

Whole recombinant *Pichia pastoris* expressing HPV16 L1 antigen is superior in inducing protection against tumor growth as compared to killed transgenic *Leishmania*

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Abbreviations: HPV, human papillomaviruses; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; KBMA, killed but metabolically active; AOX1, alcohol oxidase I gene; Yeast-HBsAg, yeast expressing hepatitis B surface antigen; *L.tar*, *Leishmania tarentolae*; DAB, 3,3'-diaminobenzidine; rL1, recombinant L1; 2-ME, mercaptoethanol; ConA, concanavalin A; SD, standard deviation

The development of an efficient vaccine against high-risk HPV types can reduce the incidence rates of cervical cancer by generating anti-tumor protective responses. Traditionally, the majority of prophylactic viral vaccines are composed of live, attenuated or inactivated viruses. Among them, the design of an effective and low-cost vaccine is critical. Inactivated vaccines especially heat-killed yeast cells have emerged as a promising approach for generating antigen-specific immunotherapy. Recent studies have indicated that yeast cell wall components possess adjuvant activities. Moreover, a non-pathogenic protozoan, *Leishmania tarentolae* (*L.tar*) has attracted a great attention as a live candidate vaccine. In current study, immunological and protective efficacy of whole recombinant killed *Pichia pastoris* and *Leishmania tarentolae* expressing HPV16 L1 capsid protein was evaluated in tumor mice model. We found that *Pichia*-L1, *L.tar*-L1 and Gardasil groups increase the IgG2a/IgG1 ratio, indicating a relative preference for the induction of Th1 immune responses. Furthermore, subcutaneous injection of killed *Pichia*-L1 generated the significant L1-specific IFN- γ immune response as well as the best protective effects in vaccinated mice as compared to killed *L.tar*-L1, killed *Pichia pastoris*, killed *L.tar* and PBS groups. Indeed, whole recombinant *Leishmania tarentolae* could not protect mice against C3 tumor mice model. These data suggest that *Pichia*-L1 may be a candidate for the control of HPV infections.

Introduction

Human papillomaviruses (HPVs) are responsible for genital warts in both women and men, especially cervical carcinoma in women.¹ Considering the high prevalence of HPV infection in women and the lack of anti-viral agents against HPV, the development of a prophylactic HPV vaccine has been an important strategy to prevent cervical cancer.² However, the design of an effective and low-cost vaccine is critical. Currently, many successful prophylactic viral vaccines have been developed. These vaccines are often based on live, attenuated or inactivated (/ killed) viruses. The killed vaccines are made as whole cell vaccines (such as Influenza, polio, rabies and hepatitis A) or as subunit vaccines.³ Advantages of killed vaccines include: a) No reversion to

virulence; b) Safe in pregnant animals; c) Safe in immuno-compromised animals and d) No shedding of the virus. However, 2 doses are often needed for initial response by killed vaccines. Killed antigens generally require an adjuvant for induction of a strong Th1 immune response in murine models.⁴ Recent studies have suggested that yeast cell wall components possess adjuvant activities.⁵ The reports have demonstrated that vaccination with *saccharomyces cerevisiae* expressing tumor-associated antigens can induce antigen-specific T-cell responses and protect animals against tumor challenge. Furthermore, heat-killed recombinant *S. cerevisiae* shows no toxicity in clinical studies.^{6,7} However, there are some restrictions in *S. cerevisiae* expression systems including a) hyperglycosylation of recombinant proteins, which is allergenic and b) limitation in various carbon sources utilized

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by this species.⁸ Recently, the *Pichia pastoris* system has been suggested for heterologous protein expression, because of the powerful genetic techniques, high expression levels, rapid growth rate and well-established fermentation technology, along with its economy of use.⁹ Subsequently, a novel protein expression system was also developed based on non-pathogenic *Leishmania tarentolae*, a protozoan parasite of lizards. This system allows not only easy handling like *E. coli* and yeast, but also full eukaryotic protein folding and the mammalian-type post-translational modification of target proteins.¹⁰⁻¹² To date, several heterologous proteins (intracellular or secreted mode) have been expressed in *L. tarentolae* such as Amylase, Cu/Zn Superoxide dismutase, Erythropoietin, Green Fluorescent Protein (GFP), HIV-1 gag, Interferon- γ , Laminin-322, Luciferase, Red Fluorescent Protein, Proprotein convertase 4, Retinoic acid receptor γ , T7 RNA polymerase, Tissue plasminogen activator, G-protein coupled receptor 56.¹¹ Currently, killed *Leishmania* vaccines have been tested against human cutaneous and visceral leishmaniasis.¹³⁻¹⁵ In addition, some studies indicated that the recombinant *Leishmania tarentolae* live vector was capable of eliciting effective humoral and cellular immune responses in mice.^{16,17}

In the present study, we evaluated and compared the ability of 2 killed vaccines (*Pichia pastoris* and *Leishmania tarentolae* strains expressing high levels of full length HPV16 L1, termed *Pichia*-L1 and *L.tar*-L1, respectively) to induce B- and T-cell mediated immune responses and to eradicate C3 tumor cells *in vivo*. In both systems, the expression cassettes including L1 gene (pLEXSY-L1 and pPICZB-L1) were integrated in *Leishmania* and *Pichia* genome through homologous recombination.^{9,16-19} Our results indicated that whole recombinant *Pichia*-L1 and *L.tar*-L1 enhance L1-specific antibody titers (IgG1 and IgG2a) and the IgG2a/IgG1 ratio in mice. Furthermore, *Pichia*-L1 stimulated the secretion of L1-specific IFN- γ from mouse splenocytes as compared to other groups. Indeed, *Pichia*-L1 could significantly elicit the protective effects against C3 tumor mice model in comparison with *L.tar*-L1 vaccinated group. These data suggest the possibility of developing whole killed *Pichia pastoris* expressing L1 as an effective vaccine to control HPV infections.

Results

In vitro pEGFP-L1 delivery using PEI

For confirmation of the L1 expression *in vitro*, PEI 25 kDa was used as a transfection reagent. GFP fluorescence was observed in cells that received 5 μ g of pEGFP-L1 plasmid. The transfection efficiency using different concentrations of PEI (NrE = 5, 7, 10) was almost similar. The percentage of L1-GFP expressing cells was \sim 71.5% as compared to negative control (\sim 2.29%) using flow cytometry.

