Recombinant *Leishmania tarentolae* encoding the HPV type 16 *E7* gene in tumor mice model

Background: Cervical cancer, the third most prevalent cause of cancer in women worldwide, is associated with HPVs. The critical role of E7 protein in HPV-related malignancies has designated it as a strong contender for generating vaccines against HPV. **Materials & methods:** In this study, we developed a novel live vaccine using recombinant *Leishmania tarentolae* expressing E7-green fluorescent protein (GFP) fusion protein for the protection of mice against HPV-associated tumors. In order to transfect *L. tarentolae* with E7-GFP fusion construct, pLEXSY-neo2 system was applied. Followed by PCR, fluorescence imaging and fluorescence-activated cell sorting analysis, integration of *E7-GFP* gene into parasites genome was confirmed. A comparative study of six groups of C57BL/6 mice was performed to analyze antigen-specific humoral and cellular immune responses against E7 encoding live and DNA vaccines. Furthermore, the anti-tumor protective effect of *L. tarentolae*-E7-GFP was compared to other vaccination strategies, namely pcDNA-E7 as the DNA vaccine and pcDNA-E7/*L. tarentolae*-E7-GFP as the prime-boost regimen. **Results:** We found that E7-GFP expressing recombinant *L. tarentolae* induces significant levels of IgG2a and IFN-γ, while there is no significant IL-5 production compared with that of other strategies and control groups before and after challenge with TC-1 tumor cells. It is noteworthy that the designed live vaccine showed the best protection and minimum tumor size among all groups against TC-1-induced tumors. **Conclusion:** Overall, the results obtained revealed that the E7-GFP recombinant *L. tarentolae* could be a potential live vaccine for induction of immune responses *in vivo*.

KEYWORDS: E7 ■ HPV ■ *Leishmania tarentolae* ■ live vaccine ■ TC-1 ■ tumor

Maryam Salehi[1,2], Tahereh Taheri[1], Elham Mohit[1], Farnaz Zahedifard[1], Negar Seyed[1], Yasaman Taslimi[1], Mandana Sattari[2], Azam Bolhassani*[1] & Sima Rafati*[1]

[1]Molecular Immunology & Vaccine Research Laboratory, Pasteur Institute of Iran, Tehran, Iran
[2]Department of Immunology, Medical School, Shahid Beheshti University of Medical Sciences & Health Services, Tehran, Iran
**Authors for correspondence:*
Tel.: +98 21 66953311 ext. 2112
Fax: +98 21 66496560
sima-rafatisy@pasteur.ac.ir
azam_bolhassani@yahoo.com

For the last few years, HPVs have been the leading cause of cervical cancer. This cancer has emerged as the third most commonly diagnosed malignancy and the fourth leading reason of cancer mortality in women worldwide [1,2]. Among all the various HPV genotypes, four high-risk types including 16, 18, 31 and 45 are associated with almost 80% of cervical cancers. Moreover, type 16 exists in half of the HPV-induced malignancies; thus it has been at the heart of current research on vaccine development against HPV [3]. The malignant stage of the HPV infection is followed by integration of the viral DNA into the host genome resulting in overexpression of the HPV oncoproteins, E6 and E7, and the immortalization stage of the infected cells. The critical role played by these proteins during the carcinogenesis is such that they are always expressed during this process [4–7]. Since *E7* has more abundant expression and conserved sequence than that of the *E6* gene, it is considered as the main stream of research focus for developing HPV vaccines [8,9]. E7 protein performs its transformation activity via interaction with pRb, one of the cell's regulatory proteins, and deactivating its function. In addition, persistent expression of the E7 protein from initial to malignant stages of cervical cancer elicits considerable humoral and cellular immune responses [10]. Moreover, in early 1990s, the immunogenicity of E7 had also been determined in humans [9,11,12]. Therefore, E7 oncoprotein serves as an appropriate target for vaccine development against HPV-associated malignancies [13]. Currently, two HPV prophylactic vaccines based on L1 virus-like particles, namely Gardasil® (Merck & Co., Inc, USA) and Cervarix® (GlaxoSmithKline, UK) have been accredited in many countries [14]. However, such vaccines provide limited type-specific protection and their mass production is less efficient with a high processing time [15–17]. The exorbitant issues linked with the currently available vaccines to prevent HPV infection as well as the loss of efficient therapeutic vaccines have provoked extensive efforts for the development of novel vaccine strategies [18]. So far, several vaccines against HPV *E6* and/or *E7* oncogenes using various approaches such as protein- or DNA-based vaccines and live vaccines have been developed [3,19–21]. It is noteworthy that among these, live vaccination strategy has attracted a great

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deal of attention. Intracellular bacteria, such as *Listeria* and *Salmonella* as well as *Vaccinia* or *Rubella* viruses, have been exploited extensively for vaccine development against HPV infection [22–26]. However, the hallmarks of these live vectors, pathogenicity, re-infection and toxicity, hinder their application as vaccine candidates in humans [3,27–30]. On the other hand, the nonpathogenic protozoan parasite, *Leishmania tarentolae*, has recently emerged as a novel candidate to generate live vaccines against intracellular pathogens. More specifically, the particular features of *L. tarentolae* such as efficient targeting of the antigen-presenting cells, protection through Th1 immune response, lack of replication within antigen-presenting cells and its immediate elimination after infection make it an appropriate vector for live vaccination [31]. A recent study indicates that recombinant *L. tarentolae* expressing the Gag protein of HIV-1 induces a favorable cell-mediated immune response and long-lasting immunity, resulting in a decrease in HIV-1 replication *ex vivo* [32]. Furthermore, the potential of *L. tarentolae* as a live vaccine recently shows that recombinant *L. tarentolae* expressing *Leishmania donovani*-specific A2 protein can be used effectively to immunize BALB/c mice against *Leishmania infantum* infection [33]. Hence, these studies suggest the unique application of such live vectors in future vaccination strategies against intracellular pathogens.

In this study, we constructed a recombinant *L. tarentolae* consistently expressing the HPV16 E7 protein and assessed its immunogenicity and tumor protection *in vivo*. Herein, we successfully verified the superior immunogenicity of E7-green fluorescent protein (GFP) encoding live vaccine strategy compared with other applied approaches (pcDNA-E7 construct as a DNA vaccine and a combination of DNA and live vaccine as a prime-boost regimen) and control groups. Finally, the anti-tumor protection potential of these vaccines was also evaluated in mice before and after challenge with E7-expressing tumor cells (TC-1). However, although the applied live vaccine showed the best response, it was not statistically significant and further manipulation in live vaccine construct are being applied to enhance its potency.

