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ORIGINAL ARTICLE

Enhancement of HCV polytope DNA vaccine efficacy by fusion to an N-terminal fragment of heat shock protein gp96

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Abstract Induction of a strong hepatitis C virus (HCV)-specific immune response plays a key role in control and clearance of the virus. A polytope (PT) DNA vaccine containing B- and T-cell epitopes could be a promising vaccination strategy against HCV, but its efficacy needs to be improved. The N-terminal domain of heat shock protein gp96 (NT(gp96)) has been shown to be a potent adjuvant for enhancing immunity. We constructed a PT DNA vaccine encoding four HCV immunodominant cytotoxic T lymphocyte epitopes (two HLA-A2- and two H2-D^d-specific motifs) from the Core, E2, NS3 and NS5B antigens in addition to a T-helper CD4+ epitope from NS3 and a B-cell epitope from E2. The NT(gp96) was fused to the C- or N-terminal end of the PT DNA (PT-NT(gp96) or NT(gp96)-PT), and their potency was compared. Cellular and humoral immune responses against the expressed peptides were evaluated in CB6F1 mice. Our results showed that immunization of mice with PT DNA vaccine fused to NT(gp96) induced significantly stronger T-cell and antibody responses than PT DNA alone. Furthermore, the adjuvant activity of NT(gp96) was more efficient in the induction of immune responses when fused to the C-terminal end of the HCV DNA polytope. In conclusion, the NT(gp96) improved the efficacy of the DNA vaccine, and this immunomodulatory effect was dependent on the position of the fusion.

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Introduction

Hepatitis C virus (HCV) is a serious threat to human health, causing more than 170 million infections worldwide. The ability to establish chronic infection is a notable feature of HCV, which persists in about 80 % of patients following acute infection. These individuals are at major risk of development of cirrhosis and hepatocellular carcinoma. HCV has a single positive-sense RNA encoding three structural (Core, E1, E2) and seven non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [1].

It is well documented that a vigorous, polyclonal and multi-specific cellular immune response against HCV antigens is required for spontaneous clearance of acute HCV infection [2]. The low frequency of virus-specific CD8+ T cells in the peripheral blood [3] is mainly attributed to the uncontrolled replication of the virus in HCV chronic infection. Therefore, a vaccination approach that is able to induce a functional antiviral T-cell response

may be a promising strategy to overcome persistent infection. Production of cross-neutralizing antibodies against envelope proteins is also necessary to prevent the attachment and entry of circulating virus into the hepatocytes [4].

The potency of multi-epitope (polytope) vaccines has been examined previously in treatment of various tumors and infectious diseases [5]. Recently, polytope DNA vaccines containing epitopes derived from structural and NS proteins of HCV were tested *in vivo* [6, 7]. However, the efficacy of these vaccines was limited due to the lack of tertiary structure and their instability during polytope expression [7]. Appropriate carriers and adjuvants could improve the immunogenicity of polytope DNA vaccines.

Heat shock proteins (HSPs) have been demonstrated to act as potent adjuvants in the immunotherapy of malignant tumors and infectious diseases. gp96, the major chaperon in the lumen of the endoplasmic reticulum involved in cross-presentation of peptides to MHC class I and class II molecules, activates specific CD8+ and CD4+ T-cells [8, 9]. Moreover, gp96 can activate dendritic cells and macrophages through induction of proinflamatory cytokines via interaction with a subset of Toll-like receptors (TLRs) [10]. Previous experiments showed that gp96 or its N-terminal domain had an adjuvant function in tumor- [11] and HBV-specific CTL and humoral immune responses [12].

In the present study, we constructed a polytope (PT) DNA vaccine encoding both structural and non-structural antigens of HCV. To investigate the influence of fusing the N-terminal domain of gp96 (NT(gp96)) to the PT on the efficacy of the PT DNA vaccine, we also constructed two fusion DNAs with NT(gp96) fused either to the C- or the N-terminal end of the polytope sequence. The immunogenicity of the polytope DNA vaccines fused to NT(gp96) was evaluated in CB6F1 mice. The impact of vaccination on the percentage of regulatory CD4+ T-cells (Tregs) was also examined in this study.

Materials and methods

Laboratory animals

Ten-week-old CB6F1 female mice (genotype H-2d/b) were purchased from Harlan Winkelmann Laboratories (Borchen, Germany) and handled according to the guidelines of the animal facility at the University Hospital, Essen. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee (Animal Care Center, University of Duisburg-Essen, Essen, Germany, and the district government of Düsseldorf, Germany).

Cells and peptides

HeLa cells (HeLa, ATCC) were cultured in monolayers in Dulbecco's modified Eagle's medium (Gibco, Germany). Murine splenocytes and hepatocytes were cultured in RPMI medium (Gibco). Cell culture media were supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Biochrom AG, Germany) and 10 U of penicillin-streptomycin (PAA Laboratories, Austria) per ml. Cell lines were maintained in a humidified 5 % CO_2 atmosphere at 37 °C.

For evaluation of the immune responses, peptides of $Core_{132\text{-}142}$ (DLMGYIPLVGA), $E2_{405\text{-}414}$ (SGPSQKIQLV) and $E2_{412\text{-}426}$ (QLINTNGSWHINSTA) with 90 % purity were synthesized (EMC Microcollections, Germany). All lyophilized peptides were dissolved in DMSO (Sigma-Aldrich, Germany) at concentration of 10 μg/μl and stored at −20 °C.

Preparation of NT(gp96)-PT and PT-NT(gp96)

HCV PT DNA, cloned in pBlueScript II SK (+), was synthesized by Biomatik (Biomatik Corporation, Canada). The PT construct was designed to encode the selected HCV epitopes: H2-D^d-restricted epitopes of $Core_{132\text{-}142}$ (DLMGYIPLVGA) [13, 14] and $E2_{405\text{-}414}$ (SGPSQK IQLV) [15], HLA-A2-restricted epitopes of $NS3_{1073\text{-}1081}$ (CINGVCWTV) [16] and $NS5B_{2727\text{-}2735}$ (GLQDCTMLV) [16], T-helper (Th) CD4 epitope from $NS3_{1248\text{-}1262}$ (GY-KVLVLNPSVAATL) [17] and a neutralizing B-cell epitope from $E2_{412\text{-}426}$ (QLINTNGSWHINSTA) [18].

To prepare the plasmid pcDNA-PT, the PT sequence was subcloned into the *Hind*III and *Bam*HI restriction sites of the plasmid pcDNA3.1(−) (Invitrogen, Germany).

