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Simultaneous use of natural adjuvants and cell penetrating peptides improves HCV NS3 antigen-specific immune responses

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ABSTRACT

To improve an effective hepatitis C virus (HCV) therapeutic vaccine, induction of a strong and long term HCV antigen-specific immune response is an important parameter. HCV non-structural protein 3 (NS3) has antigenic properties and plays a major role in viral clearance. In this study, DNA constructs encoding HCV NS3 and heat shock protein 27 (Hsp27)-NS3 genes, and the recombinant (r) NS3 and rHsp27-NS3 proteins complexed with HR9 and Cady-2 cell penetrating peptides (CPPs) were utilized to evaluate antibody, cytokine and Granzyme B secretion in mice. Herein, the formation of NS3 and Hsp27-NS3 DNA/ HR9 CPP complexes were revealed by gel retardation assay and protection against DNase and protease. Cady-2 peptide was used to form the nanoparticles with rNS3 and rHsp27-NS3 proteins. The size and charge of the nanoparticles were confirmed by SEM and Zetasizer instruments. Next, *in vitro* transfection of the nanoparticles was assessed by flow cytometry and western blotting. Finally, humoral and cellular immune responses were evaluated using different modalities in mice. Our data showed that HR9 and Cady-2 could form stable nanoparticles with DNA and proteins, respectively and enhance their delivery into HEK-293 T cells in a non-covalent approach. Furthermore, the heterologous Hsp27-NS3 DNA + HR9 prime/rHsp27-NS3 + Cady-2 protein boost elicited a higher Th1 cellular immune response with a predominant IgG2a, IgG2b, IFN-γ profile and strong Granzyme B secretion than those induced by other groups. Briefly, the combination of a natural adjuvant (Hsp27) and CPPs (HR9 and Cady-2) could significantly stimulate effective immune responses as a promising approach for development of HCV therapeutic vaccines.

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver disease and hepatocellular carcinoma (HCC) [1,2]. Generally, the main transmission routes of this infection include blood transfusion, drug injection, organ transplantation and unprotected sexual contact [3]. Among HCV proteins, the nonstructural viral protein 3 (NS3) plays a major role in the proteolytic processing of the viral polyprotein and viral RNA replication [4]. NS3 possesses several $CD4^+$ and $CD8^+$ T cell epitopes which induce strong HCV-specific T cell responses. Thus, it could be considered as an attractive antigenic candidate for viral clearance in various vaccination strategies such as DNA and protein-based vaccines [5]. However, cellular delivery of DNA and protein is a main issue for therapeutic targets. Recently, the small peptides known as protein transduction domains (PTDs) or cell-penetrating peptides (CPPs) were widely utilized to deliver a variety of biological macromolecules into cells in a non-endocytic or endocytic manner. These peptides were cationic construct composed of Arg, Lys and His amino acids, and/or amphipathic construct harboring lipophilic and hydrophilic tails [6,7]. For instance, Histidine-rich R9 (HR9: CH5-R9-H5C) containing polyhistidine and nona-arginine sequences and two cysteine residues could increase gene delivery *in vitro* [8]. Moreover, amphipathic CPPs including Cady-2 were able to form the nanoparticles with proteins and their delivery into cells [9,10]. Cady-2 (Ac-GLWWRLWWRLRSWFRL-WFRA-Cya) harbors two TrpTrp and two TrpPhe tandems, five Arg groups, but no negative charges. It constitutes highly hydrophobic and positively charged amino acids (˜ 40% and ˜ 25%, respectively) [10].

Currently, heat shock proteins (Hsps) have been known as efficient adjuvants in vaccine development. Hsps are highly conserved proteins and function as chaperones stabilizing and delivering peptides. Hsps are able to stimulate both innate and adaptive immunity. The ability of Hsps to bind antigenic peptides and deliver them to antigen presenting cells (APCs) is the basis of the generation of peptide-specific T lymphocyte responses *in vitro* and *in vivo* [11–13]. Among Hsps, small Hsp27 is a multidimensional protein which acts as a protein chaperone, an antioxidant, an inhibitor of apoptosis and an immunostimulator

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[14,15]. The expression of phosphorylated Hsp27 was useful in the screening and grading of hepatocellular carcinoma (HCC) [16].

In this study, at first, the efficiency of two cell penetrating peptides including HR9 and Cady-2 was evaluated to deliver NS3 and Hsp27-NS3 DNA and protein constructs into the eukaryotic cells, respectively. Then, BALB/c mice immunization was performed by a variety of DNA and protein formulations in three different strategies including homologous DNA prime/DNA boost, heterologous DNA prime/protein boost and homologous protein prime/protein boost. Finally, the levels of antibodies, cytokines and Granzyme B were determined two months after the last immunization using ELISA methods.

2. Materials and methods

2.1. Peptides

The HR9 (CH5-R9-H5C) and Cady-2 (Ac-GLWWRLWWRLRS WFRLWFRA-Cya) peptides were prepared by BioMatik Corporation (Canada). The peptides were dissolved in PBS for *in vivo* studies.

2.2. Generation of the recombinant DNA constructs

The immunodominant fragment of HCV NS3 gene (amino acid 1095–1379 of HCV genome, Genotype 1a, Accession No: NC_004102.1) was previously cloned into pcDNA3.1 and pEGFP-C3 expression vectors [17,18]. Moreover, the full length of Mus musculus Hsp27 gene (Accession No: NM_013560) was previously synthesized in a prokaryotic expression vector (pQE30-Hsp27 [19],). To generate pcDNA-Hsp27-NS3 fusion construct, the NS3 gene was amplified by PCR from pcDNA-NS3 using primers designed to insert *Pst*I and *Kpn*I restriction sites at the 5′ and 3′ ends of the amplified fragments, respectively.

Forward primer: 5′-CGCTGCAGGGTCCTGTCATCCAGATG-3′ (*Pst*I)

Reverse primer: 5′-CGGGTACCGATACGCTTGCCATAGAAG-3′ (*Kpn*I)

The amplified NS3 product was then cloned into the unique *Pst*I/*Kpn*I cloning sites of the pQE-Hsp27 vector. Finally, the fusion pQE-Hsp27-NS3 construct was subcloned into the pcDNA3.1 (-) using *Nhe*I/*Afl*II restriction enzymes. To prepare the pEGFP-Hsp27-NS3, the NS3 gene was ligated in *Sal*I/*Hind*III restriction sites of pQE-Hsp27 using T4 DNA ligase. The fusion of Hsp27-NS3 was digested by *Bgl*II/*Hind*III and subcloned into *Bgl*II/*Hind*III cloning sites of pEGFP-N1. Finally, all DNA constructs containing NS3 and Hsp27-NS3 fusion (*i.e.,* pEGFP-NS3, pcDNA-NS3, pEGFP-Hsp27-NS3 & pcDNA-Hsp27-NS3) were purified by an Endo-free plasmid Mega kit (Qiagen). The concentration and purity of DNA constructs were determined by NanoDrop spectrophotometer. On the other hand, for generation of pET-NS3, the NS3 fragment was previously subcloned from pcDNA-NS3 into the pET-24a using *Xho*I/*Hind*III restriction enzymes [18]. To construct the pET-Hsp27-NS3, the fusion Hsp27-NS3 construct was subcloned from pQE-Hsp27-NS3 into the pET23a using *Nhe*I/*Not*I restriction enzymes.

2.3. Generation of the recombinant Hsp27-NS3 and NS3 proteins in bacteria

The *E. coli* Rosetta strain was transformed with the recombinant pET-NS3 [18] and pET-Hsp27-NS3 plasmids. The recombinant clones were selected on Luria-Bertani (LB) agar plate and grown to an optical density of 0.7-0.8 at 600 nm in Ty2X medium. Expression of proteins was performed by 1 mM IPTG at 37 °C and evaluated at various times of incubation (*i.e.,* 2, 4 and 16 h after induction). The cell pellets were harvested and analyzed by SDS-PAGE and western blotting using anti-His tag antibody conjugated to *HRP* (Sigma, 1:10,000 v/v). The recombinant proteins were purified by affinity chromatography using a Ni-NTA agarose column under denaturing conditions according to the manufacturer's instructions (Qiagen). The purified proteins were dialyzed against PBS1 × . The endotoxin contamination was less than 0.5 EU/mg proteins as monitored by LAL assay. Finally, their concentrations were measured by NanoDrop spectrophotometry and stored at -70 °C.

