Evaluation of therapeutic potency of human papillomavirus-16 E7 DNA vaccine alone and with interleukin-18 as a genetic adjuvant

Avaliação da potência terapêutica da vacina de DNA do papilomavírus humano-16 E7 isolada e com interleucina-18 como adjuvante genético

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ABSTRACT

AIMS: Despite the existence of effective preventive vaccines for human papillomavirus (HPV), therapeutic vaccines that trigger cell-mediated immune responses are required to treat established infections and malignancies. The aim of this study was to evaluate the therapeutic potency of HPV-16 E7 deoxyribonucleic acid (DNA) vaccine alone and with interleukin (IL)-18.

METHODS: *In vitro* expressions of IL-18 were performed on human embryonic kidney 293 cells and confirmed it by Western blotting methods. DNA vaccine was available from a previous study. A total of 45 female C57BL/6 mice divided into five groups (DNA vaccine, DNA vaccine adjuvanted with IL-18, pcDNA3.1, and phosphate buffer saline) were inoculated with murine tissue culture-1 cell line of HPV related carcinoma, expressing HPV-16 E6/E7 antigens. They were then immunized subcutaneously twice at a seven-day interval. The antitumor and antigen specific-cellular immunity were assessed by lymphocyte proliferation (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide: MTT) assay, lactate dehydrogenase release assay, IL-4 assay and interferon-gamma (IFN-γ) assay. Tumor size was followed for 62 days.

RESULTS: MTT assay, which measures the lymphocyte proliferation in response to the specific antigen, increased in the co-administration and the DNA vaccine groups as compared to control and genetic adjuvant groups (p<0.001). The mice immunized with the co-administration generated significantly more IFN-γ and IL-4 than other immunized mice (p<0.001). Reduction of the tumor size in the co-administration and the DNA vaccine groups was significantly more pronounced than in the control and genetic adjuvant groups (p<0.001), but no statistically significant difference between DNA vaccine and co-administration groups (p=0.15) occurred.

CONCLUSIONS: IL-18 as a genetic adjuvant and E7 DNA vaccine alone enhanced immune responses in mouse model systems against cervical cancer. However, using of IL-18 as a genetic adjuvant with E7 DNA vaccine had no significant synergistic effect on the immune responses *in vivo*.

KEYWORDS: cellular immunity, human papillomavirus; oncogene protein, interleukin-18.

RESUMO

OBJETIVOS: Apesar da existência de vacinas preventivas eficazes contra o papilomavírus humano (HPV), são necessárias vacinas terapêuticas que desencadeiem respostas imunes mediadas por células para tratar infecções e malignidades estabelecidas. O objetivo deste estudo foi avaliar a potência terapêutica da vacina de ácido desoxirribonucleico (DNA) HPV-16 E7 isolada e com interleucina (IL)-18.

MÉTODOS: Expressões *in vitro* de IL-18 foram realizadas em células renais embrionárias humanas 293 e confirmadas por *Western blotting*. A vacina de DNA foi disponibilizada em um estudo anterior. Um total de 45 camundongos fêmeas C57BL/6 divididos em cinco grupos (vacina de DNA, vacina de DNA adjuvada com IL-18, pcDNA3.1 e solução salina tamponada com fosfato) e foram inoculados com linhagem murina-1 de carcinoma relacionado ao HPV, expressando antígenos E6 / E7 do HPV-16. Os animais foram então imunizados por via subcutânea duas vezes no intervalo de sete dias. A imunidade antitumoral e antígeno-celular específica foi avaliada pela proliferação de linfócitos (ensaio de brometo de 3- [4,5-dimetiltiazol-2-il] -2,5-difeniltetrazólio: MTT), ensaio de liberação de lactato desidrogenase, ensaio de IL-4 e ensaio de interferon-gama [IFN-γ]. O tamanho do tumor foi seguido por 62 dias.