Materials & methods

DNA constructs

The *E7* coding gene from HPV type 16 (accession number K02718, 294 bp, kindly provided by TC Wu, Johns Hopkins Medical Institutions, USA) was previously cloned into the pEGFPN1 vector (Qiagen, CLONTECH Laboratories, Inc., USA), to be fused by GFP [10]. The *E7-GFP* fragment was PCR-amplified from pEGFP-E7 plasmid using the following primers: (E7 forward, 5′-GGAAGATCTATGCATGGAGATACACCTACA-3′); and (GFP reverse, 5′-GCTCTAGATTAGGTACCCTTGTACAGCTCGC-3′) and was subsequently cloned into the pDrive cloning vector (Qiagen). After sequence confirmation, the E7-GFP was digested from the *Bgl*II and *Xba*I restriction sites of the recombinant pDrive-E7-GFP plasmid, and then it was subcloned into the *Bgl*II/*Nhe*I site of the eukaryotic protein expression vector, pLEXSY-neo2 (Kit offered by commercial supplier Jena Bioscience GmbH, Germany [101]), in order to replace the 1-kb stuffer fragment of the vector.

Parasite growth & transfections

The *L. tarentolae* Tar II parasites (ATCC 30267) were grown at pH 7.2 and 26°C in M199 medium (Sigma [Sigma-Aldrich Co. LLC, USA]) complemented with 5% heat-inactivated fetal calf serum (Gibco®, USA), 40 mM hydroxy ethyl piperazine ethane sulfonic acid (HEPES), 0.1 Mm adenosine, 5 µg/ml hemin and 50 µg/ml gentamicin. For homologous recombination into the host chromosome, the pLEXSY-E7-GFP was linearized using the *Swa*I restriction enzyme. In order to transfect the linearized gene of interest into the host, 4×10^7 log phase parasites were washed and resuspended in 400 µl of electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose; pH 7.5). Plasmid DNA (5 µg) was stored on ice for 10 min, and was electroporated using Bio-Rad Gene Pulser Ecell (Bio-Rad, USA) at 450 V and 500 µF, as previously described [34]. G418 (Sigma) was applied for selection of recombinant transfectants.

Extraction of genomic DNA & PCR confirmation of *E7-GFP* gene integration

The recombinant *L. tarentolae* genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen). The *E7*, *GFP* and *E7-GFP* genes' existence were confirmed by PCR analysis, (E7 reverse primer, 5′-ATAAGCTTTTATGGTTTCTGAGAACAGAT-3′), (GFP forward primer, 5′-ATGATATCAAGATCTATGGTGAGCAAGGGC-3′); E7 forward and GFP reverse primers were mentioned in previous sections. Likewise, integration of the *E7-GFP* into the *L. tarentolae* genome was confirmed

by PCR analysis using the following primers: (*ssu* forward primer: F3001, 5′-GATCTGGTTGATTCTGCCAGTAG-3′) and (*aprt* reverse primer: A1715, 5′-TATTCGTTGTCAGATGGCGCAC-3′) provided in pLEXSY-neo2 kit.

RNA extraction & reverse transcription PCR

RNA was extracted from promastigote form of recombinant *L. tarentolae* using a RNeasy Mini kit (Qiagen) and treated with RNase-free DNase for 30 min at 37°C to remove remaining DNA. Total RNA quality was analyzed using Agilent Bioanalyser 2100c (Agilent Technologies, Inc., USA), and subsequently the cDNA was synthesized from 1 µg of RNA using the Qiagen Omniscript reverse transcription Kit. In order to detect the E7-GFP cDNA, RT-PCR was performed by using primers as described above, at 95°C for 5 min, 40 cycles of 95°C for 15 s, 62°C for 20 s and 72°C for 40 s, and one cycle of 72°C for 15 min. The RT-PCR product was also confirmed by sequencing.

Fluorescence microscopy & flow cytometry analysis

L. tarentolae promastigotes were examined for GFP expression. Promastigotes were centrifuged at 3000 rpm for 10 min followed by its wash once using phosphate-buffered saline (PBS); cells were mounted on microscope slides. Green fluorescence was excited with a 488-nm argon-ion laser and was captured using a combination of a 510–530-nm band-pass emission filter. For flow cytometric analysis, both wild and recombinant *L. tarentolae* were prepared as mentioned above. In order to monitor the infection rate, a total of 100,000 events were counted by particle analyzing system flow cytometer (Partec, Germany) 24, 48 and 72 h post-infection. Flowjo software (Tree Star Inc., OR, USA) was employed to analyze the fluorescence-activated cell sorting (FACS) results.

Western blot analysis

Parasite promastigotes were prepared as described in earlier sections. Followed by the cells' harvest by centrifugation at 3000 rpm for 10 min and washing in PBS, the pellets were instantly lysed in 2× SDS-PAGE sample buffer on ice and later boiled for 5 min. Samples were then loaded on a 12.5% SDS-PAGE. As a next step, Bio-Rad wet blotting system was used for transferring gels onto a nitrocellulose membrane and then it was incubated overnight with blocking solution (tris-buffered saline with 0.01% Tween 20 (Sigma-Aldrich Co. LLC, USA) and 2.5% BSA). The following day, blots were first washed with 0.01% Tween20 in tris-buffered saline and then incubated with anti-HPV16 E7 monoclonal antibody as the first antibody (2:1000, US Biological, USA) for 2 h. The membranes were washed three-times and incubated for 90 min with peroxidase-conjugated goat anti-mouse IgG (1:2000, Sigma) as the secondary antibody. Extra antibodies were removed by washing as described above. Eventually, in order to detect the expected bands of the proteins, diaminobenzidine tetrahydrochloride (Sigma) was used as the substrate.

In vitro Leishmania infection

In order to assess the *in vitro* interaction between stationary-phase promastigotes and bone marrow-derived macrophages, murine RAW 264.7 cell line was transfected with recombinant *L. tarentolae*, as described previously [35]. In brief, 2×10^5 macrophages along with 2×10^6 parasites were incubated in 24-well plates for 4 h at 37°C (parasite/macrophage ratio of 10:1). Then free parasites were removed by washing, and the plates were incubated once again at 37°C for several periods of time. Infection with fluorescent *L. tarentolae* expressing the E7-GFP was monitored by fluorescence microscopy and argon ion laser equipped BD FACScalibur flow cytometer (BD Biosciences, USA). A total of 100,000 events were collected at macrophage population gated out from the whole events and analyzed using FlowJo version 7.5.3, 24, 48 and 72 h post-infection. By placing the 24-well plates on ice with cold PBS, the cells were forced to detach and collected gently. Uninfected macrophages, macrophages infected with wild-type *L. tarentolae* (negative control) and those infected with *L. tarentolae* expressing GFP only (positive control) were used for comparison. After cDNA purification, RT-PCR was performed to track the existence of the gene of interest within macrophages.

Parasite & DNA preparation for vaccination

For preparation of the DNA constructs, pcDNA3.1 and pcDNA-E7 vectors were extracted using Endofree plasmid Giga kit (Qiagen). To prepare freeze-thawed (F/T) promastigotes, parasites were harvested by centrifugation (3000 rpm, 10min, 4°C), washed twice in PBS (8 Mm Na_2HPO_4, 1.75 Mm KH_2PO_4, 0.25 Mm KCl, 137 mM NaCl) and resuspended

at a concentration of 2×10^8 parasites/ml. This preparation was frozen and thawed 17-times using liquid N_2 and a 37°C water bath. Afterwards, the protein concentration was determined by bicinchoninic acid reagent (Pierce Chemical Company, IL, USA). In order to obtain soluble *Leishmania* antigens (SLA), F/T parasites were centrifuged at 4000 rpm for 10 min; the collected supernatant was considered as SLA.