2.4. Preparation of HR9/NS3 DNA or HR9/Hsp27-NS3 DNA nanoparticles

For preparation of peptide/DNA complexes, the HR9 peptide solution was added dropwise to 1 μg of NS3 or Hsp27-NS3 plasmid DNA at different N/P ratios (*i.e.,* 0, 1, 2, 5 and 10) to a final volume of 50 μL and incubated at room temperature for 30 min. The condensation between HR9 peptide and pcDNA-NS3, pcDNA-Hsp27-NS3, pEGFP-NS3, pEGFP-Hsp27-NS3 was assessed by gel retardation assay. To assess the stability of HR9/pcDNA-NS3, HR9/pcDNA-Hsp27-NS3, HR9/pEGFP-NS3 or HR9/pEGFP-Hsp27-NS3 complexes against DNA nucleases, DNase I was added to the complexes with a final concentration of 1.37 U/ μg DNA and the mixtures were incubated at 37 °C for 1 h followed by the addition of stop solution (200 mM sodium chloride, 20 mM EDTA and 1% SDS) [20]. For evaluation of the serum stability, the nanoparticles at the N/P ratios of 0, 5 for HR9/NS3 and HR9/Hsp27-NS3 DNA were exposed to 10% serum and incubated for 5 h at 37 °C. Then, DNA plasmids were released from protein by adding 10% SDS solution for 2 h and analyzed with electrophoresis on agarose gel 1% [21]. Alternatively, the zeta-potential of pEGFP-NS3, pcDNA-NS3, pEGFP-Hsp27-NS3 and pcDNA-Hsp27-NS3 and the peptide/DNA complexes at the same N/P ratio of 5:1 were assessed by a Zetasizer Nano ZS instrument (Malvern Instruments, UK) at 25 °C. Finally, the size and morphology of the HR9/NS3 or HR9/Hsp27-NS3 DNA nanoparticles at N/P ratio of 5:1 were studied using a scanning electron microscope (SEM, KYKY-EM3200 model, China).

2.5. Generation of the Cady-2/rNS3 protein or Cady-2/rHsp27-NS3 protein nanoparticles

The Cady-2/rNS3 protein and Cady-2/rHsp27-NS3 protein complexes with molar ratios of 5:1, 10:1, 20:1 and 30:1 (1 μg for each protein) were formed in apirogenic water and incubated for 30 min at room temperature. The formation of the complexes was confirmed by SDS-PAGE, and their size and zeta potential were assessed by a Zetasizer Nano ZS instrument. In addition, their size and morphology were studied using a scanning electron microscope.

2.6. NS3 or Hsp27-NS3 DNA and protein delivery using HR9 and Cady-2 CPPs into HEK-293T cells

Human embryonic kidney cells (HEK-293 T obtained from Pasteur Institute of Iran) were grown in complete DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) at 37 °C and 5% CO_2 atmosphere. Then, the HEK-293 T cells were seeded at a density of 4–5 × 10^4 cells/well in a 24-well plate. The HR9/pEGFP-NS3 and HR9/pEGFP-Hsp27-NS3 nanoparticles were prepared at an N/P ratio of 5:1, and incubated for 30 min at room temperature. The HR9/pEGFP-NS3 and HR9/pEGFP-Hsp27-NS3 nanoparticles were added to the cells in serum-free medium. The medium was replaced after 1 h incubation at 37 °C with complete DMEM supplemented with 5% FBS. The pEGFP-NS3/TurboFect and pEGFP-C3/TurboFect, pEGFP-Hsp27-NS3/TurboFect complexes (Fermentas) were used as a positive control according to the manufacturer's instructions. The transfection efficiency was determined by fluorescence microscopy (Envert Fluorescent Ceti, Korea) and quantified by a FACS Calibur flow cytometer (Partec, Germany) at 48 h post-transfection. Similarly, after growth of the HEK-293 T cells to 80% confluency in a 24-well plate, the medium was replaced by serum-free medium and then 100 μl of rNS3, rHsp27-NS3, rNS3/Cady-2 and rHsp27-NS3/Cady-2 at a molar ratio of 20:1 was added to each well. After 1 h incubation at 37 °C, the cells were supplemented with fresh DMEM, 5% FBS in a total volume of 200 μl medium without removal of the proteins and complexes overlay, and

Table 1
Different immunization strategies using HCV NS3 and Hsp27-NS3 antigens.

Group	Modality	First injection	Second injection	Third injection
G1	Protein/protein/protein	rNS3 + CFA	rNS3 + IFA	rNS3 + IFA
G2	Protein/protein/protein	rHsp27-NS3	rHsp27-NS3	rHsp27-NS3
G3	Protein/protein/protein	rNS3 + Cady2	rNS3 + Cady2	rNS3 + Cady2
G4	Protein/protein/protein	rHsp27-NS3 + Cady2	rHsp27-NS3 + Cady2	rHsp27-NS3 + Cady2
G5	DNA/DNA/DNA	pcDNA-NS3	pcDNA-NS3	pcDNA-NS3
G6	DNA/DNA/DNA	pcDNA-NS3 + HR9	pcDNA-NS3 + HR9	pcDNA-NS3 + HR9
G7	DNA/DNA/DNA	pcDNA-Hsp27-NS3	pcDNA-Hsp27-NS3	pcDNA-Hsp27-NS3
G8	DNA/DNA/DNA	pcDNA-Hsp27-NS3 + HR9	pcDNA-Hsp27-NS3 + HR9	pcDNA-Hsp27-NS3 + HR9
G9	DNA/protein/protein	pcDNA-Hsp27-NS3	rHsp27-NS3	rHsp27-NS3
G10	DNA/protein/protein	pcDNA-Hsp27-NS3 + HR9	rHsp27-NS3 + Cady-2	rHsp27-NS3 + Cady-2
G11	DNA/protein/protein	pcDNA-NS3 + HR9	rNS3 + IFA	rNS3 + IFA
G12	DNA/protein/protein	pcDNA-NS3 + HR9	rHsp27-NS3	rHsp27-NS3
G13	DNA/protein/protein	pcDNA-NS3 + HR9	rNS3 + Cady2	rNS3 + Cady2
G14	control	PBS	PBS	PBS
G15	control	HR9	HR9	HR9
G16	control	Cady-2	Cady-2	Cady-2
G17	control	pcDNA3.1	pcDNA3.1	pcDNA3.1
G18	control	Adjuvant (CFA)	Adjuvant (IFA)	Adjuvant (IFA)

[*] r: recombinant.

were further incubated for 3 h. Then, the cells were treated with trypsin, harvested by centrifugation and resuspended in PBS 1 × . The delivery of rNS3 and rHsp27-NS3 proteins was confirmed by western blotting using anti-His tag antibody under standard procedures. Briefly, HEK-293 T cells were lysed in lysis buffer (10% glycerol, 0.5 mM EDTA, 1 mM DTT, 2 mM natrium fluoride, 0.2% Triton X-100 in PBS pH = 7.4) supplemented with protease inhibitor. Proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane (Millipore). The anti-His tag antibody (Sigma; 1:10,000 v/v) was used to confirm protein entry into cells. The immunoreactive protein bands were visualized using peroxidase substrate (3, 3′-diaminobenzidine or DAB, Sigma). TurboFect/protein complexes were prepared according to manufacturer's instructions (Pro-Ject™ Reagent, Germany) as a positive control, as well [9].

2.7. Cytotoxicity assay

The MTT proliferation assay was utilized to evaluate the cytotoxicity of the pEGFP-NS3, pEGFP-Hsp27-NS3, HR9/pEGFP-Hsp27-NS3 and HR9/pEGFP-NS3 nanoparticles at different ratios of 2:1, 5:1 and 10:1, and various molar concentrations of HR9 peptide (2, 5 and 10) as well as 70% ethanol as a positive control in the non-malignant HEK-293 T cell line. Briefly, the cells were seeded at a density of 1×10^4 cells/well in a 96-well culture plate. After an overnight period, the cells were treated with different doses of the HR9 peptide, HR9/pEGFP-NS3 and HR9/pEGFP-Hsp27-NS3 nanoparticles for 48 h. Then, the cells were incubated with the MTT yellow dye (Sigma, 5 mg/ml in PBS) at 37 °C in a humidified 5% CO_2 for 3 h. The purple formazan product was dissolved in DMSO and the absorbance was measured at 570 nm by an ELISA reader. Similarly, the MTT assay was performed for different concentrations of Cady-2 peptide (5, 10, 20 and 30 μM), and various molar ratios of the Cady-2/ rNS3 and Cady-2/ rHsp27-NS3 complexes (5:1, 10:1, 20:1 and 30:1). The non-treated cells were used as a negative control. MTT assay was performed in triplicate.