RESULTADOS: O ensaio MTT, que mede a proliferação de linfócitos em resposta ao antígeno específico, aumentou nos grupos de coadministração e de vacina de DNA em comparação aos grupos controle e adjuvante genético (p <0,001). Os camundongos imunizados com a coadministração geraram significativamente mais IFN-γ e IL-4 do que os outros camundongos imunizados (p<0,001). A redução do tamanho do tumor nos grupos de coadministração e de vacina de DNA foi significativamente mais acentuada do que nos grupos controle e adjuvante genético (p<0,001), mas não houve nenhuma diferença estatisticamente significativa entre os grupos vacina de DNA e coadministração (p=0,15).

CONCLUSÕES: A IL-18 como adjuvante genético e a vacina de DNA E7 aumentaram as respostas imunes em sistemas modelo de camundongos contra o câncer cervical. No entanto, o uso de IL-18 como adjuvante genético com a vacina de DNA E7 não teve efeito sinérgico significativo nas respostas imunes *in vivo*.

DESCRITORES: imunidade celular; papiloma vírus humano; proteínas oncogênicas; interleucina-18.

Abbreviations: DNA, deoxyribonucleic acid; DNA vaccine, E7 gene+pcDNA3.1; EL4, murine lymphoma cell line; E7, Early 7, protein of human papillomavirus type 16; HEK-293 cells, human embryonic kidney-293 cells; HPV, human papillomavirus; IFN-γ, interferon-gamma; IGIF, interferon-gamma inducing factor; IL, interleukin; LDH, lactate dehydrogenase; μg, microgram; min, minute/s; μl, microliter; ml, milliliter; mM, millimolar; mr, mouse recombinant; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nm, nanometer; PBS, phosphate buffer saline; pcDNA3.1, eukaryotic expression plasmid as a vector; pRb, protein retinoblastoma; RPMI, Roswell Park Memorial Institute; TC-1, tissue culture-1 cell line; Th1, T helper type 1; Th2, T helper type 2; U, unit; 2ME, 2 mercaptoethanol.

INTRODUCTION

Cervical cancer is the fourth most common cause of cancer and deaths among women worldwide. In addition, it is still the main cause of cancer-related death in the developing countries [1]. Cervical cancer is highly linked to persistent infection due to the high-risk human papilloma virus (HPV), such as types 16 and 18 [2]. In HPV-associated cervical cancers, the viral deoxyribonucleic acid (DNA) integration into the host genome causes an upregulation of HPV oncogenes (E6 and E7) which disrupts the cell cycle and interferes with apoptosis [3]. The expression of HPV-16 E6 and E7 genes in an appropriate host's cells is essential for the process of cellular immortalization [3, 4].

HPV E7 is an acidic polypeptide composed of about 100 amino acids and is functionally similar to other viral oncoproteins, *Adenovirus* E1A and SV40 large T antigen. The E7 oncoprotein can bind to the hypo-phosphorylated form of the retinoblastoma protein and degrade it. Degradation of this retinoblastoma phosphoprotein, Rb, is leaded to release repression of the E2F transcription factor and allows cells to progress through G1 into S phase [4-6]. However, HPV-16 E7 can induce apoptosis in the absence of E6 that is associated with nuclear breakdown [6].

Interleukin (IL)-18 belongs to the IL-1 family of pro-inflammatory and immune regulatory cytokines. IL-18 may be used as a vaccine adjuvant because it can enhance the immune responses in DNA vaccines via inducing the interferon-gamma (IFN-γ) production and promote T lymphocytes and natural killer (NK) cells by IFN-γ. The therapeutic effects of IL-18 were reported in different carcinogenesis mouse models [7].

