

A Live Vector Expressing HPV16 L1 Generates an Adjuvant-Induced Antibody Response In-vivo

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Abstract

Background: The association between human papillomavirus (HPV) infections and cervical cancer has suggested the design of prophylactic and therapeutic vaccines against genital warts. The HPV capsid has made of two L1 and L2 coat proteins that have produced late in viral infections. Regarding to the recent studies, two commercial prophylactic vaccines have based on L1 viral like particles (VLPs) could strongly induce antibody responses, and protect human body from HPV infections. However, the use of these HPV vaccines has hindered due to their high cost and some limitations. Currently, among various vaccination strategies, live vector-based vaccines have attracted a great attention.

Objectives: Herein, a non-pathogenic strain of the protozoan organism known as *Leishmania tarentolae* has utilized to induce potent humoral immunity in mice model.

Materials and Methods: At first, cloning of HPV16 L1 gene into *Leishmania* expression vector has performed and confirmed by PCR and digestion with restriction enzymes. The promastigotes of *Leishmania tarentolae* (*L.tar*) have transfected with linearized DNA construct by electroporation. Protein expression has analyzed by SDS-PAGE and western blotting. Then, the immunogenicity of *leishmania* expressing L1 protein (*L.tar-L1*) has assessed in mice model.

Results: Our data has indicated that subcutaneous immunization of mice with the recombinant *L.tar-L1* has led to enhance the levels of IgG1 and IgG2a in comparison with control groups. Furthermore, there was no significant increase in antibody levels between two and three times of immunizations.

Conclusions: The recombinant live vector was able to induce humoral immunity in mice without need of any adjuvant. However, further studies have required to increase its efficiency.

Keywords: HPV, L1 Protein, Live Vaccine

1. Background

Cervical cancer, as the most common malignancies in women, has associated with human papillomavirus (HPV) infection (1, 2). The HPV genotypes have accompanied by genital infection included low-risk types (e.g. HPV 6 and 11), and high-risk types (e.g. HPV 16 and 18) generating ~ 90% of anogenital warts, and ~ 70% of cervical cancers, respectively (1, 3). Epidemiological studies have shown that primary prevention of cervical cancer by a prophylactic vaccine against HPV infections could significantly decrease the incidence of cervical cancer (1). HPV genome has coded eight early proteins (E1-E8) and two late coat proteins (L1 and L2) (1, 4). One of the best strategies for generating a preventive HPV

vaccine has included the use of VLPs (virus-like particles) composed of the major capsid L1 protein, alone (5). Two prophylactic VLP-based vaccines have commercialized worldwide known as Cervarix® and Gardasil® (3, 6). The main disadvantages of the VLP vaccines were the high cost of preparation, and the existence of suitable cold chain. These drawbacks have hampered their extensive use in developing or resource-poor countries which had 80% cervical cancer cases (5, 7). Thus, the studies have focused on other approaches such as protein or live vector-based vaccines (7-10). Among them, live vaccines could open an efficient way for prevention of infectious diseases. For instance, intracellular bacte-

ria, such as *Salmonella*, *Shigella*, *Listeria*, and *Lactococci* as well as *Rubella* or *Vaccinia* viruses, have broadly used to develop HPV vaccine (11-14). However, the pathogenicity, re-infection, and toxicity of these live vectors have prevented their application as vaccine candidates in humans (7, 8).

Recently, a trypanosomatid protozoan parasite, *Leishmania tarentolae*, has used as an efficient gene expression system for production of the recombinant proteins (15-17). In particular, *Leishmania tarentolae*, as a live vector was able to efficiently target the antigen-presenting cells (APCs), and stimulate immune has responded without replication within APCs (18, 19). For example, the recombinant *Leishmania tarentolae* expressing the HIV-1 Gag protein has elicited a potent cellular immune response, leading to a reduction of HIV replication *ex vivo* (19).