2.8. Mice immunization

Five to seven week-old female BALB/c mice (n = 4 per group) were obtained from the breeding stock maintained at Pasteur Institute of Iran. All mice were maintained under specific pathogen-free conditions and all procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals (Pasteur Institute guideline). Eighteen (18) groups were subcutaneously immunized at the footpad with different modalities including DNA/DNA, protein/protein and DNA/protein strategies (Table 1). Mice were immunized on days 0, 14 and 28 with 50 μg of the naked DNA construct, the DNA (10 μg)/HR9 nanoparticles (N: P = 5:1), the recombinant protein (10 μg) emulsified in Freund's adjuvant at ratio of 50:50 v/v, and the recombinant protein (10 μg)/ Cady-2 nanoparticles (molar ratio = 1: 20 for protein: peptide).

2.9. Evaluation of antibody responses

Three weeks and two months after third injection, pooled sera were prepared from the whole blood samples of each group. The levels of NS3-specific antibodies (total IgG, IgG1, IgG2a, IgG2b; Southern biotechnology Association) in the sera were determined using indirect ELISA. The coated antigen was the recombinant NS3 protein (˜10 μg/mL) diluted in PBS1 × .

2.10. In vitro cytokine assay

Two months after the last immunization, four mice from each group were sacrificed and the spleens were removed. The red blood cell depleted pooled and/or single splenocytes (2×10^6 cells/ml) were cultured in U-bottomed, 96-well plates for 72 h in the presence of 10 μg/mL of rNS3, RPMI 5% (negative control), and 5 μg/mL of concanavalin A (ConA, positive control) in complete culture medium. The rest of the splenocytes were simultaneously used to assess Granzyme B release. The presence of IFN-γ and IL-5 in supernatants was measured using a sandwich-based ELISA system (R & D) according to the manufacturer's instructions. The detection limit was 2 pg/ mL for IFN-γ and 7 pg/ mL for IL-5.

2.11. Granzyme B (GrB) ELISA assay

P815 target cells (T) were seeded in triplicate into U-bottomed, 96-well plates (2×10^4 cells/ well) incubated with NS3 antigen (˜ 30 μg/mL) for 24 h. The splenocytes (Effector cells: E) previously provided in section 2.10 were counted using trypan blue and added to the target cells at E: T ratio of 100:1 in which maximal release of Granzyme B was observed. The target and effector cells were co-cultured in complete RPMI-1640 supplemented with 10% heat-inactivated FCS at 37 °C and 5% CO_2 under humidified conditions. The wells containing effector cells were considered for possible spontaneous release of Granzyme B. After 6 h incubation, microplates were centrifuged at 250 *g* for 5 min at 4 °C and the supernatants were harvested. The concentration of Granzyme B in these samples was measured by ELISA (eBioscience) according to the manufacturer's instruction.

2.12. Statistical analysis

The differences between the control and test groups were assessed using one-way ANOVA (Graph-pad Prism, GraphPad Software, USA). Results were expressed as mean ± standard deviation. Similar results were obtained in two independent experiments. A *p*-value < 0.05 was statistically considered significant.

3. Results

3.1. Characteristics of the HR9/NS3DNA or HR9/Hsp27-NS3DNA nanoparticles

At first, the recombinant pcDNA-Hsp27-NS3, pEGFP-Hsp27-NS3 and pET-Hsp27-NS3 plasmids were confirmed as a clear band of ˜1581 bp (861 bp for NS3 and 720 bp for Hsp27) related to Hsp27-NS3 fusion construct after digestion on agarose gel (data not shown). Moreover, the accuracy of Hsp27-NS3 fusion cloned in pQE30 was confirmed by sequencing. Next, to determine the interaction between HR9 and NS3 DNA, the recombinant pEGFP-NS3 and pcDNA-NS3 was mixed with the increasing amounts of HR9 CPP to form various N/P ratios (*i.e.,* 0, 1, 2, 5 & 10). Both pcDNA-NS3 and pEGFP-NS3 did not migrate into the agarose gel at an N/P ratio of 2:1, indicating the formation of the HR9/pEGFP-NS3 and HR9/pcDNA-NS3 complexes (data not shown). The pEGFP-C3/HR9 and pcDNA3.1/HR9 complexes were also formed at an N/P ratio of 1:1. Moreover, both pcDNA-Hsp27-NS3 and pEGFP-Hsp27-NS3 did not migrate into the agarose gel at an N/P ratio of 5:1 indicating the formation of the peptide/DNA complexes. For stability assay, after DNase I treatment, the naked NS3 and Hsp27-NS3DNA was quickly degraded, while the HR9/NS3DNA and HR9/Hsp27-NS3DNA complexes protected the DNA from DNase I degradation at the N/P ratios more than 2:1 and 5:1, respectively (data not shown). For serum protection assay, the same N/P ratio of 5:1 was selected for the HR9/NS3 DNA and HR9/Hsp27-NS3 DNA complexes. Our data showed that recovered DNA from nanoparticles remained intact on agarose gel in the presence of serum after 5 h incubation with FBS. In contrast, unprotected plasmid DNA was degraded (data not shown). To analyze the size (/diameter) and surface charge of nanoparticles, the NS3 DNA, Hsp27-NS3 DNA, HR9/NS3 DNA and HR9/Hsp27-NS3 DNA complexes at the same N/P ratio (5:1) were measured using a Zetasizer (Table 2). The pEGFP-NS3, pcDNA-NS3, pcDNA-Hsp27-NS3 and pEGFP-Hsp27-NS3 exhibited negative charges, while the HR9/pEGFP-NS3, HR9/pcDNA-NS3, HR9/pcDNA-Hsp27-NS3 and HR9/pEGFP-Hsp27-NS3 complexes displayed positive charges. These data suggests that electropositive charges of HR9 CPP/NS3 DNA complexes can be considered as an important factor for transport across the negative-charged cytoplasmic membrane of HEK-293 T cells. SEM analysis of the nanoparticles indicated a spherical shape with a size of ˜ 100–150 nm for the HR9/pEGFP-NS3 nanoparticles (N/P: 5:1) and a size of ˜ 100–180 nm for the HR9/pcDNA-NS3 nanoparticles at 25 °C (Fig. 1 A & B). Moreover, a spherical shape was observed with a size of ˜ 200–300 nm for the HR9/pcDNA-Hsp27-NS3 nanoparticles (N/P: 5:1) and a size of ˜ 150–250 nm for the HR9/pEGFP-Hsp27-NS3 nanoparticles at 25 °C (Fig. 1 C & D).

Table 2
The size (/diameter) and surface charge of plasmid DNA, proteins and nanoparticles measured using a Zetasizer.

Construct	Z. Average	Surface charge
pEGFP-NS3	˜ 396 nm	−16.8 mV
pcDNA-NS3	˜ 672 nm	−13.6 mV
HR9/ pEGFP-NS3	˜ 853 nm	+8.82 mV
HR9/pcDNA-NS3	˜ 181 nm	+6.35 mV
pcDNA-Hsp27-NS3	˜ 889 nm	−8.55 mV
HR9/ pcDNA-Hsp27-NS3	˜ 465 nm	+12.7 mV
pEGFP-Hsp27-NS3	˜ 552 nm	−7.31 mV
HR9/ pEGFP-Hsp27-NS3	˜ 440 nm	+7.87 mV
rNS3 protein	˜ 156 nm	+11.3 mV
rHsp27-NS3 protein	˜ 240 nm	+6.97 mV
Cady-2/rNS3 protein	˜ 211 nm	+9.11 mV
Cady-2/rHsp27-NS3 protein	˜ 291 nm	+2.72 mV

3.2. Characteristics of the Cady-2/rNS3 or Cady-2/rHsp27-NS3 nanoparticles

At first, both the recombinant NS3 [as previously performed in Ref. [18]] and Hsp27-NS3 proteins could be expressed in *E. coli* Rosetta strain at 4 h after IPTG induction, and purified under denaturing conditions. The results indicated that the purified NS3 and Hsp27-NS3 proteins migrated as clear bands of ˜32 kDa and ˜59 kDa in SDS-PAGE (Supplementary 1). Furthermore, the recombinant proteins were detectable using anti-His antibody in western blotting. The recombinant NS3 and Hsp27-NS3 proteins had a concentration range between 0.6 and 0.8 mg/mL. Then, the formation of carrier-cargo complexes (Cady-2/rNS3 or Cady-2/rHsp27-NS3) was confirmed by SDS-PAGE. As observed in Fig. 2, chemical dissociation was detected as a dominant band of ˜ 32 kDa and ˜ 59 kDa related to the NS3 and Hsp27-NS3 proteins along with the Cady-2 peptide band in SDS-PAGE indicating formation of complexes over a range of molar ratios. In addition, the formation of discrete nanoparticles for Cady-2/rNS3 and Cady-2/rHsp27-NS3 at molar ratio of 20:1 was confirmed by SEM analysis with an average size of about 80–120 nm and 100–150 nm, respectively (Fig. 3). The diameter and surface charge of nanoparticles were measured using a Zetasizer. As shown in Table 2, Hsp27 increased the diameter (Z. Average) and decreased positive charge in water.