To generate NT (gp96)-PT, the region of nucleotides 1 to 1014 of gp96 (designed as NT(gp96)) was first amplified from pQE30-NT(gp96) [19] using the forward primer 5′-ATT**GGATCC**ACCATGGAAGATGACGTTGAA - 3′ and the reverse primer 5′- GGC**GAGCTC**GGTACCTT TGTAGAAGGCTTT- 3′ and then inserted into the *Bgl*II and *Sac*I restriction sites of pEGFP-N3 (Invitrogen).

The *Bam*HI and *Sac*I restriction sites in the forward and reverse primers are shown in bold, and the Kozak sequence is underlined. The PT sequence was then cloned into unique *Hind*III and *Bam*HI cloning sites of pEGFP-NT (gp96). pcDNA3.1-NT (gp96)-PT was prepared by subcloning NT (gp96)-PT into the unique *Nhe*I and *Bam*HI cloning sites of pcDNA3.1(−).

To prepare the PT-NT(gp96) fusion construct, the PT sequence was first amplified using primers designed to generate *Nhe*I restriction site at its 5′ end and cloned into *Nhe*I and *Bam*HI multiple cloning sites of pcDNA3.1(−). In the same manner, *Bam*HI and *Kpn*I restriction enzymes

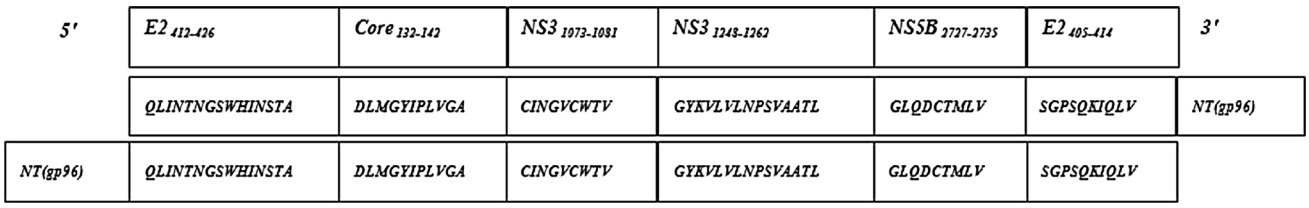

Fig. 1 Schematic illustration of two polytope fusion constructs containing six different epitopes of HCV in different blocks

were used for cloning of NT(gp96). Finally, PT-NT(gp96) was subcloned into the *Nhe*I and *Kpn*I sites of the pEGFP-N3 expression vector.

The accuracy of the constructs was confirmed by DNA sequencing (Eurofins MWG-Operon, Germany). pcDNA-NT(gp96)-PT, pcDNA-PT-NT(gp96), and pcDNA-PT were purified using an EndoFree Plasmid Giga Kit (QIAGEN, Germany). A schematic diagram of the constructs is presented in Fig. 1.

In vitro expression of PT-NT (gp96) and NT (gp96)-PT constructs in HeLa cells

HeLa cells were transfected separately with 1 μg of pEGFP-PT-NT(gp96) or pEGFP-NT(gp96)-PT plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. A Lipofectamine/plasmid complex was prepared with 5 μL of Lipofectamine. Expression of the fusion proteins was confirmed by observation of the EGFP signal under a fluorescence microscope at 24 h post-transfection. In addition, the cells were collected at 24 h post-transfection, washed with 1x PBS, and analyzed by flow cytometry to determine the proportion of GFP positive cells (FL1 channel). Untransfected cells were used as a control.

Cytotoxicity studies

The cytotoxicity of PT, PT-NT(gp96) and NT(gp96)-PT to HeLa cells was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay [20]. Cells were seeded in a 96-well plate at a density of 10^5 cells/well in 100 μL DMEM supplemented with 10 % fetal bovine serum (FBS) and incubated overnight at 37 °C in a 5 % CO_2 atmosphere. Cells were treated with pcDNA-PT, pcDNA- PT-NT(gp96) and pcDNA-NT(gp96)-PT in concentrations ranging from 6.25 to 100 μg/100 μl in DMEM (supplemented with 2% FBS) for 24, 48 and 72 h. Then, 20 μl of MTT dye solution (5 mg/ml in PBS) was added to each well and plates were incubated for 4 h at 37 °C. The supernatant of each well was removed, and formazan crystals were dissolved in 200 μl of dimethylsulfoxide (DMSO). The absorbance values were measured using a microplate reader at 570 nm. The percentage of cells that were viable was determined by comparing the optical density of plasmid-treated cells to that of normal cells (control).

Mice immunization

Mice were pretreated by intramuscular injection of 100 μl of cardiotoxin (10 μM in phosphate-buffered saline [PBS]; Latoxan, France) into tibialis anterior muscle of both hind limbs one week before the plasmid immunization. Mice were then injected intramuscularly with 100 μg of plasmids in PBS at 2-week intervals as described previously [21], and two weeks after the last immunization, they were sacrificed. Naïve untreated mice were used as controls.

Isolation of mouse splenic and hepatic lymphocytes

Preparation of a single-cell suspension of murine splenocytes was performed by homogenization according to a procedure described previously [22]. Hepatic lymphocytes were also isolated from the liver using previously published methods [22]. Briefly, livers were perfused with prewarmed PBS (to flush blood from the hepatic vasculature) and were forced through a 70-mm nylon cell strainer (BD Falcon, USA). After washing, cell pellets were resuspended for 30 min at 37 °C in prewarmed enzyme solution containing collagenase type II and DNase type I (Sigma-Aldrich) in HBSS supplemented with FBS. Cells were then layered on 40 % Percoll solution (Sigma-Aldrich) in RPMI supplemented with penicillin-streptomycin for density separation, and then centrifuged at 2000 rpm for 10 minutes. Cells were washed and suspended in Buffer EL (QIAGEN) to lyse red blood cells. Cell yields and viability were determined by trypan blue exclusion microscopy.

Intracellular cytokine staining and flow cytometry analysis

Up to 1×10^6 lymphocytes isolated from mice were seeded per well in 96-well plates in 200 μl of RPMI 1640 and stimulated of HCV peptides ($E2_{405-414}$ [SGPSQKIQLV] or $Core_{132-142}$ [DLMGYIPLVGA]) at a concentration of 2 μg/

ml and incubated for 6 days (in the presence of 10 U of recombinant murine IL-2 [Roche, UK] per ml) or 6 h at 37 °C in a 5 % CO_2 atmosphere. Cells with no peptide served as negative controls.