Mice & immunization

This study was carried out using six groups of 8–10-week-old female C57BL/6 mice (n = 10) namely, group 1 (pcDNA3.1/pcDNA3.1), group 2 (PBS/PBS), group 3 (pcDNA-E7/pcDNA-E7), group 4 (*L. tarentolae-E7-GFP*/*L. tarentolae-E7-GFP*), group 5 (*L. tarentolae*-GFP/*L. tarentolae*-GFP) and group 6 (pcDNA-E7/*L. tarentolae-E7-GFP*), obtained from the breeding stock maintained at the Pasteur Institute of Iran. Mice were kept in plastic cages, provided with free access to tap water and standard rodent pellets in a centralized air-conditioned facility which was maintained under a constant 12:12 h light–dark cycle at room temperature with relative humidity (50–60%). The present study was approved by ethical committee at Pasteur Institute of Iran. Also, the maintenance of the animals and their blood sample collections were carried out under the strict supervision of animal care office of Pasteur Institute of Iran. For priming, total number of 2×10^7 stationary phase recombinant *L. tarentolae* promastigotes expressing E7-GFP protein was injected into group 4. Groups 3 and 6 received 100 µg of pcDNA-E7. Control groups, 1, 2 and 5 were immunized with 100 µg of pcDNA3.1 (-) plasmid, PBS only and *L. tarentolae* expressing GFP, respectively. Three weeks after priming, as booster immunization, the same schedule was performed except for group 6 (prime/boost regimen), which received E7-GFP expressing *L. tarentolae*. All vaccinations including tests and control groups were administered subcutaneously (sc.), immortalized lung epithelial cells of C57BL/6 mice expressing HPV-16 E7, TC-1 cells (ATCC: CRL-2785), were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum. Cultures were incubated at 37°C in the presence of 5% CO_2. Three weeks after booster, all groups were sc. challenged with 1×10^5 TC-1 cells/mouse on the preshaved right flank of mice.

Humoral immune responses

Mice sera were collected from all groups before and 3 weeks after challenge to be analyzed by ELISA to assess specific antibodies, including IgG1 and IgG2a against recombinant E7 (rE7, 5 µg/ml), SLA (10 µg/ml) and F/T (10 µg/ml). Pooled sera for each group were stored at -20°C. In brief, 96-well plates (NUNC) were coated with rE7, SLA and F/T in PBS and incubated at 4°C overnight. To block the plates, 100 µl of 1% BSA in PBS for 2 h at 37°C was applied. Then, serum samples (100 µl, 1:50 dilutions) was added and incubated for 2 h at 37°C. After three washes, biotinylated rabbit anti-mouse IgG1 (1:10000, Southern biotech) or IgG2a (1:500, Southern biotech) were added and incubated for 2 h at 37°C, and then plates were incubated for 1 h at 37°C with streptavidine-conjugated horseradish peroxidase (1:1000, Sigma). After four washes, plates were treated with 100 µl/well *O*-phenylenediamine (OPD, Sigma) as substrate. Finally, the reactions were ended with 100 µl/well of 4M H_2SO_4 and the absorbance level was measured at 492 nm.

Cytokine assay

Prior and three weeks post challenge, three mice from each group were sacrificed and spleens were homogenized. Splenocytes derived from immunized mice were then lysed using ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2-EDTA), followed by its wash and re-suspension in complete RPMI medium (RPMI-1640 supplemented with 5% fetal calf serum, 1% L-glutamine, 1% HEPES, 0.1% 2ME, 0.1% gentamicin). In the presence of SLA and F/T (50 µg/ml), rE7 (20 µg/ml) and medium alone, a density of 2×10^6 cells/well was seeded in the plates. As the positive control, concanavalin A (ConA; 5 µg/ml) was used in all experiments. Plates were incubated at 37°C in 5% CO_2 humidified atmosphere. After 3 days, the supernatants of splenocyte cultures were collected in order to measure the IFN-γ and IL-5 production using sandwich-based ELISA kits (R&D, MN, USA) and according to the manufacturer's instructions. The lower detection limits of IFN-γ and IL-5 were 2 and 7 pg/ml, respectively. All the experiments were performed in duplicates.

In vivo tumor growth inspection

A controlled procedure was adopted where after 2 weeks of TC-1 challenge, tumor growth was inspected visually and with palpation; tumor size was also measured with calipers twice a week for a period of 2 months as previously described [36]. The mean diameter of the tumor was schemed in cubic centimeters against various time points.

Tumor volumes were calculated using the formula: (length × width^2)/2 [37]. For the comparison of tumor volume between groups, the mean and SD of tumor size for each group were also determined. According to the ethical guidelines of Pasteur Institute of Iran, tumor mice were killed when approaching death.

Statistical analysis

The statistical significance of immune responses before and after challenge was determined by one-way ANOVA analysis using Graph-Pad Prism® 5.0 for Windows (GraphPad Software Inc.). In addition, for analyzing the tumor growth, both one-way ANOVA and un-paired Student's t-test were applied. For all such experiments, $p < 0.05$ was considered significant and the data was shown as mean ± SD.

Results

Production of recombinant *L. tarentolae* expressing the *E7* gene

In order to verify stable expression of E7 by *L. tarentolae*, pLEXSY-E7-GFP vector was introduced into *L. tarentolae* by electroporation as described in the 'Materials & methods' section. The PCR amplification of the integrated fragment can be attributed to the successful insertion of the *E7-GFP* into the *L. tarentolae* genome (Figure 1A). In addition, expression of E7 in *L. tarentolae* was confirmed at the mRNA level through reverse transcription PCR analysis using promastigote cDNA as a template. Detection of the desired PCR fragment confirmed the expression of E7 mRNA in E7-GFP recombinant *L. tarentolae* (Figure 1B). Similarly, western blot analysis was also performed on parasite total protein using E7-specific monoclonal antibody. As shown in (Figure 1C), E7 protein is expressed in log-phase of recombinant strain. Further to earlier confirmations, fluorescence microscopy analysis was also performed as depicted in (Figure 1D). E7-GFP fusion gene (*E7* gene attached to EGFP in the DNA plasmid) is consistently expressed in recombinant parasite after integration into its genome (Figure 1D). Furthermore, the fluorescence activity of log-phase E7-GFP-expressing parasites assessed by