Development of the recombinant *L. tarentolae* strain expressing HPV16 L1 protein

To generate *L. tarentolae* expressing L1-GFP protein, the 2265 bp fragment encoding the L1-GFP was cloned into

pLEXSY-neo2. Then, the linearized recombinant plasmid was electroporated into wild type *L. tarentolae*. The transformants were selected by plating on solid medium containing G418. Integration of gene in the genomic DNA of the recombinant cells was confirmed by PCR analysis. The expected 1 kb band was only observed from transformed cells indicating the correct integration of expression construct into the *ssu* locus. The expected PCR products of L1 and L1-GFP were appeared as \sim 1515 bp and \sim 2265 bp fragment for L1-GFP positive clones, respectively. To verify mRNA synthesis, RT-PCR was performed on total mRNA extracted from wild and transgenic cells. The desired bands for L1 and L1-GFP were obtained in recombinant *Leishmania* promastigotes (data not shown). In addition, the expression of L1-GFP using GFP was monitored by Epi-fluorescent microscopy and flow cytometry. The expression of genes was readily evident from the intense green fluorescence of the parasites. Fluorescence-activated cell sorting (FACS) analysis indicated a clear quantitative separation between transfected and wild type parasites. L1-GFP expression in transgenic *L. tarentolae* was detectable in 65% of the cells as compared to GFP expression in *L.tar*-GFP (\sim 98.7%). The stability of protein expression was monitored over a period of 4 months post-electroporation (data not shown). Furthermore, L1 expression was also detectable in cell extracts of transgenic parasites compared to wild type by western blotting. As shown in **Figure 1A**, the dominant band of \sim 82 kDa was detected in transgenic parasites expressing L1-GFP. No such corresponding band was revealed in the wild-type promastigotes.

Expression of HPV16 L1 protein in *Pichia pastoris* under the control of the AOX promoter

To generate *Pichia pastoris* expressing L1 protein, the 1543 bp fragment encoding L1 was cloned into pPICZ-B. Then, the recombinant pPICZB-L1 was linearized and electroporated into GS115 yeast strain. The transformants were selected by plating on solid medium containing 500 μ g/ml zeocin. Integration of genes in the genomic DNA of the recombinant cells was confirmed by PCR analysis using AOX primers. The expected 2100 bp band was only observed in transgenic cells indicating the correct integration of expression construct into the genome in contrast with negative clones (\sim 600 bp). In addition, a 55 kDa L1 protein (+5 kDa His-tag) was detected in the lysates of *Pichia pastoris* strain using protein gel blotting. L1 band was not detected in negative control extract (GS115 transformed with empty pPICZB vector) and in the non-induced clone, showing that the expression of the heterologous protein was specifically induced in the presence of methanol as indicated in **Figure 1B**.

Generation of the recombinant L1 protein, killed *leishmania* and killed yeast

The HPV16 L1 protein was expressed and analyzed in SDS-PAGE by Coomassie Blue staining as previously described.²⁰ The recombinant GST-L1 and GST proteins migrated as 82 kDa and 27 kDa proteins in SDS-PAGE, respectively. These recombinant proteins (rGST and rGST-L1) were purified,

concentrated, assessed and kept in -70°C for next use in antibody and cytokine assays. Preparation of the killed *Leishmania* with different approaches showed that the protein content in 0.1% formalin is more intact than that in other methods especially autoclaving. **Figure 1C** demonstrates western blot analysis of total proteins detected in various killing methods.

Whole recombinant yeast and *Leishmania* indicated the mixture of IgG1 and IgG2a isotypes with high intensity toward IgG2a response

C57BL/6 mice were subcutaneously vaccinated 3 times at 2-week intervals with 5 mg of *Pichia*-L1 and yeast, 100 μg of *L. tar*-L1 and *L. tar* as well as one fifth of a dose of Gardasil. The quantity of anti-HPV16 L1 antibodies (IgG1 and IgG2a) in the sera of the vaccinated mice was determined by ELISA at different times (after second and third immunizations as well as 7 days after C3 challenge). Vaccination with *Pichia*-L1 and *L. tar*-L1 generated L1-specific IgG2a and IgG1 antibodies as compared to control groups especially before challenge ($P < 0.05$). The same result was also obtained for the group immunized with Gardasil ($P > 0.05$). Indeed, anti-L1 IgG2a and IgG1 isotypes increased significantly after the second immunization in test groups. It is interesting that IgG1 levels were reduced in the sera of groups 1 to 3 after C3 challenge (**Fig. 2**). As known, Th bias of the immune response can be analyzed by determination of IgG isotypes. Herein, the significant increases in L1-specific IgG1 and IgG2a isotypes were observed in mice immunized with yeast-L1, *L. tar*-L1 and Gardasil. Moreover, groups 1 to 3 greatly enhanced the IgG2a/ IgG1 ratio compared to control groups ($P < 0.05$) indicating a relative preference for the induction of Th1 immune responses (**Table 1**).

Pichia-L1 stimulates the secretion of HPV16 L1-specific IFN- γ

Protection following successful immunization requires the generation and maintenance of antigen-specific CD4⁺ Th cells. Herein, splenocytes from immunized mice were assayed for the secretion of IFN- γ (Th1-like) and IL-4 (Th2-like) cytokines upon re-stimulation with rL1, rVLP and rGST, before C3 tumor challenge. As shown in **Figure 3**, splenocytes from mice immunized with *Pichia*-L1 produced higher levels of L1-specific IFN- γ

(~ 152 pg/ml) as well as VLP-specific IFN- γ (~ 101 pg/ml), confirming the induction of Th1 response. However, neither IFN- γ nor IL-4 was detected in mice immunized with yeast or *L. tar* vectors. Moreover, mice immunized with killed *Pichia*-L1 (group 1) exhibited approximately a 3.2-fold higher L1-specific and VLP-specific IFN- γ responses as compared to the IFN- γ response measured following vaccination with killed *L. tar*-L1 (group 2) ($P < 0.05$). Overall, killed *Pichia*-L1 stimulated the most potent L1-specific IFN- γ responses of vaccine candidates. There were