Prior to intracellular cytokine staining, the cells were cultured for 5–6 h in the presence of anti-CD28 (1 mg/mL) (clone 37.51; BD Pharmingen, Germany) and 5 mg/ml of brefeldin A (Sigma-Aldrich). Cells were then stained with anti-CD8 (clone 56.6-7; eBioscience, Germany), and anti-CD4 (clone L3T4; BD Pharmingen) antibodies, permeabilized and fixed with perm/wash reagents (eBioscience), stained with anti-IFNγ (clone XMG1.2; BD Pharmingen), anti-TNFα (clone MP6-XT22; eBioscience,), and anti-IL-2 (clone JES6-5H4, eBioscience) at 4 °C for 30 minutes, and analyzed immediately on a Gallios flow cytometer (Becton Coulter). A minimum of 100,000 events were acquired per sample. Subsequent analysis was performed using FlowJo software (Tree Star, Inc, Ashland, Ore). Dead cells were excluded from analyses using Fixable Viability Dye (FVD) (eBioscience) [23].

For Treg cell analysis, splenocytes from treated mice were stained with anti-CD4, anti-CD25 (clone PC 61.5, eBiosceience), and anti-Foxp3 antibodies (clone FJK-16 s; eBiosceience). Then, Treg cells were counted relative to total CD4+ cells by flow cytometry.

Determination of IgG isotypes

Two weeks after the last immunization, mice were bled via retro-orbital puncture. Specific total IgG and its subclasses (IgG1 and IgG2a) were measured in the sera of the mice by ELISA as described previously [19]. Briefly, an ELISA plate was coated with 100 µl of $E2_{412\text{-}426}$ peptide (10 µg/ml) in 0.5 M carbonate/bicarbonate buffer, pH 9.6. The plate was rinsed with PBS containing 0.05 % Tween 20 (PBS-T) and incubated with blocking buffer (5 % skim milk in PBS) for 2 h at 37 °C. After washing with PBS-T, diluted sera (1:50) in PBS buffer containing 1 % BSA and 0.05 % Tween-20 were added, and the plate was incubated at 37 °C for 2 h, followed by washing and incubation at 37 °C for 2 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, or IgG2a (1:4000; Southern Biotechnology Association, USA). Detection was done with Tetramethylbenzidine (TMB) substrate and the absorbance was measured at 450 nm.

Statistical analysis

GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, Calif) was used for plotting graphs and statistical analysis. Statistical differences were evaluated using Student's *t*-test and Mann-Whitney analysis. A *P*-value <0.05 was considered significant.

Results

Monitoring of PT-NT(gp96) and NT(gp96)-PT expression in HeLa cells

We first designed and constructed a PT DNA vaccine that encoded HCV immunodominant CTL epitopes (HLA-A2 and H2-D^d restricted) from Core, NS3 and NS5B, a Th CD4+ epitope from NS3 and a B-cell epitope from E2. In the next step, NT(gp96) was fused to the 5′ or 3′ terminus of the PT. To test whether these two fused constructs could be expressed properly *in vitro*, they were cloned into pEGFP-N3 reporter plasmid at the N-terminus of enhanced green fluorescent protein (EGFP). The clones that were obtained were named pEGFP-NT(gp96)-PT and pEGFP-PT-NT(gp96). HeLa cells were transfected with pEGFP-NT(gp96)-PT, pEGFP-PT-NT(gp96), pEGFP-N3 (as a positive control) and pcDNA3.1 (as a negative control). Then, expression was evaluated by detection of GFP fluorescence using fluorescence microscopy at 24 h after transfection. As shown in Fig. 2a and b, PT-NT(gp96) and NT(gp96)-PT were efficiently expressed in HeLa cells, and the antigen expression levels of these fusion DNAs were almost the same. GFP expression of the positive control was also confirmed (Fig. 2c). In the negative control, fluorescence emission was not observed (Fig. 2d). The experiments were performed twice, and similar results were obtained.

Quantification data obtained by flow cytometry showed that the expression of GFP in pEGFP-PT-NT(gp96) (Fig. 3a), pEGFP-NT(gp96)-PT (Fig. 3b) and pEGFP-N3 (Fig. 3c) was 10.1 %, 9.87 % and 16.1 %, respectively.

Cytotoxicity studies

The cytotoxicity of the DNA constructs was assessed in HeLa cells by MTT assay. More than 80 % of cells were observed to be viable at all time points and all concentrations of PT, PT-NT(gp96) and NT(gp96)-PT constructs (Fig. 4a, b, and c). No significant differences were found in the viability of cells after exposure to DNA constructs.

Vaccination with HCV polytope DNA fused to NT(gp96) enhanced HCV-specific CD8+ T-cell-mediated immune responses

We evaluated the immunogenic effects of the NT(gp96) fusion *in vivo* by immunization of CB6F1 mice. In the first step, we compared the effector functions of HCV epitope-specific CD8+ T cells in the spleens of mice immunized with the polytope and the two NT(gp96)/PT fusion DNA vaccines (pcDNA-PT-NT(gp96) and pcDNA-NT(gp96)-PT). Twelve female CB6F1 mice were randomly divided