3.3. In vitro cytotoxicity of the peptide/cargo complexes

According to MTT results, the HR9 peptide and the HR9/pEGFP-NS3, HR9/pEGFP-Hsp27-NS3, HR9/pcDNA-NS3 and HR9/pcDNA-Hsp27-NS3 complexes at N/P ratio of 5:1 did not show any considerable cytotoxic effect compared to untreated cells over a period of 48 h (cell viability: 90–96%). Moreover, the Cady-2 peptide and the Cady-2/rNS3 and Cady-2/rHsp27-NS3 complexes at N/P ratio of 20:1 did not reveal any considerable cytotoxic effect compared to untreated cells over a period of 48 h (cell viability: 90–95%). The cell viability was 95–98% for untreated cells. However, the cells treated with 70% ethanol indicated significantly strong decrease in viability (˜12%) compared to other groups ($p < 0.05$). Generally, these peptides and their complexes with DNA or protein were not toxic at the used doses *in vitro* and *in vivo*.

3.4. Delivery of the HR9/DNA and Cady-2/protein complexes into HEK-293T cells

The ability of HR9 peptide complexed with pEGFP-NS3 and pEGFP-Hsp27-NS3 at an N/P ratio of 5 was evaluated to penetrate HEK-293 T mammalian cells by flow cytometry and fluorescence microscopy using GFP reporter marker at 48 h post-transfection. These results showed that HR9 was able to deliver plasmid DNA into mammalian cells indicating HCV NS3 gene expression. The delivery of pEGFP-NS3 was detected in approximately 42.26% ± 1.19 and 20.51% ± 0.94 of HEK-293 T cells treated with TurboFect and HR9 peptide, respectively. The percentage of GFP-positive cells was 80.63 ± 2.76 and 23.10% ± 1.99 after transfection with pEGFP-C3 + TurboFect (as a positive control) and pEGFP-C3 + HR9 complexes, respectively. The results indicated that HR9 was able to deliver the plasmid NS3 DNA non-covalently into HEK-293 T cells. In addition, HR9 and TurboFect could deliver pEGFP-Hsp27-NS3 with the transfection rates of about 33.34% ± 0.94 and 57.96% ± 0.62, respectively. Fluorescent microscopy showed no

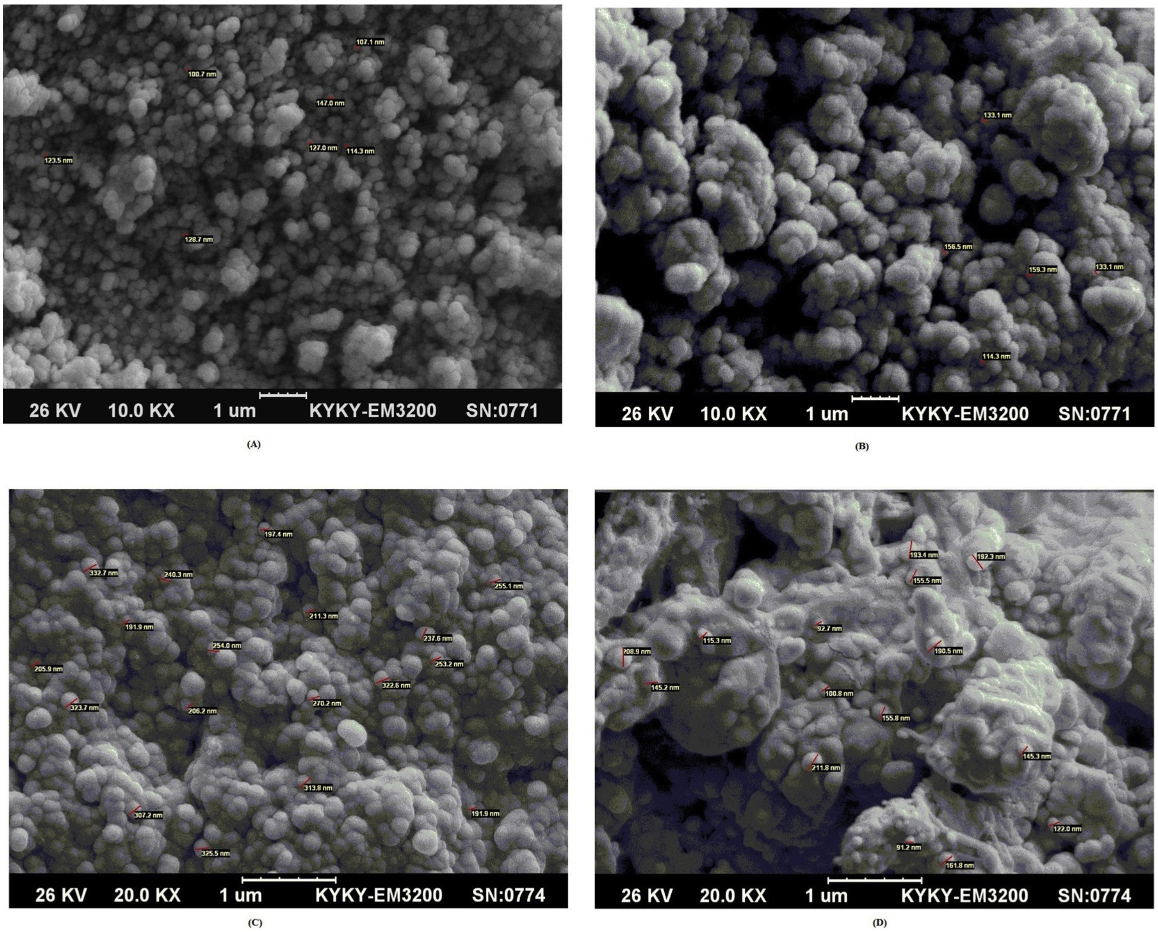

Fig. 1. The SEM micrograph of the spherical HR9/ pEGFP-NS3 nanoparticles (A), HR9/ pcDNA-NS3 nanoparticles (B), HR9/ pcDNA-Hsp27-NS3 nanoparticles (C) and HR9/ pEGFP-Hsp27-NS3 nanoparticles (D) formed at N/P = 5:1 at 25 °C.

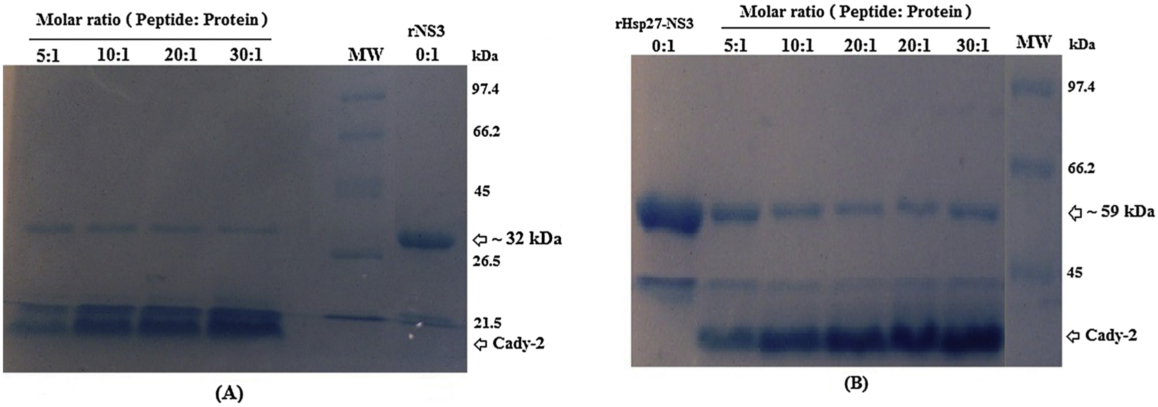

Fig. 2. Analysis of Cady-2/rNS3 (A) and Cady-2/rHsp27-NS3 (B) complexes at different molar ratios using SDS-PAGE; MW is molecular weight marker (Fermentas).

detectable signal in the cells treated with the plasmid DNA alone. In contrast, green fluorescence was observed in the cells treated with HR9/NS3 and HR9/Hsp27-NS3 DNA complexes (Fig. 4). These results indicated that HR9/NS3 DNA and HR9/Hsp27-NS3 nanoparticles had the ability to enter cells.

