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A non-pathogenic *Leishmania tarentolae* vector based- HCV polytope DNA vaccine elicits potent and long lasting Th1 and CTL responses in BALB/c mice model

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ABSTRACT

Despite successful anti-viral (DAAs) treatment of Hepatitis C virus (HCV) infection, recent data indicated the need for an effective vaccine. Preexisting anti-vector immunity is an obstacle for application of live vectors for antigen delivery and development of effective T-cell based HCV vaccines. Herein, we report construction of recombinant *Leishmania tarentolae*, a lizard (non-human) parasite, expressing an HCV polytope DNA, PT-NT(gp96), encoding for several immunogenic HCV epitopes and evaluation of its immunogenicity in three different prime/boost immunization groups (G) of BALB/c mice. Homologous prime/boost immunization by *L.tarentolae*-PT-NT(gp96) either with or without CpG (G1 and G2 respectively) and heterologous immunization with a PT-NT(gp96) encoding-pCDNA plasmid followed by *L.tarentolae*-PT-NT (G3) was undertaken. Immune responses were measured three and nine weeks (W) post immunization. Splenocytes (cultured with antigen-stimulant) of mice in G1 showed the highest percentage of specific CTL-cytolytic activity compared to G2 and G3 at both short (W3:70.98% versus 41.29% and 13.12%) and long (W9: 50% versus 24.5% and 20%) term periods, accompanied with high levels of secreted IFN-γ. Comparison of IFN-γ, IL-4, IL-17 and TNF-α cytokines levels obtained from the supernatant of antigen-stimulated splenocytes as well as antibodies level (as IgG1/IgG2a ratio; obtained from sera of immunized mice) indicated higher Th1 oriented responses for G1, G2 groups and balanced Th1-Th17 for G3. Results indicated the potential of *L.tarentolae* (+ CpG), as a non-pathogenic live vaccine vector, for delivery and enhancement of immune responses against HCV-polytope antigens.

1. Introduction

It is estimated that around 70 million people are living with HCV worldwide. Hepatitis C virus is one of the major causes of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC), accounting for 500,000 annual deaths each year (Hill et al., 2017). The recent development of effective direct-acting antivirals (DAAs) has remarkably improved the treatment of HCV chronic infection. However, important challenges including; need of mass global screening to identify infected cases, high cost of DAA, drug resistant strains, host factors, side effects and risk of reinfections as well as progression of liver disease (to even HCC) following curative therapy is remained. These challenges sound that HCV infection control on global scale needs an effective vaccine along with treatment (Bartenschlager et al., 2018; Walker and Grakoui, 2015). But no approved vaccine against HCV infection is available to date. High genetic diversity and immune evasion of the virus are the major obstacles for HCV vaccine development. However, the spontaneous clearance of primary acute HCV infection in about 30% of individuals via immune responses encourages the vaccine studies (Agrawal et al., 2017). Indeed, prior studies in humans and chimpanzees elucidated the key role of strong, broad and long lasting antibodies as well as cellular responses for spontaneous resolution of infection (Bailey et al., 2019). In this context, major contribution of CD4 + Th1 and CD8 + CTL cellular responses, in form of cytotoxic or non-cytotoxic (such as the secretion of antiviral cytokines: IFN-γ or TNF-α) was suggested (J. Shi et al., 2017; Verstrepen et al., 2015). Accordingly,

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majority of the prior vaccine studies were conducted towards invention of T-cell vaccines via utilization of the conserved sequences of HCV structural (core, envelope proteins; E1, E2) and Nonstructural proteins (NS3, NS4, NS5) using different strategies and various vaccine modalities and platforms including; virus like particles (VLPs) and recombinant virus vectors, (Bailey et al., 2019; Tan et al., 2017). To this end, and specially due to the presence of immunosuppressive and immunopathogenic regions in HCV proteins, application of the so called multi-epitope (polytope) strategy to select epitopes from conserved regions of several HCV proteins (with optimization of their codons) was emerged (Memarnejadian et al., 2009; Mohammadzadeh et al., 2016; L. Shi et al., 2006). These types of antigen formulations were shown to be particularly effective when used as DNA vaccines that effectively engage both MHC class I and MHC class II pathways allowing for the induction of both CD8+ and CD4 + T-cells, especially in prime-boost immunization approaches (Ikram et al., 2018; Rollier et al., 2016). The drawback of such vaccines however, is elicitation of weak immune responses which might be overcome by employing different strategies such as use of various adjuvants, prime-boost immunization regimens and routes as well as application of proper platforms for vaccine delivery (Porter and Raviprakash, 2017; Bailey et al., 2019).

Among vaccine delivery-platforms, replication-defective virus vectors are recognized as one of the most promising tools. Indeed, currently a T-cell viral vector-based HCV vaccine encoding for virus nonstructural proteins (NS3 to NS5)and is the only HCV vaccine ever tested in non-infected humans, gets use of two different replication-defective virus vectors (Adenovirus and Modified virus Ankara; MVA) in a Prime–boost immunization strategy (https://clinicaltrials.gov/ct2/show/NCT01436357). However, application of these kinds of vectors for vaccine delivery is usually hampered by the presence of the pre-existing anti-vector immunity in the general population that might induce the neutralization of the vector up on immunization, a major obstacle to overcome.

Recently *Leishmania tarentolae* is introduced as a new live vector candidate for vaccine delivery against intracellular pathogens. Of note, *L. tarentolae* is a lizard parasite that is not a pathogen for humans and thus there is no problem of the preexisting anti-vector immunity concerning its application as a vaccine delivery system (Breton et al., 2007; Montakhab-Yeganeh et al., 2017; Salehi et al., 2012). Moreover, it targets APCs (macrophages and dendritic cells) and secondary lymphoid organs without replicating within the targeted cells. Therefore, in contrast to replication-defective virus vectors, *L. tarentolae,* needs no extra modification on its genome to make it a non-replicating vector (Breton et al., 2005). Accordingly, *L. tarentolae* allows insertion of large amount of exogenous DNA. Another important pro of this organism is the ability of rapid growth in cell free media with minimal nutritional requirements (Basile and Peticca, 2009). The potency of *L. tarentolae* to target APCs might increase the antigen presentation through MHCI and MHCII pathways. This characteristic might induce stronger CD4 + Th cell and CD8 + CTL responses which are essential for the control of viral infections. It also stimulates the process of dendritic cell maturation and induces CD4 + T cell proliferation and IFN-γ production which leads to a Th1-skewed phenotype (Kumar and Samant, 2016). Only a few prior studies addressed the application of *L. tarentolae* for the vaccine delivery. The efficiency of this system for induction of high levels of IFN-γ, Th1 responses and protection against *Leishmania* infection in mice and dog models is reported (Abdossamadi et al., 2017; Shahbazi et al., 2015). In addition, immunization studies by either *L. tarentolae*-expressing HIV-1 Gag (Breton et al., 2007) or Human Papiloma virus (HPV)-16 E7 proteins (Salehi et al., 2012) indicated the induction of strong cell mediated and Th1-directed immunity in mice model. But to our knowledge, application of this vector for delivery of HCV antigens is not reported to date. We proposed that *L. tarentolae* with characteristics such as non-pathogenic to humans and potential of inducing strong cellular immunity might be a worthy platform as a live vector for delivery of HCV antigens. To address this concern, in the present study, we used a previously reported HCV polytope (PT) DNA construct, PT-NT (gp96), encoding for several HCV CD8 + CTL and T-helper CD4+ epitopes fused to the N-terminal domain of heat shock protein gp96 (NT(gp96)) (Pishraft-Sabet et al., 2015. The generated *L. tarentolae* PT-NT (gp96) was used in different immunization studies including homologous prime-boost (live/live) strategies alone or in combination with CpG as adjuvant or heterologous prime-boost (DNA/live) strategy with a DNA plasmid encoding the same antigen (pcDNA-PT-NT (gp96)).