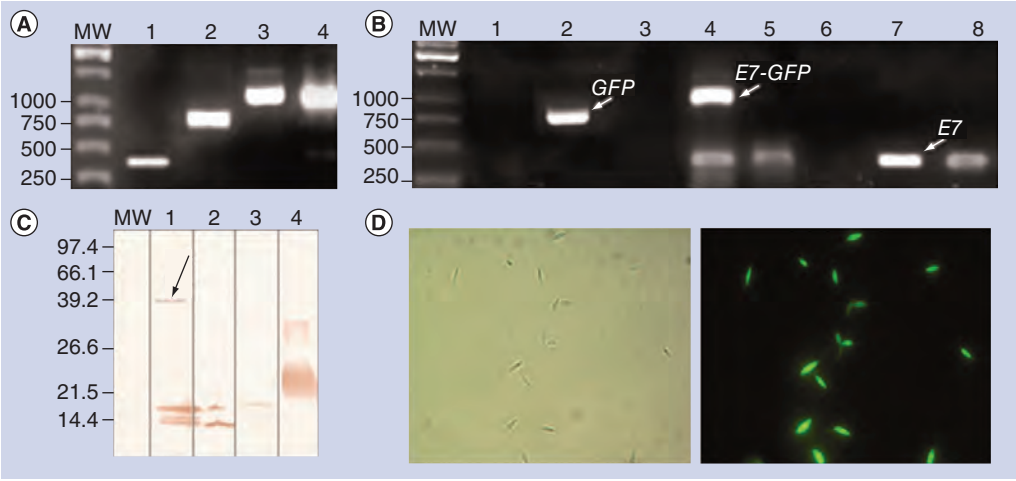

Figure 1. E7-GFP integration and expression in *Leishmania tarentolae* strain. *Leishmania tarentolae* was transfected with *E7-GFP* fusion gene in pLEXSY plasmid; the genomic DNA and RNA were then extracted for further confirmation. **(A)** Results of PCR reaction on *L. tarentolae* genome using E7 and GFP primers. Columns 1, 2 and 3 demonstrate the *E7*, *GFP* and *E7-GFP* genes amplification, respectively. Column 4 is the PCR amplification of the integrated fragment, which includes *E7-GFP*, using integration primers provided by pLEXSY-neo2 kit. **(B)** Amplification of the expected fragments in cDNA samples. Promastigotes' RNA was extracted and cDNA was synthesized from 1 µg of RNA using Qiagen Omniscript® reverse transcription kit. Column 1 (PCR amplification of the extracted RNA), columns 2 and 3 (amplification of *GFP* gene and its negative control), columns 4 and 5 (*E7-GFP* amplification and its negative control), column 6 (reverse transcription PCR negative control), columns 7 and 8 (*E7* amplification and its negative control) are shown in the order designated. **(C)** Western blot analysis for E7 protein using the anti-HPV16 E7 monoclonal antibody. The 40-kD band determines the E7-GFP protein expression (column 1). Recombinant E7 (column 4) and GFP expressing *L. tarentolae* (column 2) were used as positive controls. Column 3 shows the *L. tarentolae* as negative control. **(D)** Fluorescence microscopic images of GFP expression in recombinant *L. tarentolae* 48 h post-infection; both bright field (right) and fluorescence (left) are shown.
GFP: Green fluorescent protein; MW: Molecular weight.

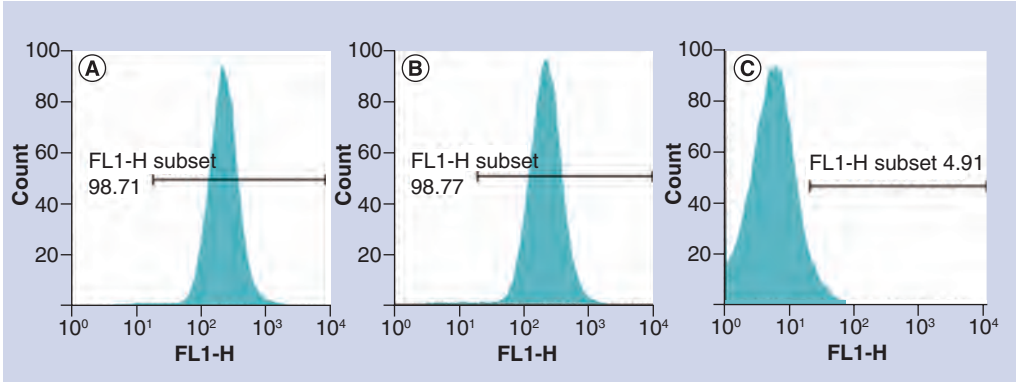

Figure 2. Quantitative ratios of recombinant parasites indicated by flow cytometry 48 h post-infection. (A) Recombinant *Leishmania tarentolae* expressing E7-green fluorescent protein. **(B)** Recombinant *L. tarentolae* expressing green fluorescent protein (positive control). **(C)** Wild-type *L. tarentolae* strain (negative control). Similar results were obtained following experiment repetition on various days. Infection rate was monitored by particle analyzing system flow cytometer counting a total of 100,000 events per sample.

FACS as illustrated in Figure 2, confirmed our previous findings.

E7-GFP *in vitro* expression in murine cell line transfected with recombinant *L. tarentolae*

Consistent with the *in vivo* immunization, murine RAW 264.7 cell line was transfected with stationary-phase *L. tarentolae* expressing E7-GFP protein, *L. tarentolae* expressing GFP as a positive control and *L. tarentolae* as a negative control. Subsequently, GFP expression was assessed using FACSCalibur (Figure 3A). Results obtained by fluorescence microscopy also confirmed the E7-GFP expression in infected macrophages (Figure 3B). The expression of the E7-GFP at mRNA level was evaluated followed by RNA extraction from transfected macrophages (Figure 3C).

Immunization with live recombinant *L. tarentolae*-E7-GFP induces high levels of both E7-specific IFN-γ & IgG2a in mice

Different groups of mice were sc. immunized with recombinant *L. tarentolae* encoding E7-GFP and GFP (control), pcDNA-E7 and pcDNA3.1 (control), PBS (control) and a combination of designed live and DNA vaccines as the prime boost regimen. Vaccinations were performed twice after 3 weeks. As a next step, 3 weeks after booster, mice were sc. challenged with TC-1 tumor cells. Obtained results were compared among three vaccination strategies and their controls. As shown in Figure 4A & B, specific IgG2a and IgG1 antibodies against rE7 (for all groups), F/T and SLA (for all groups except DNA vaccines) were measured by ELISA before and 3 weeks after challenge. No significant levels of IgG1 production were observed among all three strategies compared to their controls ($p > 0.05$) except for group 5 (GFP control), which showed significant values for IgG1. While analyzing the IgG2a levels, the group of mice that received *L. tarentolae*-E7-GFP (group 4) showed significant values against all three coated antigens compared to that of the two other strategies and control groups before and after challenge ($p < 0.05$) However, insignificant levels of IgG2a production against rE7 were observed for the DNA vaccination strategy using pcDNA-E7 in comparison with other strategies and its control group (pcDNA 3.1) both before and after challenge ($p > 0.05$) (Figure 4). However, the prime-boost regimen (group 6) showed significant level of IgG2a compared with PBS control and DNA-vaccinated groups before and after challenge, and it did not show any significant difference compared with live vaccination strategy (group 4).