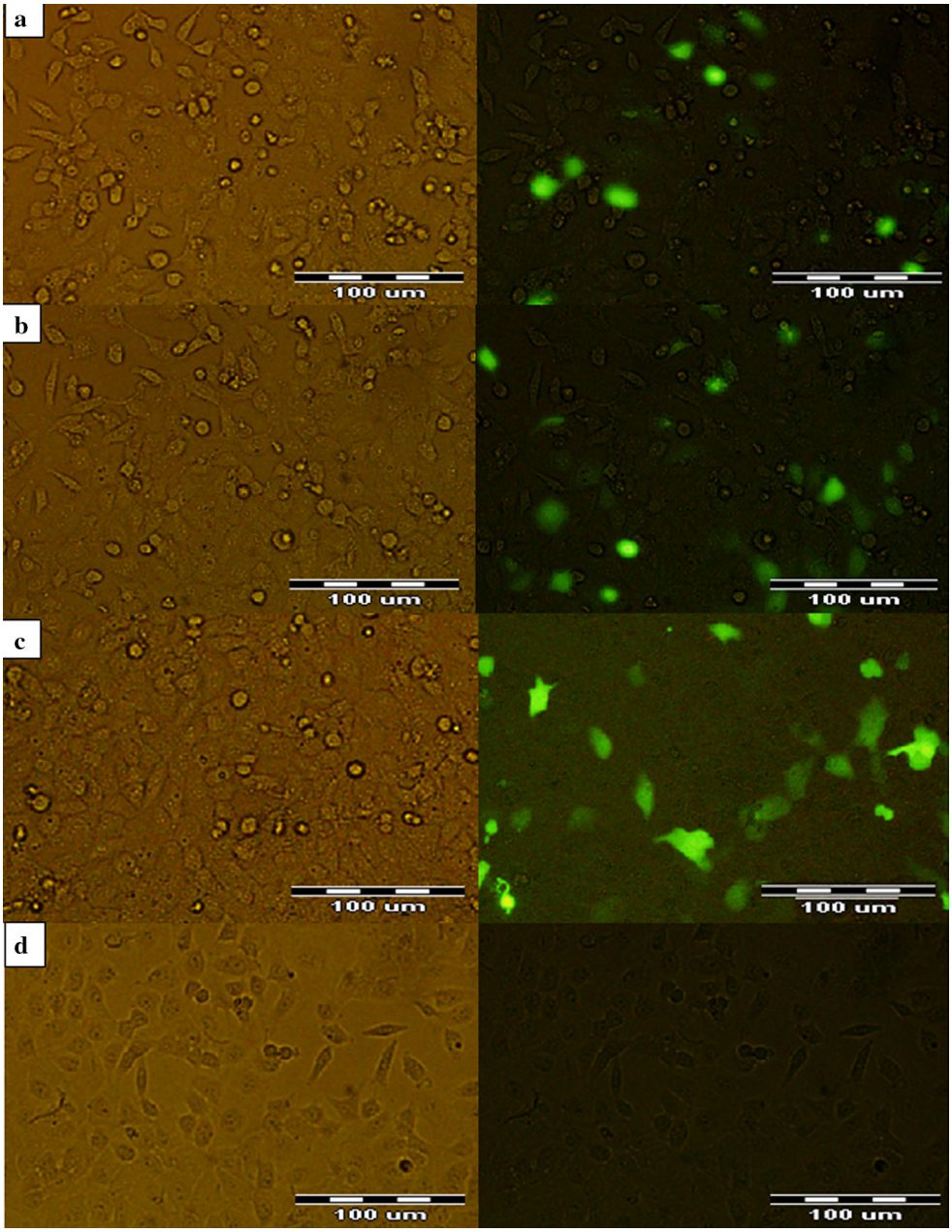

Fig. 2 Fluorescent microscopy images. HeLa cells were treated with 1 μg of pEGFP-PT-NT(gp96), pEGFP-NT(gp96)-PT, pEGFP-N3 (positive control) and pcDNA3.1 (negative control) plasmids complexed with 5 μl of Lipofectamine. **a**, **b**, **c** GFP expression of the transfected cells (before and after glinting of flurescence) with pEGFP-PT-NT(gp96), pEGFP- NT(gp96)-PT, and pEGFP-N3, respectively. **d** Image from transfected cells with pcDNA3.1

into three groups of four mice. The mice were injected intramuscularly with 100 μg of pcDNA-PT, pcDNA-PT-NT(gp96), and pcDNA-NT(gp96)-PT, three times at 2-week intervals, according to vaccination schedule shown in Fig. 5a. Naïve mice of corresponding age were used as controls. Two weeks after the last immunization, the frequency of CD8+ T-cells expressing antiviral cytokines such as IFNγ and TNFα was measured by flow cytometry in splenocytes expanded *in vitro* for 6 days with H2-D^d epitopes ($E2_{405-414}$ and $Core_{132-142}$).

Our results showed that immunization of mice with NT(gp96) fusion constructs induced significant HCV E2 epitope-specific IFNγ+ (Fig. 5c) and TNFα+ (Fig. 5d) CD8+ T-cell responses, compared to the background values obtained in naïve controls ($p < 0.05$). (Representative dot plots of IFNγ+ CD8+ T-cells are shown in Fig. 5b.) By contrast, the E2-specific CD8+ T-cell response induced by immunization with PT DNA vaccine in the spleen was very weak and not statistically significant compared to naïve controls. The frequency of CD8+