2. Materials and methods

2.1. DNA constructs

The pEGFP-PT-NT (gp96) vector harboring the HCV polytope (PT) DNA construct that encoded for immunodominant CD8 + T cell epitopes (HLA-A2 and H2-Dd) from Core (132–142), NS3 (1073–1081), NS5B (2727–2735) as well as a CD4+ epitope from NS3 (1248–1262) and a B-cell epitope from E2 (412–426) fused to the N-terminal domain of heat shock protein gp96 (NT(gp96)) was described before (Pishraft-Sabet et al., 2015). This vector was used as PCR template for amplification of the PT-NT (gp96) fragment using the following primers (forward NR1: 5′-ATT GCT AGC CTC GAG ACC ATG GGC CAA CTG-3′) including *NheI–SlaI* restriction sites (underlined) and (reverse NR2: 5′-GGC GGT ACC TTT GTA GAA GGC TTT GTA TTC-3′) including *KpnI* restriction site (underlined). The PCR-amplified PT-NT (gp96) fragment was cloned into the *KpnI* and *NheI* sites of pEGFP-N3 vector (Clontech, USA). Subsequently, the PT-NT(gp96)-EGFP fragment was subcloned into the *NheI* and *NotI* sites of pcDNA3.1 (+) vector (Invitrogen, Germany) to generate pcDNA-PT-NT(gp96)-EGFP plasmid as a DNA vaccine. The pcDNA-PT-NT(gp96)-EGFP was purified by ion exchange chromatography with Endo Free Plasmid Giga Kit (QIAGEN, Germany). The pLEXSY-Neo2 vector (EGE-233, Jena bioscience, Germany) was used as integrative vector for transfecting the EGFP gene and PT-NT(gp96)-EGFP fragment into *L. tarentolae*. First, the EGFP gene was amplified from pEGFP-N3 vector using the following primers (forward EGFP1: 5′- GCC ACC AGA TCT ATG GTG AGC AAG GGC -3′) including *BgLII* restriction site (underlined) and (reverse EGFP2: 5′- GCC GCT GGT ACC TTA CTT GTA CAG CTC GTC -3′) including *KpnI* restriction site (underlined). Subsequently, the PCR-amplified EGFP gene was cloned in to the *BgLII* and *KpnI* sites of pLEXY-Neo2 vector to generate PLEXY-EGFP. Finally, PT-NT(gp96)-EGFP fragment was subcloned into the *SlaI* and *NotI* sites of pLEXSY-Neo2 vector to generate pLEXSY-PT-NT(gp96)-EGFP. Precision and accuracy of the constructs was confirmed by restriction analyses and DNA sequencing. Molecular and cloning procedures were based on routine protocols (Sambrook and Russell, 2006) and/or manufacturer recommendations.

2.2. Parasite growth and transfections

The *L. tarentolae* Tar II (ATCC30.267) strain was grown at 26 °C in M199 medium (pH 7.2) supplemented with 40 mM HEPES, 5 μg/ml hemin, 0.1 mM adenosine, 50 μg/ml gentamicine and 5% heat inactivated fetal serum. *Leishmania* transfection was carried out by electroporation as previously described (Saljoughian et al., 2013). Briefly, the pLEXSY-PT-NT(gp96)-EGFP was first linearized using *SwaI* restriction enzyme and then 2 μg was added to the 4×10^7 log-phase parasites in 300 μl of electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose; pH 7.5), stored on ice for 10 min and electroporated at 450 v and 500 MF (BioRad Gene Pulser Ecell, Germany) as described previously (Saljoughian et al., 2013). Subsequently, promastigotes were incubated at 26 °C for 24 h in M199 medium supplemented with 10% FCS without G418 and then were grown on solid media (2x M199 medium containing 2% noble agar, 10% FCS, and 50 μg /ml G418). After 7–10 days several colonies were randomly selected and propagated in liquid M199 medium supplemented with 10% FCS and different concentration of G418 (the concentration of drug was gradually increased to 200 μg/ml).

2.2.1. Confirmation of PT-NT(gp96)-EGFP gene integration in to transgenic L. tarentolae

Integration of the expression cassette into the proper site of the parasite genome (*ssu* locus) was confirmed by diagnostic PCR, according to the manufacturer instructions (LEXSY kits, Jena bioscience, Germany). Briefly, the recombinant *L. tarentolae* genomic DNA was extracted by GF-1 Genomic DNA extraction kit (Vivantis, Malaysia) and used as template. The diagnostic PCR was performed by using the following primers: F3001 (forward): 5′-GAT CTG GTT GAT TCT GCC AGT AG-3′ and A1715 (reverse): 5′-TAT TCG TTG TCA GAT GGC GCA C-3′) that hybridize with the 5´ssu sequence of *L. tarentolae* and 5´UTR of the target gene, respectively. In case of the integration of the expression cassette into the ssu locus of *L. tarentolae*, the diagnostic PCR would yield a 1 kb fragment. The genomic DNA of *L. tarentolae* wild type was used as a control. To detect the PT-NT (gp96) cDNA, PCR reaction was performed with specific primers. The accuracy of the PCR products was further confirmed by DNA sequencing.

2.2.2. Fluorescence microscopy

Promastigote forms of *L. tarentolae*-PT-NT(gp96)-EGFP, after centrifugation at 3000 rpm for 10 min, washed and resuspended in PBS and analyzed for EGFP expression by Epi-fluorescence microscopy (Nikon, E200, ACT-1 soft water, Digital sight camera, Japan). The EGFP-expressing *L. tarentolae* was used as a positive control.

2.2.3. Western blot(WB) analysis

WB analysis was performed by conventional protocols (Sambrook and Russell, 2006). Briefly, the promastigotes of *L. tarentolae*-PT-NT(gp96)-EGFP and *L. tarentolae*-EGFP were centrifuged at 3000 rpm for 10 min, washed and mixed with 6x SDS-PAGE sample buffer (4.5 mM Tris–HCl, pH 6.8, 10%, v/v glycerol, 2%, w/v SDS, 5%, v/v 2-mercaptoethanol, 0.05%, w/v bromophenol blue), boiled for 5 min and loaded on a 15% SDS-PAGE. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche) using the Bio-Rad wet blotting system. Following incubation with blocking solution (TBS; with 0.1% Tween 20 and 2.5% BSA) membranes were washed and probed with anti-GFP antibody (1:5000; polyclonal antibody to GFP-HRP; Acris antibodies GmbH), for 2 h at 37 °C. Reactive bands were visualized using an enhanced chemiluminescence (ECL) kit (Clarity™ Western ECL Substrates, Bio-Rad) and light emission was detected by exposing the membrane to a Hyperfilm ECL (GE-Amersham).

2.3. In vitro expression of PT-NT (gp96) in COS-7 cell line

Up to 5×10^4 COS-7 cells per well of a four well plate (Greiner, Germany) was incubated until the cells reached a confluence of 75%. The COS-7 cells were transfected with 5 µg of either pEGFP-PT-NT (gp96), pEGFP-N3 (positive control) or pcDNA3.1 (negative control) plasmids (in separate reactions) using LINPEI-25 kDa (10 µM; poly sciences, Europe) as described previously (Sabet et al., 2014). Expression of the proteins was confirmed by observation of the EGFP signal under fluorescence microscope (Nikon E200, USA) at 24 h post transfection. To determine the proportion of GFP positive cells (FL1 channel), the cells were collected at 24 h post-transfection, washed with PBS, and analyzed by flow cytometry (Partec, Germany).