The delivery of rNS3/Cady-2 and rHsp27-NS3/Cady-2 nanoparticles was investigated in the HEK-293 T cells compared to the cells transfected by NS3 or Hsp27-NS3 protein alone using western blotting at 3 h after transfection. The dominant bands of ˜ 32 kDa and ˜ 59 kDa were detected in transfected cells with rNS3/Cady-2 and rHsp27-NS3/Cady-2 using the anti-His tag antibody (Fig. 5). The corresponding bands were not detected in the untransfected cells or transfected with proteins alone indicating that Cady-2 could transfer NS3 and Hsp27-NS3 proteins into the cells.

3.5. Evaluation of antibody responses

Mice immunization was performed by various regimens as shown in Table 1. The proper dilutions of serum and antibody were determined to evaluate antibody responses. The antibody detection in serially diluted sera revealed that the IgG1 levels against NS3-coated antigen start to decrease from 1:500, although the IgG2a level reduced from 1:200

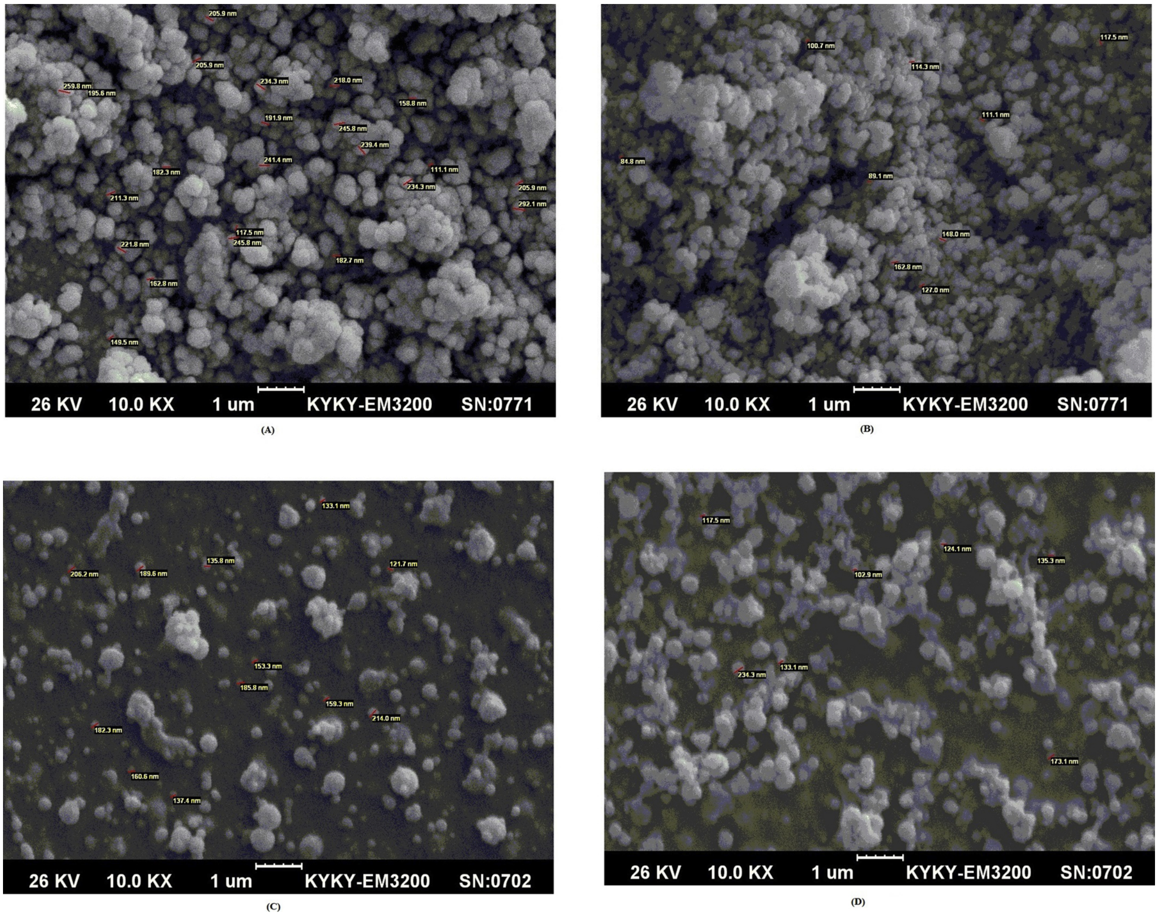

Fig. 3. SEM electron microscopy of rNS3 (A), Cady-2/rNS3 (B), rHsp27-NS3 (C) and Cady-2/rHsp27-NS3 (D); SEM micrograph of spherical nanoparticles formed at a molar ratio of 20:1 at 10,000 × magnification.

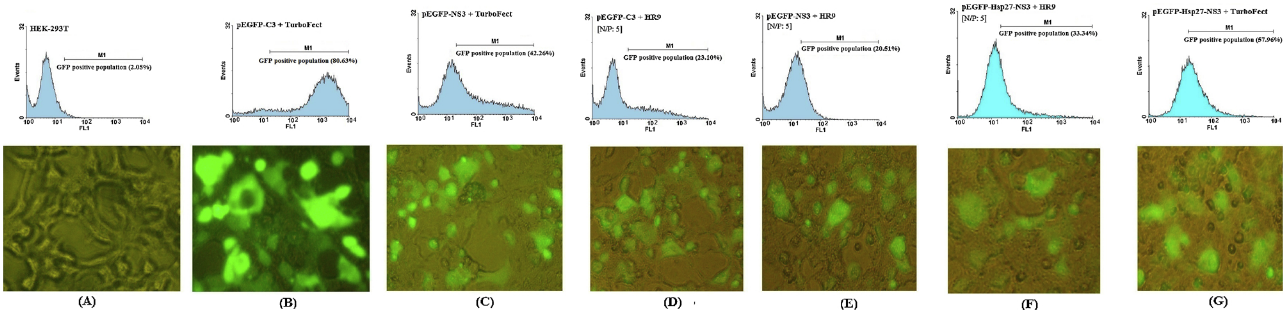

Fig. 4. HR9 CPP-mediated NS3 and Hsp27-NS3 DNA delivery in HEK-293 T cells by Fluorescent microscopy and Flow cytometry: A) HEK-293 T as a negative control; B) pEGFP-C3/TurboFect as a positive control; C) pEGFP-NS3/TurboFect; D) pEGFP-C3/HR9 at an N/P ratio of 5:1; E) pEGFP-NS3/HR9 at an N/P ratio of 5:1; F) pEGFP-Hsp27-NS3/TurboFect; G) pEGFP-Hsp27-NS3/HR9 at an N/P ratio of 5:1.

serum dilution; thus, the ratio of 1:100 dilution was selected in all experiments. Furthermore, the ratio of IgG1, IgG2a, IgG2b and total IgG (*i.e.,* 1:2000, 1:5000, 1:10,000, 1:15,000 and 1:20,000 dilutions for each antibody) was set up in experiments. The OD_{450} was decreased between 1:15,000 and 1:20,000; thus, the 1:10,000 dilutions were used for all antibodies (data not shown). Then, the levels of total IgG and the related subclasses in mice sera against the rNS3 protein were assessed in different groups three weeks (Supplementary 2) and two months after the last immunization. Due to the similarity of antibody responses in two different times, we showed the levels of antibodies two months after the second booster indicating the stability of antibody secretion after a long time (Fig. 6). Our data showed that the level of total IgG in group immunized with homologous rHsp27-NS3 + Cady-2 prime/ rHsp27-NS3 + Cady-2 boost protein regimen (G4) did not show significant differences as compared to group immunized with heterologous pcDNA-Hsp27-NS3 + HR9 prime/ rHsp27-NS3 + Cady-2 boost (G10; $p > 0.05$). In protein regimens, groups receiving Hsp27 fused to rNS3 (G2 and G4) generated a higher total IgG than groups receiving rNS3, alone (G1 and G3; $p < 0.05$). In DNA regimens, the level of total IgG in group receiving homologous pcDNA-Hsp27-NS3 + HR9 prime/ pcDNA-Hsp27-NS3 + HR9 boost (G8) was significantly higher than other DNA groups ($p < 0.001$). All groups immunized with homologous DNA showed higher levels of total IgG compared to group immunized with NS3 DNA alone (G5, $p < 0.001$). In heterologous prime/boost regimens, there are no considerable differences in total IgG level between groups receiving pcDNA-NS3 + HR9/ rHsp27-NS3 (G12), pcDNA-NS3 + HR9/rNS3 + Cady-2 (G13) and pcDNA-NS3 + HR9/ rNS3 + IFA (G11, $p > 0.05$, Fig. 6A). Moreover, the results showed

