

# Enhanced Immune Responses of a Hepatitis C Virus Core DNA Vaccine by Co-Inoculating Interleukin-12 Expressing Vector in Mice

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## ABSTRACT

**Introduction:** Hepatitis C virus (HCV) infection is a worldwide problem without an effective vaccine with more than 170 million chronically infected people worldwide. DNA vaccines expressing antigenic portions of the virus with adjuvants have recently been developed as a novel vaccination technology. In the present study, a DNA vaccine expressing HCV core protein was developed with IL12 as a genetic adjuvant and different aspects of cell immune responses due to this vaccine were evaluated in an animal model of the infection. **Material and Methods:** HCV core gene was inserted into pcDNA3.1(-) eukaryotic expression vector and the recombinant plasmid was transformed into DH5 $\alpha$  competent cells and a large scale DNA vaccine was prepared. The expression of the vector was verified in CHO cell line. Female C57BL/6 mice were immunized 3 times with 90 ng doses of the DNA vaccine on days 0, 14 and 28. Two weeks after the last immunization, the immune responses against HCV core antigen were assessed by lymphocyte proliferation, CTL cytotoxicity and cytokines secretion assays. **Results:** The results showed that the co-administration of IL12, as a genetic adjuvant, increased the ability of HCV core DNA vaccine to enhance cytolytic T lymphocyte activity, lymphocyte proliferation and shifting of the immune response toward a T helper type 1 pattern and altogether improved the protective immunity. **Conclusion:** This study demonstrated that intramuscular injection of HCV core DNA vaccine with a genetic adjuvant induced significant cellular immune responses in C57BL/6 mice.

**KEYWORDS:** HCV core DNA vaccine, Interleukin-12, Cellular immunity, C57BL/6 mice

## INTRODUCTION

Although DNA vaccines have the potential to be a safe and effective alternative to conventional vaccine modalities [1], increasing their immunogenicity still remains the most important challenge [2]. A DNA vaccine induces robust T-cell responses, leading to development of T-cell-dependent antibody formation [3]. Cytokine adjuvants have been co-delivered with DNA vaccines as a mean of increasing their immunogenicity [4]. Interleukin-12 (IL12) is one of many cytokines which have been shown to significantly modulate the inflammatory processes [5]. Preliminary studies have suggested that IL12 may induce suitable immune responses when co-administered with immunogenic DNA [5]. HCV infection is a major public health problem and approximately

3% of the world population are infected with HCV [6, 7]. Among those, approximately 50-85% are afflicted with chronic hepatitis which in the long term may lead to liver cirrhosis and hepatocellular carcinoma [8]. The development of an effective vaccine against HCV infection faces a variety of obstacles [9]. For instance, the HCV genome has a high mutagenicity rate and due to the heterogeneous nature of the genome [10], it would be very essential to choose a conserved antigen candidate. Among different HCV genes, the core antigen exhibits the most conserved viral antigen (about 95% homology at the amino acid level has been recognized between the different viral genotypes/subtypes, so far identified) [11, 12]. Therefore, designing an HCV vaccine based on the core could be considered as a suitable antigenic candidate for vaccine development against HCV infection. The core antigen also contains some well-known T-cell and B-cell epitopes [9-14]. Here we assessed an HCV core DNA vaccine in a C57BL/6 mouse model of the infection. Furthermore, to optimize and improve the immunogenicity of this DNA

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vaccine candidate, the use of IL12 as an adjuvant was also evaluated.

## MATERIALS and METHODS

### Plasmid construction and amplification

pcDNA3.1-HCVcore plasmid containing an expression cassette of HCV core gene under human cytomegalovirus immediate-early promoter control was constructed. HCV core gene was amplified using sera of HCV infected patients (genotype 1) as described previously [15]. Purified PCR products were cloned into pTZ57R/T Vector (Fermentas, Germany) which were then confirmed by sequencing. The digested gene was subcloned into pcDNA3.1(-) plasmid. All plasmids were transferred into *Escherichia coli* DH5 $\alpha$  strain using a standard heat-shock method and colonies were allowed to grow at 37°C on Luria Bertani (LB) agar supplemented with 50  $\mu$ g/ml ampicillin. The selected colonies were extracted using Qiagen plasmid extraction kit (Qiagen, Hiden, Germany) according to the manufacturer's instructions. Plasmid pcDNA3.1-IL12 was kindly provided by Dr. T. Sakai (University of Tokushima, Japan).