2.4. Parasite and antigen preparation

Promastigote forms of L. tar-PT-NT(gp96)-EGFP, harvested in the stationary phases were centrifuged (3000 rpm, 4 °C, 10 min), washed in PBS and suspended at a concentration of 2×10^8 parasites/ml. To prepare frozen and thawed (F/T) L. tar-PT-NT(gp96)-EGFP antigen, the promastigotes were washed and exposed to liquid nitrogen and water bath (37 °C) for 15 times. For preparing the recombinant PT-NT(gp96)-EGFP protein, the bacterial culture containing pQE-PT-NT(gp96)-EGFP was grown to an optical density 0.6 at 600 nm. Expression of protein was induced with 1 mM IPTG for overnight at 16 °C and analyzed by 10% SDS-PAGE. The recombinant PT-NT(gp96)-EGFP was purified by affinity chromatography using 6x His-tag according to the manufactures protocol (The QIAexpressionist™, QIAGEN). Protein concentrations were determined using BCA assay kit (pierce, Thermo Scientific).

2.5. Mice and vaccination regimens

Two different prime-boost regimens were carried out in seven groups of 6-week-old female BALB/c mice (n = 12 per group) which were obtained from the breeding stock maintained at Pasteur Institute of Iran. The mice were kept in plastic cages with standard rodent pellet and appropriate water in a centralized air conditioned facility under a constant 12:12 h light-dark cycle at room temperature and 55–60% relative humidity. In this study, all performed experimental protocols were approved by the Ethical Committee of School of Public Health, Tehran University of Medical Sciences (Code of Ethics: 94-02 – 27-28841-203029).

Group 1 (G1) was immunized with 2×10^6 stationary phase *L. tarentolae*-PT-NT(gp96)-EGFP in combination with CpG adjuvant (5′-TCCA TGACGTTCCTGACGTT-3′) for both prime and boost immunizations (Hereafter; live/live + CpG). Group 2 (G2) was immunized with 2×10^6 *L. tarentolae*-PT-NT(gp96)-EGFP with non-CpG (5′-TCCAGGACTTCTCTC AGGTT-3′) for both prime and boost immunizations (Hereafter; live/live). Group 3 (G3) was vaccinated with 100 µg of pcDNA-PT-NT(gp96)-EGFP as prime but 2×10^6 *L. tarentolae*-PT-NT(gp96)-EGFP for boost (Hereafter; DNA/live) immunization. Group 4 (G4) was immunized with 2×10^6 *L. tarentolae* in combination with CpG for both prime and boost immunizations (control group; hereafter; control live/live + CpG). Group 5 (G5) was vaccinated with 2×10^6 *L. tarentolae* with non-CpG for both prime and boost immunizations (control group; hereafter; control live/live + non-CpG). Group 6 (G6) was vaccinated with empty vector pcDNA3.1 as prime but 2×10^6 *L. tarentolae* as a boost immunization (control group; hereafter; control DNA/live) and the last group (PBS) received PBS as control. All groups were vaccinated in the foot pad at a three week interval as summarized in Table 1.

2.6. In vivo CTL assay

Splenocytes from naïve BALB/c female mice were adjusted to 2×10^8 cells/ml after lysis of erythrocytes and divided in two fractions. The first fraction was pulsed with rPT-NT(gp96)-EGFP at 10 µg/ml final concentration for 90 min at 37 °C and the second fraction was left unpulsed.

Subsequently, based on the standard protocols, Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Cell Trace ™ CFSE Cell Proliferation Kit, Thermo Fisher Scientific) was added at 5 µM to pulsed cells (CFSE ^{high}) and at 0.5 µM (1/10 concemtration) to unstimulated (no pulse) cells (CFSE ^{low}) for 15 min. Both fractions were washed and 2×10^8 cells a 1:1 mixture of CFSE ^{high}/CFSE ^{low} was transferred to mice through the tail vein injection. After 14 h, the mice were sacrificed and splenocytes were analyzed by flow cytometry to detect the CFSE-labeled target cells. The percentage of specific lysis was calculated using

Table 1
Immunization schedule in different mice groups.

Groups	Prime 1st Week	Boost 3rd Week	Strategy
G1	*L. tarentolae*-PT-NT (gp96)-EGFP + CpG ODN	*L. tarentolae*-PT-NT (gp96)-EGFP + CpG ODN	Live/Live
G2	*L. tarentolae*-PT-NT (gp96)-EGFP + non-CpG	*L. tarentolae*-PT-NT (gp96)-EGFP + non-CpG	Live/Live
G3	pcDNA-PT-NT (gp96)-EGFP	*L. tarentolae*-PT-NT (gp96)-EGFP	DNA/Live
G4	*L. tarentolae* + CpG ODN	*L. tarentolae* + CpG ODN	Control
G5	*L. tarentolae* + non-CpG	*L. tarentolae* + non-CpG	Control
G6	pcDNA3.1(+)	*L. tarentolae*	Control
PBS	PBS	PBS	Control

the formula [1-($R_{immunized}/R_{PBS}$] ×100 where R= % $CFSE^{high}$/%$CFSE^{low}$ for each mouse (Fournillier et al., 2007).

2.7. Cytokine assays

Three mice of each group were sacrificed at 3 and 9 weeks after last immunization (W3 and W9) and the spleens were homogenized using a tissue grinder. Erythrocytes were lysed using ammonium-chloride-potassium (ACK) lysis buffer (NH4Cl 0.15 mM, KHCO3 1 mM and Na2EDTA 0.1 Mm; pH 7.2) and washed in DMEM. Subsequently, splenocytes were suspended at 2 × 106 cells/ml in complete DMEM (5% FCS, 0.1% L-glutamine, 1% HEPES, 0.1% 2ME, and 0.1% gentamicin) and incubated with rPT-NT (gp96) (5 μg/ml), *L. tarentolae*-PT-NT(gp96)-EGFP F/T (10 μg/ml) at 37 °C in 5% CO2. Concanavalin A (ConA, 5 μg/ml) incubated cells and cells with no treatment (medium alone) were used as positive and negative controls, respectively. The supernatants of splenocyte cultures were collected after 24 h for TNF-α assay, 72 h for IL-17 and IL-4 assays, and 96 h for IFN-γ assay. The cytokine levels were measured using a sandwich ELISA according to the manufacturer manual (R&D, Quantikine®ELISA, USA). All assays were performed in duplicates. The optical density (OD) was measured at 450 nm with ELISA reader (TECAN, USA) and the concentration was calculated according to the standard curve.

2.8. Antigen-specific antibody responses

To evaluate the humoral immune responses, blood samples were collected from the orbital plexus of each mouse before immunization and at W3 and W9. Specific antibody levels of IgG1 and IgG2a and the ratio of IgG2a/IgG1 isotype responses against *L. tarentolae*-PT-NT(gp96)-EGFP (F/T) (10 μg/ml) and rPT-NT (gp96) (5 μg/ml) were measured by ELISA as described previously (Sabet et al., 2014). Briefly, 96-well maxisorb plates (Greiner, Germany) were coated with antigen overnight at 4 °C. Plates were blocked with 300 μl of 5% skim milk in PBS at 37 °C for 2 h. After three washes, 100 μl of pooled sera of each group was added (1:300) and incubated at 37 °C for 2 h. Subsequently, goat anti-mouse IgG1-HRP or IgG2a-HRP (1:7000, Southern Biotech Canada) was added to each plate and incubated at 37 °C for 2 h. The plates were washed and incubated 30 min with 100 μl of TMB as substrate. The reaction was stopped with sulfuric acid and absorbance was measured at 450 nm.