In order to evaluate cellular immune response, levels of IFN-γ and IL-5 production were also analyzed in the splenocytes of all groups prior and 3 weeks post-challenge. Levels of IL-5 production in live, DNA and DNA/live vaccinated groups compared to control groups (groups 1, 2 and 5) did not show any significant differences (data not shown). Besides, results of IFN-γ production before and 3 weeks after challenge, for the *L. tarentolae*-expressing E7-GFP group reached the highest level compared with prime/boost vaccination (group 6), DNA vaccination (group 3) and control groups including PBS (groups 2) and *L. tarentolae-GFP* (groups 5) with respect to rE7, F/T and SLA (Figure 5A & B). Similarly, the levels of IFN-γ production in

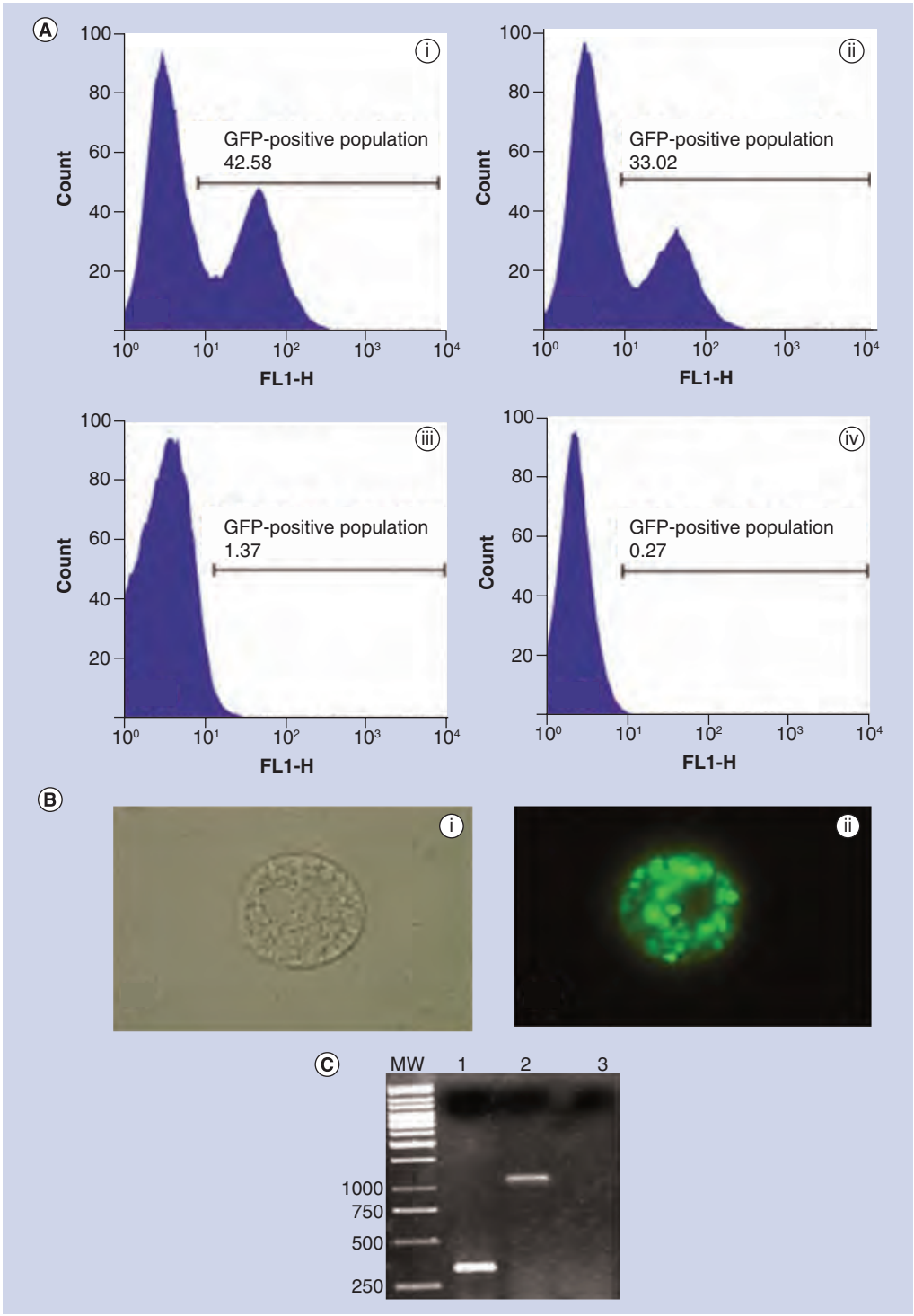

Figure 3. *In vitro* evaluation of E7-GFP expression. (A) Fluorescence-activated cell sorting analysis of RAW 264.7 cells transfected with stationary-phase parasites 48 h post-infection: **(i)** *Leishmania tarentolae-E7-GFP*, **(ii)** *L. tarentolae*-GFP (positive control), **(iii)** *L. tarentolae* (negative control) and **(iv)** macrophages only. **(B)** Fluorescence microscopy images of E7-GFP expression in transfected macrophages. Both the nonfluorescent (**i**, white light) and fluorescent (**ii**) fields are shown. The promastigotes have infected the macrophages, expressing the GFP fused to E7. **(C)** Reverse transcription PCR analysis on synthesized cDNA of infected macrophages. Column 1: *E7* gene amplification; column 2: *E7-GFP* fusion PCR; column 3: control RNA; the 1080-bp band of *E7-GFP* as well as the 300-bp *E7* amplification confirm *E7* existence and expression within macrophages.
GFP: Green fluorescent protein; MW: Molecular weight.