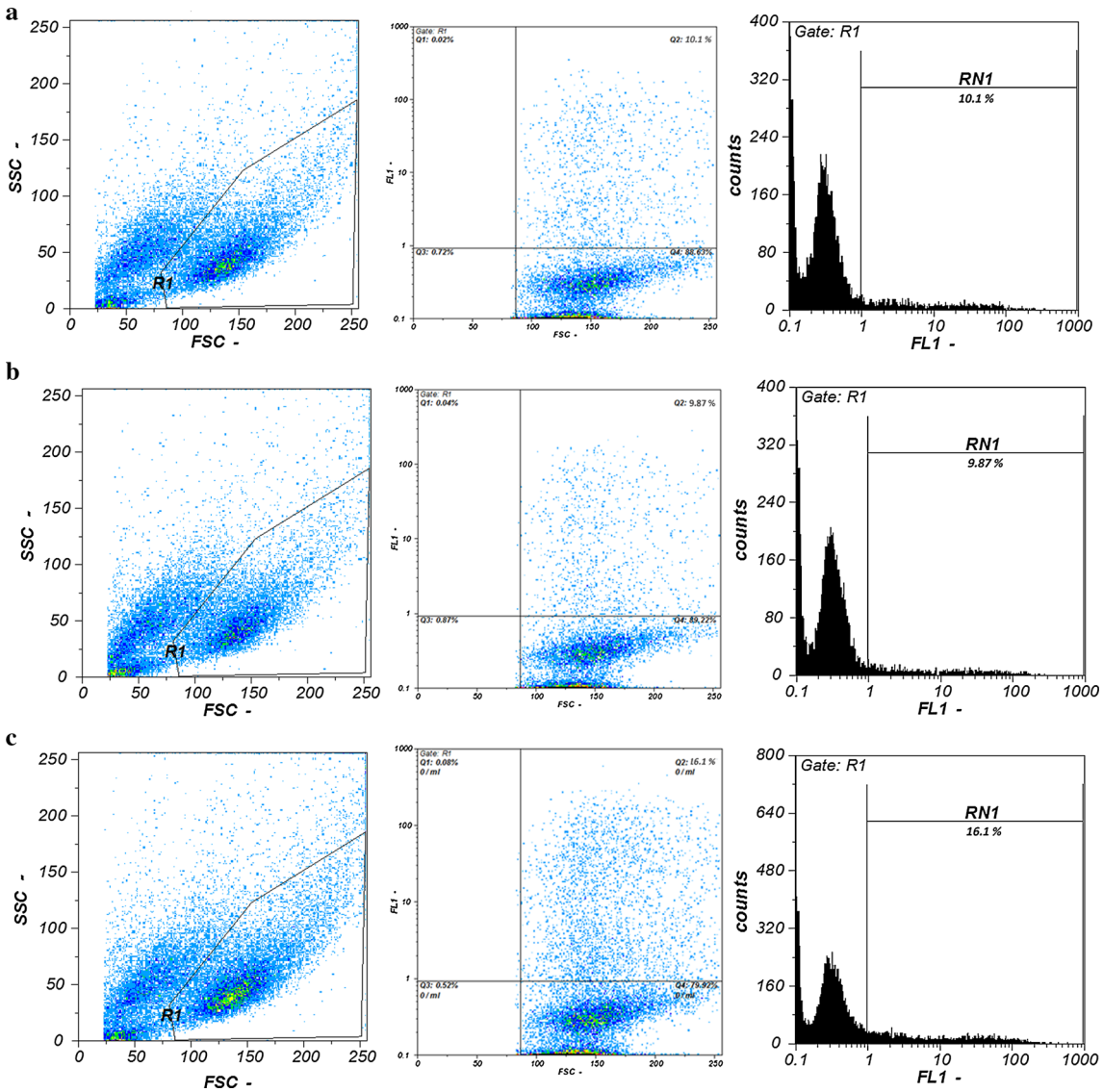

Fig. 3 Percentage of EGFP-positive cells tranfected with pEGFP-PT-NT(gp96) **(a)**, pEGFP-NT(gp96)-PT **(b)** and pEGFP-N3 **(c)**

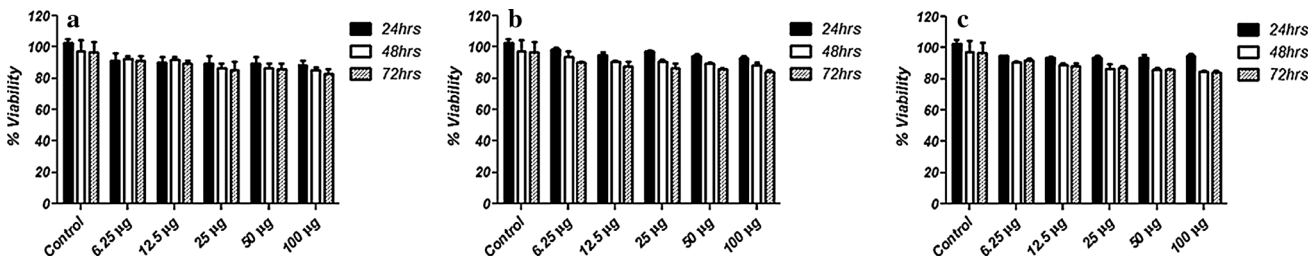

Fig. 4 Cytotoxicity analysis of PT **(a)**, PT-NT(gp96) **(b)** and NT(gp96)-PT **(c)** constructs in HeLa cells. The cells were treated with 6.25 to 100 µg of PT, PT-NT(gp96) and NT(gp96)-PT constructs per 100 µl for 24, 48, or 72 h. Cell viability was determined using MTT dye solution. Data represent the mean ± SD of three separate experiments. Statistical differences were analyzed by ANOVA

T cells producing IFNγ and TNFα was significantly higher in mice vaccinated with PT-NT(gp96) and NT(gp96)-PT compared to PT. The mean value of IFNγ responses was approximately 6.8 % and 5 % in the PT-NT(gp96)- and NT(gp96)-PT-immunized group, respectively, and these values were significantly higher than