2.9. Statistical analysis

Statistical analyses were performed using Graph pad Prism version 7.03 for windows (Graph pad Software Inc. 2017, La jolla, California, USA). All data were analyzed with one way ANOVA (Multiple-comparison HSD-Tukey test) and when required with a student's *t*-test. Assays were performed in duplicate and for at least two times. $p < 0.05$ were considered statically significant. The results were expressed as mean ± SEM. To ensure the blind condition for prevention of any bias in the interpretation of the results, the mice handling procedures like injections and blood sampling were under taken by a trained veterinarian who was unaware of the study design and the nature of the injected material. The same goes for the results analysis by the statistician.

3. Results

3.1. Evaluation of the PT-NT(gp96)-EGFP expression in COS7 cell line

The primary evaluation of the expression potential of the PT-NT(gp96)-EGFP construct in Eukaryotic cells was performed in COS-7 cells that were separately transfected with pEGFP-PT-NT(gp96), pEGFP-N3 (as a positive control) and pcDNA3.1 (as a negative control) vectors. As shown in Fig. 1, Flourescence microscopy indicated the proper expression of the EGFP by pEGFP-PT-NT(gp96)-EGFP vector compared to the positive control in COS-7 cells at 24 h post transfection. In the negative control, fluorescence emission was not observed. Quantification data obtained by flow cytometry indicated that the expression of EGFP in pEGFP-N3 (Fig. 1A) and pEGFP-PT-NT (gp96) (Fig. 1B) was 12% and 10%, respectively. The lower signal intensity observed in transfected COS-7 cells with pEGFP-PT-NT(gp96)-EGFP compared to the control, might be due to the fusion of PT-NT (gp96) before EGFP gene that changed the EGFP translation potency.

3.2. Generation of a recombinant L. tarentolae expressing the PT-NT(gp96)-EGFP construct

Recombinant *L. tarentolae* stably expressing the PT-NT (gp96) along with EGFP gene was constructed by introducing the linearized pLEXSY-PT-NT(gp96)-EGFP into the 18S rRNA *ssu* locus of *L. tarentolae* as described in the materials and methods section (Fig. 2A). The integration of PT-NT(gp96)-EGFP cassette into the *ssu* locus of the recombinant parasite genome was confirmed by amplification of a 1kb fragment (Fig. 2C). The presence of PT-NT (gp96)-EGFP gene in transgenic *L. tarentolae* was confirmed at the DNA level by PCR amplification of a 1200 bp fragment corresponding to PT-NT (gp96)-EGFP from the genomic extracts of the recombinant *L. tarentolae*-PT-NT (gp96)-EGFP (Fig. 2B). Expression of EGFP in *L. tarentolae*-PT-NT(gp96)-EGFP and *L. tarentolae*-EGFP parasites was confirmed by fluorescence microscopy (Fig. 2C) through direct observation of the green fluorescence and by Western blot analyses through detection of an immunoreactive band of 84 kDa in *L. tarentolae*-PT-NT(gp96)-EGFP using an anti-GFP antibody (Fig. 2D). Overall, these results indicated the accurate integration of the PT-NT(gp96)-EGFP fragment into *L. tarentolae* genome and its continuous expression.

3.3. Live/Live + CpG vaccination induced strong and sustained in vivo lytic activity

To examine the rPT-NT (gp96)-specific CTL responses, CFSE-labeled target splenocytes pulsed with rPT-NT (gp96) were transferred in to all mice groups at W3 and W9. Specific cell lysis was measured by flow cytometry. G1 and G2 immunized with live/live vaccines showed higher specific lysis than G3 immunized with DNA/live vaccine ($p < 0.0001$). At W3, 70.98 ± 3.73% and 41.29 ± 1.53% specific killing of target cells in G1 and G2 was observed, respectively (Fig. 3). The lowest cytolytic activity (13.12 ± 2.16%) was shown in G3 group. We also analyzed the specific CTL-mediated killing of target cells at W9. In memory phase (W9), the specific killing of the target cells in G1 and G2 was 50 ± 1.54% and 24.5 ± 4.21%, respectively (Fig. 3). In contrast to G1 and G2 groups, G3 showed an increase of cytotoxic activity up to 20% at W9. Taken together, our results demonstrate that live/live vaccine in the presence of CpG ODN could induce high levels of the potent and sustained $CD8^+$ CTL activity in vivo.

3.4. DNA/live vaccination induced multiple cytokine profiles

After stimulating of splenocytes isolated from vaccinated and control mice by rPT-NT(gp96)-EGFP and *L. tarentolae*-PT-NT(gp96)-EGFP F/T as recall antigens, their supernatant were used for cytokine measurements. IFN-γ production levels (as an indication of the Th1-oriented immunity) were evaluated at W3 for all mice groups. As shown in Fig. 4A, G1 compared to G2 ($p < 0.0001$) and the control groups G4 ($p < 0.0001$) and PBS ($p < 0.001$), showed a significantly higher levels of IFN-γ production while G2 showed only a minor but still significant difference compared to the corresponding control group (G5) ($p < 0.05$). Moreover, G3 group showed the highest rPT-NT(gp96)-EGFP-specific IFN-γ secretion in comparison to the other vaccinated groups including G1 and G2 and the control groups G6 and PBS ($p < 0.001$, Fig. 4A). Interestingly, the significant differences observed between IFN-γ production of G1-G3 with control groups G4-G6 and PBS ($p < 0.0001, p < 0.001, p < 0.01$) at W9 were similar to those found at W3 (Fig. 4A). We further examined whether immunized groups

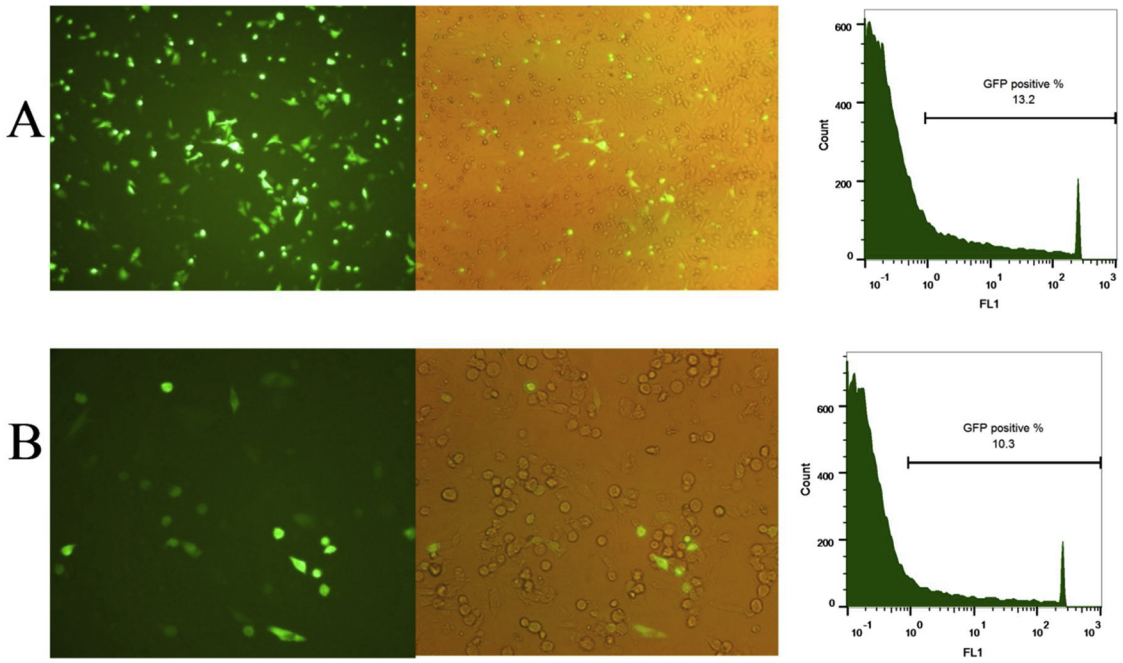

Fig. 1. In vitro expression of PT-NT(gp96)-EGFP in COS-7 cells. Recombinant pEFGP-PT-NT (gp96) and pEFGP-N3 plasmids were transiently transfected into COS-7 cells using Polyethylenimine. (A) and (B) GFP expression of the transfected cells (before and after glinting of florescence) with pEFGP-N3 (as positive control) and pEGFP-PT-NT (gp96), respectively.