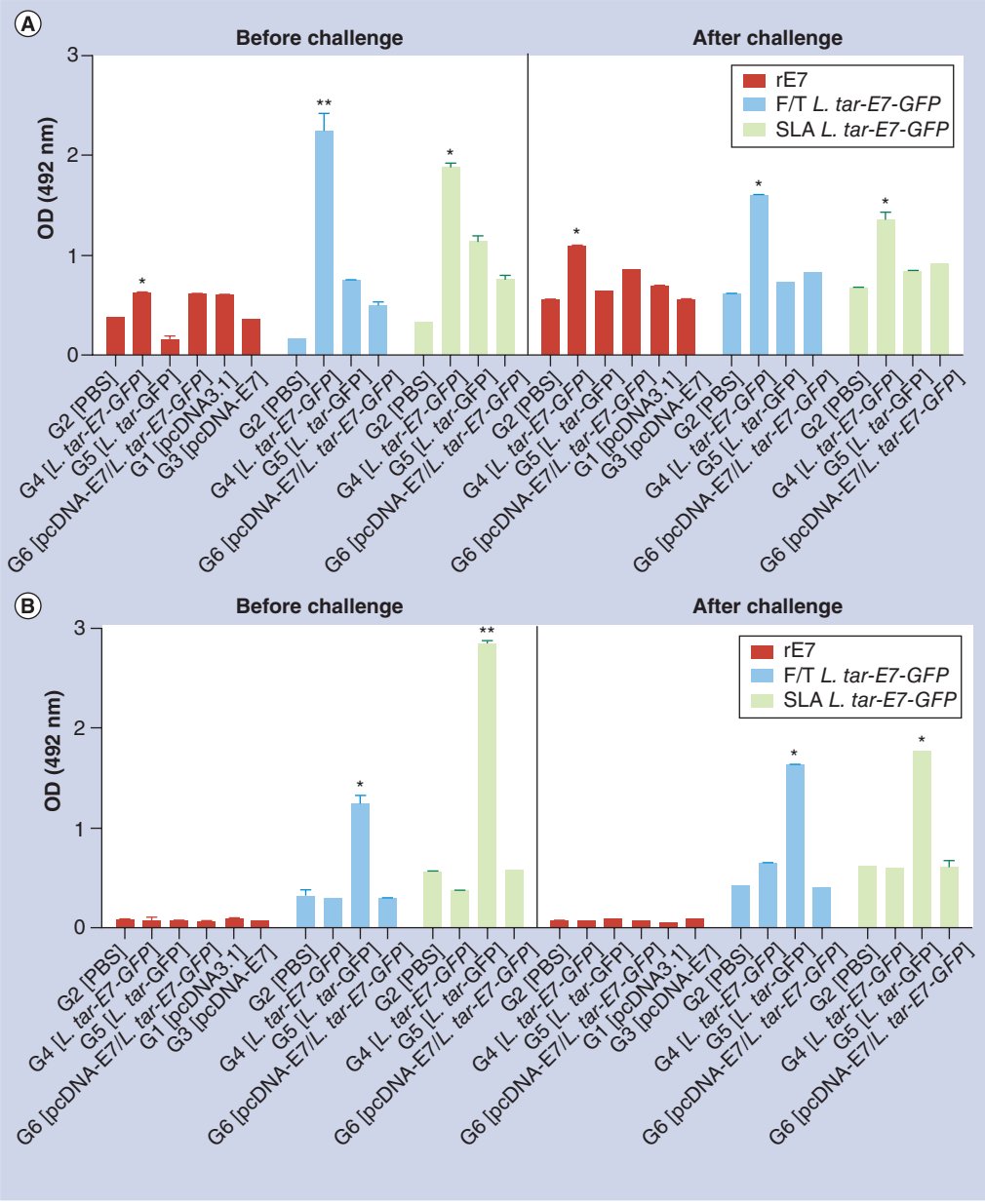

Figure 4. Analysis of specific humoral immune responses in C57BL/6-vaccinated mice.
Groups of mice (n = 10) were immunized twice at intervals of 3 weeks, with E7-GFP recombinant *Leishmania tarentolae*, pcDNA-E7 and a combination of both as prime-boost regimen. The control groups, including PBS, pcDNA3.1 and GFP-expressing *L. tarentolae*, were applied for comparison. Mice were subcutaneously challenged with TC-1 tumor cells on the right flank, 3 weeks after booster. Before and after challenge collected sera from all groups were pooled and diluted to 1:50 ratio. Recombinant E7 protein, *L. tarentolae* soluble *Leishmania* and freeze-thawed antigens were used to coat the plates and ELISA was performed in duplicate for all samples. **(A)** IgG2a and **(B)** IgG1 levels were measured against rE7 (to compare all three strategies), freeze-thawed and soluble *Leishmania* antigens (to compare *L. tar-E7-GFP* with prime-boost regimen). The data demonstrate before and after challenge comparisons. The rates of OD are depicted according to mean ± SD. Asterisks indicate the significance of live vaccine group (*L. tar-E7-GFP*) versus its controls (*L. tar*-GFP and PBS) and other groups (*$p < 0.05$, **$p < 0.01$).
GFP: Green fluorescent protein; OD: Optical density; PBS: Phosphate-buffered saline.

DNA/live vaccination group against all three antigens were significant in comparison with the PBS group both before and after challenge, however, it was much lower than that of the live/live vaccination group (group 4). It is noteworthy that after challenge, results showed an obvious decrease in the amounts of IFN-γ in all groups due to three antigens (rE7, F/T and SLA) except

for the live group (group 4), this was attributed to its minimum decrease and the maximum durability (FIGURE 5B).

Protection assessment of E7-GFP expressing vaccines against TC-1-induced tumors

In order to test and compare the protective efficacy of all three E7-GFP expressing vaccines against TC-1 tumor cells, C57BL/6 mice were sc. challenged with the TC-1 E7 expressing tumors cells 3 weeks after booster. Measurement of tumor size was initiated 2 weeks after the challenge, and the data was recorded for a period of 60 days as shown in FIGURE 6. A comparative study indicates that there was no statistically significant difference among the various strategies employed, which includes live/live (group 4), DNA/DNA (group 3) or DNA/live (group 6) with respect to each other and the control groups. However, groups of mice receiving the live (*L. tar-E7-GFP*) and DNA vaccines (pcDNA E7), showed the least tumor size. This result suggests that the prophylactic immunization with both E7-GFP expressing live and DNA vaccines needs further investigation in order to enhance their effect and promote significant anti-tumor protection.

Discussion

In this article, we introduced and developed a novel live vaccine using recombinant *L. tarentolae* expressing E7-GFP fusion protein to immunize mice against HPV-associated tumors. For this, the pLEXSY-neo2 system was applied to transfect *L. tarentolae* with E7-GFP fusion and to integrate this construct into a parasite genome. PCR methodology, fluorescence imaging, FACS and western blot analysis of recombinant *L. tarentolae* confirmed *E7-GFP* integration and expression in the parasite. Since the applications of viral and bacterial vectors for vaccine development against HPV have always been a serious concern for human safety, live nonpathogenic *L. tarentolae* holds great promise for the development of such live vaccines, as it shows no risk of infection and potent immune response elicitation in humans [31]. Live recombinant vaccination has been always considered as a crucial strategy in development of HPV vaccines; this is mainly due to having greater immunogenicity compared to subunit vaccines and showing the same features as the natural infection [13,22,38,39].

In this study, the protective effects of three vaccination strategies (*L. tarentolae-E7-GFP* as the live vaccine, pcDNA-E7 as the DNA vaccine and *L. tarentolae-E7-GFP*/pcDNA-E7 as the prime-boost regimen) were compared with each other and their controls in six groups of C57BL/6 mice (n = 10). We found that the live vaccination strategy (*L. tarentolae-E7-GFP*) elicits significant levels of humoral (IgG2a) and cellular (IFN-γ) immune responses compared with that of its control groups (*L. tarentolae-GFP* and PBS). This significant IgG2a and IFN-γ production in *L. tarentolae-E7-GFP* group was confirmed repeatedly against various antigens including rE7, F/T and SLA before TC-1 challenge (as