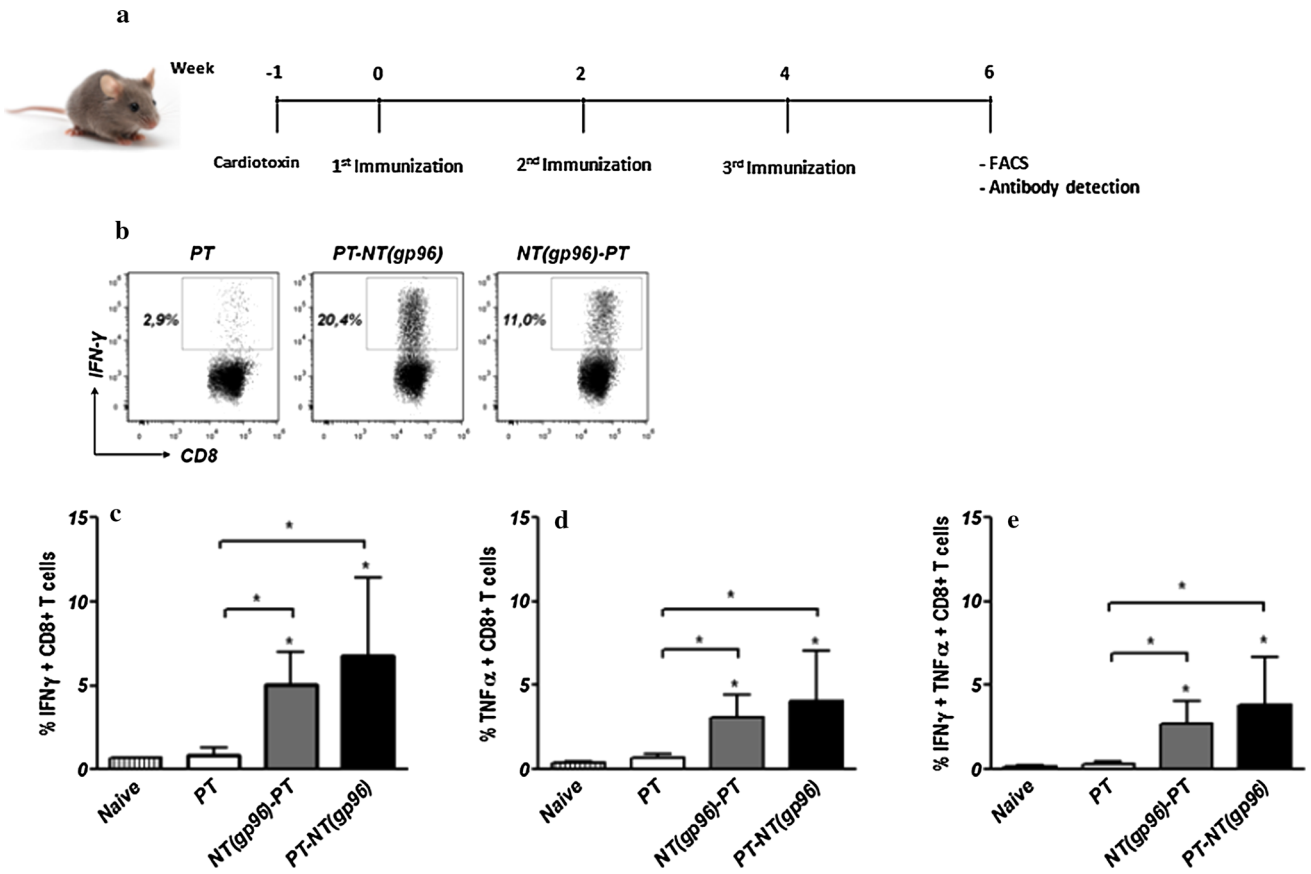

Fig. 5 Analysis of the cellular immune responses induced by plasmids containing the polytope and two fusion constructs in the spleen of immunized mice. **a** Vaccination schedule. The CB6F1 mice were immunized three times intramuscularly with 100 μg of pcDNA-PT, pcDNA-PT-NT(gp96) and pcDNA-NT(gp96)-PT at 2-week intervals. **b** Dot plots of splenic CD8+ T cells stimulated with $E2_{405-414}$ peptide. The values shown indicate the percentage of IFNγ+ CD8+ T-cells in the CD8+ T-cell population. **c, d, e** Frequencies of IFNγ+, TNFα+ and IFNγ+ TNFα+ CD8+ T cells from splenocytes expanded *in vitro* for 6 days with the $E2_{405-414}$ peptide. The bars represent mean values and standard errors of the mean obtained for each group of mice. Asterisks indicate statistically significant differences: *, $p < 0.05$. The asterisks shown directly above the bars, indicate statistically significant differences between plasmid-vaccinated groups and the naïve control group of mice

those obtained for the PT-immunized group (0.9 %) ($p < 0.05$). However, the differences found between PT-NT(gp96) and NT(gp96)-PT were not statistically significant (Fig. 5c). Similar results were observed for TNFα production. The percentage of TNFα+ CD8+ T cells directed against the $E2_{405-414}$ peptide was significantly higher in vaccinated mice with PT-NT(gp96) and NT(gp96)-PT (4 % and 3 %, respectively) compared to PT (0.8 %) ($p < 0.05$) (Fig. 5d).

Polyfunctional analysis of CD8+ T-cells producing IFNγ and TNFα revealed that the majority of CD8+ T cells induced by vaccination produced both cytokines simultaneously. Indeed, the mean value of CD8+ T cells producing both IFNγ and TNFα was 4.1 % and 2.7 % in PT-NT(gp96)- and NT(gp96)-PT-immunized mice, respectively, and these values were significantly higher than the value obtained for the PT vaccinated group (0.3 %) ($p < 0.05$) (Fig. 5e).

In addition, we measured the immune responses directed to the $Core_{132-142}$ epitope. Surprisingly, we did not observe any response against this peptide in immunized mice (data not shown).

Fusion with NT(gp96) enhanced PT-specific CD8+ T-cell responses in the liver

Since the liver is the major compartment of HCV replication, we compared the magnitude of the intrahepatic CD8+ T-cell response elicited by the polytope and the two NT(gp96)/PT fusion DNA vaccines. For this purpose, we isolated liver-infiltrating lymphocytes two weeks after the last immunization and stimulated the cells *in vitro* with the $E2_{405-414}$ peptide for 6 h. The predominant cytokine secreted by intrahepatic CD8+ T cells was IFNγ (Fig 6b; representative dot plots are shown in Fig. 6a), followed by TNFα (Fig. 6c) and IL-2 (Fig. 6d). The mean frequencies

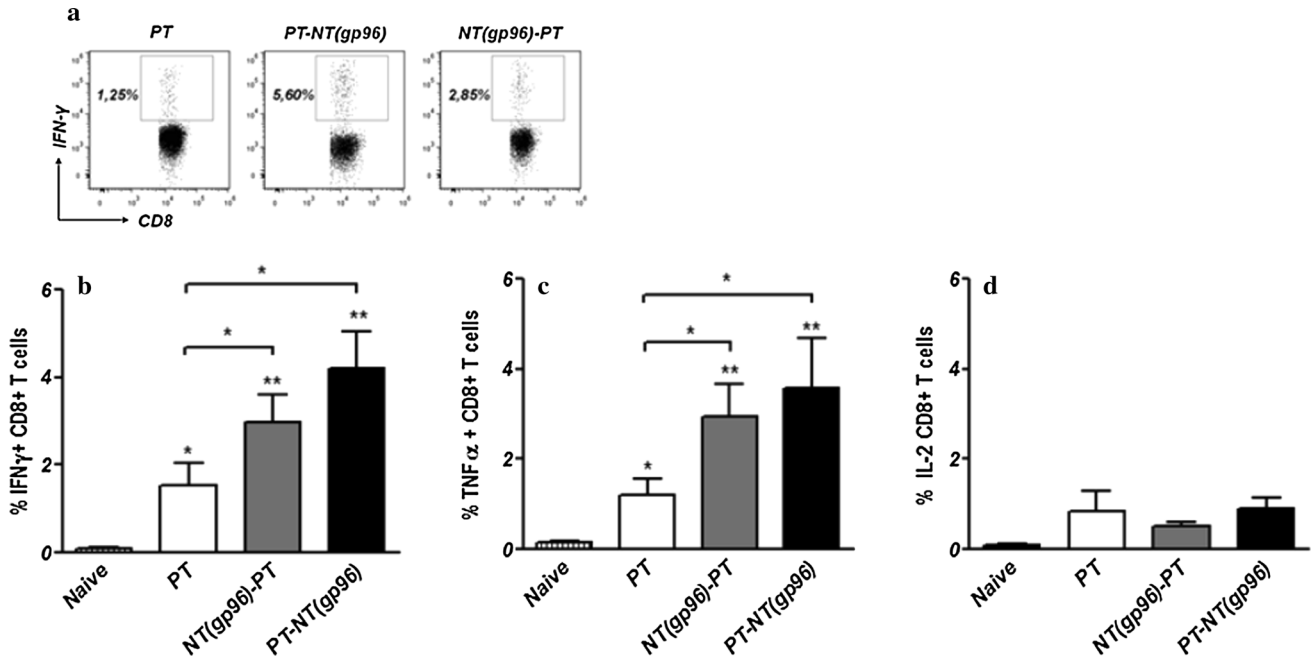

Fig. 6 Analysis of the cellular immune responses induced by plasmids containing the polytope and two fusion constructs in the liver of immunized mice. The hepatocytes of the vaccinated CB6F1 mice were stimulated for 6 hours with $E2_{405-414}$ peptide. **a** Dot plots of intrahepatic CD8+ T cells stimulated with $E2_{405-414}$ peptide. The values shown indicate the percentage of IFNγ+ CD8+ T cells in the CD8+ T-cell population. **b**, **c**, **d** Frequencies of IFNγ+, TNFα+, and IL-2+ CD8+ T cells from hepatocytes stimulated *in vitro* for 6 h with the epitope $E2_{405-414}$. The bars represent the mean values and standard errors of the mean obtained for each group of mice. Asterisks indicate statistically significant differences: *, $p < 0.05$; **, $p < 0.005$. The asterisks shown directly above the bars indicate statistically significant differences between plasmid-vaccinated groups and the naïve control group of mice