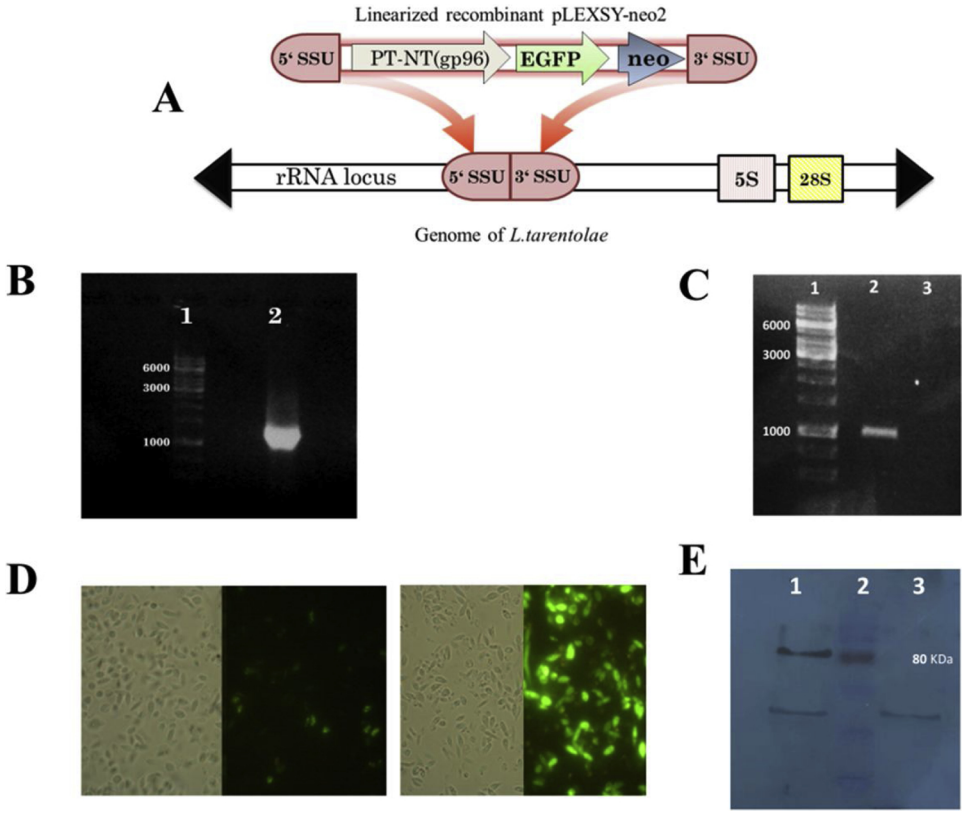

Fig. 2. Generation of *recombinant L. tarentolae* expressing PT-NT(gp96)-EGFP fusion protein. (A) Schematic representation of the linearized pLEXSY-PT-NT(gp96)-EGFP construct containing two regions with homology to the rRNA locus of *L. tarentolae* (5′ssu and 3′ssu) and integration by recombination via the two homologous regions (5′ssu and 3′ssu), following transfection of the construct into *L. tarentolae.* (B) Amplification of the 1.2 kb band indicated the presence of PT-NT(gp96) within the genome of recombinant *L. tarentolae*. Wild type *L. tarentolae* was used as negative control (lane 3). (C) Amplification of the 1 kb band confirmed the integration of PT-NT(gp96)-EGFP cassette into the ssu locus of *L. tarentolae genome*. (D) Expression of EGFP *L. tarentolae*-PT-NT(gp96)-EGFP promastigotes (left) and *L. tarentolae*-EGFP promastigotes (right) before and after glinting of fluorescence. (E) Western blot analysis for evaluating the expression of PT-NT(gp96)-EGFP protein by recombinant *L. tarentolae*-PT-NT(gp96)-EGFP using an anti-GFP antibody. Presence of the 84-kDa band in lane 1 indicated the expression of the PT-NT(gp96)-EGFP protein, the 80-kDa band in lane 2 represents the MW marker. No band was observed in lane 3 which is the wild type *L. tarentolae* (negative control).

produced Th2-associated cytokine IL-4. There was no significant difference in IL-4 level between G1 and G2 ($p < 0.5$) and that of the control groups G4, G5 and PBS ($p < 0.5$, Fig. 4B). However, the G3 showed a significant decrease in the level of rPT-NT(gp96)-EGFP-specific IL-4 compared to the control group G6 ($p < 0.05$). These results indicated that DNA/live prime-boost strategy enhanced the immune responses toward the Th1 type. As shown in Fig. 4B, there were no significant differences for IL-4 levels between G1-G3 compared to control groups G4-G6 at W9. Accordingly, the IFN-γ /IL-4 ratios for vaccinated groups also showed a bias toward the Th1 response. This ratio in G1 was significantly higher than in G2 at W3 and W9 ($p < 0.01$, $p < 0.001$, data not shown). Interestingly, the highest ratio belonged to G3 that showed significant differences at W3 ($p < 0.0001$) and W9 ($p < 0.0001$) as compared to both groups G1 and G2.

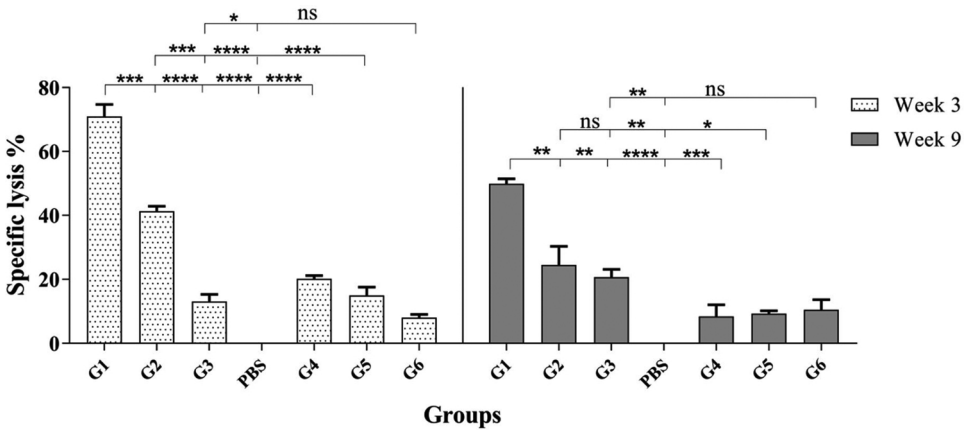

Fig. 3. Cytotoxic potential of live/live and DNA/live vaccine induced $CD8^+$ T cells in different experimental mice groups. Three and nine weeks after the last immunization, antigen-pulsed CFSE high and un-pulsed CFSE low target cells were injected into all mice groups. Mice were sacrificed 14 h after cell transfer and the percentage of specific lysis was evaluated for each mouse group. Statistical analyses were done by one-way ANOVA and Data show mean ± SEM of two independent experiments with n = 3. (n = 2). ($p < 0.05$ denoted as *, $p < 0.01$ denoted as **, $p < 0.001$ denoted as ***, $p < 0.0001$ denoted as **** and non-significant denoted as ns).

