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The Antitumor Effect of a Non-transforming E7 Protein Combined with a TLR7 Agonist

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ABSTRACT

Despite great efforts in developing peptide-based therapeutic vaccines against human papillomavirus (HPV)-induced cervical cancers, they have failed to elicit strong and sustainable immune responses. Here, we evaluated the vaccine potential of an HPV16 three mutant of E7 (E7GGG) (D21G/C24G/E26G) protein combined with Aldara (topical imiquimod) adjuvant in a TC-1 mouse tumor model.

The HPV16-E7GGG, with eliminated transforming properties but retained antigenicity, and E7 wild-type were inserted into pET28, expressed in the *E coli* system, and purified using Ni-NTA chromatography. The E7GGG and E7 wild-type proteins were combined with Aldara adjuvant and injected into C57BL mice.

We determined the ability of HPV16-E7GGG in combination with Aldara adjuvant to induce robust immune responses by IgG total development, IL-4, IL-17, and IFN-γ induction, CTL activity, and inhibit tumor growth in the murine TC-1 model in different immunized groups.

The generated recombinant HPV16-E7GGG induced humoral and cellular immune responses in a T_H1-mediated pathway, specifically with the (E7GGG) (D21G/C24G/E26G) antigen combined with Aldara, which could be a suitable therapeutic vaccine candidate against HPV.

Keywords: Epitopes; Human papillomavirus 16; Imiquimod; Uterine cervical neoplasms; Vaccines

INTRODUCTION

Cervical cancer is one of the most commonly diagnosed cancers and the leading cause of cancer death in women, with 569,847 new cases annually.[1] Human papillomavirus (HPV) infection is sexually transmitted and is responsible for several malignancies, including cervical squamous cell carcinoma. Infection with several types of HPV, such as 16, 18, 45, 31, 33, 52, 58, and 35, is the main risk factor for cervical cancer, with HPV16 and HPV18 infections accounting for about 70% of the total cases.[2] Currently, 4 preventive HPV vaccines, Cervarix, Gardasil, Gardasil9, and Cecolin, are available

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to induce antibodies against the L1 capsid protein. However, the prophylactic vaccines have no therapeutic effects on eliminating the infection in patients already infected with HPV.[3] To this end, various therapeutic vaccines have been developed to treat established cervical cancers by eliciting effective cellular immune responses against HPV-infected cells.[4] Several strategies have been applied to develop efficient therapeutic vaccines, including DNA-based, synthetic peptides/recombinant subunit proteins, and vector-based, where protein/peptide-based vaccines attracted more attention owing to their stability, safety, and facile production.[5]

HPV E7 oncogenic protein, a pivotal protein for the beginning and development of malignancy and expressed in premalignant and aggressive lesions, is considered the ideal target in designing and developing therapeutic vaccines.[6] This protein interacts with a significant number of cellular proteins, and these interactions can be based on each of the domains or shared between them. For example, conserved region (CR) 2 and CR3 regions are responsible for the interaction with the family of retinoblastoma proteins (pRb, p107, and p130), leading to the degradation of the pRb tumor suppressor protein.[7] The most important mutation in the CR2 domain is C24G, which disrupts the interaction of E7 with pRb and p107 proteins, thus reducing the power of protein transformation.[8] To avoid the safety concerns associated with the administration of an oncoprotein in humans, a mutant form of the E7 protein, E7GGG (amino acids number 21(D), 24(C), and, 26(E) were changed to glycine amino acid, respectively), which cannot interact with pRb, reversing its oncogenic property, could be a more suitable vaccine antigen candidate.[9] Thus, targeting this mutant antigen with therapeutic vaccines to induce cytotoxic T lymphocytes (CTLs) may help to eliminate infected and malignant cells.[10] However, therapeutic vaccines based on proteins/peptides have low immunogenicity, so they need appropriate adjuvants or immunomodulators to induce a strong cellular immune response.[11]