Various approaches are investigating to enhance the DNA vaccine efficacy, usually involving the co-expression of cytokine genes [8]. The DNA vaccines are a new approach to antigen-specific immunotherapy, as they are well tolerated and have an excellent safety profile. Immunization with DNA plasmid can induce both cell-mediated and humoral immune responses, which makes them an appropriated choice for vaccine [9]. One of the major concerns about DNA vaccines is their limited potency, therefore different strategies are used to increase their potency. The co-administration of cytokines can enhance and modulate the immune response in the desired direction [10, 11]. IL-18 is an extremely potent adjuvant when combined with a variety of cell-based or molecularly defined anticancer vaccines [7]. Some studies mentioned that using IL-18 in the animal model can suppress sarcoma, lung and breast cancers, lymphoma and melanoma [12-14], while other studies mentioned that IL-18 can promote cancer, such as hepatocellular carcinoma and gastric cancer cells [15, 16]. These studies show a double effect of IL-18 on cancers.

The aims of the present study were to evaluate the antitumor immune responses and tumor size in a mouse model by DNA vaccine, genetic adjuvant and co-administration.

METHODS

Plasmid construction

Mouse recombinant IL-18 cloned in pcDNA3.1 (mrIL-18) as a genetic adjuvant was purchased from BioMatik (Waltham, MA, USA) and the recombinant DNA vaccine (pcDNA3.1/ HPV16-E7) was obtained from our previous study [17]. The DH5α strain of *Escherichia coli* competent cells were transformed with confirmed DNA vaccine and mrIL-18 vectors separately in Luria-Bertani medium. Then, the plasmid was extracted using the plasmid mini kit (Qiagen, Hilden, Germany) based on the manufacturer's instructions. DNA concentrations were determined by measuring absorption at 260 nm. The amplification and purification of DNA were previously described [17]. The absence of *E. coli* DNA or RNA contamination was checked by agarose gel electrophoresis. The presence of the DNA vaccine and mrIL-18 genes in the constructed vectors was confirmed by restricting enzyme digestion. The restriction enzymes that were used for the DNA vaccine were *EcoRI* and *XhoI*; and *HindIII* and *BamHI* for the adjuvant. Large-scale preparations of endotoxin-free DNA vaccine plasmids and vector control plasmid DNA (pcDNA3.1) were obtained for immunization studies using the EndoFree® Plasmid Maxi Kit (Qiagen, Hilden, Germany).

Transfection and western blot analysis

Human embryonic kidney-293 cell line (HEK-293) transfected with pcDNA3.1 expressing mrIL-18 DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the mentioned protocol. For *in vitro* confirmation, western blotting was performed on the cell lysate of HEK-293 cells with lysis buffer (Promega, USA). The cell lysates were separated on NuPAGE Bis-Tris and transferred to polyvinylidene difluoride membranes (Amersham, Bioscience), regarding the manufacturer's instruction. The cell lysate of un-transfected HEK-293 cells was used as a negative control. After blocking the membranes in 5% non-fat dry milk overnight, the mrIL-18 antibodies (anti-IL-18 antibody, Millipore) also pre-incubated in 5% non-fat dry milk was added to the membrane at a concentration of 1-1.50 mg/ml and incubated for one hour. After three 15-min. washes with Tris-buffered saline 0.2% with Tween 20, the secondary antibody (horseradish peroxidase-conjugated (HRP)-conjugated rabbit) anti-goat IgG (Sigma-Aldrich, UK) was incubated with the membranes for one hour. After another wash, the antibody binding was visualized using a TMB reagent (Thermo-Fisher Scientific, Waltham, USA) based on the manufacturer's instructions. The expression of DNA vaccine encoding HPV-16 E7 confirmed from our previous study [17].

Experimental animals and cell lines

All the experiments were carried on based on the Animal Care and Use Protocol of Tehran University of Medical Sciences, Iran, regarding to the Ethics Approval Protocol number IR.TUMS.VCR.REC.1395.84.

The origin of cell lines tissue culture-1 (TC-1) and murine lymphoma (EL4) used in the study were from C57BL/6 mice (H2b). These cell lines were purchased from the cell bank of Pasteur Institute of Iran. Cells were cultured in RPMI-1640 medium (Gibco, MA, USA) supplemented with 10% heat-inactivated fetal calf serum, insulin, growth factor, 2 mM L-glutamine, 1 mM pyruvate, 0.1 mM minimal essential medium with nonessential amino acids, 100 U of penicillin/ml and 100 µg of streptomycin/ml, and incubated at 37°C, 5% CO_2.