Measurement of IL-17 in mice groups at W3 indicated similar levels of the cytokine in groups G1, G2, G4 and PBS, but significantly higher levels in G3 than G6 and PBS groups ($p < 0.0001$, Fig. 4C). At W9, except G2 and PBS groups which showed significant differences in IL-17 levels ($p < 0.05$), the other immunized and control groups had similar levels of this cytokine. The IL-17/ IFN-γ ratio was also calculated for all groups to reveal the presence of the Th-17- shifted cell phenotype. At W3, the ratio of IL-17/IFN-γ showed a significant increase in G3 that was significantly higher than that of G1 and G2. Indeed, heterologous regimen (G3) skewed immune responses toward Th-17 phenotype in comparison to homologous regimen (G1 and G2).

The level of TNF-α production as a major proinflammatory cytokine was also measured in all mice groups. At W3 and W9, there were significant differences in TNF-α level in G1 and G2 with PBS group. However, the differences for G1 and G2 compared to G4 and G5 were not significant (Fig. 4D). At W3, G3 produced significantly the highest TNF-α level ($p < 0.0001$) but a sharp decrease of TNF-α was observed in this group at W9.

3.5. Live/Live + CpG vaccine induced early and sustained antibody responses with dominant Th1 phenotype

The IgG1 and IgG2a levels against rPT-NT (gp96)-EGFP and *L. tarentolae* - PT-NT (gp96)-EGFP F/T antigens in the sera of the immunized and control mice groups at W3 and W9 were assessed by ELISA. The level of *L. tarentolae*-PT-NT(gp96)-EGFP F/T specific IgG1 in the vaccinated group G1 was significantly lower than the G4 control group at both W3 and W9 ($p < 0.0001$), but only at W9 for that of the G2 compared to the G5 control group ($p < 0.0001$) (Fig. 5A). As shown in Fig. 5B, *L. tarentolae*-PT-NT(gp96)-EGFP F/T specific IgG2a was higher in group G1 and G2 compared to the PBS group at both W3 and W9. Immunization by Live/live (without CpG) strategy induced similar levels of *L. tarentolae*-PT-NT(gp96)-EGFP F/T specific IgG1 and IgG2a in G2 at W3, (possible indication of a more balanced Th1-Th2 phenotype). However, the ratio of IgG2a/IgG1 increased to < 1 at W9, suggesting higher Th1 polarized immune responses in time (Fig. 5E). At both W3 and W9, G1 group showed the highest ratio of IgG2a/IgG1 compared to all other groups indicating potentially dominant Th1 responses for this group of mice (Live/Live + CpG) in early and long term post immunization periods. At W3, rPT-NT(gp96)-EGFP specific IgG1 was significantly lower in G3 compared to the control group (G6) ($p < 0.0001$) (Fig. 5C). At W9, an increased amount of rPT-NT(gp96)-EGFP specific IgG1 and IgG2a was observed in G3 and the levels of both isotypes were significantly higher in this group compared to the control groups ($p < 0.001$, $p < 0.0001$) (Fig. 5C, 5D). Accordingly, the rPT-NT (gp96)-EGFP specific IgG2a/IgG1 ratio in G3 was < 1, suggesting a shift toward Th2 response. Overall, in G1 and G2 groups immunized with *L. tarentolae*-PT-NT(gp96)-EGFP with and without CpG (live/live), respectively, a higher level of IgG2a/IgG1 ratio was found in comparison to G3 immunized with pcDNA-PT-NT(gp96)-EGFP/*L. tarentolae*-PT-NT(gp96)-EGFP (DNA/live) regimen (Fig. 5F).

4. Discussion

In the present study we generated a recombinant *L. tarentolae* strain stably expressing PT-NT(gp96)-EGFP and evaluated its efficiency as a candidate live vaccine for delivery of HCV antigens, in different prime-boost strategies. We showed that vaccination with this live recombinant *L. tarentolae* in the presence or absence CpG in a prime-boost regimen was immunogenic and more importantly induced a long term response. In this study, *L. tarentolae* was used for the first time as a vaccine delivery system for HCV antigens. Besides being non-pathogenic to humans, the other major reasons behind selecting *L. tarentolae* as a vector for vaccine delivery are its ability to target macrophage and dendritic cells which enhances for antigen presentation and no incidence of preexisting immunity in humans to neutralize the vector, which is a major concern of widely used vectors in immunization studies (Breton et al., 2005; Taheri et al., 2016).

Given the fact that the most significant indicator for functional effector T cells is their ability to lyse the target cells, the cytotoxic activity in mice vaccinated by live/live in combination with CpG (G1), live/live (G2) and DNA/live (G3) was tested. As shown in Fig. 3, the immune response of in vivo CTL assay from highest to lowest was live/live + CpG (71%) > live/live (40%) > DNA/live (13%). It appears that in homologous prime-boost regimen, recombinant *L. tarentolae* as priming vehicle, facilitated induction of CTL responses by activating DCs to induce high level of antigen expression and efficient antigen delivery to MHC I, while its use as booster further expanded the CTL response (Fig. 3). Of note, in our study, the mean specific lysis percentage (70.98 ± 3.73%) in mice immunized with live/live + CpG is about 1.4 fold higher than that of a recent report on a NS34-NS5B encoding MVA (50.75 ± 6.86%) (Fournillier et al., 2007). In another study, a similar percentage of cytolytic activity (76.7 ± 13%) to that of our results at 14 days post vaccination is reported. However, at 60 days post vaccination this percentage decreased significantly (19.3 ± 4.6) and was 2.5 fold lower than that of ours (50 ± 1.54%) (Mikkelsen et al., 2011), indicating the efficiency of *L. tarentolae* for induction of long lasting CTLs.

Although in vivo CTL and / or tumor challenge studies are the usual assays for evaluation of protective cellular immune responses (Liao et al., 2008), cytokine profiles of Th1 (IFN-γ, TNF-α) and Th17 (IL-17) might be considered as reliable indicators to evaluate vaccine induced protective responses. As shown in Fig. 4, our results indicated that Live/Live + CpG (G1) immunization strategy induced significantly higher levels of IFN-γ than G2 group. Furthermore, IFN-γ /IL-4 ratio in G1 was > 1 and 1.5 fold higher than G2 group at W3. These findings