2. Objectives

In current study, a recombinant *Leishmania tarentolae* expressing the HPV16 L1 protein (*L.tar-L1*) has generated and its immunogenicity has assessed in C57BL/6 mice model. Our results have shown that the recombinant *L.tar-L1* could significantly increase both levels of IgG1 and IgG2a antibodies without the differences between two and three times of immunizations.

3. Materials and Methods

3.1. Preparation of Plasmid DNA Constructs

For the production of HPV16 L1-expressing vector, the L1 gene was subcloned from pcDNA-L1 into the *XhoI* and *KpnI* cloning sites of pLEXSY-I-blecherry3 expression vector (Jena bioscience, Germany). The obtained pLEXSY-I-L1 construct has purified in large-scale using Midi-kit (Qiagen). The accuracy of pLEXSY-I-L1 has confirmed using DNA sequencing. Then, pLEXSY-I-L1 has linearized by *SwaI* enzyme for integration into the chromosomal *odc* locus of the LEXSY host (T7-TR).

3.2. Parasite Transfection

The promastigotes of *Leishmania tarentolae* (*L. tar*) have grown in brain-heart infusion medium (BHI, Sigma) at pH = 7.2 and 26°C. For transfection, the linearized pLEXSY-I-L1 (8 µg) was electroporated into 4×10^7 log phase parasites at 450 V and 500 µF using Bio-Rad Gene Pulse-Ecell. The electroporated parasites have cultured on the BHI-agar plate containing 50 µg/mL of bleomycin for selection of recombinant transfectants (Jena Bioscience, Germany).

3.3. Genomic Analysis

The genomic DNA of recombinant *L.tar-L1* has extract-

ed using the DNeasy® blood and tissue kit (GF-1). Integration of pLEXSY-L1 into the genome has confirmed by diagnostic PCR using L1 primers and also *odc* forward/*aprt* reverse primers as mentioned in Jena bioscience manual.

3.4. Protein Analysis

The protein expression has induced by tetracycline according to Jena bioscience manual. Parasite promastigotes have harvested by centrifugation at 3000 rpm for 10 minutes and washed in PBS. The pellets have lysed in 2X SDS-PAGE sample buffer on ice and then boiled for 5 minutes. The samples have loaded on a 12.5% SDS-PAGE. After that, western blot analysis has performed using an anti-HPV16 L1 monoclonal antibody (MD2H11, kindly provided by Professor Martin Muller, German Cancer Research Center; 1:5000 v/v) under standard procedures. In order to detect the expected band of L1 protein, 3, 3'-diaminobenzidine (DAB, Sigma) has used as a substrate.

3.5. Mice Immunization

Five groups of 6 - 8-week-old female C57BL/6 mice (n = 5) have obtained from breeding stock maintained at the Pasteur Institute of Iran. All mice have maintained under specific pathogen-free conditions and all procedures have performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. Mice have immunized two or three times with two-week intervals as: group 1 (*L.tar-L1/L.tar-L1*), group 2 (*L.tar-L1/L.tar-L1/ L.tar-L1*), group 3 (*L.tar/L.tar*: control), group 4 (*L.tar/L.tar/ L.tar*: control), and group 5 (PBS: control), respectively. Total number of 2×10^7 stationary phase recombinant *L. tar* or *L.tar-L1* promastigotes, have injected subcutaneously into groups 1 - 4.

3.6. Assessment of Humoral Immune Responses

Mice sera from each group have collected two weeks after the last injections. The levels of IgG1 and IgG2a have assessed using ELISA. Briefly, a 96-well flat-bottom ELISA plate (Greiner) has coated overnight at 4°C with killed *L.tar-L1* protein as an antigen (prepared in 0.1% formalin (20), 10µg/mL) diluted in PBS (pH = 7.2). Then, the plate has rinsed with washing buffer (0.5% (v/v) Tween-20 in PBS), incubated with blocking buffer (1% BSA in PBS) for 2 hours at 37°C. The pooled sera have diluted 1:50 in dilution buffer (0.5% (v/v) Tween-20 in blocking buffer), added to the plate and incubated for 2 hours at 37°C. After rinsing with washing buffer, the plate has incubated with biotin-conjugated goat anti-mouse IgG1 or IgG2a (diluted 1:1000 in 1% BSA/PBS-Tween, Southern biotechnology Association Inc, USA) for 2 hours at 37°C. Then, the plates have washed and incubated with streptavi-