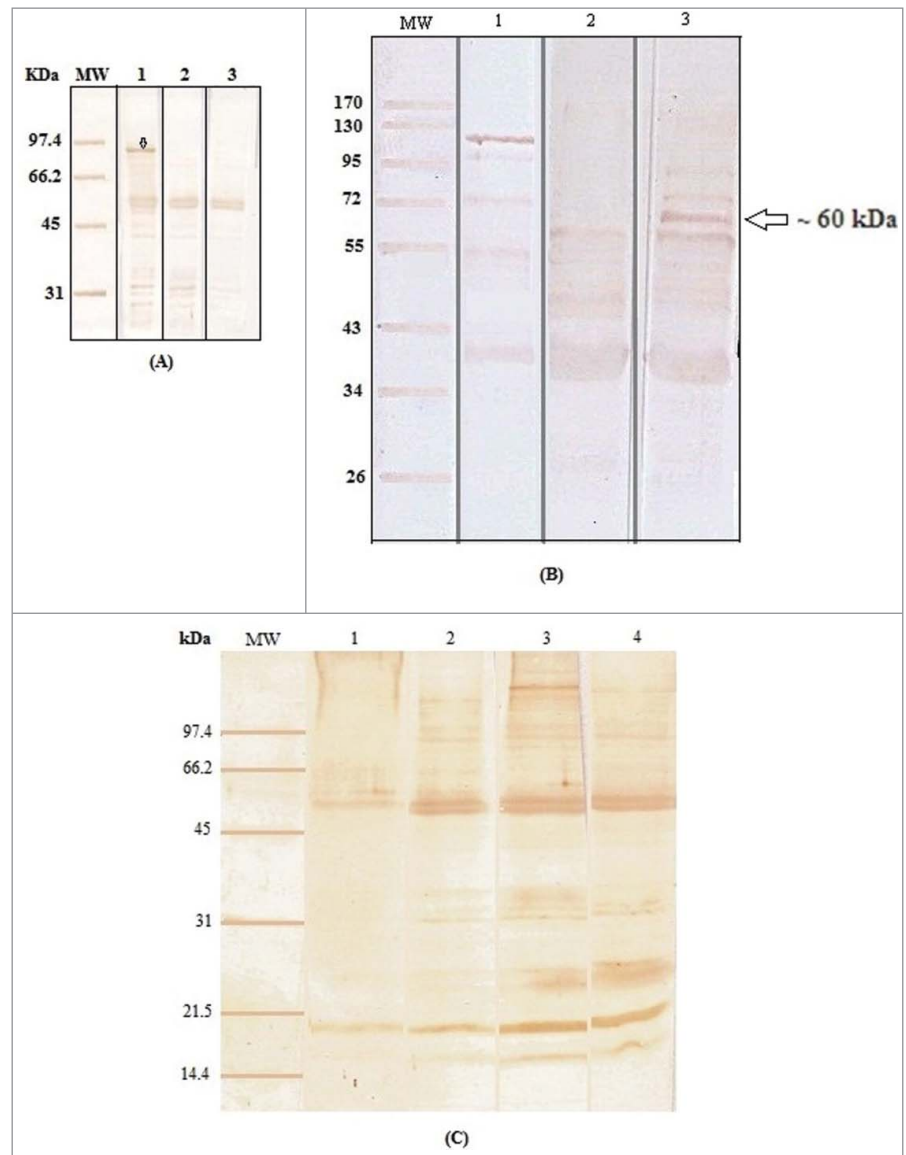


Figure 1. A) Detection of L1-GFP protein in the recombinant *Leishmania* by western blotting. Western blot analysis using an anti-L1 antibody showed high levels of expression of the full-length L1-GFP protein (~ 82 kDa, line 1) in contrast with *L. tar*-GFP (line 2) and wild type *L. tar* (line 3). These data were obtained using anti-GFP antibody, while GFP was detected at 27 kDa for *L. tar*-GFP as a positive control; **(B)** Identification of HPV16 L1 expressed in *Pichia*-L1 by protein gel blotting. line 1: yeast vector sample without L1-specific band; line 2: Before induction of *Pichia*-L1; line 3: After induction of *Pichia*-L1 (~ 72 h); **(C)** Killed promastigotes of *L. tarentolae* in stationary phase (day 7): Line 1: autoclaved; Line 2: heat-killed in 60°C ; Line 3: suspension in formalin and Line 4: freeze-thawed parasites. MW: Protein molecular weight marker.

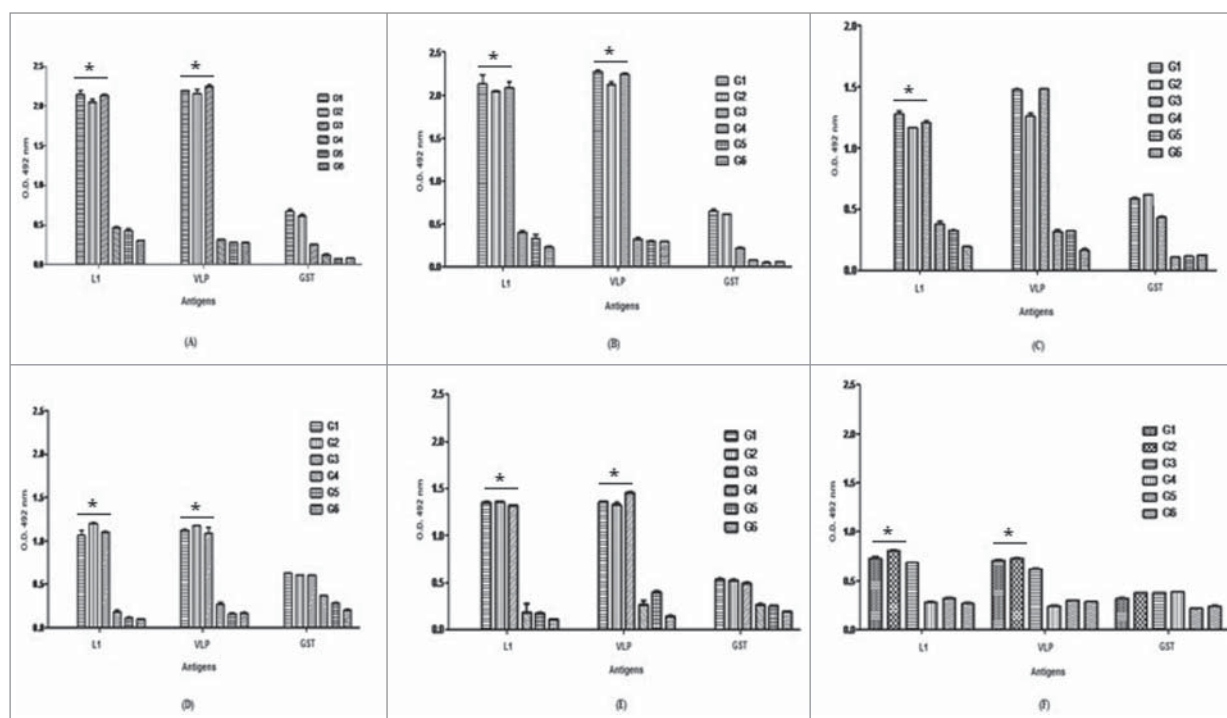


Figure 2. Analysis of IgG2a (A–C) and IgG1-specific antibody levels (D–F) with respect to rL1, rVLP and rGST per group using ELISA. Serum samples were obtained from immunized mice after second (A & D), third (B & E) immunization and 7 days after C3 challenge (C & F). The significant increases in L1-specific IgG1 and IgG2a isotypes were observed in mice immunized with *Pichia*-L1, *L.tar*-L1 and Gardasil as compared to control groups 4 to 6 ($P < 0.05$). Data shows antibody titers of pooled mice sera with independent experiments and error bars indicate SD. N = 8 per group.