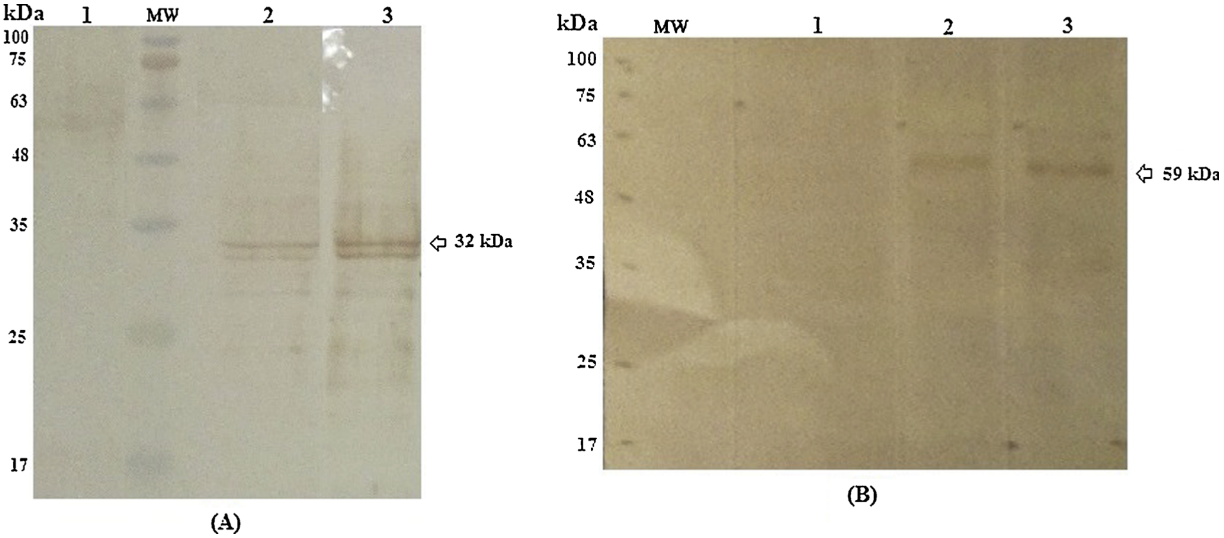

Fig. 5. Delivery efficiency of NS3 and Hsp27-NS3 proteins using Cady-2 at a molar ratio of 1:20 (protein/Cady-2) in HEK-293 T cells for 3 h after transfection by Western blotting: A) untransfected cell (Lane 1), the transfected cells with rNS3/ TurboFect complex (Lane 2), the transfected cells with rNS3/ Cady-2 complex (Lane 3); B) untransfected cell (Lane 1), the transfected cells with rHsp27-NS3/ TurboFect complex (Lane 2), the transfected cells with rHsp27-NS3/ Cady-2 complex (Lane 3). MW is the molecular weight marker (prestained protein ladder, 10–170 kDa, Fermentas).

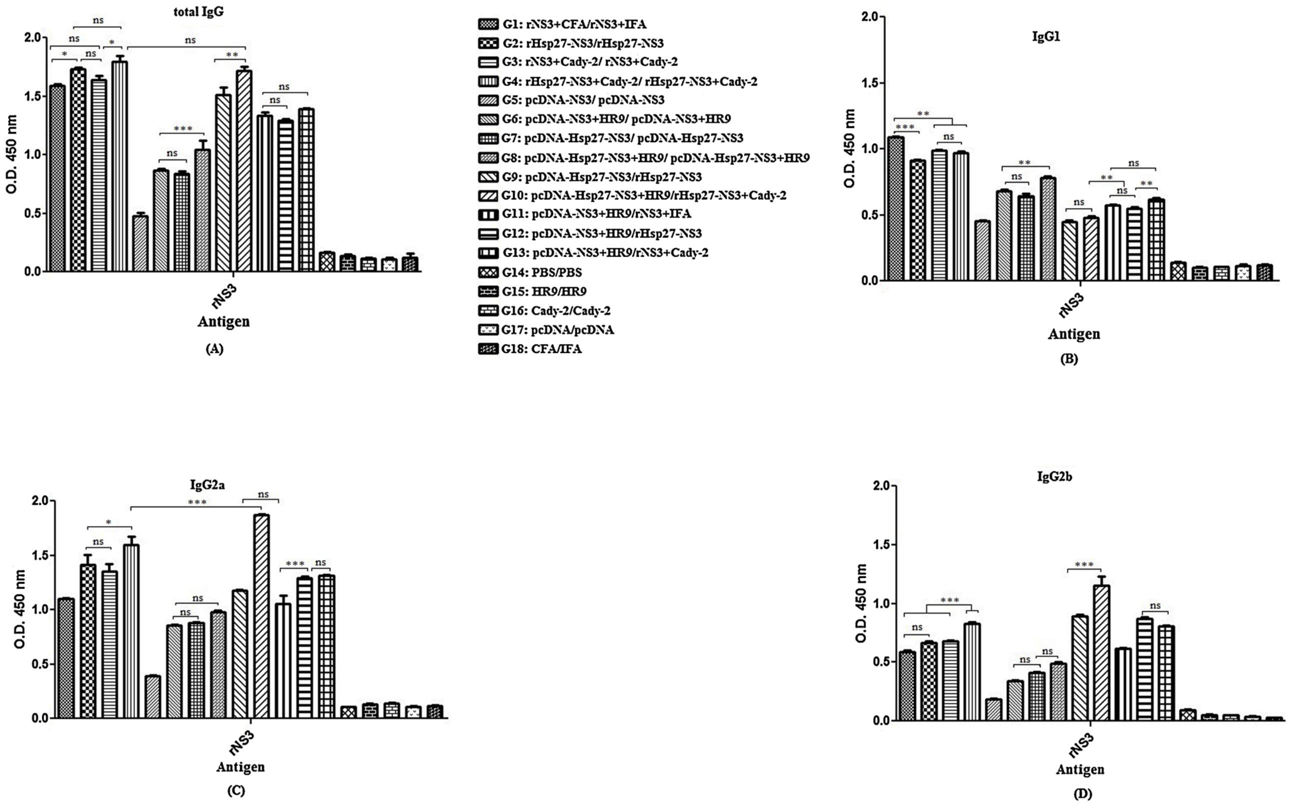

Fig. 6. Evaluation of antibody responses (total IgG, IgG1, IgG2a and IgG2b; 1:10,000 dilutions) against NS3 antigen in different regimens: Mice sera were prepared from whole blood samples of each group (n = 4) two months after the last immunization. All analyses were performed in duplicate for each sample. The results from the 1:100 sera dilutions are shown as mean absorbance at 450 nm ± SD.

that the level of IgG1 in group immunized with rNS3 + CFA prime/ rNS3 + IFA boost (G1) was significantly higher than that in other groups ($p < 0.05$). Among groups receiving DNA regimens, group immunized with pcDNA-Hsp27-NS3 + HR9 prime/ pcDNA-Hsp27-NS3 + HR9 boost (G8) generated a higher IgG1 level than other DNA groups ($p < 0.01$). However, groups receiving heterologous prime/boost regimens showed lower levels of IgG1 as compared to other groups ($p < 0.05$, Fig. 6B). Other results showed that IgG2a level was higher in group immunized with heterologous pcDNA-Hsp27-NS3 + HR9 prime/rHsp27-NS3 + Cady-2 boost (G10) than that in other groups ($p < 0.001$). In protein regimens, the level of IgG2a in group immunized with homologous rHsp27-NS3 + Cady-2 prime/ rHsp27-NS3 + Cady-2 boost protein regimen (G4) was higher than other protein groups ($p < 0.05$). In DNA regimens, groups 6, 7 and 8 receiving pcDNA-NS3 + HR9, pcDNA-Hsp27-NS3 and pcDNA-Hsp27-NS3 + HR9, respectively produced higher IgG2a levels than group 5 receiving pcDNA-NS3 ($p < 0.001$,

Fig. 6C). The levels of IgG2b showed similar results with IgG2a among these groups (Fig. 6D). Our data indicated that Cady-2 and Hsp27 could elicit more effective NS3-specific IgG2a and IgG2b responses than Freund's adjuvant in heterologous prime/boost and also protein regimens in mouse model ($p < 0.05$, Fig. 6 C & D). However, the levels of IgG2a and IgG2b in the sera of mice immunized with the heterologous Hsp27-NS3 DNA + HR9 prime/rHsp27-NS3 protein + Cady-2 boost (G10) was significantly higher than that in other groups ($p < 0.05$) indicating high potency of heterologous prime/boost strategy. In addition, the Freund's adjuvant could induce high total IgG and IgG1 in groups immunized with protein/protein and DNA/protein modalities indicating its importance for stimulation of humoral immunity *versus* cellular immunity. These studies showed that the ratio of mean IgG2a/ IgG1 was higher in groups immunized by pcDNA-Hsp27-NS3 + HR9/ rHsp27-NS3 + Cady-2 (G10, ˜ 4 fold) and then rHsp27-NS3 + Cady-2/ rHsp27-NS3 + Cady-2 (G4, ˜1.6 fold) as compared to other groups indicating direction of immune system toward Th1 response.