### Transfection, and expression analysis

Twenty hours before transfection, Chinese Hamster Ovary (CHO) cells were cultured in RPMI 1640 (Gibco BRL, Paisley, UK), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM L-glutamine (Sigma, Germany) at 70% confluence. The sub confluent CHO eukaryotic cells were transfected with the recombinant plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 hours, the cell monolayers were washed three times with Phosphate Buffered Saline (PBS) and scraped into 1 ml of PBS. The cells were then recovered by centrifugation at 12,000  $\times$  g for 15 min and stored at -20°C. The proteins in the lysate were quantitated. The samples were then loaded onto a 10% polyacrylamide/SDS gel (PAGE) and the proteins were isolated by electrophoresis and transferred onto a PVDF membrane. After fixation with 95% alcohol, washing with PBS-Tween (PBST), and blocking with PBST containing 5% dried skim milk, the membranes were incubated with 1:500 dilution of monoclonal mouse anti-HCV core antigen antibody (Abcam Cambridge, UK) at the room temperature for 2 h. Thereafter, the membranes were extensively washed with PBST and incubated with goat anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma, St. Louis, Mo., USA) in secondary antibody solution at room temperature for one hour. Color was developed by incubating the membrane in alkaline phosphate buffer containing tetramethylbenzidine substrate solution.

### Immunization of mice

All experiments were carried out on female 6-8 weeks-old C57BL/6 mice, purchased from Pasteur Institute of Iran. Mice were housed for one week before the experiments, given free access to food and water and were maintained in a light/dark cycle. All experiments were carried out in accordance with the animal care and use protocol of Golestan University of Medical Sciences of Iran. The mice were divided into five groups, each containing 7 mice. To test the HCV core DNA vaccine and to increase the efficiency of the vaccine, the quadriceps muscles were

injected with 90  $\mu$ g of each plasmid. Mice were injected according to the regimen described below:

Group 1: pcDNA3.1 plasmid without gene (negative control)

Group 2: pcDNA3.1 -IL12 plasmid

Group 3: pcDNA3.1 plasmid without gene + pcDNA3.1-IL12 Plasmid

Group 4: pcDNA3.1-HCV core plasmid

Group 5: pcDNA3.1-HCV core plasmid + pcDNA3.1-IL12 plasmid

Mice were immunized three times on days 0, 14 and 28. The mice were sacrificed a week following the third immunization.

### Splenocytes proliferation index

One week after the third immunization, a single-cell suspension of mononuclear cells obtained from the immunized mice was used for the lymphocyte proliferation assay. The suspension of isolated spleen cells was treated with lysis buffer (0.15 M NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA, pH 7.2) in order to eliminate the red blood cells. The splenocytes at a concentration of  $2 \times 10^5$  cells/well were cultured in 96-well flat-bottom culture plates (NalgeNunc International, Denmark) in the presence of 1  $\mu$ g/ml HCV core antigen, 5  $\mu$ g/ml phytohemagglutinin (Sigma Chemical Co., Munich, Germany) or media. The preparations were cultured in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 1% HEPES, 0.1% 2-mercaptoethanol and 0.1% penicillin/streptomycin. After 48 h of incubation, 10  $\mu$ g/ml of MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; Sigma chemicals) were added to each well and incubated for four hours at 37°C in 5% CO<sub>2</sub>. Dimethyl Sulfoxide (DMSO) solution; 100  $\mu$ l was also added to dissolve formed formazan crystals. The plates were then read with a microtiter reader (Eppendorf, Germany) at 540 nm and the results were expressed as a stimulation index (SI) [15]. The SI was determined as follows: optical density (OD) values of stimulated cells (Cs) minus relative cell numbers of unstimulated cells (Cu) divided by relative OD values of unstimulated cells:  $SI = (Cs - Cu)/Cu$ .

All tests were performed in triplicate for each mouse.