no significant differences for IL-4 production in different groups compared to that in control groups at before challenge ($P > 0.05$). The levels of IFN- γ and IL-4 with respect to ConA, as a positive control were between 400–600 pg/ml and 100–200 pg/ml in all groups, respectively (data not shown).

Vaccination with *Pichia*-L1 enhances protection of mice against C3 tumors

To evaluate the anti-tumor effects of killed vaccine candidates, *in vivo* tumor protection experiments were performed by challenging vaccinated mice with C3 tumor cells. The data showed that ~60% of mice receiving *Pichia*-L1 (group 1) remained tumor-free 50 days after the C3 challenge, in contrast with zero percent (0%) for mice receiving *L.tar*-L1 (group 2). In addition, all of the mice receiving killed *L.tar* vector and PBS developed a tumor growth at 7 days after tumor challenge (Fig. 4). Control groups (5 and 6) showed progressive tumor growth. Vaccination with Gardasil significantly resulted in prevention of TC-1 tumor

growth (~80% survival rates) in comparison with other groups ($P < 0.05$, Fig. 4). The growth of tumor delayed about 20 days in mice vaccinated with Gardasil and 7 days in group 1 vaccinated with *Pichia*-L1 after challenge. Interestingly, group 4 receiving yeast vector showed ~20% tumor-free mice as compared to mice immunized with *L.tar* (group 5), PBS (group 6) and *L.tar*-L1 (group 2), indicating the protective efficacy and adjuvant activity of killed yeast components. Immunization with whole killed *Pichia*-L1 significantly retarded the tumor growth rate compared to other groups between days 35 and 50 except to group 3 vaccinated with Gardasil ($P < 0.05$, Fig. 4).

Discussion

Availability of a safe and immunogenic vaccine would represent the best strategy for control of viral diseases such as human papillomavirus.^{2,3,21} The studies support the idea that an

Table 1. The mean of IgG2a/IgG1 ratios against rL1 and rVLP for test groups after second and third immunizations (Booster 1 and 2) and post-challenge

Group	Booster 1		Booster 2		Challenge	
	L1	VLP	L1	VLP	L1	VLP
1	2.03 ± 0.05	1.95 ± 0.00	1.58 ± 0.01	1.67 ± 0.01	1.76 ± 0.02	2.08 ± 0.00
2	1.70 ± 0.01	1.84 ± 0.01	1.51 ± 0.01	1.60 ± 0.02	1.45 ± 0.00	1.74 ± 0.02
3	1.94 ± 0.02	2.07 ± 0.02	1.58 ± 0.02	1.54 ± 0.01	1.76 ± 0.01	2.40 ± 0.00

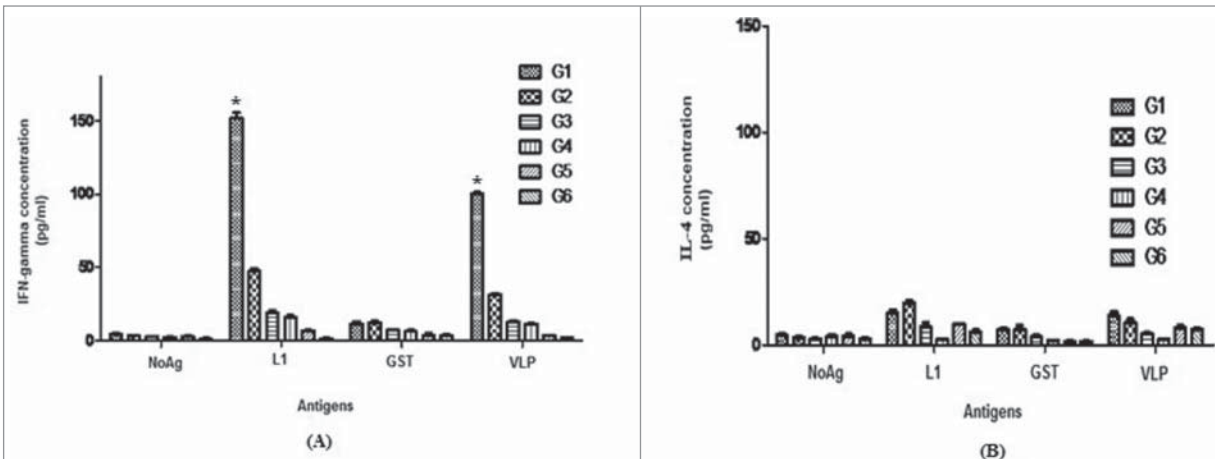


Figure 3. The levels of IFN- γ (A) and IL-4 (B) were measured by a sandwich ELISA in culture supernatants of splenocytes following *in vitro* re-stimulation with the recombinant proteins of GST-L1, GST and HPV16 VLP L1. Secretion of L1 (VLP)-specific IFN-gamma from spleen cells in the *Pichia*-L1 group is significantly higher than other groups ($P < 0.05$). There were no significant differences for IL-4 production in different groups at before challenge ($P > 0.05$). Data are presented as the mean \pm SD.