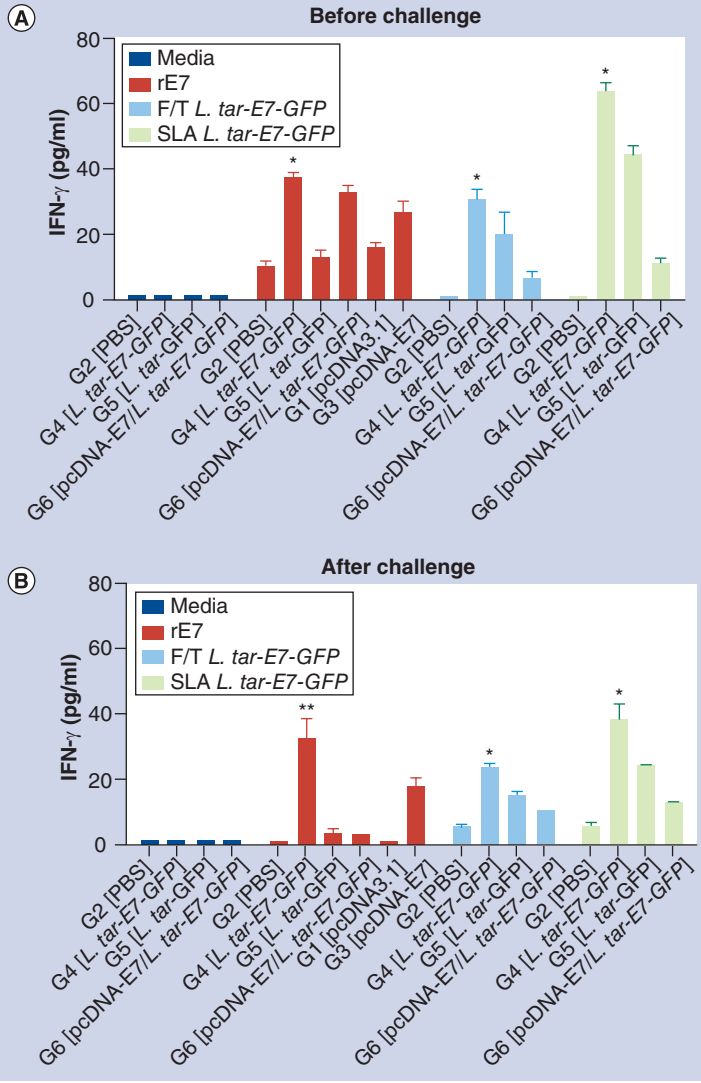

Figure 5. Comparison of IFN-γ response in different groups of immunized C57BL/6 mice. Following subcutaneous immunization with live and DNA vaccines expressing GFP or *E7-GFP*, three mice from each group were sacrificed **(A)** before and **(B)** 3 weeks after challenge and their splenocytes were stimulated by rE7 (for all groups), freeze-thawed and soluble *Leishmania* antigens (for all groups except DNA vaccines, groups 1 and 3). IFN-γ levels were measured in the supernatant of splenocytes by sandwich-based ELISA in duplicates. Concanavalin A was used as the positive control and the data represents as the mean ± standard error. Asterisks indicate the significance of live vaccine group (*L. tar-E7-GFP*) versus its control group (*L. tar-GFP*) (*p < 0.05, **p < 0.01).
GFP: Green fluorescent protein; PBS: Phosphate-buffered saline.

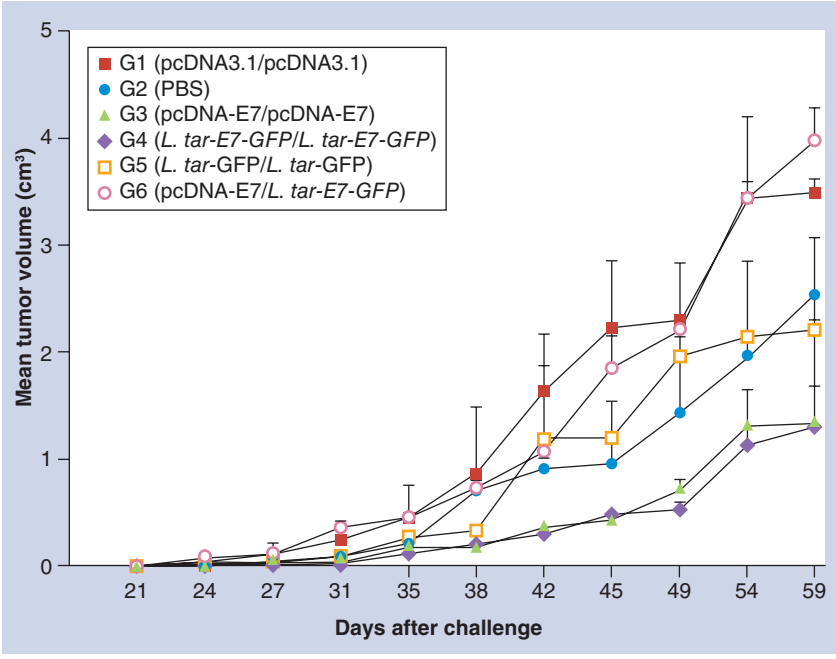

Figure 6. *In vivo* tumor protection of recombinant vaccines against TC-1 tumor challenge. In order to test tumor protection, ten mice per group were immunized with *Leishmania tarentolae-E7-GFP* and *L. tarentolae-GFP* as live vaccination, pcDNA-E7 and plasmid without insert as DNA vaccination and a combination of both (pcDNA-E7/*L. tarentolae-E7-GFP*) as prime-boost regimen. Mice were boosted with the same regimen as the first injection; 3 weeks later they were subcutaneously challenged with 1 × 10^5 TC-1 cells/mouse on the right flank. The tumor growth was monitored biweekly for 2 months.

shown in Figures 4A & 5A). Furthermore, similar patterns were also observed after challenge as shown in Figure 4A. This was attributed due to the induction of potent type 1 immune responses in our live vaccination group. Notably, this is in good agreement with the earlier work which indicates that high levels of IFN-γ and IgG2a production link with Th1 responses [40]. On the other hand, when we compared live vaccine group with the control groups, insignificant levels of IgG1 subclass and IL-5 production were observed that corresponds to the Th2 responses [41,42]. This confirms our findings that E7-GFP expressing live recombinant vaccine elicits a strong Th1-mediated immunity compared with that of control groups.

Further to our confirmations, E7-GFP expressing live vaccination (group 4) was compared to both other strategies, including DNA/DNA regimen (group 3) and DNA/live regimen (group 6) using recombinant E7 protein as coated antigen in ELISA plates. Before challenge results showed that although each individual applied strategy elicited a significant amount of IFN-γ compared with that of their respective controls, no superiority was observed over each other. However, after challenge with TC-1 cells, E7-GFP expressing live vaccine showed significant values of IFN-γ production compared with that of DNA and prime-boost vaccines, while pcDNA-E7 vaccine also showed dominance over prime-boost strategy. Moreover, the production of IgG2a in response to rE7 was also consistent with the cytokine results. These data indicate the superiority of the live vaccine group in inducing specific immune responses over its counterparts before and after challenge.

The applied control group for our live vaccine, GFP-expressing *L. tarentolae*, produced an IgG1-dominant response as compared with other groups. Previous studies have shown similar results in which the GFP is able to potently induce a strong IgG1 antibody response in mice, which is correlated with a Th2 profile [43,44]. While IgG1-dominant, the induced immune responses also consisted of other subclasses such as IgG2a [45]. Furthermore, a similar study has demonstrated that GFP immunogenicity could be dose-dependent and only in very low amounts it is considered to be nonimmunogenic [46]. Importantly, while concurrent anti-GFP immune responses do occur, GFP has been characterized as a neutral selection marker for immunization purposes, which does not significantly interfere with anti-tumor responses [47]. In our case, although GFP did not interfere in the E7 anti-tumor immune response, it elicited antibody responses, in particular the IgG1 subclass. This is in accordance with the significant levels of E7-induced Th1 responses (both IgG2a and IFN-γ) in the live vaccination group along with a lack of IgG1 production in this group before and after challenge. This explains the major role of E7 in shifting the Th1/Th2 balance toward type 1 immune responses in the presence of GFP.