### In vitro cytotoxic activity of splenocytes

The cytolytic activity of the splenocytes was determined by lactate dehydrogenase (LDH) release assay. One week after the last immunization, a single-cell suspension of the splenocytes was prepared and used as effector cells. A precise number of  $4 \times 10^4$  EL4 cells (EL4 was established in tissue culture from a lymphoma induced in a C57BL/6 mouse by 9, 10-dimethyl-1, 2-benzanthracene) in a volume of 100  $\mu$ l were incubated with effector cells (100  $\mu$ l) at different effect/target ratios. For preparation of the target cells, EL4 cells were stimulated with 1  $\mu$ g/ml HCV core antigen. Released LDH due to the cell lysis was measured by LDH release assaying kit (Takara, Japan) according to the manufacturer's instruction. For low and high control wells (spontaneous releasing and maximum releasing, respectively) instead of effector cell suspension, 100  $\mu$ l of assay medium or 2% Triton X-100 in assay medium were added. All experiments were done in triplicates and percentage of specific cytotoxicity was determined by the following formula [16].

Cytotoxicity (%) = [(Test sample - Low sample) / (High sample - Low sample)]  $\times$  100

## The cytokine assays

One week after the last immunization, spleens of each mouse were removed and homogenized in RPMI 1640 medium, supplemented with 10% FCS and the above-mentioned antibiotics. The red blood cells were osmotically lysed using ammonium chloride buffer (NH<sub>4</sub>Cl 0.16 M, Tris 0.17 M). Cells were washed twice with RPMI 1640 medium supplemented with 10% FCS and were counted while their viability was determined by trypan blue exclusion (0.4% w/v). A total of  $1 \times 10^6$  spleen cells were added to each well of a 24-well plate. Three wells were considered for each mouse. The cells were re-stimulated in vitro with 1  $\mu$ g/ml HCV core antigen. Plates were incubated at 37°C in 5% CO<sub>2</sub>. The supernatants were removed 48 h after the stimulation and were kept at -70°C for evaluation of the secreted IFNG and interleukin-4 (IL4) levels. The concentrations of IFNG and IL4 in the supernatants were estimated using a commercial ELISA kit (R & D systems, USA).

## Statistical analyses

The results are depicted as the mean  $\pm$  SD of triplicate determinations. Statistical analyses were performed using ANOVA. A value of  $p < 0.05$  was considered to be statistically significant. All statistical analyses were accomplished using SPSS 18 software (SPSS Inc., Chicago, IL, USA).

## RESULTS

### Construction of HCV-core DNA vaccine

To evaluate the expression of HCV core gene in the transfected CHO cells, Western blot analysis using monoclonal mouse anti-HCV core antibody was used. Non-transfected CHO cell lysate was used as a negative control. The transient expression of pcDNA3.1-HCV core was verified using Western blot analysis. The CHO lysate showed a single band at approximately 21 kDa for the core in the Western blot (Fig. 1A). The purity and identity of the DNA vaccines following a large scale preparation of the plasmids were confirmed by electrophoresis on 1% agarose gel (Fig. 1B).

### LDH cytolytic activity

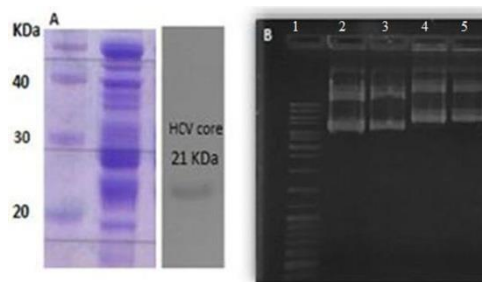
As shown in fig. 2, lymphocytes in vaccinated mice with HCV core gene group ( $82.41 \pm 13.20$ ) and HCV core + IL12-encoding gene group ( $87.27 \pm 12.14$ ) showed significant increases in cytotoxic activity against the target EL4 cell compared to IL12 and the negative control. There was no significant difference between HCV core and HCV core + IL12.

### Lymphocyte proliferation assay

As shown in Fig. 3, the mice immunized with HCV core+IL12 ( $1.39 \pm 0.158$ ) and IL12 control groups ( $2.12 \pm 0.06$ ), have induced much better proliferation response than the core gene and the negative control groups. Also the results demonstrated significant difference in the lymphocytes proliferation index in the IL12 group in comparison to the core+IL12 group ( $p > 0.05$ ; Fig. 3).