Six to seven weeks female C57BL/6 mice were obtained from the Pasteur Institute of Iran. The mice were housed for 10 days before the experiments in a standard condition with free access to food and water. A total of 45 mice in five groups (nine per each group) had different challenges with DNA vaccines and adjuvant. The groups were DNA vaccine (were given only pcDNA3.1/HPV16-E7 gene), genetic adjuvant (were given only pcDNA3.1/mrIL-18 gene), co-administration (were given pcDNA3.1/HPV16-E7 gene + pcDNA3.1/mrIL-18 gene), pcDNA3.1, and phosphate buffer saline (PBS).

Tumor challenge and mice immunization

For the *in vivo* therapeutic experiments, C57BL/6 mice were injected subcutaneously with a suspension of 100 µl PBS containing TC-1 cells (5×10^5 cells/mouse) in their left flank, and then grouped into five cages (n=9). After two weeks, the subcutaneous tumors were obvious and palpable in mice. The mice were immunized subcutaneously with 100 µg of each recombinant plasmid twice at 7-day intervals [17, 18]. Two groups of mice received the same volume of PBS (100 µl) and pcDNA3.1 (100 µg) according to the same protocol as the control groups.

Following of tumors size was started two weeks after TC-1 injection. The smallest diameter (a) and biggest diameter (b) of the tumor were blinded measured, in a coded fashion twice a week until 62 days after the first injection of the vaccines. Then, the tumor volume was estimated based on Carlsson's formula: $V=(ab^2)/2$ [19].

Three mice per group were sacrificed one week after the second immunization and the spleens of each animal were removed under the sterile conditions. Then, cell proliferation, cytolytic activity, and cytokine secretion were assayed. All tests were performed in triplicate for each mouse.

Lymphocyte proliferation assay (MTT assay)

After the treatment of the spleen with ammonium chloride-potassium lysis buffer for one minute to deplete erythrocytes, the splenocytes were cultured (2×10^5 cells) in 96-well flat-bottom culture plates (Nunc, Denmark) in triplicate with RPMI-1640 supplemented with 1% fetal calf serum, 1% L-glutamine, 1% 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, and 0.1% penicillin/streptomycin.

Cultured cells were incubated with E7-specific H-2Db CTL epitope (Biomatik, Canada) at a purity of 99%, with either a medium alone or T cell mitogen phytohemagglutinin added as negative and positive controls in each well, at 37°C in 5% CO_2, respectively.

After 48 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, UK) was added in a concentration of 2 µg/µl per well and incubated for additional five hours at 37°C

in 5% CO_2. Dimethyl sulfoxide (100 µl) was added to dissolve the produced formazan crystals. The plates were read at 540 nm, and the results were expressed as the stimulation index (SI). The SI was determined as follows: OD values of stimulated cells minus relative cell numbers of unstimulated cells divided by relative OD values of unstimulated cells. All tests were performed in triplicate for each mouse.

Cytotoxicity assay (LDH assay)

The kinetics of cytolytic activity of E7 specific T cells in tumor-bearing mice was assayed by LDH kit (Thermo Fisher Scientific, Waltham, MA, USA) in 96-well round-bottom plates. In this test, we had two different cells including effector and target of which the effector cells lyse the target one and release LDH from killed cells and this kit assay this factor in an optical way by enzyme linked immunoassay reader or spectrophotometry. A precise number of 2×10^4 EL4 cells (used as the target cells) were cultured in RPMI-1640 medium without the indicator with 1% fetal bovine serum and added 3 µg of E7 peptide (BioMatik, Canada) and incubated in incubator 37ºC for 12-14 hours. Splenocyte that was obtained from mouse spleen (used as the effector cells) was co-cultured with EL4 cell line. After 12-14 hours from co-cultures, supernatant for each sample was collected and detected by LDH kit (LDH, Takara, South Korea) based on manufacturer protocol. Target/effector ratio was 1/50 (20). Each sample was carried on in triplicate. The color change was detected and the cytotoxicity was determined by the following equation:

$$\text{cytotoxicity (\%)} = \frac{(\text{experimental value} - \text{effecter cell control}) - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

Three controls were used for the cytotoxicity assay. A high control meant that the total LDH released from the target cells with all EL4 cells was lysed by a medium with 10% Triton X-100. A low control meant that there was a natural release of LDH from the target cells, which were obtained by adding only EL4 cells in the assay medium. A background control was used to assay the natural release of LDH from the medium. The assay for all samples, including the controls, was performed in triplicates.