3.6. Cytokine assay

For determination of Th1 and Th2 immune responses, IFN-gamma and IL-5 cytokines were assessed in mice splenocytes, respectively. Our data indicated that all mice immunization with different modalities effectively increased the IFN-γ secretion as compared to control groups ($p < 0.05$, Fig. 7A). Furthermore, mice immunization with rHsp27-NS3 + Cady-2/rHsp27-NS3 + Cady-2 indicated significant differences in IFN-γ secretion with other groups ($p < 0.05$). On the other hand, the level of IFN-γ in DNA immunization was significantly higher in mice immunized with pcDNA-Hsp27-NS3 + HR9 prime/ pcDNA-Hsp27-NS3 + HR9 boost (G8) compared to the groups injected with other DNA regimens ($p < 0.05$, Fig. 7A). In heterologous prime/boost immunization, the levels of IFN-γ in groups immunized by pcDNA-Hsp27-NS3 + HR9/rHsp27-NS3 + Cady-2 (G10) and then pcDNA-NS3 + HR9/ rNS3 + Cady-2 (G13) were higher than other groups indicating a major role of Cady-2 for protein delivery. The order of the highest IFN-γ secretion was G4 > G3 > G2 > G1 = G10. The secretion of IFN-γ was similar between G1 (rNS3 + Freund's adjuvant) and G10 (Hsp27-NS3 DNA + HR9/ rHsp27-NS3 + Cady-2, $p > 0.05$). There are no significant differences between the levels of rNS3-specific IL-5 secretion in the groups injected with the heterologous DNA prime/protein boost and control groups ($p > 0.05$, Fig. 7B). All groups immunized by homologous DNA and protein regimens were significant as compared to control groups ($p < 0.001$). However, there is no significant secretion between groups immunized with DNA and protein regimens ($p > 0.05$). On the other hand, the ratio of mean IFN-γ/ IL-5 for G1, G2, G3, G4 and G10 was about 31, 35, 40, 35 and 73 pg/mL, respectively. This result demonstrated the direction of immune responses for G10 toward Th1 response with respect to the highest IFN/IL-5 ratio.

3.7. Granzyme B secretion

For determination of *in vitro* CTL activity, the secretion of Granzyme B was measured two months after the last immunization. The data showed that the mice immunized with the heterologous pcDNA-Hsp27-NS3 + HR9 prime/rHsp27-NS3 + Cady-2 boost (G10) showed higher levels of Granzyme B than other groups ($p < 0.001$). On the other hand, the groups immunized with rHsp27-NS3 + Cady-2 prime/rHsp27-NS3 + Cady-2 boost (G4) produced significantly higher concentration of Granzyme B than the groups immunized with other protein regimens (G1, G2 and G3, $p < 0.05$, Fig. 8). Moreover, the secretion of Granzyme B was significantly higher in three groups immunized with DNA (G6, G7, G8) compared to G5 (*i.e.,* pcDNA-NS3, alone; $p < 0.001$).

4. Discussion

Many studies were performed to improve the cellular uptake of therapeutic agents [22]. Recently, small cationic and/or amphipathic peptides have attracted a special attention as non-viral delivery systems due to their ability to mediate uptake of a variety of macromolecular cargos [23,24]. Cationic CPPs can penetrate into the cells without eliciting a cellular response. At least eight positive charges were essential for effective uptake of various cationic CPPs [25]. Amand et al. demonstrated that increased arginine content in small peptides such as HR9 can improve proteoglycan-dependent uptake [26]. Moreover, amphipathic peptides including Cady-2 and Pep-1 were able to deliver some proteins into mammalian cells [10]. In this study, we evaluated the ability of two cell penetrating peptides, HR9 and Cady-2, for *in vitro* and *in vivo* delivery of HCV NS3 DNA and protein as an antigenic agent, respectively. The previous findings showed that NS3 protein is an attractive candidate for development of therapeutic vaccines that induce T cell-mediated immune responses against HCV infections. In general, some prophylactic and therapeutic vaccines against HCV harboring NS3 were developed in chimpanzees and clinical trials [27]. A study demonstrated that HCV core protein increased both $CD4^+$ and $CD8^+$ T_{regs} which help to persistent infection, whereas HCV NS3 stimulated both $CD4^+$ and $CD8^+$ effector T cells which lead to viral clearance [28]. Another study indicated that plasmid DNA encoding the NS4A protein in a construct harboring NS3 reduced the immunogenicity of NS3, whereas the use of a cytolytic protein, perforin (PRF), increased NS3 immunogenicity in C57BL/6 mice [29]. Jiao et al. showed that an immunodominant epitope recognized by the NS3-specific Th cells was located at amino acid 1251–1259 of NS3 protein which was completely

Fig. 7. IFN-γ and IL-5 levels in immunized groups with various formulations (A and B): Pooled splenocyte cultures were prepared from four mice in each group (n = 4 per group) and re-stimulated with rNS3 *in vitro*. The levels of IFN-γ and IL-5 were determined in the supernatants with ELISA as mean absorbance at 405 nm ± SD for each set of samples. All analyses were performed in duplicate for each sample. The detection limit was 2 and 7 pg/ mL for IFN-γ and IL-5, respectively.

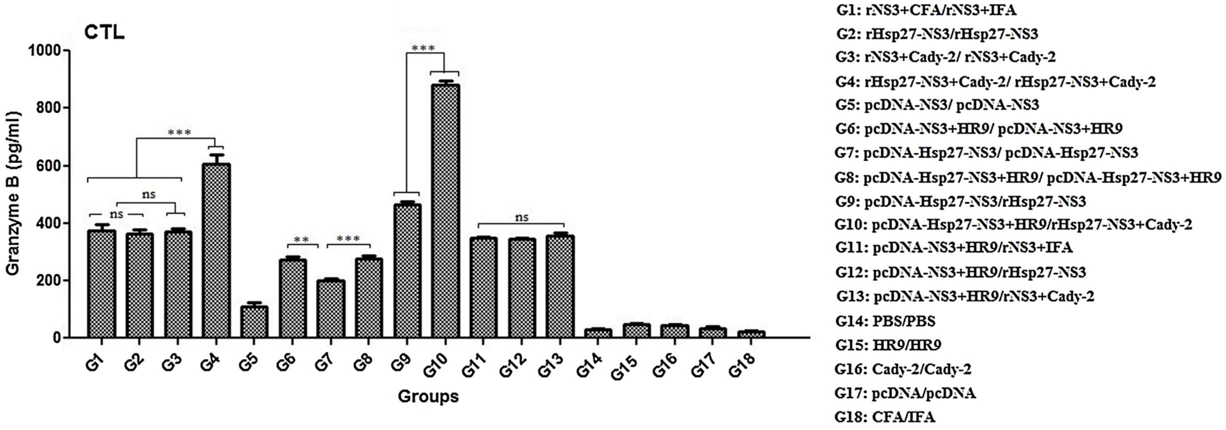

Fig. 8. Granzyme B concentration measured by ELISA using pooled splenocytes from four mice in each group (n = 4 per group): Mice injected with pcDNA-Hsp27-NS3 + HR9 prime/rHsp27-NS3 + Cady-2 boost (G10) secreted significantly higher level of Granzyme B than all other groups. All analyses were performed in triplicate for each sample. The results represent mean values calculated from triplicate samples as well as the standard deviation (SD) as error bars.