Toll-like receptor (TLR) agonists are used as strong adjuvants in cancer therapy. Imiquimod is an imidazquinoline amine analog similar to guanosine, containing an antiviral activity that induces immune responses via binding to TLR7.[12] Regarding HPV infections, imiquimod induces the secretion of proinflammatory cytokines, such as interferon-alpha (IFN-α), interleukin (IL)-6, and tumor necrosis factor-alpha (TNF-α). It alters the local immune response in favor of the clearance of persistent HPV infection.[13] Topical treatment of imiquimod onto the skin of mice was shown to induce the maturation of plasmacytoid dendritic cells through the expression of costimulatory molecules on their cell surface, including CD40, CD80, and CD86, leading to the induction of an inflammatory response in the skin and production of proinflammatory cytokines, such as IFN-α and IFN-γ.[14] In addition to the effect on immune cells, Aldara (imiquimod 5% cream) had direct proapoptotic effects on tumor cells.[15] It is worth noting that the US Food and Drug Administration (FDA) approved Aldara for the treatment of superficial basal cell carcinoma, actinic keratosis, molluscum contagiosum, and genital and perianal warts.[16] In a study, treatment with Aldara, 3 times a week for 16 weeks, resulted in 16 to 50% wart clearance.[17] Aldara, as an adjuvant in the formulation of therapeutic vaccine candidates, has been used against several cancers, such as HPV-induced cervical squamous cell carcinoma[18,19] and melanoma.[14,20] Moreover, topical treatment with Aldara not only provides a self-administered and non-invasive strategy, but it is also a cost-effective method with low cost and excellent cosmetic outcome.[21] Therefore, it seems that Aldara adjuvant is an effective candidate in the design and development of cancer vaccines.

In this study, we chose the mutant E7GGG protein with the least oncogenic properties and cell transformation potential while preserving its main antigenic epitopes and the potential to generate strong immune responses. The mutant E7GGG protein was expressed in *Escherichia coli*, and cellular immune responses against HPV16 E7GGG adjuvanted with Aldara were evaluated in a mouse model.

MATERIALS AND METHODS

Construction, Expression, and Purification of HPV16 E7 Proteins

The full-length amino acid sequence of the HPV16 E7 protein (98 amino acids) was obtained from GenBank (Accession No. AXB50332.1). Three mutations in the conserved region 2 (CR2), D21G/C24G/E26G,[22] were applied to the HPV16 E7 wild-type protein to construct E7GGG. The coding sequences of E7GGG and wild-type E7 were inserted into the pET-28a expression vector, and after confirming the final construct by colony-PCR and double digestion using *Bam*HI, *Eco*RI, *Sal*I, and *Hin*dIII (Thermo Scientific, US) restriction enzyme sites, the vector was

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transformed into *E coli* BL21(DE3). The expression of E7GGG and E7 proteins was induced in *E coli* BL21(DE3) cells for 3 hours with 0.2 mM IPTG (isopropyl-β-D-thio-galactoside). The protein samples were treated through a 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (Bio-Rad system) using horse-radish peroxidase (HRP)-conjugated monoclonal anti-6x His tag antibody (Santa Cruz Biotechnology, UK) at 1:100 dilution according to the standard protocol.[23] The expressed proteins (E7 and E7GGG) were purified using the nickel-nitrilotriacetic acid column under denaturing conditions based on the producer protocol (Qiagen, Germany).[24] Finally, the purified proteins were dialyzed against phosphate buffer saline (PBS) using a 12 kDa dialysis membrane and stored at −70°C. The concentration of the proteins was determined by the Bradford method.[25] The endotoxin level of the purified E7 and E7GGG proteins was appraised by the chromogenic limulus amoebocyte lysate (LAL) method (BioWhittaker, UK) according to the manufacturer's protocols.

Animal Immunization

Six to eight-week-old female C57BL/6 mice were purchased from the Pasteur Institute of Iran (Karaj, Iran). The mice were randomly divided into three groups (9 per group) and were injected subcutaneously three times on days 0, 7, and 14. As shown in Table 1, in 2 groups, the injection site was topically pretreated with Aldara (imiquimod 5% cream), and then 10 μg of either E7 or E7GGG proteins were injected. Mice injected with PBS and pretreated with Aldara were considered the control group. After each injection, Aldara alone was applied topically to the tumor site (every other day). Nine days after the third immunization, retroorbital blood samples were collected for testing. The mice were euthanized, and the spleens were removed for analysis. All animal experiments were conducted according to the guidelines of the Pasteur Institute of Iran's Ethics Committee (IR.PII.REC.1394.44).