Systemic cytokine assay

The mononuclear cells from the spleens of immunized mice at a concentration of 10^6 cells/well in 96 well plates were incubated for three days in a total volume of 200 µl of RPMI-1640 supplemented with 1% fetal bovine serum, 1% L-glutamine, 1% 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, 0.1% 2ME, 0.1% penicillin/streptomycin. Then it was pulsed with the E7-specific epitope (E7, amino acids 49-57) at a concentration of 2 µg/µl at 37°C in 5% CO_2. The cell supernatants were collected and assayed for the presence of IFN-g, TNF-a (eBioscience, San Diego, CA), and IL-4 (R&D Systems, Minneapolis, MN) using commercially available Sandwich-based enzyme linked immunoassay kits and following the manufacturer's instructions. All tests were performed in triplicate for each mouse (three mice per group).

Statistical analysis

All values were expressed as mean ± standard deviation. To compare tumor volumes estimated on Carlsson's formula $V=(ab2)/2$, statistical analyses were performed using the Student's t-test. To compare results between the different groups, Univariate Analysis of Variance test and one way ANOVA test were used. The software SPSS Statistics (version 19.0) was utilized for the statistical analyses. Differences were considered statistically significant when p-value was <0.05.

RESULTS

Results of *in vitro* expression of the recombinant plasmid containing IL-18

Western blot analysis detected *in vitro* mrIL-18 protein expression using a cross-reactive anti-goat IL-18 monoclonal antibody as a secondary antibody and demonstrated the presence of a single 18-kDa band as the predicted size of the mrIL-18 protein (**Figure 1**).

Figure 1. *In vitro* expression of the recombinant vector containing mrIL-18 DNA. Western blot analysis on HEK-293 cells lysate transfected with pcDNA3.1/mrIL-18 showed a strong band at the molecular mass of approximately 18 kDa, which was not detectable in HEK-293 cells lysate well.

Co-administration and DNA vaccine groups were the main groups that increased T cell proliferation more than the other groups

Lymphocyte proliferation assay (MTT assay), which measures the lymphocyte proliferation in response to the specific antigen, increased in the immunized mice of the co-administration and the DNA vaccine groups as compared to mice in control and genetic adjuvant groups (p<0.001). There was no significant difference between co-administration and the DNA vaccine groups. There were statistically significant differences between genetic adjuvant and both PBS (p=0.005) and pcDNA3.1 (p=0.01) as the control groups. This data confirmed that use of DNA vaccine or co-administration alone could induce protective antitumor immune responses that protect vaccinated mice from the TC-1 tumor challenge, but was not seen a synergism between genetic adjuvant and DNA vaccine (**Figure 2**).