din-horseradish peroxidase diluted in PBS (1:100,000; Sigma) at 37°C for 1 hour. Detection has done with 100 μ L of O-Phenylenediamine (OPD, Sigma) as the substrate in citrate phosphate buffer (pH = 4.5), followed by incubation for 30 minutes at 37°C. The enzyme reaction has stopped by 1 M H₂SO₄ and the absorbance has measured at 492 nm.

3.7. Statistical Analysis

Statistical analysis has performed using Prism 5.0 software (GraphPad, San Diego, California, USA). One-way ANOVA has performed to analyze humoral immune responses. For all comparisons, P < 0.05 has considered statistically significant. Data have presented as mean \pm standard deviation (SD).

4. Results

4.1. Generation of *Leishmania tarentolae* Expressing L1 Protein (*L.tar-L1*)

To generate *L.tar-L1*, firstly, the 1515 bp fragment encoding the L1 has cloned into pLEXY vector. Then, the linearized pLEXY-L1 has electroporated into *L. tar*. Figure 1 has shown the linearized pLEXY-L1 with *SwaI* and separation of ~2 kb band for formation of 5' *odc* and 3' *odc* regions (~6700 bp) and homologous recombination into the host chromosome. After obtaining the recombinant clones, integration of gene in the genomic DNA has confirmed by PCR analysis. The expected 1 kb band has only observed from transgenic cells suggesting the correct integration of linearized plasmid into the genome (Figure 2 A). The PCR product of L1 has also appeared as ~1515 bp fragment for L1 positive clones (Figure 2 A). Furthermore, western blotting by an anti-L1 antibody has indicated the expression of L1 protein (~60 kDa) for *L.tar-L1* versus wild type *L.tar* (Figure 2 B).

4.2. *L.tar-L1* Induces Both IgG1 and IgG2a Isotypes in Mice

C57BL/6 mice have subcutaneously immunized two and three times at 2-weeks interval with 2×10^7 *L.tar-L1* and *L.tar*. The levels of IgG1 and IgG2a in the mice sera have determined by ELISA two weeks after third immunization. Vaccination with *L.tar-L1* has generated the mixture of IgG2a and IgG1 antibodies against killed *L.tar-L1* with high intensity toward IgG2a response in comparison with control groups after two and three immunizations (P < 0.05). It was interesting that antibody levels have not changed with increasing the number of injections as observed in groups 2 and 4 in comparison with groups 1 and 3, respectively (Figure 3).

Figure 1. Generation of 5' *odc* and 3' *odc* Regions for Homologous Recombination