It is worth mentioning that the group of mice receiving the E7-GFP expressing *L. tarentolae* live vaccine (group 4) showed superior durability of IFN-γ responses to rE7 protein after challenge. This was mainly due to minimum relative decline in its production (50%), whereas, the remaining groups showed drastic decrease in their responses accordingly. Furthermore, we noticed that pcDNA-E7 injected mice (group 3) showed a relative stability after group 4. While priming the immune system with *L. tarentolae-E7-GFP* and boosting with pcDNA-E7 (group 6), resulted in the lowest duration for IFN-γ responses after challenge. More specifically, the obtained data was further validated with the help of an advance comparison between live and DNA/live groups by applying F/T and SLA as coating antigens. Interestingly, both IFN-γ and IgG2a results before and after

challenge, again confirmed the dominance of the E7-GFP expressing *L. tarentolae*. Here we provide evidence that live/live approach is the most significant strategy in induction of a potent Th1-mediated immune response compared with that of two other applied strategies.

An earlier study indicated that the HPV-16 E7-expressing murine tumor model, TC-1 [48], has been exploited successfully for the prophylactic and therapeutic analysis of HPV-associated tumors [13,49,50]. The live (*L. tar-E7-GFP*) and DNA (pcDNA-E7) vaccinated groups, although not statistically significant, showed the best anti-tumor responses compared with that of DNA/live vaccinated and control groups. This could be attributed to the similar IFN-γ durability after challenge for both DNA and live groups. The minimum observed tumor size in the case of group 4 could be indicative of significant levels of Th1-mediated responses, including high production of IFN-γ and IgG2a before and after challenge. According to the tumor protection data, a long-term memory response that could prevent tumor outgrowth in group 4 was not induced sufficiently (i.e., the IFN-γ and IgG2a durability was not enough to provide a desirable protection. Additionally, DNA/live strategy demonstrated the lowest level of tumor protection; this is in line with the fact that the IFN-γ production for this strategy was not significant before and after challenge.

The live vaccine, *L. tarentolae-E7-GFP* may be a suitable candidate to prevent HPV type 16 infections, however, certain issues linked with this strategy need to be addressed carefully. Enhancement of tumor-specific immunity with merging the principal tumor antigens with immune system enhancers, such as adjutants and cytokines, has been demonstrated in several studies [50–55]. Based on our latest work, we believe that lack of protection against tumors can be explained due to the short-term memory response after challenge which can be improved by manipulating the vaccine construct. Application of immune enhancers in the design of *L. tarentolae*-E7-GFP live vaccine can potentially help to improve its protective efficacy. It is also possible that the variation introduced in the TC-1 dose might have some positive impact on the protection capabilities of the designed vaccines. Different doses of TC-1 cells, 1×10^4 to 1×10^6 cells/mouse have been previously used for tumor induction in mice models [48,50]. The live vaccine under discussion has shown trivial effect to control the tumor, when a dose of approximately 1×10^5 cells/mouse was sc. administered. We believe that an optimal dose of TC-1 cells could help to further improve the overall effects of this novel vaccine. Furthermore, HPV tumor protection has been investigated in viral and bacterial vaccines based on various routes of immunization. In one recent study, mucosal co-administration of live *Lactococcus lactis* expressing E7 and IL-12 showed full anti-tumor protection and prolonged immunity [54]. Moreover, in a number of related studies, the intraperitoneal immunization of different live vaccines has also demonstrated strong protection against HPV-associated or other infections [33,56,57]. As demonstrated in several studies, sc. immunization against HPV infection or tumor can also elicit significant immune response and anti-tumor protection [58–61]. Furthermore, it is believed that sc. is among the most applicable means of vaccine delivery in human [62]. Although other routes such as intraperitoneal can elicit strong protection, the lack of persuasive results from several clinical trials and also acute effects of intraperitoneal immunization versus sc. have confined the usage of this route in clinic [63,64]. Therefore, manipulating the vaccine construct to enhance its immunogenicity and usage of different TC-1 doses while applying sc. delivery, could yield a potential improvement in HPV tumor protection of this novel live vaccine in future studies.

Future perspective

A number of therapeutic vaccines have been developed targeting E6 and E7 including live vector vaccines, peptide/protein-based vaccines, cell-based vaccines and nucleic acid-based vaccines, each with advantages and disadvantages. Among different vaccine strategies, the use of plasmid DNA and live recombinant vectors are the most promising, either alone or as part of heterologous prime-boost combinations. However, live vectors inherently pose a potential safety risk, especially to immunocompromised individuals. Therefore, there is an urgent need to develop new live-vaccine vectors that are capable of enhancing antigen presentation and eliciting potent immune responses without the risk of developing disease in humans. In this study, for first time, we investigated the potential of a nonpathogenic protozoan, *L. tarentolae*, as a live vaccine candidate vector to efficiently target the immune system. This strain has been shown to be nonpathogenic when injected into immunodeficient severe combined immunodeficiency mice. *L. tarentolae*, has the capability to express heterologous genes. Therefore,

different approaches could be applied in order to enhance the protective effect of this strategy by using different immune system enhancers such as adjuvants as well as cytokines.

Acknowledgements

The authors wish to thank A Javadi (Pasteur Institute of Iran, Department of Immunology) and Sh. Alizadeh (Pasteur institute of Iran, Molecular Immunology and Vaccine Research Laboratory) for their technical assistance.

Financial & competing interests disclosure

The authors acknowledge the financial support from the Iran National Science Foundation (ID 89000588) for experimental works. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All mouse experiments including maintenance, animals' handling program and blood sample collection were approved by Institutional Animal Care and Research Advisory Committee of Pasteur Institute of Iran (Education Office dated January, 2010), based on the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education (MOHME) of Iran that was issued in 2005.

Executive summary

- We developed a novel live vaccine using recombinant *Leishmania tarentolae* to express E7-GFP fusion protein against HPV-associated tumors.
- Three subcutaneously administered vaccination strategies, namely *L. tarentolae-E7-GFP* as the live vaccine, pcDNA-E7 as the DNA vaccine and *L. tarentolae-E7-GFP*/pcDNA-E7 as the prime-boost regimen were studied. A comparative study was performed in order to estimate the vaccines' ability of generating antigen-specific humoral and cellular immune responses.
- The protection efficacy of the developed vaccines was also evaluated against TC-1-induced tumor.
- E7-GFP recombinant *L. tarentolae* strain positively induced significant levels of IgG2a and IFN-γ compared with the control (GFP recombinant *L. tarentolae*) and other strategies before and after challenge with TC-1 tumor cells.
- No significant differences for IL-5 and IgG1 production were observed between test and control groups except for group 5 receiving the *L. tarentolae-GFP* expressing vaccine.
- Although the anti-tumor effect of the live vaccine group (*L. tarentolae-E7-GFP*) was not significant compared with the control and other strategies, it showed the best efficacy against tumor outgrowth.
- The protective properties of DNA and live strategies were equivalent in the current tumor mouse model.
- In brief, we conclude that the *L. tarentolae-E7-GFP* live immunization was the most effective strategy for the induction of immune responses.

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