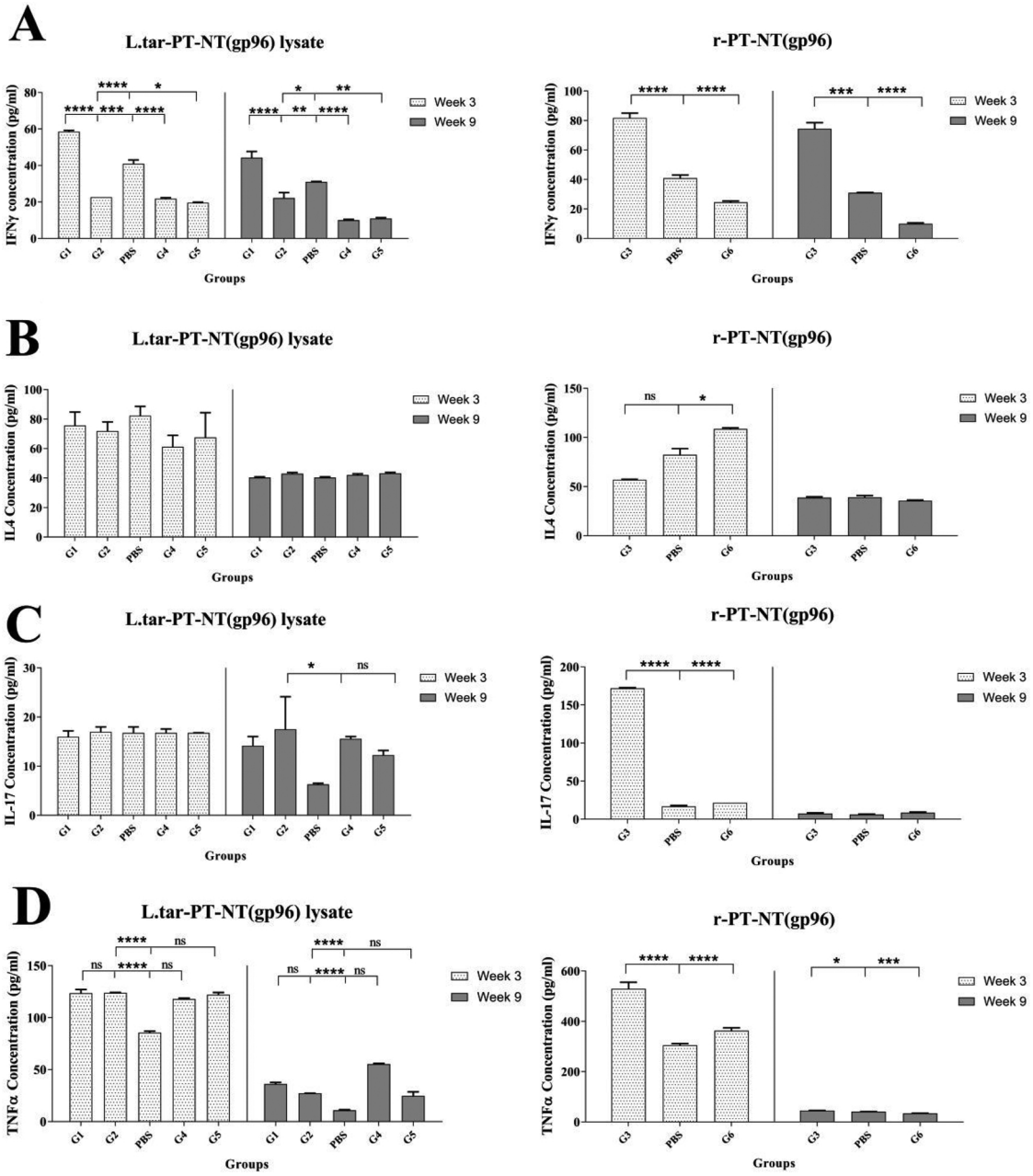

Fig. 4. Cytokine levels detected in vaccinated and control BALB/c mice. Cytokine secretion by splenocytes was assessed at 3 and 9 weeks after the last immunization (number of mice/time point n = 3). Splenocytes were cultured in duplicate and stimulated in vitro with rPT-NT(gp96)-EGFP and *L. tarentolae*-PT-NT(gp96)-EGFP F/T (all at 10 μg/ml). or ConA as positive control (not shown). IFN-γ (panel A), IL-4 (panel B), IL-17 (panel C) and TNF-α (panel D) levels in cell supernatants were measured by ELISA. Bars represent the mean ± SEM in pg/ml for all cytokines. All measurements were in duplicate. One-way ANOVA was used for statistical analysis ($p < 0.05$ denoted as *, $p < 0.01$ denoted as **, $p < 0.001$ denoted as ***, $p < 0.0001$ denoted as **** and non-significant denoted as ns).

suggested the positive/additive adjuvant role of CpG ODN that might be due to interaction with TLR9 on macrophage and DCs, as previously reported (Bonam et al., 2017; Kovacs-Nolan et al., 2009). Interestingly, the level of IFN-γ in our immunization study was similar to that of the prior reports on application of the *L. tarentolae* vector for delivery of the viral antigens (Hosseinzadeh et al., 2013; Salehi et al., 2012), while it was lower than that reported by the same groups for delivery of parasite antigens (Abdossamadi et al., 2017; Mizbani et al., 2009; Saljoughian et al., 2013). The enhanced induction of IFN-γ in the *L. tarentolae* vector-mediated delivery of parasitic antigens compared to viral antigens might be due to closer phylogenetic relationships in the preceding and thus higher biological adaptation with the vector.

As shown in Fig. 5, despite eliciting the lowest cytotoxic activity (13%), DNA-live vaccinated mice (G3) showed the highest level of IFN-γ accompanied with the lowest level of IL-4 compared to G1 and G2 groups (Fig. 4A, B), indicating a dominant Th1-shifted immune response for this group. This observation could be expected, since our DNA vaccine contains CpG motif that not only interact with TLR-9 on macrophage and DCs, but also lead to efficient induction of CD4 + T cells to produce IFN-γ by prime immunization, while boost-immunization by *L. tarentolae* might further enhance this Th1 cytokine profile. Consistent with our observation, several prior studies reported the higher level of IFN-γ production by vaccine-induced CD4 + T cells following heterologous rather than homologous prime-boost immunization strategy (Kardani et al., 2016; Rollier et al., 2016). Apart from IFN- γ/IL-4 ratios that indicated profound Th1 shifted responses in