Tumorigenicity in Mice

The TC-1 cell line was purchased from the Iran National Cell Bank (Tehran, Iran) and used for tumorigenicity in mice. The TC-1 cell line originated from primary lung epithelial cells of C57BL/6 mice after cotransformation with oncogenes Ras and HPV16 E6 and E7. The TC-1 cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, Germany), penicillin (100 μg/mL), and streptomycin (100 μg/mL) (Gibco, Germany) at 37°C in 5% CO_2.

To generate tumor tissue in mice, first, a mouse was injected subcutaneously into the right flank with 2×10^5 TC-1 cells. After the tumor size reached 20 mm^3, the mouse was euthanized. The tumor was removed, cut into 2 mm^3 fragments, and each fragment was implanted subcutaneously in the right flank of other mice. One day later, mice were immunized subcutaneously with different immunogens as shown in Table 1. Tumor sizes were monitored every 3 days with manual calipers, and tumor volumes were calculated according to Carlsson's formula: (length × $width^2$)/2. Mice were sacrificed when tumor volumes exceeded 400 mm^3.

Table 1. A group of mice immunized with different immunogens.

Group	Immunogen (dose)
1	E7GGG (10 μg) + Aldara
2	Wild-type E7 (10 μg) + Aldara
3	PBS + Aldara

Evaluation of Lymphocyte Proliferation

Lymphocyte proliferation was evaluated using the bromodeoxyuridine (BrdU) cell proliferation assay kit (Roche, Germany) according to the manufacturer's instructions. Nine days after the third immunization, mice were sacrificed, and the spleen of immunized mice was removed under sterile conditions and suspended in cold, sterile PBS. Red blood cells were lysed with lysis buffer, and a single-cell suspension was adjusted to 3×10^6 cells/mL in RPMI 1640 supplemented with 4 mM L-glutamine, 5% FBS, 100 μg/mL streptomycin, and 100 IU/mL penicillin. Diluted cell suspensions were dispensed into 96-well flat-bottom culture plates (Nunc-Thermo Fisher, US) and stimulated with 10 μg/mL of

the wild-type E7 protein. Unstimulated wells and culture medium were used as negative controls and blanks, respectively. After 72 hours of cell culture, BrdU reagent was added to each well, and plates were incubated at 37°C for 18 hours. Then, the plates were centrifuged, the supernatant was carefully aspirated, and in the following, the plates were dried, fixed, and incubated for 30 min. The plates were aspirated, and anti-BrdU was added before 2 hours of incubation. After 5 times washing with PBS, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well and incubated, followed by stopping the reaction by adding 2N H_2SO_4. The absorbance at OD_{450} nm was measured for each well. The optical density of the blank wells was subtracted from all other wells, and then the stimulation index (SI) was calculated according to the formula: SI=A_{450} of the stimulated wells/A_{450} of the unstimulated wells for an individual mouse. All experiments were done in triplicate.

Cytokines Release Assessment

Nine days after the third immunization, a total number of 3×10^6 spleen cells were seeded in a 24-well plate in RPMI 1640 and stimulated in vitro by adding 10 µg/mL of E7 protein and incubated at 37°C in 5% CO_2 for 72 hours. The cell culture supernatants were removed, and the concentrations of interferon-gamma (IFN-γ), interleukin (IL)-4, and IL-17 cytokines were evaluated by an enzyme-linked immunosorbent assay (ELISA) kit (Mabtech, Sweden) according to the manufacturer's instructions. Immunoreactivity was detected by measuring the optical density at 450 nm using an ELISA reader (Bio-Tek, USA).

Humoral Immune Response Assessment

Specific antibodies were determined using the indirect ELISA method. Briefly, 10 µg/mL E7 protein in PBS was added to 96-well ELISA Maxisorp wells (Nunc, Naperville, IL) and incubated overnight at 4°C. The wells were washed 3 times with PBST (containing 0.05% Tween 20) and blocked using 5% skimmed milk for 1 h at 37°C. The plates were washed with PBST, and 1:100 to 1:12800 dilutions of sera were added to each well and incubated at 37°C for 90 minutes. The wells were washed five times with PBST and incubated for 1 h, and a 1:10000 dilution of anti-mouse conjugated to HRP (Sigma, USA) was added to each well. After 5 times washing and incubation for 30 min with TMB (Sigma, USA) substrate in the dark, the reaction was stopped with 2N H_2SO_4, and color density was measured at OD_{450} nm with an ELISA plate reader (Bio-Tek, USA). Detection of specific IgG1 and IgG2a subclasses was performed using secondary goat anti-mouse IgG1 and IgG2a antibodies (Sigma, USA) according to the manufacturer's instructions.