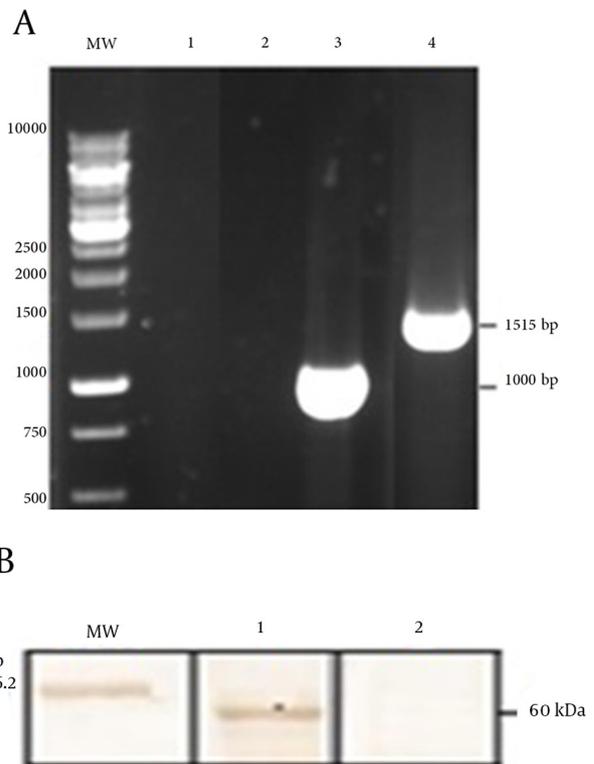
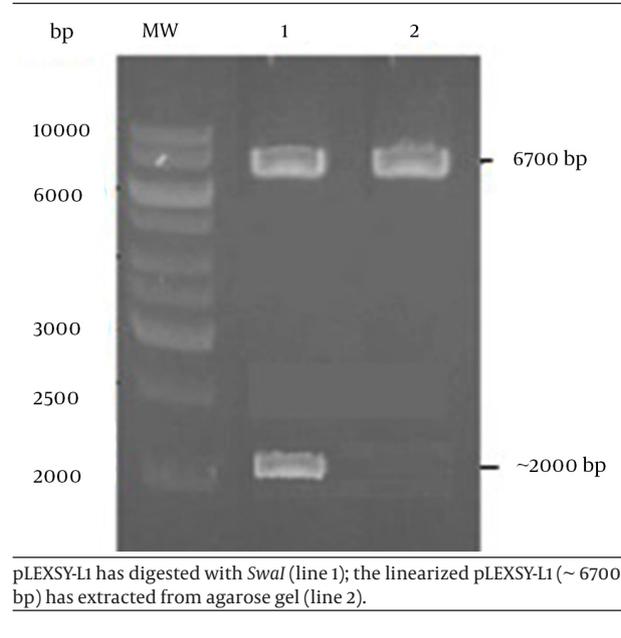
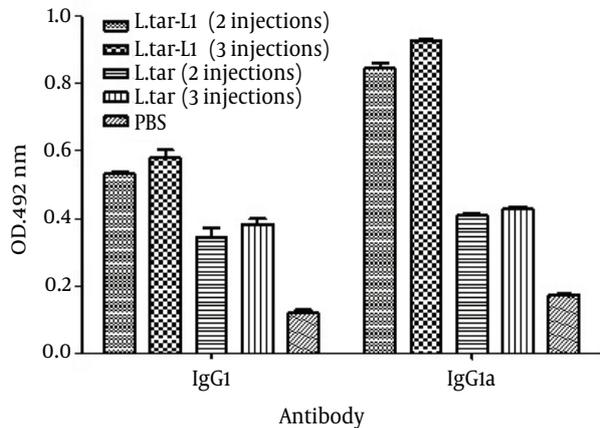


Figure 2. A) Confirmation of the Correct Integration of Linearized pLEXY-L1 Into the Genome: the Amplification of Wild Type *L.tar* Genome With *odc/aprt* Primers (Line 1) and Also L1 Primers (Line 2); the Amplification of *L.tar-L1* With *odc/aprt* Primers (Line 3) and Also L1 Primers (Line 4); B) Western Blot Analysis for L1 Protein has Been Using the Anti-HPV16 L1 Monoclonal Antibody: The 60 kDa Band of the L1 Protein (Line 1) Against no Band for *L. tar* as a Negative Control (Line 2)

Figure 3. Analysis of the Levels of IgG2a and IgG1 With Respect to *L.tar-L1* per Group Using ELISA



Serum samples have obtained from immunized mice two weeks after third immunization. The significant increases in *L.tar-L1*-specific IgG1 and IgG2a isotypes have observed in mice immunized with *L.tar-L1* in comparison with control groups ($P < 0.05$). Data has shown antibody titers of pooled mice sera with independent experiments and error bars have indicated SD. $N = 5$ per group.