conserved within HCV1a, 1b, 1c, 2a and 2b genotypes [30]. Thus, NS3 is a valuable candidate in therapeutic vaccine development. Our previous study indicated that small Hsp20 conjugated with NS3 protein significantly enhanced the levels of IgG2a, IgG2b and IFN-γ directed toward Th1 responses compared to other groups [18]. On the other hand, another study done by our group showed that the combination of Hsp27 with the recombinant hPP10-E7 protein in homologous protein/protein (hPP10-E7 + Hsp27) and heterologous DNA/protein (pcDNA-E7 + MPG/ hPP10-E7 + Hsp27) significantly enhanced the human papillomavirus (HPV) E7-specific T cell responses and also displayed complete protection more than 60 days after treatment in tumor mouse model. The efficiency of small Hsp27 (HspB1) as an adjuvant mixed with HPV E7 antigen was importantly higher than Hsp20 (HspB6) as another adjuvant [31]. Thus, we used small Hsp27 as an effective adjuvant conjugated to HCV NS3 antigen in this study. Herein, small Hsp27 was used as an adjuvant as well as Freund's adjuvant for increasing antigen-specific immune responses. In this line, the recombinant Hsp27-NS3 DNA and protein were generated in bacterial systems. The recombinant NS3 and Hsp27-NS3 DNA and protein were mixed with HR9 and Cady-2 for formation of nanoparticles. The results showed that HR9 at N/P ratio of 5:1 and Cady-2 at molar ratio of 20:1 (peptide: protein) could form stable nanoparticles through non-covalent binding with NS3/ Hsp27-NS3 DNA and NS3/ Hsp27-NS3 protein, respectively. The MTT assay indicated that the HR9 peptide complexed with DNA or Cady-2 complexed with protein at the used concentration did not induce any significant cytotoxic effect compared to untreated cells. The delivery of pEGFP-NS3 and pEGFP-Hsp27-NS3 was detected in approximately 42.26% ± 1.19 and 57.96% ± 0.62 of HEK-293 T cells treated with TurboFect, respectively indicating the effect of Hsp27 for further delivery of NS3 gene. Moreover, the pEGFP-NS3 and pEGFP-Hsp27-NS3 delivery was detected in approximately 20.51% ± 0.94 and 33.34% ± 0.94 of HEK-293 T cells treated with the HR9 at N/P ratio of 5:1, respectively confirming the effect of Hsp27 in gene delivery as well as HR9 peptide. A study indicated that two arginine-rich CPPs including HR9 and IR9 were able to enter cargos including plasmid DNA, red fluorescent proteins (RFPs) and semiconductor quantum dots (QDs) into rotifers [32]. Liu et al. demonstrated that HR9 peptide stably and non-covalently combined with QDs was able to enter into cells in a short time (4 min). Dimethyl sulphoxide, ethanol and oleic acid increased HR9-mediated direct translocation of QDs across *cell membrane* [33]. Moreover, three arginine-rich CPPs (SR9, HR9 and PR9) could form stable non-covalently complexes with plasmid DNA as an efficient and safe carrier for transgenesis in eukaryotic protozoans [34]. It was shown that nona-arginine (R9) CPP enhanced the efficiency of QD uptake by A549 cancerous cells [8,35,36]. Mechanistic studies revealed that HR9/DNA complexes mediate the direct membrane translocation pathway for gene delivery [37]. Arginine-rich CPPs were useful and non-toxic tools for insect transgenesis [38]. Some researchers revealed that zeta-potential of CPP/cargo nanoparticles plays an important role in determining delivery efficiency, while particle sizes of CPP/cargo nanoparticles have a minor effect in cell permeability. Furthermore, the route of internalization is not affected by cell type, but is dependent on the nature of both CPPs and related cargoes [39]. A study showed that combination of CPP/cargo could block the endocytosis pathway and translocate directly into cells. These CPPs have benefits such as ease of synthesis, high internalization and low cytotoxicity [40]. Our study confirmed the non-toxic effects of HR9 and Cady-2 in nanoparticles.

Our previous study showed that Hsp27 linked to HIV-1 Nef could increase its delivery in HEK-293 T cells as a suitable carrier in DNA vaccine design [19]. In the current study, we confirmed the Hsp27 role as a suitable carrier as well as HR9 for HCV NS3 DNA construct. Subsequently, evaluation of the HCV NS3-specific antibody and T cell immune responses was performed in BALB/c mice. Our data showed that heterologous pcDNA-Hsp27-NS3 + HR9 prime/rHsp27-NS3 + Cady-2 boost regimen could elicit an increased level of IgG2a, IgG2b, Granzyme B and IFN-gamma/IL-5 ratio compared to other groups. Although, groups immunized with homologous proteins especially rHsp27-NS3 + Cady-2/rHsp27-NS3 + Cady-2 (G4) increased significantly the secretion of IFN-gamma compared to pcDNA-Hsp27-NS3 + HR9 prime/rHsp27-NS3 + Cady-2 boost regimen (G10), but the ratio of IFN-gamma/IL-5 was considerably higher in G10 than that in other groups. Moreover, the ratio of IgG2a/IgG1 was higher in G10 and then G4 as compared to other groups. These results indicated to direct immune system toward Th1 *versus* Th2 responses in group immunized with heterologous prime/boost regimen (G10). In addition, the highest Granzyme B secretion was observed in this group (G10) indicating *in vitro* CTL activity. It seems that the use of Hsp27 and Cady-2 is more effective than HR9 peptide in stimulating IgG2a, IgG2b, IFN-gamma and Granzyme B secretion and subsequently Th1 response as observed in group immunized with rHsp27-NS3 + Cady-2/rHsp27-NS3 + Cady-2 (G4).

Milani et al. demonstrated that the recombinant Hsp27-Nef fusion protein could induce Th1 immune responses against HIV-1 Nef antigen [15]. Moreover, the use of Tat (PTD)-Nef antigen in prime-boost strategy along with Gp96 adjuvant (a heat shock protein) and Cady-2 CPP could increase HIV Nef-specific immune responses in mice [41]. Hsp27 (/HspB1) is an ATP-independent chaperone that confers protection against apoptosis through various mechanisms including a direct interaction with cytochrome *c*. [42]. Its level was up-regulated when cells were exposed to conditions that alter protein folding. Hsp27 possesses anti-apoptotic and antioxidant properties [43,44]. In a study, dendritic cells pulsed with Hsp27 and Hsp90AA1 peptides were used to stimulate peripheral blood mononuclear cells (PBMCs) from myeloma patients to generate peptide-specific CTLs. The findings showed that Hsp peptide-specific CTLs effectively reduced tumor growth in the xenograft mouse model of myeloma [45]. Other study demonstrated that Hsp27 acts as a direct inhibitor of actin polymerization and protectant against BBB disruption after ischemia/reperfusion [46].

At present, two vaccines have achieved into preclinical and clinical trials: a) A recombinant form of the virus envelope glycoproteins E1 and E2 aimed at inducing neutralizing antibodies and CD4 helper T cells; b) A vector-based vaccine encoding the virus NS proteins (NS3-NS5) using chimpanzee adenovirus priming and modified vaccinia Ankara (MVA) boost aimed at inducing virus-specific polyfunctional CD4 and CD8 T cells in healthy volunteers. This vaccine is currently in phase II clinical trials (NCT01436357) [47]. However, development of an effective non-viral vaccine against HCV infection is of a great importance. In this line, some nanoparticle vaccines were improved against HCV infections. For instance, gelatin nanoparticles enhanced delivery of HCV recombinant NS2 gene [48]. On the other hand, challenge experiments with *Vaccinia* virus expressing HCV epitopes emphasized the use of neutral liposomes as HCV vaccine [49,50].

Many studies showed that strong and persistent $CD8^+$ and $CD4^+$ T-cell responses are critical in HCV clearance, as well as cytokine-induced factors that can directly inhibit virus replication [51,52]. *In vitro* CTL activity could be determined by Granzyme B secretion. Of course, there are some challenges in this connection; *e.g.,* Shafer-Weaver et al. indicated that the release of Granzyme B by cytolytic lymphocytes upon effector-target interaction may be a more specific indicator of CTL and NK cytotoxic ability than IFN-γ secretion [53]. On the other hand, Lin et al. reported that Granzyme B secretion by human memory $CD4^+$ T cells is less strictly regulated compared to memory $CD8^+$ T cells [54]. Our study showed that group receiving pcDNA-Hsp27-NS3 + HR9 prime/rHsp27-NS3 + Cady-2 boost regimen (G10) generated higher Granzyme B secretion and IFN-gamma/IL-5 ratio than other groups.

In conclusion, non-viral delivery systems including peptide-based systems are effective for cellular uptake with minimal toxicity and low levels of immunogenicity. Our data showed that heterologous prime/boost (G10: Hsp27-NS3 DNA + HR9/ rHsp27-NS3 protein + Cady-2) induced high levels of IgG2a, IgG2b, IFN-γ and Granzyme B and low levels of IgG1 and IL-5 directed toward Th1 responses as compared to other immunization strategies. Subsequently, homologous rHsp27-NS3 + Cady-2/ rHsp27-NS3 + Cady-2 (G4) regimen was also more effective than other groups indicating the important roles of Hsp27 and Cady-2 as an adjuvant and a carrier, respectively. Briefly, the combination of a natural adjuvant (Hsp27) and CPPs (HR9 and Cady-2) could significantly stimulate immune responses as a promising approach for development of HCV therapeutic vaccines.

Author contributions

A.B. designed the project. S.A. and A.B. conducted the experiments. A.B., S.I. and S.M.S. analyzed the results. S.A. and A.B. wrote the main manuscript. A.B. critically revised the manuscript for important intellectual content. All authors have approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.06.011.

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