Statistical Analysis

Prism 7.0 (GraphPad Inc., USA) software was used for statistical analysis. The analysis of variance was done to determine the difference in the immune responses between the different immunization groups. The statistical significance of tumor volumes was analyzed with the Mann-Whitney U-test. The data were expressed as mean±SEM. A p-value<0.05 was considered statistically significant.

RESULTS

Expression and Purification of HPV16 E7 Protein

Induction of *E coli* BL21 (DE3) harboring either pET-28a-E7GGG or pET-28a-E7 plasmids with IPTG resulted in the expression of proteins with a molecular weight of 19 and 17 kDa, respectively (Figure 1A). Western blot results confirmed the induction of both proteins. Additionally, purification by Ni-NTA chromatography in denaturing conditions resulted in homogenous protein bands with yields around 1.8 and 1.4 mg/mL, respectively (Figure 1B). Evaluation of the endotoxin level of the purified proteins indicated values less than 0.01 EU/mL, which was appropriate for the aim of immunization.

In vivo Tumor Regression Assay

Mice were implanted with TC-1 tumor cells, and tumor size was measured after immunization. Our results showed that the tumors slowly expanded by day 9. From day 9 onwards, the TC-1 tumor volume increased gradually in HPV wild-type E7 + Aldara recipients; thus, tumor volume decreased in wild-type E7 + Aldara compared to E7GGG with Aldara (p<0.05). Finally, the results showed that mice injected with either wild-type E7 or E7GGG with topical Aldara could significantly reduce tumor growth compared to the control group (p<0.0001) (Figure 2). It seems that the combination of Aldara adjuvant with proteins had the effect that led to tumor growth suppression.

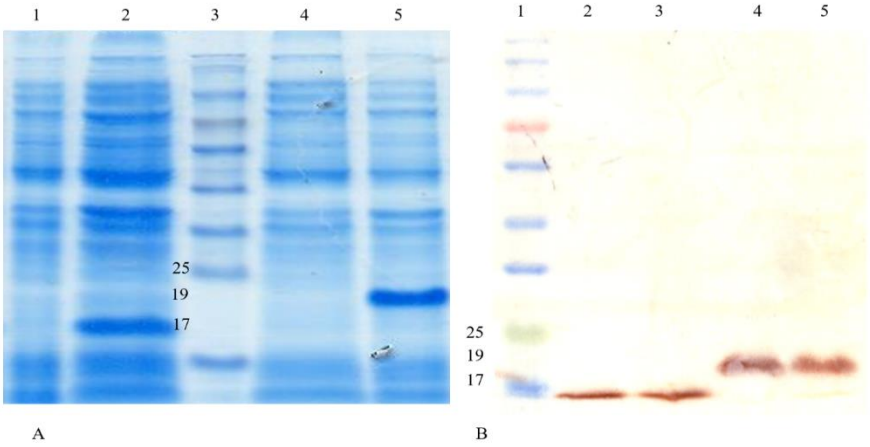

Figure 1. Characterization of the *Escherichia coli*-derived E7GGG and wild-type E7 proteins by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses. A) SDS-PAGE analysis before and after induction of *E. coli* harboring pET28a-E7GGG and pET28a-E7 wild-type plasmids by IPTG revealed protein bands of about 17 and 19 kDa corresponding to the expressed wild-type E7 and E7GGG, respectively. Lanes 1&5: uninduced cell lysates of the *E coli* BL-21(DE3) cells harboring pET28a-E7 wild-type and pET28a-E7GGG, respectively. Lanes 2&4: Cell lysates of *E coli* cells harboring pET28a-E7 wild-type and pET28a-E7GGG, respectively, after 3 hours of IPTG-induction. Lane 3: molecular weight marker (Thermo Fisher, USA). B) Western blotting results for the expressed wild-type E7 and E7GGG, Lane 1: molecular weight marker (Cinacoln, Iran). Lanes 2 and 3: purified wild-type E7 protein. Lanes 4 and 5: purified E7GGG protein.