5. Discussion

In this article, we have represented the immunological properties of a novel live vector expressing the L1 protein in mice model. Herein, the linearized pLEXSY-L1 construct has generated and transfected in *Leishmania tarentolae* for integration into the genome. PCR technique and western blot analysis of the recombinant *L.tar-L1* has confirmed L1 integration and its expression in the parasite as a 60 kDa fragment. Since, the use of bacterial and viral vectors for development of HPV vaccine have shown an important problem for human safety, live non-pathogenic *L. tarentolae* has suggested as a live vaccine without the risk of infection (19). The studies indicated that live vaccines mainly have greater immunogenicity in comparison with subunit vaccines (11, 16). In current study, the humoral immune responses have induced by the recombinant live *Leishmania tarentolae* expressing HPV16 L1 (*L. tar-L1*) has evaluated and compared with controls (*L.tar*, and PBS). In addition, we have compared the immunological effects of two and three immunizations with *L.tar-L1* and also *L.tar*

in C57BL/6 mice. Our results have indicated high levels of IgG1 and IgG2a levels with high intensity toward IgG2a response in comparison with control groups after two and three immunizations. Furthermore, the number of immunizations has not shown any changes in enhancement of their potency. Our previous studies have also shown that a novel live vaccine using recombinant *Leishmania tarentolae* expressing HPV16 E7 protein could generate the protection of mice against HPV-associated tumors. Indeed, the immunization with live recombinant *L.tar-E7* induced high levels of IgG2a in mice. In contrast with our recent data for *L.tar-L1*, no significant levels of IgG1 have observed for *L.tar-E7* (8). In addition, subcutaneous administration of mice with both the recombinant *L.tar-E7-NT* (gp96) and *L.tar-E7-CT* (gp96) fusion proteins have led to enhance the levels of IgG2a before and after challenge with TC-1 tumor cells. But, the levels of IgG1 have not increased in these groups (21). These results have shown that the antigen could influence type of immune responses, e.g. L1 was able to induce both IgG1 and IgG2a significantly against *L.tar* or PBS, while E7, E7-NT (gp96) and E7-CT (gp96) were able to elicit IgG2a, alone. For this reason, the L1 has suggested as a target antigen inducing high antibody levels for prophylactic vaccines and E7 has proposed as a target antigen inducing potent cellular immune response for therapeutic vaccines.

Except to *Leishmania tarentolae* has used as live vector, the studies have shown that *Toxoplasma gondii* and *Neospora caninum* (22, 23) have closely related apicomplexan parasites. The surface antigen of *T. gondii* (TgSAG1) was a major immunodominant antigen and, therefore, has considered being a good candidate for the development of an effective recombinant vaccine against toxoplasmosis. In a study, *N. caninum* stably expressing the TgSAG1 gene (Nc/TgSAG1) has induced TgSAG1-specific Th1-dominant immune responses and protected the mice from a lethal challenge infection with *T. gondii*. These results have indicated that *N. caninum* might be used for the production of a live recombinant vector vaccine against toxoplasmosis in animals (22). Generally, the non-pathogenic live parasites could stimulate antigen-specific immune responses. They have possessed the high adjuvant properties for increasing the potency of a vaccine. However, it was necessary to optimize these systems and enhancement of antigen expression.

Our study has demonstrated that live *Leishmania tarentolae* could be used as an appropriate carrier to deliver HPV16 L1 antigen and induce potent humoral immune responses. Moreover, this live vector would be safe for human, as a potent vaccination strategy against HPV and other intracellular pathogens. The live *Leishmania tarentolae* has been capable of stimulating humoral immunity in mice without the need of any adjuvant.

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Footnotes

Authors' Contribution: Azam Bolhassani has designed and headed the study. Zeinab Shirbaghaee, Azam Bolhassani, Fatemeh Motevalli, and Negar Zohrei have performed and analyzed the data as well as they prepared the manuscript. Abbas Mirshafiey have made substantial written contributions to the manuscript.

Conflict of Interest: The authors have declared that they had no competing interests.

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