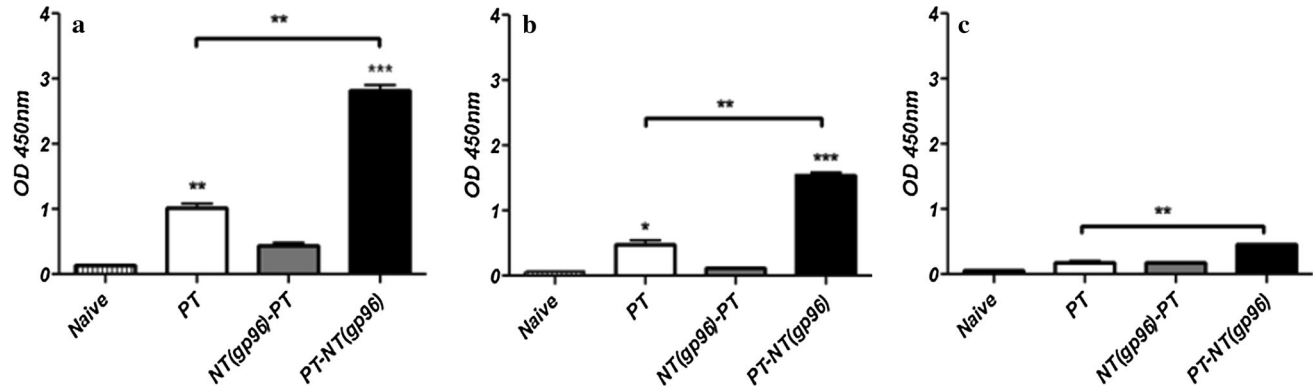

Fig. 7 Humoral immune responses induced by plasmid containing the polytope and two fusion constructs. Specific IgG **(a)**, IgG1 **(b)** and IgG2a **(c)** antibodies against $E2_{412-426}$ peptide were detected in immunized mice. The bars represent the mean values and standard errors of the mean obtained for each group of mice. Asterisks indicate statistically significant differences: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. The asterisks shown directly above the bars indicate statistically significant differences between plasmid-vaccinated groups and the naïve control group of mice

of IFNγ+ or TNFα+ CD8+ T cells in three immunized groups were significantly higher than the corresponding values obtained for naïve mice. The results showed that immunization with PT-NT(gp96) and NT(gp96)-PT induced a stronger immune response than PT. The percentages of IFNγ+ and TNFα+ CD8+ T cells detected in the liver of mice immunized with PT-NT(gp96) (4.2 % and 3.6 %, respectively) and NT(gp96)-PT (3 % and 2.3 %, respectively) were significantly higher than the mean percentages detected in the PT-immunized group (1.6 % and 1.2 %, respectively) ($p < 0.05$). It should be noted that the level of IL-2 production in all three groups of mice was low, and no statistically significant differences between the groups were observed.

The immune responses directed to $Core_{132-142}$ epitope in liver CD8+ T-cells were not detectable (data not shown).

Vaccination with HCV polytope DNA fused to NT(gp96) enhanced humoral immune responses

We determined the immunostimulatory effect of NT(gp96) fusion on the induction of HCV-specific humoral responses. The level of antibody in the sera of the mice was measured by ELISA 2 weeks after three injections. As shown in Fig. 7a, the level of IgG antibody in the group of the mice immunized with PT-NT(gp96) was significantly higher than in the mice injected with PT ($p < 0.005$), and those were significantly higher than the background value obtained from naïve mice ($p < 0.005$ and $p < 0.0005$ for PT and PT-NT(gp96), respectively). However, in the mice vaccinated with NT(gp96)-PT, the antibody response was below the detection limit.

Detection of IgG isotypes in the sera of the mice demonstrated that the PT and PT-NT(gp96) immunization regimens induced predominantly IgG2a antibodies (Fig. 7b), followed by IgG1 (Fig. 7c). Only mice immunized with PT-NT(gp96) showed a significantly higher level compared to the PT-immunized group ($p < 0.005$).

Immunization with PT-NT(gp96) DNA reduces the percentage of regulatory CD4+ T-cells

Regulatory CD4+ T cells (Tregs) are known to suppress the effector functions of antigen-specific T cells. In addition, they are specifically involved in downregulation of HCV-specific T-cell responses in chronic infection [24, 25]. Therefore, we investigated whether immunization with the fusion PT-NT(gp96) DNA vaccine could reduce the percentage of regulatory T cells *in vivo*. For this purpose, five CB6F1 mice were immunized with PT-NT(gp96) two times at two-week intervals according to the protocol described above. Two weeks after the second injection, the percentage of Tregs was evaluated in the spleen of immunized mice and naïve controls.

Interestingly, we found a significantly lower mean percentage of regulatory T cells in spleens of mice immunized with PT-NT(gp96) compared to the naïve mice, $p < 0.05$) (Fig. 8). These data indicated that immunization with PT-NT(gp96) vaccine was able to decrease the percentage of regulatory T cells and might facilitate the induction of HCV-specific humoral and cellular immune responses.

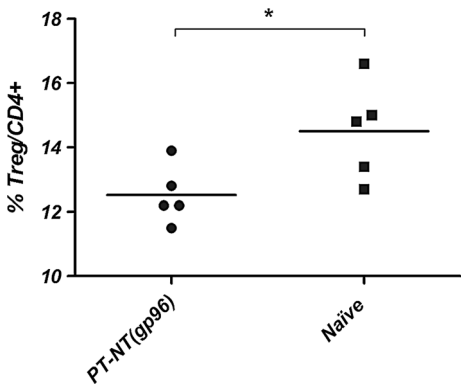

Fig. 8 CD4+ FoxP3+ Treg downregulation by immunization with PT-NT(gp96). Splenocytes from mice were stained with CD4+, CD8+, CD25+, FVD and intracellular FoxP3+. The percentage of CD4+ FoxP3+ Treg was measured in two groups of mice. The bars represent the mean values and standard errors of the mean obtained for each group of mice. Asterisks indicate statistically significant differences: *, $p < 0.05$

Discussion

The use of multi-epitope DNA vaccines is a promising approach for inducing a safe and effective immunity against highly variable pathogens like HCV, but their efficacy should be improved. Antigen fusion with HSPs such as gp96 or their fragments has been shown to increase the immunogenicity of the conjugated protein [26–28]. In the present study, we examined the adjuvant properties of the N-terminal fragment of gp96 in enhancing the immunogenicity of an HCV-specific polytope DNA vaccine in mice.