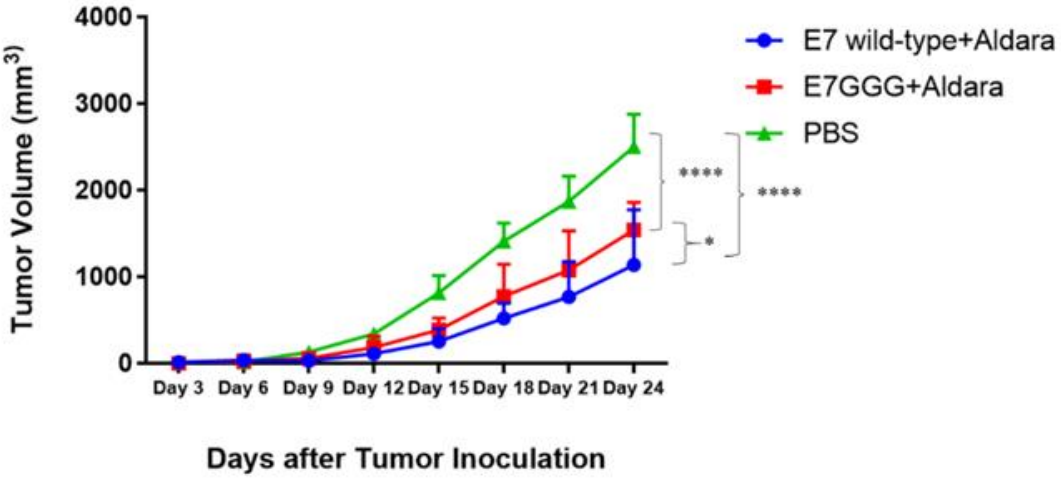

Figure 2. Tumor challenge analyses. C57BL/6 mice were injected with a vaccine combination as described in Materials and Methods, and TC-1 tumor cell growth inhibition by wild-type E7 and E7GGG with Aldara in tumor-bearing mice per group (9 per group). Tumor volume was measured 3 times a week. The data are presented for absorbance at 450 nm ± SD (p<0.05:* and p<0.0001**).**

Evaluation of Lymphocyte Proliferation Response

The lymphocyte proliferation of experimental groups was analyzed by the BrdU/ELISA method. Antigen recall was carried out after 72 h, and then lymphoproliferative activity was evaluated and reported as the SI of individual mice. As shown in Figure 3, the mice immunized with wild-type E7 and E7GGG with Aldara elicited significantly more lymphocyte proliferation compared to the control group (p<0.0001), and E7-wild-type+Aldara could proliferate the lymphocytes a little more compared to E7GGG+Aldara (p<0.05). This observation indicated that proteins are well combined with Aldara adjuvant, leading to the proper release of protein antigen.

The Levels of IFN-γ, IL-4, and IL-17 Cytokines

As the indicator of T_H1 response, IFN-γ production was measured as shown in Figure 4A. The result, similar to lymphocyte proliferation, indicated that the TLR7 agonist (Aldara) adjuvant enhanced the T_H1-skewed response. Similarly, the highest level of IFN-γ secretion was reached in mice immunized by E7 wild-type and E7GGG with Aldara compared to the control group (p<0.0001). Mice immunized by E7 wild-type adjuvanted with Aldara induced significantly higher IFN-γ secretion compared to the E7GGG with Aldara group (p<0.005). This observation indicated the critical role of a few mutations in E7GGG in the level of IFN-γ secretion.

IL-4, as a sign of the T_H2 response, was assessed and compared among different mouse groups. As shown in Figure 4B, both adjuvanted proteins with Aldara induced higher IL-4 compared to the control group (p<0.0001). No significant differences were found between the wild-type E7+ Aldara and E7GGG+ Aldara groups.

Our data also revealed a significant difference in the level of IL-17 secretion between the immunization with both wild-type E7 and E7GGG with Aldara groups compared to the control group (p<0.0001). Moreover, treatment with E7GGG+ Aldara significantly increased IL-17 levels compared with wild-type E7+ Aldara (p<0.005) (Figure 4C).