HPV16L1 VLP-based vaccine will induce the strongly neutralizing antibodies necessary for effective prophylaxis. This was the basis for the development of 2 highly efficient VLP-based HPV vaccines named Gardasil (Merck MSD) and Cervarix (GlaxoSmithKline)^{2,3,22,23} The major drawbacks of these vaccines are the high production costs, the need for a cold chain and sterile needles for the intramuscular administration.²³ Therefore, the production, assembly and characterization of HPV16 L1 structural protein in *P. pastoris*, has been studied by a Brazilian team for the development of cheap and efficient prophylactic vaccine in future.⁹

Currently, inactivated whole pathogen vaccines are the most common form of vaccine.²⁴ However, these vaccines typically require multiple doses and regular boosters. Moreover, their efficacy frequently depends on use of potent adjuvants.²⁴⁻²⁶ Recently, a new class of vaccines termed killed but metabolically active (KBMA) was produced. KBMA vaccines are whole pathogenic or attenuated organisms killed through photochemical inactivation and cannot cause disease, yet retain sufficient metabolic activity to initiate a potent immune response.²⁷ In current study, the immunogenicity and protective effects of 2 different vaccine modalities such as whole killed *Pichia pastoris* and *Leishmania tarentolae* expressing HPV16 L1 were evaluated in tumor mice model. Our goal of this study is not development of a vaccine for tumor therapy. The novelty of the study is limited to use killed vectors expressing L1 protein for protective vaccine design. At first, we constructed a *Pichia*-L1 vaccine using the pPICZ-B vector and *L.tar*-L1 vaccine using the pLEXSY vector. Herein, the efficient and tightly regulated promoter from the

alcohol oxidase I gene (AOX1) was used to drive the expression of HPV16 L1 gene in *Pichia pastoris*.

From the yeast expression systems, *Saccharomyces cerevisiae* has been considered to be safe for consumption in humans without any side effects and a good expression system for heterologous proteins.²⁸ Recent studies in mice showed that the recombinant *saccharomyces cerevisiae* induced potent antigen-specific proliferative and cytotoxic T cell responses associated with Th1-type cytokine secretion. Furthermore, prophylactic administration of the recombinant *saccharomyces cerevisiae* led to eradication of tumor cells in mice model.²⁹ The studies suggest that oral vaccination with the yeast expressing *apxIIA* gene could induce the protection against porcine *pleuropneumonia* by eliciting both mucosal and systemic immune responses.²⁸ Moreover, the protection against systemic murine coccidioidomycosis was observed by vaccination with heat-killed *Saccharomyces cerevisiae*.³⁰

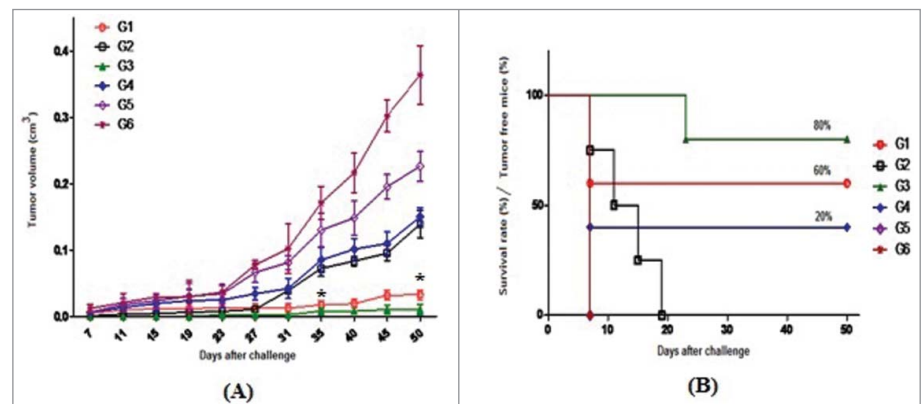


Figure 4. Prophylactic anti-tumor effect of various killed vaccines: At 2 weeks after the last immunization, mice were subcutaneously challenged with 0.5×10^6 C3 tumor cells. Tumor growth was then measured twice a week and tumor volumes calculated (A); In addition, the survival rate was determined as a Kaplan-Meier plot (B). Mice in PBS and *L.tar*-vaccinated groups experienced rapid and exponential tumor growth, which was delayed by only 7 days after C3 challenge. Mice in the group 1 (*Pichia*-L1) had the significant differences with other groups except to group 3 (Gardasil) between days 35 and 50 after C3 challenge ($*P < 0.05$). Tumor volume is presented as the mean \pm SD.

In this study, mice immunization was done by different modalities at 3 times. Then, the induction of L1 antibody titres and also T-cell responses were investigated for analyzing protection against tumor formation by C3 tumor cells, a cell line containing the entire HPV16 genome.³¹ We showed that a tumor-specific Th1-type response was established with whole killed *Pichia*-L1 vaccinated mice. As known, Th1 response is characterized by the production of IFN- γ and an enhancement of IgG2a and Th2 response is determined by the production of IL-4 and an enhancement of IgG1. Analysis of serum antibody isotypes revealed that *Pichia*-L1 not only enhances the levels of IgG1 and IgG2a, but also increases the IgG2a/IgG1 ratio, indicating a relative preference for the induction of Th1 immune responses. Furthermore, analysis of L1-specific cytokines secreted by spleen cells showed that *Pichia*-L1 increases the secretion of IFN- γ from spleen cells with no production of IFN- γ observed in mice receiving yeast vector, indicating that the cytokines are L1-specific. Similar data has been obtained by immunization with heat-killed whole recombinant *Hansenula polymorpha* yeast expressing hepatitis B surface antigen (yeast-HBsAg) in mice model. Indeed, yeast-HBsAg enhanced both HBsAg-specific Th1 and Th2 immune responses, while alum as an adjuvant only increased Th2 immune responses, suggesting that yeast-HBsAg may be an ideal candidate for an effective vaccine against chronic HBV infection.⁵

On the other hand, *Leishmania tarentolae* is a promising host for large-scale recombinant protein production for both pharmaceutical purpose and structural studies.^{11,32-34} In the present study, *Leishmania tarentolae* was transfected with an expression cassette containing HPV16 L1 gene. This expression cassette harboring L1 gene was integrated in *Leishmania* 18S rRNA genes through homologous recombination.³²⁻³⁴ In this study, vaccination with killed parasites (*L.tar*-L1) generated L1-specific antibody and cellular responses, but it could not induce any protection in C57BL/6 mice model as compared to whole killed *Pichia*-L1. The reasons for this difference have not been still known. It may be related to differences in the quality of the early immune responses to killed parasites or yeast. Because, the modulation of the early responses with appropriate adjuvants could enhance efficacy of killed parasite vaccines. For instance, the researchers demonstrated that CpG ODN, when used as a vaccine adjuvant with heat-killed leishmanial antigens, could induce long-term protection against an intracellular infection.¹⁵ Our data confirm the potent adjuvant activities of yeast cell wall components in comparison with parasite components as previously described.³⁵ Indeed, yeast can be easily engineered to express multiple antigens and its inherent adjuvant properties avoid the need for additional adjuvants.⁵ In addition, Formalin-killed promastigotes have been used as antigens in vaccine candidates against leishmaniasis.⁴ In current study, we also used formalin-killed promastigotes of *Leishmania tarentolae* expressing HPV16 L1 for the first time. Most studies have focused on the use of autoclaved *L. major* promastigotes with or without adjuvants against leishmaniasis. Regarding to our data, autoclaving of parasites in stationary phase denatures proteins and this may interfere with the appropriate conformational structure of important antigen epitopes resulting in less efficacious vaccines as described by others.^{4,14}