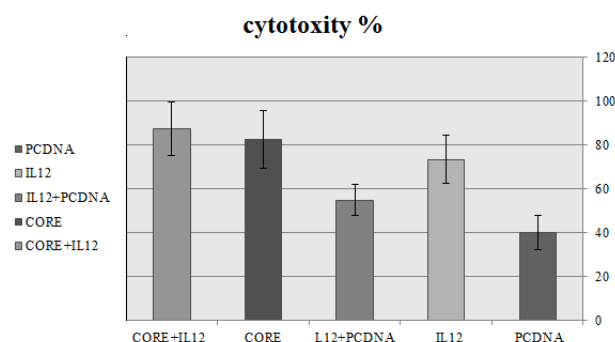
### The cytokines assays

As shown in Fig. 4, all HCV core+IL12 and IL12 DNA vaccinated mice had significantly higher levels of IFNG and IL4 production compared to the negative control groups ( $p < 0.05$ ). Furthermore, the combination of HCV core and IL12-encoding DNA vaccine stimulated IFNG and IL4 production more than IL12, alone (Fig. 4).

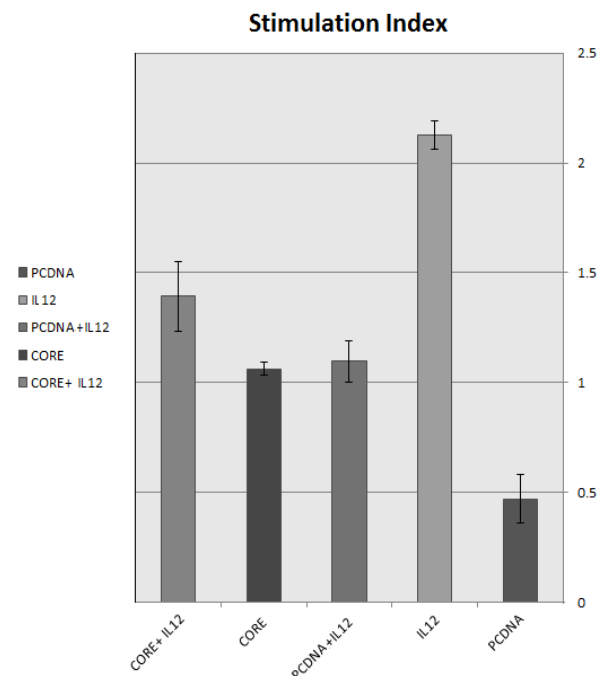


**Fig. 1.A.** SDS-PAGE and Western blot analyses of HCV-core expression in CHO cells.

**Fig. 1.B.** Electrophoresis pcDNA3.1-HCV-core gene: pcDNA3.1, HCV core gene; lane 1-10: Kbp gene ruler from Fermentas; lane 2, 3: plasmid gene pcDNA3.1; lane 4, 5: HCV-core gene.

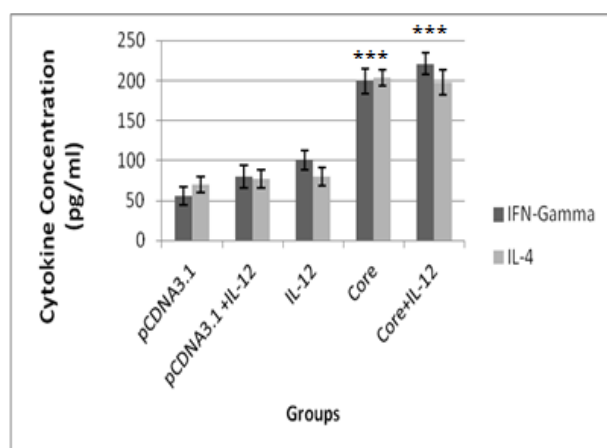


**Fig. 2.** CTL assays to demonstrate lymphocytes in mice vaccinated with different DNA vaccines using quantitative measurement of LDH release. Data were collected from LDH results at various E/T ratios (25:1, 50:1 and 100:1) and expressed as cytotoxicity%  $\pm$ SD. The data shown here are from 3 independent experiments in triplicates.



**Fig. 3.** One week after the last immunization, the spleens were removed and the proliferation of lymphocytes was evaluated by MTT assay. Values are the mean  $\pm$  SEM of 3 experiments. Mice immunized with IL12 gene induced a large proliferation response compared to the other groups ( $p < 0.05$ ).