Evaluation of the IFN-γ, IL-4, and IL-17 secretion indicated that the combination of Aldara with wild-type E7 and E7GGG resulted in a balanced T_H1/T_H2 polarization of the immune response.

Humoral Responses Assessment in Immunized Mice

As shown in Figure 5, evaluation of the humoral response by ELISA indicated that mice immunized with wild-type E7 + Aldara and E7GGG + Aldara induced significantly higher responses compared to the control group (p<0.0005). There was no significant difference between humoral responses for wild-type E7 + Aldara and E7GGG + Aldara groups, although wild-type E7 compared to E7GGG resulted in a slightly more antibody response (Figure 5A). As shown in Figure 5B, assessment of IgG subclasses indicated a balanced IgG1/IgG2a for groups receiving wild-type E7 + Aldara and E7GGG + Aldara, which implies a balanced T_H1/T_H2 polarization of the immune response.

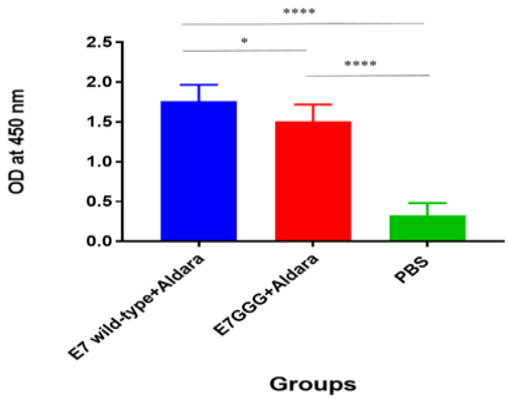

Figure 3. Lymphocyte proliferation response. After immunization, mice were euthanized, splenocytes of mice (9 per group) were collected, and proliferation was stimulated in vitro for 72 h. Proliferation was measured using the BrdU kit as described in the materials and methods. The data are presented as the mean of duplicate absorbance at 450 nm ± standard deviation SD (*p<0.05:* * and *p<0.0001***).**

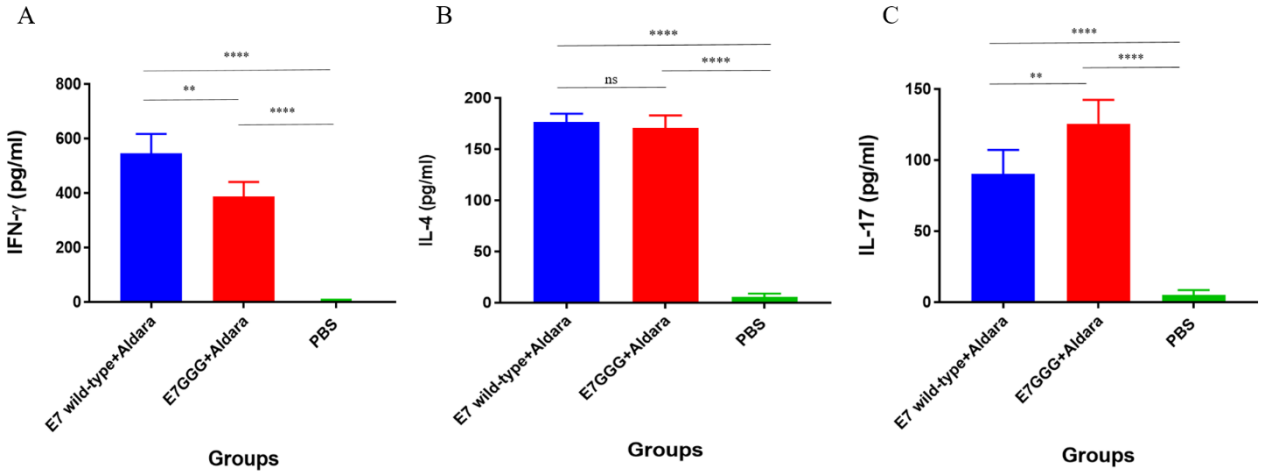

Figure 4. Cytokine secretion levels. The level of IFN-γ (A), IL-4 (B) and IL-17 (C) were determined by the splenocytes of immunized mice (9 per group) as mean absorbance at 450 nm ± standard deviation for each set of samples (p<0.005:, p<0.0005***, p<0.0001**** and ns: no significant).**

