Such protein might not be subjected to proper post-translational modifications interfering with the detectability and the specific antibody recognition. While its reactivity to patients 5 and 20 might reflect high titer to antibodies raised against linear epitopes that are not affected by misfolding or modifications. Second, the messenger RNA (mRNA) instability or the production of mRNA transcription inhibitors might result in an impaired expression [35]. This can lead to mRNA accumulation or destruction before reaching the endoplasmic reticulum affecting peptide presentation levels [36]. In general, the activity of the donors including D3 might be further improved under the effect of boosting elements through the co-expression of stimulatory molecules, cytokines, or adjuvants [37].

The immunization procedure in mice was designed in order to assess the very early cellular responses *in vivo*. Mice were injected with low consistent doses of the constructs and scarified early enough to assess the primary T-cell response only. The groups that received EC showed an early significant increase in $CD4^+$ T-lymphocytes over the CC control group, while there was no significant change in $CD8^+$ T-cells (Fig. 7). The primed $CD4^+$ T-lymphocyte has been considered a promising and most desirable response from HCV vaccine development approaches. The $CD4^+$ T-cell potential stimulation *in vivo* is a crucial factor associated with acute infection resolution [5]. The stimulation of $CD4^+$ over $CD8^+$ T-cells was previously reported in E1/E2 antigenic peptides expression vaccine development approach tested in both mammalian cell lines and animal models [38]. Although the primed T-lymphocytes response should be assessed for HCV-specificity, the response was promising to further study the response in both mice models and human PBMCs stimulation in antigen naïve as well as previously infected patients.

However, some limitations should be noted. First, the sample size of five (either donors or mice) should be increased to decrease the possibility of bias. Second, testing the *in vivo* stimulated antibodies in a neutralization assay against HCV pseudo-particles would have assessed the pan genotype nAb production that supports the vaccine efficiency. Another limitation was the lack of previous studies on the individual mRNA to protein level and DNA vaccine regulations lead to gaps in the interpretations.

In conclusion, the study opens up a promising HCV DNA vaccine candidate based on envelope 1 and 2 conserved epitopes that might result in a natural humoral immune response developed by natural infection. The described vaccine candidate can elicit an early $CD4^+$ T-cell response *in vivo*, which is the desired response to mimic the spontaneous clearance events that will prime $CD8^+$ T-cell development. The study stresses that the difference in the human individual immune response to vaccines might be related to individual differences in antigen processing and presentation. We also postulate that the individual antigen expression and presentation might be independent of the antigenic reactivity levels. This emphasizes the importance of studying the factors that would support vaccine fitness and increased efficiency before modifying the vaccine design. This would aid in the selection of proper boosting elements and the fitness of a broader population group.

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Supplementary Materials

Supplementary materials are available at Clinical and Experimental Vaccine Research website (http://www.ecevr.org).

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