Our results showed that ~60% of mice receiving *Pichia*-L1 remained tumor-free 50 days after the C3 challenge, in contrast with zero percent (0%) for mice receiving *L.tar*-L1. Furthermore, immunization with whole killed *Pichia*-L1 significantly retarded the tumor growth rate compared to other groups especially *L.tar*-L1 vaccinated mice. However, even when an immune response is effectively induced in a vaccinated group, the immunological outcomes do not necessarily correlate with clinical improvement as reported by others.³⁶ In addition, the route of immunogen administration influences how and where an immune response is started.²⁸ More importantly, the induced T-cell responses may play an important role in establishing long-lived B-cell immune memory. Our data indicated that *Pichia*-L1 could significantly enhance the anti-tumor immunity against the growth of C3 tumors; although, Gardasil represented a higher survival rate in mice. The studies showed that the quadrivalent HPV vaccine (Gardasil) results in protective immunity against type-specific HPV infection using the production of a systemic neutralizing anti-HPV response indicating the importance of this response.^{22,23} The exact role of different immune mechanisms in the protective efficacy of the HPV L1 VLP vaccine remains to be determined. Therefore, a HPV-pseudovirion neutralizing assay would be necessary to evaluate the vaccine candidates used in this experiment, in future.

Tumor protection studies comparing live versus heat-killed yeast cells were performed with yeast expressing ovalbumin and with yeast expressing the HIV-Gag protein.⁷ The results demonstrated that protective immunity was equivalently elicited with heat-killed yeast compared to live yeast and was also not dependent on the antigen expressed. However, the use of heat-inactivated yeast eliminates potential safety risks associated with the administration of live cells⁷ confirming our results. Determination of exact dose with high stability, manipulation of vector and the use of other strains for higher protein expression would be critical for selecting *Pichia*-L1 as a vaccine candidate. The durability of the protection by killed whole yeast vaccines is unknown and should be determined in future. Furthermore, the comparison of live *pichia*-L1 and killed *pichia*-L1 will be suitable for determination of the best strategy.

In summary, our data demonstrated that vaccination with *Pichia*-L1 not only induces Th1 immune responses, but also has protective effects as compared to *L.tar*-L1 vaccinated mice. Indeed, whole recombinant *Leishmania tarentolae* could not protect mice against C3 tumor mice model. The potential advantages of this approach are low cost and the absence of additional adjuvant. Therefore, *Pichia*-L1 may be a novel approach for prevention and control of HPV infections.

Materials and Methods

In vitro expression of HPV16 L1 in COS-7 cells

For the generation of L1-expressing plasmid [pEGFP-L1], the L1 DNA was amplified from pUF-L1 (kindly provided by Prof. Martin Muller, German Cancer Research Center) by PCR using the primer sets as following:

Forward L1: 5' ATA TCT AGA CTC GAG ATG AGC CTG TGG CTG CCC 3'
XbaI SlaI

Reverse L1: 5' TAT GGT ACC AAG CTT CAG CTT CCT CTT CCT C 3'
KpnI HindIII

The amplified L1 DNA was then cloned into the unique *XbaI* and *HindIII* cloning sites of the pEGFP-N1 expression vector (Clontech, CA). DNA constructs containing L1-GFP was purified in large-scale using Midi-kit (Qiagen). The presence of the inserted L1 fragment was confirmed by PCR and restriction enzyme digestion as detected on a 0.8% agarose gel electrophoresis. The accuracy of this construct was confirmed by DNA sequencing. DNA concentration was determined by the absorbance measured at 260 nm.

After DNA preparation, COS-7 cells were incubated at 37°C in the presence of 5% CO₂ into the 4-well plates (Greiner) in complete RPMI medium supplemented with 5% FCS overnight. PEI/DNA complexes were generated by mixing LIN-PEI 25 kDa (10 μM, NrE = 5, 7 and 10, Polysciences) with 5 μg of pEGFP-L1 plasmid and pEGFP-N1 as a positive control in HBS buffer (HEPES buffered saline) and incubated at room temperature for 15 min. COS-7 cells were used as a negative control. PEI/DNA complexes were added to 0.5 × 10⁵ COS-7 cells in serum-free media. The medium was replaced after 6 h incubation at 37°C with complete RPMI medium supplemented with 5% FCS. Transfection efficiency of each construct was determined by the fluorescence microscopy (Nikon, E200, Japan) and flow cytometry analysis (Partec GmbH) at 24 and 48 h after transfection.

Generation of *Leishmania tarentolae* expressing HPV16 L1 through homologous recombination

For the generation of L1-GFP-expressing plasmid [pLEXSY-L1-GFP], the L1-GFP gene (~2265 bp) was subcloned from pEGFP-L1 into the *XbaI* (*SlaI*) and *NotI* cloning sites of pLEXSY-neo2 expression vector (Jena bioscience). DNA construct containing L1-GFP was purified in large-scale using Midi-kit (Qiagen). For gene integration in *leishmania* genome, approximately 8 μg of the expression plasmid was digested with *SwaI* restriction sites and the linearized product was gel purified (QIAquick gel extraction kit) for transfection in *L.tarentolae*. Briefly, 4 × 10⁷ log-phase parasites re-suspended in ice-cold electroporation buffer (pH = 7.5) containing 5–6 μg of linearized pLEXSY-L1-GFP stored on ice for 10 min and electroporated using Bio-Rad Gene PulserEcell under conditions of 500 mF, 450 V and pulse time ~5–6 ms. The electroporated promastigotes were plated on solid media containing 50 μg/ml of G418. Clones were selected on Noble agar plates and further propagated in liquid M199 10% medium in the absence of G418^{16, 17, 19} (LEXSYcon2 Expression Kit, Jena Bioscience). Expression of L1-GFP protein in promastigote stage of the recombinant *Leishmania* species was evaluated by Epi-fluorescent microscopy and flow cytometry. Integration of the linearized expression cassettes (pLEXSY-L1-GFP) into the *ssu* locus of genome was confirmed by diagnostic PCR using genomic DNA of wild type and

transgenic strain of *L. tarentolae* (*L.tar-L1*) as template (LEXSYcon2 Expression Kit, Jena Bioscience). Furthermore, RNA samples were extracted from promastigote form of wild type and transgenic parasites using RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. The cDNA synthesis was performed using Omniscript Reverse Transcriptase kit (Qiagen) from 1 μg of RNA. PCR analysis was performed to amplify L1 and L1-GFP under standard conditions.