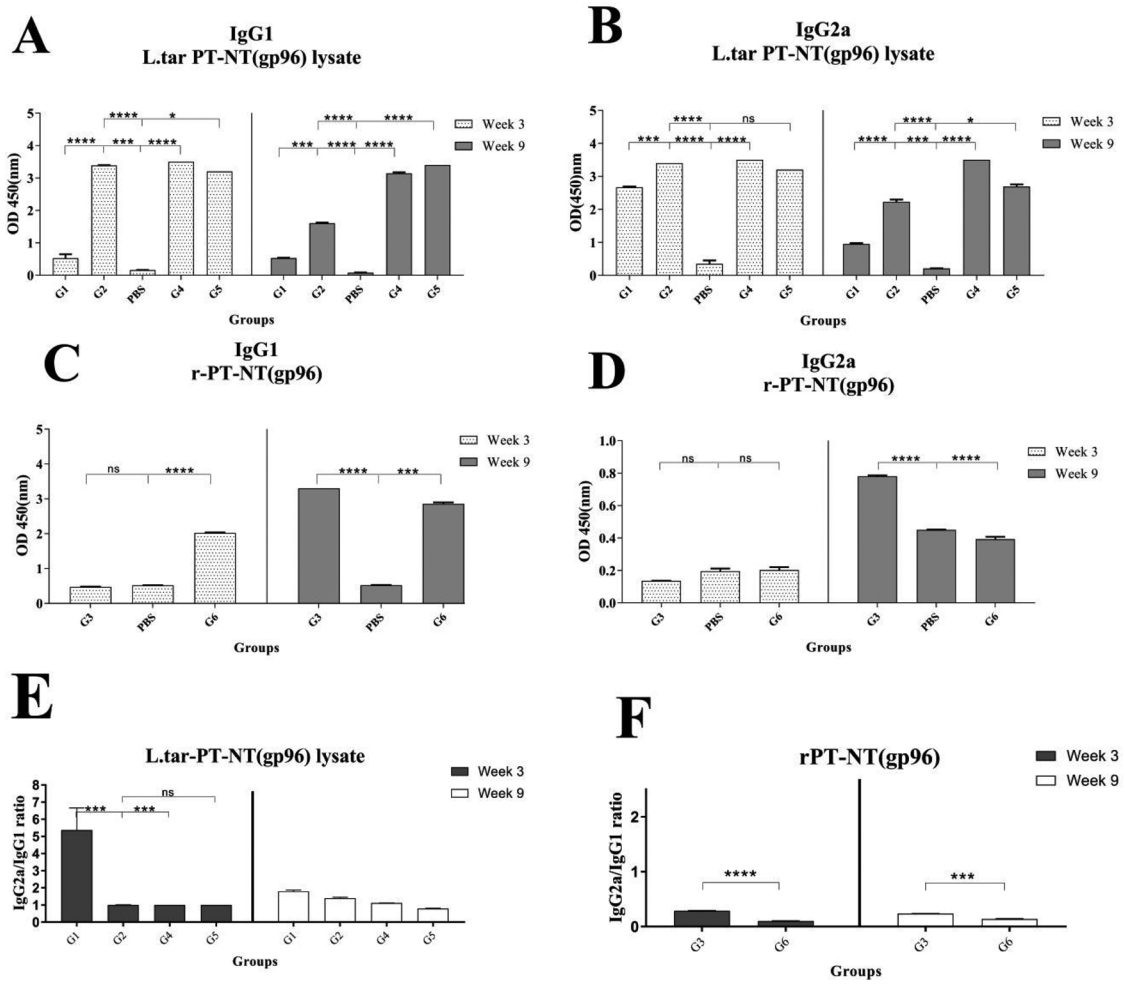

Fig. 5. Antigen specific IgG1 and IgG2a antibody responses in immunized and control mice. Sera were obtained in individual mice from each group and pooled (n = 3). Sera were tested for anti *L. tarentolae*-PT-NT(gp96)-EGFP F/T in G1 and G2 and for anti rPT-NT(gp96)-EGFP in G3 at 3 and 9 weeks after the last immunizations by an isotype-specific ELISA. Panel (A) and (C) show specific IgG1, panel (B) and (D) show specific IgG2a and panel (E) and (F) show the IgG2a/IgG1 ratio. All measurements were done in duplicate. The asterisk indicates the significant difference between values at the indicated time points as determined by one-way ANOVA or Student's *t*-test ($p < 0.05$ denoted as *, $p < 0.01$ denoted as **, $p < 0.001$ denoted as ***, $p < 0.0001$ denoted as **** and ns denoted as non-significant).

G3, the IL-17/IFN-γ ratio in this group also showed a shift toward Th17 responses (balanced Th1-Th17 response). Indeed, priming with DNA vaccine might have induced the production of IL-1 and IL-6 that skewed CD4 + T cells toward a Th17 cell phenotype. The strong IL-17 response in G3 group (Fig. 4C) might have a protection role against HCV infection by the induction of pro inflammatory and inflammatory cytokines which facilitate the entry of effector Th1 cells and neutrophils to the site of immunization. IL-17 appears to act as a double-sward and several studies indicate the potential role of this cytokine in promoting immunopathology. However, only few reports in the literature studied the potential relation of the IL-17 cytokine and vaccine/infection-derived immunity against HCV (Parlane et al., 2011) and thus the beneficial or detrimental function of IL-17 in HCV infection is not yet clear (Katebi et al., 2015; Parlane et al., 2011). Collectively, our results indicated that higher CD8 + CTL responses were elicited in mice immunized by homologous prime-boost strategy using live vector (G1,G2), while strong Th1 and Th17 cytokines profile was observed in mice immunized by DNA/live vaccine (G3). These differences might be in part due to the nature of antigen presentation via the influence of the priming vector, the low or high level antigen expression, and the associated early inflammatory signals delivered by the primary vectors to the immune system.

Since long term memory CD8 + T cells and cytokine production are an essential requirement for HCV vaccine (Hofmann et al., 2018), we followed the PT-NT (gp96) specific cytolytic activity, cytokines profile and Ab responses for up to 60 days. As shown in Fig. 3, results of the cytotoxicity and IFN-γ production, 60 days after the last immunization, indicated a long lasting immunological memory state in mice groups immunized by homologous live vector strategy (G1 and G2). This observation is consistent with a report on development of long term immunity via the production of effector memory T cells by immunization of mice with recombinant *L. tarentolae* expressing HIV-1 gag protein (Breton et al., 2007). Our results also suggest that PT-NT (gp96) specific T cell population contain both effector and memory T cells which are producers of IFN-γ and cytotoxic granules. Of note, in our study, long lasting response was significantly stronger in G1 group compared to G2 (Fig. 4A) which might indicate the positive/additive adjuvant role of CpG for induction of long lasting cellular immune responses, as previously described (Kovacs-Nolan et al., 2009; Yokokawa et al., 2018).

Concerning the induction of humoral responses and IgG subtypes, as shown in Fig. 5, G1 group indicated significantly higher ratio of IgG2a/IgG1 in comparison to G2 and G3. This shows a Th1 polarization of immune response in this group. In fact, our results for the values of IgG2a/IgG1 ratio in G1 (> 1) and G2(= 1) were in agreement with a recent report on use of the *L. tarentolae* for delivery of PpSP15 antigen, an immune protective protein against *Leishmania major* infection from

the sand fly *Phlebotomus papatasi* (Katebi et al., 2015). Interestingly, our result for the value of IgG2a/IgG1 ratio in G3 (< 1) was also consistent with another recent report for a similar heterologous prime-boost immunization strategy with HPV-E7 protein encoding pCDNA plasmid and *L. tarentolae* (pcDNA-E7/L.tar-E7) (Salehi et al., 2012). Indeed, in contrast to the broad and enhanced Th1 and Th17 responses, the humoral immune response in G3 was not so strong, while IgG2a/IgG1 ratio was also < 1, indicating a Th1 bias (Fig. 3F). The reason for this could be that the pathway of cellular mediated immune response to viral proteins is different from that of antibody response (Liao et al., 2008). Overall, our results suggested that immune response induced by G1 (live/live + CpG) were more efficient than G2 and G3. However, the present study cannot clarify whether homologues live /live regimen in the presence of CpG is more effective than the heterologous DNA /live regimen if the latter is used in combination with CpG. In fact, it is necessary to evaluate our DNA/live vaccine in combination with CpG to elucidate its potential adjuvant effect on this heterologous regimen. In addition, more precise results might be obtained by: *i*) increasing the number of mice in each group; *ii*) determining the frequency and phenotype of cytokine secreting CD4+ and CD8 + T cells by flow cytometry; *iii*) determining the neutralization potency of the induced antibodies in a "neutralization assay" and *iv*) determining the antiviral protection value of live/live + CpG vaccine induced-T cells by challenge in an HCV animal model (transgenic mice).

5. Conclusion

To the best of our knowledge, in the present study, we provided the first report on application of *L. tarentolae* as a live vector for delivery of HCV antigens. Our findings suggested *L. tarentolae* as an efficient vector for a vaccination approaches against HCV and other viral infections which require T cell mediated immunity for protection. Moreover, combination of CpG ODN with this live recombinant *L. tarentolae* could enhance PT-NT(gp96)-EGFP-specific T cell responses and induce long lasting immunity. Overall, the present results represent live/live + CpG vaccine as a valuable and efficient immunization strategy, although additional studies must be performed to measure the extent of its protection in animal models.

Conflicts of interest

The authors declare that they have no competing interests.

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