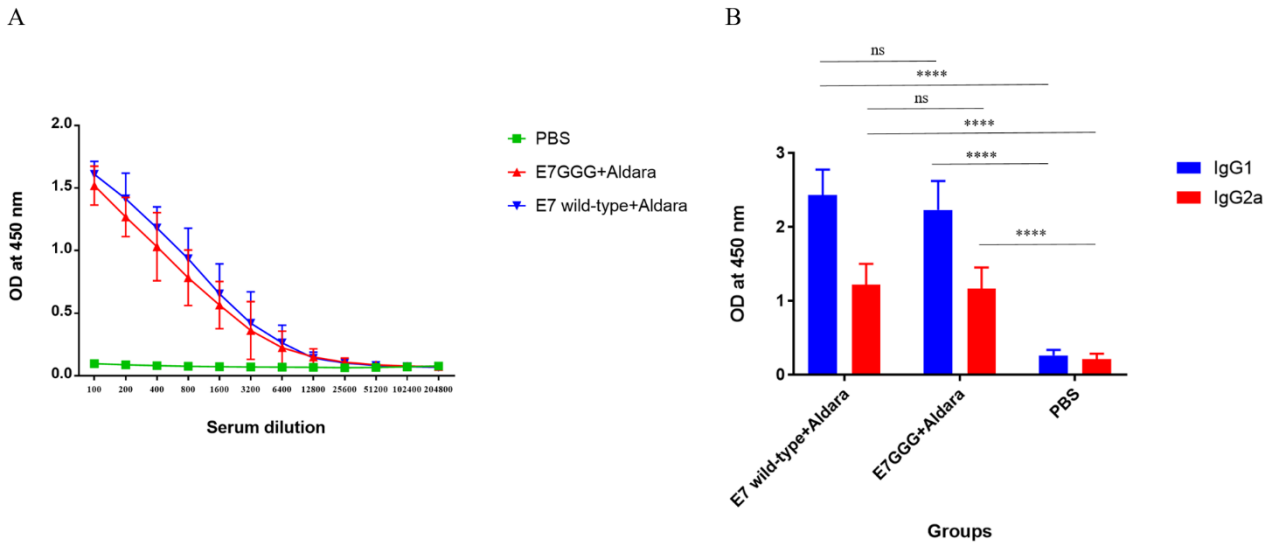

Figure 5. Evaluation of total IgG and IgG subclasses (IgG1 and IgG2a) in immunized mice. Nine days after the last immunization, sera were collected, and total IgG and subclasses were evaluated by ELISA. The OD_{450} values observed for each mouse in 1:100 to 1:204800 diluted sera for total IgG and 1:1000 for subclasses were included. Data are represented as means±standard error of the means (SEM) of duplicate samples from 5 mice per group (p<0.0001** and ns: no significant).**

DISCUSSION

In the current study, we showed that the E7GGG protein, containing three substitutions in D21G/C24G/E26G, with similar antigenicity to wild-type HPV16-E7 as a powerful antigen, in combination with imiquimod, had a crucial role in eliciting CTL activity and regressive TC-1 tumors in immunized mice.

Concerning the E7 protein CR2 region, the most important of these mutations is C24G, which disrupts the interaction of the E7 protein with pRb and p107 proteins (E7 protein without Rb binding site) and thus reduces the power of the protein's cell transformation potential[26] Therefore, it seems that the E7GGG protein can be used as a suitable therapeutic vaccine antigen to replace the wild-type E7 protein. To replace the E7GGG protein

with the E7 wild-type as a vaccine candidate, its immunogenicity should be similar to the E7 wild-type, so both proteins with Aldara adjuvant were investigated and compared in immunological tests. Expression of the E7GGG and wild-type E7 in *E. coli* produced 17 and 19 kDa proteins, respectively, that were efficiently purified under denaturing conditions (Figure 1). Moreover, the obtained protein stability was consistent with a prior report on the expression of both epitopes.[27]

As shown in Figure 2, wild-type E7 compared to E7GGG protein with Aldara significantly resulted in regression of the tumor due to stimulation of HPV16-specific cytotoxic T-lymphocyte responses ($p<0.05$) that were also shown through the IFN-γ secretion assay (Figure 4). As an adjuvant, imiquimod has been used in various therapeutic vaccines to induce strong immune responses. For instance, a phase II clinical trial showed that imiquimod treatment resulted in regression in the majority of intraepithelial lesions of the vulva and intraepithelial lesions of the vagina.[13] Imiquimod provided approximately 80% to 90% complete and permanent eradication in basal cell carcinoma.[28] The tumor regression results of our study, in accordance with Franconi et al[29] showed that our vaccine formulation (E7 wild-type or E7GGG with Aldara) effectively inhibited tumor growth.