For protein analysis, samples from both wild type and transgenic *L. tarentolae* were separated by SDS-PAGE in a 12.5% (w/v) polyacrylamide gel as previously described^{16, 17, 19} Western blot analysis was performed to confirm L1-GFP protein expression using anti-HPV16 L1 monoclonal antibody (MD2H11, kindly provided by Prof. Martin Muller, German Cancer Research Center; 1:10000 v/v) or anti-GFP polyclonal antibody (1:5000 v/v; Acris antibodies GmbH) under standard procedures. The immunoreactive protein bands were visualized using peroxidase substrate named 3, 3'-diaminobenzidine (DAB, Sigma).

Generation of *Pichia pastoris* expressing HPV16 L1 through homologous recombination

HPV16 L1 gene was amplified by PCR from the plasmid vector pUF-L1 using Pfu DNA polymerase and the primer sets [F/R1 and then F/R2] as following:

F: 5' TCT GAATTC ACC ATG GGC CTG TGG CTG CCC AGC 3'
EcoRI Kozak

R1: 5' ATG ATG ATG ATG ATG ATG CAG CTT CCT CTT CTT CCT C 3'
6 His tag

R2: 5' TGC TCT AGA TCA TTA ATG ATG ATG ATG ATG ATG C 3'
XbaI Stop 6 His tag

The resulting L1 fragment (~1543 bp) was then cloned into the unique *EcoRI* and *XbaI* cloning sites of the linearized pPICZB vector (3270 bp, Invitrogen). Plasmid extraction was done by alkaline lysis and confirmed by PCR and digestion with *EcoRI*/*XbaI*. The accuracy of gene direction was confirmed using *Bgl* II enzyme by generating 2 fragments (~2949 and ~1864 bp). DNA sequence analysis was also performed using AOX1 primer set (AOX1-F & R; *Pichia* expression kit, Invitrogen) on an automated DNA sequencer using the dideoxy termination method. DNA construct containing HPV16 L1 was purified in large-scale using Midi-kit (Qiagen). After confirmation, the pPICZB-L1 construct was linearized by *SacI* for gene integration into *Pichia* genome. Seven micrograms of pPICZB/L1 was added to 80 μl of competent yeast cells and transferred into a 0.2 cm-gap electroporation cuvette that pre-cooled on ice. *Pichia pastoris* strains (GS115) were transformed by electroporation at 1.5 kV, 200 Ω and 25 μF with a Gene Pulser II system (Bio-Rad). After the pulse, 1 ml cold 1 M sorbitol was added and the suspension was transferred into a sterile 2 ml Eppendorf tube. Cells were incubated for 1–2 h at 30°C without shaking. Aliquots of 200 μl were spread onto agar plates containing YPD supplemented with 500 μg/ml zeocin and incubated for 3 days at 30°C (*Pichia* expression kit, Invitrogen). The transformants were cultured in

selection medium YPD and screened for the existence of the L1 gene by PCR using AOX1 primers according to *Pichia* Expression Kit manual. Yeast colonies that were positive for L1 DNA were inoculated in 10 ml of YPD medium supplemented with 500 µg/ml zeocin and grown overnight with shaking (250 rpm) at 30°C. Fresh BMMY medium supplemented with 500 µg/ml zeocin was inoculated 1:10 with the overnight culture and grown at 30°C. Methanol was added to final concentration of 1% (v/v) to induce protein expression and cultures were grown at 30°C with shaking (250 rpm) for 3 days. To maintain the induction of the recombinant protein, 100% methanol was added every 24 h to the culture to a final concentration of 1%. Then, protein expression was performed by SDS-PAGE and Western blotting. Yeast cells were harvested by centrifugation at 5000 rpm for 5 min and re-suspended in 300 µl of breaking buffer. An equal volume of micro glass beads (0.5 mm) was added to the tubes and a total of 8 cycles of vortexing and incubation on ice were performed (30 s each). Samples were centrifuged at 5000 rpm for 10 min and the supernatant was transferred to a new micro-centrifuge tube for protein analysis. Protein samples were analyzed by SDS-PAGE in a gel containing 12.5% (w/v) polyacrylamide for *Pichia* and *Pichia*-L1, followed by staining with Coomassie brilliant blue. For protein gel blot analysis, the proteins resolved on the gel were transferred onto protran nitro-cellulose transfer membrane (Schleicher and Schuell Bioscience, Dassel, Germany). The membrane was pre-equilibrated with TBST solution [10 mM Tris-HCl (pH = 7.4), 150 mM NaCl and 0.1% Tween 20) containing 2.5% bovine serum albumin (BSA) for overnight and then reacted with anti-HPV16 L1 monoclonal antibody (MD2H11, Germany; 1:10000 v/v) for 2 h at room temperature. After three washes with TBST, the membrane was incubated with anti-mouse IgG-HRP (Sigma, 1:5000) for 1.5 h at room temperature. The immunoreactive protein bands were visualized using DAB substrate.

Purification and assay of the recombinant protein

For the production of recombinant GST and GST-L1 proteins, the bacterial cultures containing pGEX-L1 were grown to an optical density of 0.8–1 at 600 nm and protein expression was induced with 1 mM IPTG for 2 h at 37°C. Protein samples were analyzed by SDS-PAGE in 12.5% (W/V) polyacrylamide gel. The recombinant L1 protein (rL1) was purified by Zn⁺² reverse staining method as described before.²⁰ The purified protein fraction was concentrated by ultrafiltration and dialyzed against PBS. Protein concentration was measured using BCA assay kit (Pierce, Rockford, USA). The recombinant protein was kept at –70°C until use. For monitoring humoral and cellular immune responses, the purified rL1 protein and VLP (previously provided by Prof. Martin Muller, German Cancer Research Center³⁷) were used as a coated antigen in ELISA.