We used the MTT test to assess the effects of the PT-NT(gp96), NT(gp96)-PT and PT DNAs on cell viability. Our results showed that the DNA constructs had no cytotoxic effects on cells, and cell viability remained greater than 80 %.

Our data demonstrated that the immunostimulatory potency of PT was greatly enhanced by the fusion with NT(gp96). It is known that gp96 cross-presents chaperoned antigens and activates professional APCs to stimulate specific CTL responses. CD91 serves as a receptor for gp96 and facilitates the cross-priming effects [29]. The interaction of gp96 with stimulatory receptors of APCs may explain the enhancement of the PT-specific CD8+ T-cell response in mice vaccinated with PT-NT(gp96) and NT(gp96)-PT compared to mice immunized with PT alone. Our findings are in agreement with previous reports that demonstrated that fusion of HBV-specific antigens to the N-terminal domain of gp96 gene could improve the potency of HBV DNA vaccines [12, 30]. Mohit et al. [31] also showed that a DNA vaccine encoding the E7 antigen of human papillomavirus fused to NT(gp96) generated a significantly stronger E7-specific immune response than a vaccine containing E7 gene alone. Our data also showed that the vaccination with PT-NT(gp96) DNA vaccine significantly decreased the percentage of regulatory CD4+ T

cells. This effect could also contribute to further enhancement of PT-specific immune responses. This is in keeping with a study by Wang et al. [32], who suggested that NT(gp96) led to a decrease in the Treg population in the vaccinated group.

Previous experiments showed that the resolution of acute hepatitis C in a chimpanzee model was related to an early intrahepatic CTL response directed against HCV proteins [33]. Interestingly, our results revealed that fusion of NT(gp96) to PT DNA vaccine also enhanced intrahepatic CTL responses. Moreover, these responses were multifunctional. We found an increase in the frequency of IFNγ/TNFα double producers in mice vaccinated with two fusion DNAs compared to the PT-immunized group. These are encouraging data for HCV vaccine design, as it has been reported that induction of multifunctional effector T cells, which produce multiple cytokines simultaneously, are critical for immune defense against pathogens [34] and vaccine development [35, 36].

It has been suggested that an effective vaccine for HCV should prime anti-envelope neutralizing antibodies (nAbs) in addition to inducing a broad cellular immune response [37]. This kind of vaccine could increase the chance of clearance of the virus after exposure. It has been shown that the $E2_{412\text{-}426}$ epitope is conserved between HCV genotypes and can induce the production of nAbs [18]. In this study, we observed significant improvement of the humoral immune responses against the E2 epitope through the NT(gp96) fusion. Our findings are consistent with the data obtained by Li et al. [28], which showed that NT(gp96) greatly enhanced the humoral immune response induced by HBsAg. Moreover, Chen et al. [38] provided evidence for involvement of NT(gp96) in antigen-specific humoral immune responses elicited by B-cell epitopes of porcine reproductive and respiratory syndrome virus in swine. In this study, we showed that IgG2a was the dominant IgG isotype produced in the immunized mice. Our experiment revealed that immunization with PT-NT(gp96) DNA was able to produce a significantly higher amount of IgG2a antibody compared to PT DNA. These results showed that our vaccines preferentially primed Th1-type immune responses.

In addition, our findings indicated that position of the NT(gp96) fusion could affect the quantity of the induced PT-specific cellular and humoral immune responses. We showed that fusion of NT(gp96) to the C-terminus of the PT (PT-NT(gp96)) resulted in enhancement of the function of CTL responses. However, this difference was not statistically significant. The results are consistent with the findings that a C-terminal fusion of gp96 (CT(gp96)) to Her2 (Her2-CT(gp96)) could enhance the efficacy of a Her2 DNA vaccine [39]. In addition, Yan et al. [30] found that the fusion of NT(gp96) to the 5′ end of the HBV S gene negatively affected CTL immune responses. Moreover, *in vitro* expression of PT-NT(gp96) and NT(gp96)-PT in HeLa cells indicated that the two constructs had similar expression. Therefore, it seems that the enhanced immune responses observed for PT-NT(gp96) were not a consequence of increased expression.

Strikingly, in this study N-terminal and C-terminal fusion had different effects on the humoral immune response. HCV-specific antibody production was nearly abolished by the NT(gp96)-PT fusion, whereas an increased antibody response to PT-NT(gp96) was observed. We suggest that N-terminal fusion of NT(gp96) to PT results in a conformational change or steric hindrance, which reduces the efficacy of the PT DNA vaccine. Moreover, the position of the fusion may affect the pattern of ubiquitination of our fusion constructs [40].

One of the H2-D^d epitopes in our PT DNA vaccine was derived from HCV Core protein ($Core_{132\text{-}142}$). Even though *in silico* prediction tools were used to design the PT to provide proper proteosomal processing, we did not detect a CTL immune response against the Core epitope. This could be due to (1) higher binding affinity of E2 compared to Core epitope, leading to an immunodominant effect of E2 on the Core epitope, or (2) a failure in appropriate trimming of the PT in the endoplasmic reticulum.

In conclusion, our data demonstrate that NT(gp96) is able to enhance cellular and humoral immune responses. More-potent adjuvant activities of the N-terminal domain of gp96 were shown when the adjuvant was fused downstream of the HCV DNA PT, thus demonstrating the importance of the position of the fusion in the DNA vaccine. Further studies are needed to determine whether differences in conformation and ubiquitination patterns due to differences in the position of the adjuvant in the polypeptide led to differences in the immune response. Moreover, preclinical studies will be done to analyze the CTL immune responses against HLA-A2-restricted epitopes incorporated in PT in HLA-A2 transgenic mice. These future studies may provide useful data to motivate further evaluation of our PT DNA vaccine candidate in clinical settings.

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Conflict of interest The authors declare that they have no conflict of interest.

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