Our data showed that two groups receiving the E7GGG and wild-type E7 proteins in combination with Aldara adjuvant developed significant immune responses, but mice immunized with wild-type E7 + Aldara showed the highest lymphocyte proliferation and INF-γ levels compared with E7GGG + Aldara (Figure 3 and Figure 4A). These data showed the pivotal role of Aldara in combination with the protein immunogens for the induction of cellular immunity. These results are in agreement with the Mashhadi et al study,[27] which showed the induction of cellular responses against E7GGG combined with Montanide 266 adjuvant. Paula Di Sciulloa et al found that administration of imiquimod alone significantly enhanced immune response to a tumor vaccine and produced an elevated number of $CD4^+$ T cells and an IFN-γ response along with specific antibodies.[30] Imiquimod has been studied in different types of therapeutic vaccines. In another study, Kokcu et al investigated the positive effects of imiquimod therapy on the proliferative activity of epithelial ovarian cancer cells in vitro.[31]

Aldara induces NLRP1 inflammasome activation in human keratinocytes. In addition, Aldara induces the production of IL-23 by dermal myeloid cells, which in turn causes the secretion of IL-17 by dermal cells, thus IL-17 affects keratinocytes, which leads to their proliferation.[32] The level of IL-17 cytokine secretion in mice immunized with E7GGG + Aldara was significantly higher than in the wild-type E7 + Aldara group, which shows that the three mutations in the E7 protein may have increased the pro-inflammatory activity of imiquimod. The results of our study, in agreement with the others, indicate an enhancement in cellular immune response and antitumor agent elicited by agonistic activity mediates TLR-7 signaling cascades, resulting in NF-κB activation and induction of pro-inflammatory cytokines. Some secondary effects can also be explained by NF-κB activation, that can result in the induction of T_H1 cellular immune response,[33] NLRP1 inflammasome activation in keratinocytes, and induction of IL-17.[34] Moreover, Aldara could link innate and adaptive immune responses by stimulating the production of type I IFN, leading to elevated T_H1-mediated cellular anti-tumor and anti-viral immunity.[35] It is worth noting that transcutaneous immunization using nano-formulated imiquimod provokes superior $CD4^+$ and $CD8^+$ T-cell responses than Aldara against virus infection in a TLR7/MyD88-dependent pathway and IL-1R-independent pathway.[36]

We observed that the response of IgG2a subclasses in mice vaccinated with E7GGG or E7 wild-type with Aldara increased significantly compared to the control group. Dang et al evaluated the immunogenicity of imiquimod in a mouse model via systemic and mucosal routes and found that IgG2a production against HPV-E7,[37] in accordance with the immunogenic results of our study, was potently induced.

These data show that the use of Aldara adjuvant creates a strong cellular immunity that leads to the suppression and inhibition of tumor growth. The results of vaccination with E7GGG and E7 wild-type showed that E7GGG, as a less proliferative E7 counterpart, can be used as an efficient vaccine candidate. In agreement with our study, in a TC-1 tumor model, immunization using E7GGG formulated with Montanide 266 in an oil-in-water emulsion enhanced antigen-specific $CD8^+$ T cells with cytolytic potential (CTLs) and provided both prophylactic and therapeutic benefit.[27]

In conclusion, the immunogenicity of E7GGG as a proper antigen in combination with Aldara was assessed in mice. Our results showed that the E7GGG protein could be considered a candidate antigen for the

therapeutic vaccine development that increased humoral and cellular immune responses and resulted in tumor growth suppression.

STATEMENT OF ETHICS

Procedures were approved by the ethics committee of the Pasteur Institute of Iran, Tehran, and according to the 1964 Helsinki Declaration. (Ethics Code: IR.PII.REC.1394.44).

FUNDING

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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