Preparation of killed yeast and parasites

After washing of *Pichia*-L1 and yeast (GS115 as a control) for 3 times with PBS, the cells were heat-killed at 60°C for 2 h and then stored at –20°C until use. For parasites, the promastigotes of wild (*L.tar*) and transgenic parasites (*L.tar*-L1) were harvested

at stationary phase (7 days) and washed 2 times in sterile PBS. Parasites were killed by several approaches for selecting the best method: 1) repeated freeze-thawing (15 rounds); 2) heat-killing at 60°C for 2 h; 3) Autoclaving and 4) suspension in 0.1% formalin overnight and then washing 3 times in PBS.⁴ In each approach, the dead promastigotes were counted by haemocytometer and re-suspended in a concentration of 1×10^8 / mL in sterile PBS and kept at –70°C prior to use. SDS-PAGE and Western blot analyses were done using Balb/c serum (1: 200 dilution) twice injected by parasites with 3 week intervals as first antibody and anti-mouse IgG-HRP (Sigma, 1:5000) as a secondary antibody. Finally, total protein concentrations were measured using BCA assay kit.

Cells

C3 cancerous cell line (Kindly provided by Prof. Martin Kast, Departments of Molecular Microbiology & Immunology and Obstetrics & Gynecology, University of Southern California, USA), a cell line containing the entire HPV16 genome (confirmed for L1 and L2, Hitzeroth et al., 2009) was cultured in complete RPMI 1640 supplemented with 5% FCS, 2 mM glutamine, 10 mM HEPES and 40 µg/ml gentamycin. On the day of tumor challenge, C3 cells were harvested by trypsinization, counted ($\sim 0.5 \times 10^6$) and finally re-suspended in PBS.

Mice immunization

Female C57BL/6 mice, 6–8-weeks old, were obtained from breeding stock maintained at the Pasteur Institute of Iran. All mice were maintained under specific pathogen-free conditions and all procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. Six groups of 8 mice were immunized including: *Pichia*-L1, *L.tar*-L1, Gardasil, *Pichia* (control), *L.tar* (control), and PBS (control), respectively.

In vivo tumor protection

For the tumor protection experiment, C57BL/6 mice (n = 8) were subcutaneously vaccinated 3 times at 2-week intervals with 5 mg of *Pichia*-L1 and *Pichia* (groups 1 and 4), 100 µg of *L.tar*-L1 and *L.tar* (groups 2 and 5) as well as one fifth of a dose of Gardasil as a positive control (group 3). Group 6 was injected with PBS as a negative control. Two weeks after the last vaccination, mice were subcutaneously challenged in the right flank with 0.5×10^6 C3 tumor cells. Tumor growth and survival rates were monitored by palpation twice a week. At each time point, tumor size was determined by measuring the smallest diameter (a) and the biggest diameter (b) by caliper. Tumor volume was calculated using the formula: $V = (a^2b)/2$.²¹

The doses of different regimens were chosen after a pilot study and also other studies^{15,30,38} In our preliminary studies, the tumor growth was measured after mice immunization with different doses of 2.5, 5 and 10 mg of *Pichia*-L1 and 50 and 100 µg of *L.tar*-L1. Then, the concentrations of interest were selected based on low toxicity and decrease of tumor growth.

Monitoring humoral immune response

Pooled sera were prepared after retro-orbital bleeding from the whole blood samples of each group after the second and third injections and post-challenge. L1-specific IgG1 and IgG2a in the sera were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, a 96-well flat-bottom ELISA plate (Greiner) was coated overnight at 4°C with rL1 (5 µg/ml), rGST (5 µg/ml) or VLP L1 (0.2 ng/ml) proteins diluted in PBS (pH = 7.2). Then, the plate was rinsed with washing buffer (0.5% (v/v) Tween-20 in PBS), incubated with blocking buffer (1% BSA in PBS) for 2 h at 37°C. The pooled sera were diluted 1:50 in dilution buffer (0.5% (v/v) Tween-20 in blocking buffer), added to the plate and incubated for 2 h at 37°C. After rinsing with washing buffer, the plate was 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 h at 37°C. Then, the plates were washed and incubated with streptavidin-horseradish peroxidase diluted in PBS (1:100,000; Sigma) at 37°C for 1 h. Detection was done with 100 µl of O-Phenylenediamine (Sigma) as the substrate in citrate phosphate buffer (pH = 4.5), followed by incubation for 30 min at 37°C. The enzyme reaction was stopped by 1 M H₂SO₄ and the absorbance was measured at 492 nm.

Cytokine assay

To determine the T-helper phenotype of lymphocytes activated by L1, GST and VLP antigens, we investigated the pattern of IFN-γ and IL-4 cytokine production in spleen. Two weeks after third injection (before C3 challenge), 2 mice from each group were killed and the spleens were removed. An amount of 2 × 10⁶ cells/ml of red blood cell-depleted pooled splenocytes from immunized mice of each group were re-suspended in complete RPMI medium 1640 supplemented with 5% FCS, 2 mM glutamine, 5 × 10⁻⁵ mM mercaptoethanol (2-ME), 10 mM HEPES and 40 µg/ml gentamycin. Cells were incubated in U-bottomed, 96-well plates (Costar, USA) in the presence of 10 µg/ml of rL1, 10 µg/ml of rGST and 2 µg/ml of VLP L1 protein. RPMI 5% and 5 µg/ml of concanavalin A (ConA) were

used as negative and positive controls, respectively. Cells were cultured for 3 days at 37°C and 5% CO₂. Supernatants were then collected and frozen at -70°C, until the samples were analyzed. The presence of IFN-γ and IL-4 was measured using a DuoSet ELISA system (R&D Systems, USA) according to the manufacturer's instructions. All data were represented as mean ± SD of duplicate for each set of samples. The detection limit was 2 pg/ml for IFN-γ and 7 pg/ml for IL-4.

Statistical analysis

Statistical analysis was performed using Prism 5.0 software (GraphPad, San Diego, California, USA). One-way ANOVA and Student's t-test were performed to analyze cellular and humoral immune responses as well as tumor volume measurements among the various immunization groups and compare individual data points, respectively. In the tumor protection experiment, the percentage of tumor-free mice in different groups (Survival of animals) was analyzed with log-rank test on Kaplan-Meier curves. For all comparisons, *p* < 0.05 was considered statistically significant. Data are presented as mean ± standard deviation (SD).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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