**Fig. 4.** Determination of Th1 and Th2 cytokines. Collected supernatants were screened for the presence of IFNG and IL4 to determine the phenotype of the immune responses (Th1 vs. Th2). The data are presented as the mean  $\pm$  SD. Each sample was examined in triplicate. \*\*\* ( $p < 0.001$ ) indicates a statistically significant difference between the indicated groups as determined by one way ANOVA. Mice vaccinated with the core and the core-IL12 treatments showed significant differences when compared to the negative control groups (IL12 and pcDNA;  $p < 0.05$ ).

## DISCUSSION

An effective HCV vaccine would reduce the number of new infections and thereby could reduce the burden of the disease on the healthcare systems. However, there are many impediments to the development of an effective HCV vaccine including the existence of multiple HCV genotypes, limited availability of animal models and the complex nature of the immunological response to HCV [17]. DNA vaccines are well-suited for eliciting strong T cell responses. Unlike immunization with recombinant proteins which tend to produce Th2 responses, DNA vaccines are able to induce strong Th1 responses due to the ability of their antigens that are delivered and processed intracellularly [18]. However, reliable results in this regard have not yet been obtained in human clinical trials [19, 20]. In this study, we investigated the potential enhancement of DNA vaccine's immunogenicity (especially the CTL response) of HCV core gene via co-delivery of the IL12-encoding gene as a genetic adjuvant and modulator.

A recent study has demonstrated that HCV genotype 1a plays an important role in liver damage by affecting some of the cellular genes such as caspases [21]. Therefore, vaccines designed using the genotype 1a genes can be more protective compared to the other genotypes. However, the T cell proliferative responses against the structural proteins are shown to be typically weak [22]. Meanwhile, studies in humans and chimpanzees indicate thus far that an ideal vaccine should induce broad humoral, T helper, and cytotoxic T-cell responses [23, 24]. In previous studies, immune responses to a plasmid encoding HCV core protein have been demonstrated. The results from intramuscular inoculation of mice have indicated that the DNA vaccine has generated HCV core-specific antibody responses, lympho proliferative responses and CTL activities [25-27]. Several studies have shown the generation of CTL activities in mice immunized with HCV core plasmids [25-27]. One approach to augment the immunogenicity of DNA vaccines is employing cytokines as genetic adjuvants [28, 29]. Results obtained by Geissler et al. who co-injected GMCSF, IL2 or IL4 expressing plasmids with the core DNA showed this approach could result in a 2 to 3-fold increases in

the anti-core antibody seroconversion rates; however, only the co-injection of an IL2-encoding plasmid led to improved CD4+T cell proliferation and CTL activities [30]. Other studies have shown that the major cytokines associated with the development of antiviral cell-mediated immunity (CMI) are IL2 and IFNG while cytokines such as IL4, IL5, IL6, IL9 and IL13 can inhibit the development of CMI [31]. Among these cytokines, IL12-encoding genes were shown to be more powerful than others [32]. IL12 displays a variety of biological activities, such as enhancing the proliferation on natural killer (NK) cells and T cells, inducing the production of IFNG, enhancing the cytolytic activities by NK and T cells, and differentiating the CD4+ T helper type 1 (Th1) cells [33-36]. Therefore, IL12 has been considered to be a potent adjuvant for enhancing the cell-mediated immunity including the CTL responses.

It has been reported that the co-inoculation of an IL12 expression plasmid together with another plasmid expressing recombinant antigens could enhance the epitope-specific CTL induction in mice [37, 38]. Our results showed that mice of all 3 experimental groups (i.e. IL12, HCV core and HCV core-IL12 groups) had significantly higher CTL activities, lymphocyte proliferation and IFNG production, compared to mice of the pcDNA group (i.e. the negative control group). However, CTL activities were significantly higher in HCV core and HCV core-IL12 groups compared to the IL12 group. The results of HCV core-IL12 group suggest that the administration of IL12 in combination with an HCV core DNA vaccine increases the ability of the HCV DNA vaccine to induce cellular immunity and polarizes the specific immune responses toward cell-mediated immunity and a Th1 pattern.

In conclusion, based on our results HCV core protein appears as a suitable candidate to be induced in potential HCV DNA vaccines however the responses induced to the DNA vaccines is probably transient and weak. Moreover, one approach to enhance the immunogenicity of DNA vaccines may be the employment of genetic adjuvants such as IL12.

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## CONFLICT of INTEREST

The authors declare that they have no conflict of interest.

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