DNA vaccine: pcDNA3.1/HPV16-E7, IL-18: adjuvant (pcDNA3.1/IL-18), DNA vaccine+ IL-18: co-administration (pcDNA3.1/HPV16-E7+ pcDNA3.1/IL-18).
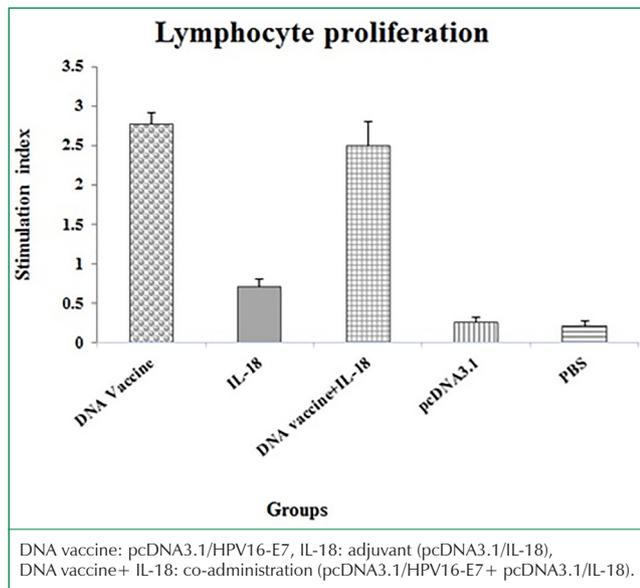
Figure 2. Stimulation index values of vaccinated mice cells in different groups. Results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test showed that the co-administration and the DNA vaccine groups increased the proliferation of T cells higher than the other studied groups (p<0.001). Also, the adjuvant group had a significant difference as compared with the control groups (p=0.01).

Increasing the cytotoxic activity in DNA vaccine and co-administration groups compared to other groups

Cell-mediated immune responses against HPV-16/E7 expressing cells (TC-1) were measured by cytotoxicity of CD8 T cells by LDH cytotoxicity assay. There was a statistically significant difference between the DNA vaccine, co-administration and genetic adjuvant group compared with other groups (p<0.01), but there was no significant difference between DNA vaccine and co-administration group (**Figure 3**).

DNA vaccine: pcDNA3.1/HPV16-E7, IL-18: adjuvant (pcDNA3.1/IL-18), DNA vaccine+ IL-18: co-administration (pcDNA3.1/HPV16-E7+ pcDNA3.1/IL-18).
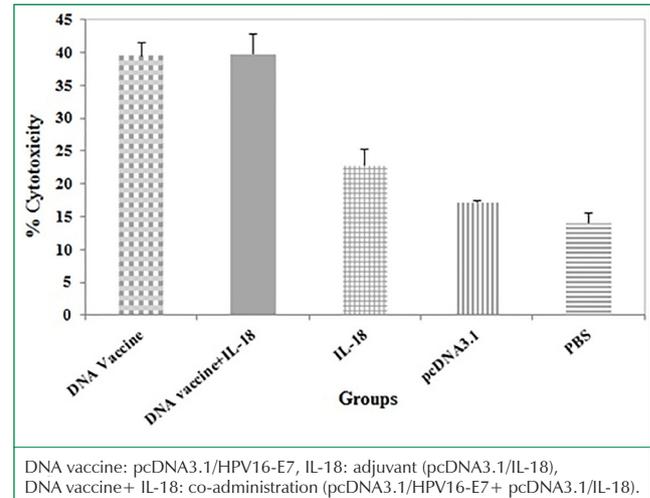
Figure 3. Quantitative measurement of LDH release from EL4 cells due to cytotoxic activity of the spleen lymphocytes (as effector cells). Data were collected from LDH results at E/T ratio of 50:1 and expressed as percent cytotoxicity ± side deviation. DNA vaccine and co-administration groups had the highest cytotoxic activity among all the studied groups (p<0.01); but there is no statistically significant difference between these two groups (p=0.8).

Cytokine assay

The E7-specific IFN-γ (T helper type 1 [Th1] cytokine) and IL-4 (T helper type 2 [Th2] cytokine) in splenocytes from mice immunized with HPV-16 DNA vaccine with or without mrIL-18 adjuvant are shown in **Figure 5**. The mice immunized with the co-administration generated significantly more IFN-γ and IL-4 than other immunized mice (p<0.001). In the other words, the splenocytes from these mice produced the largest amounts of IFN-γ and IL-4. These data showed that both cellular and humoral immune activated against E7 antigen by using co-administration (**Figure 4**).

In vivo Tumor Challenge Assays (compared tumor size between groups)

We investigated whether the DNA vaccine, genetic adjuvant, and co-administration could stimulate a protective antitumor effect and tumor regression
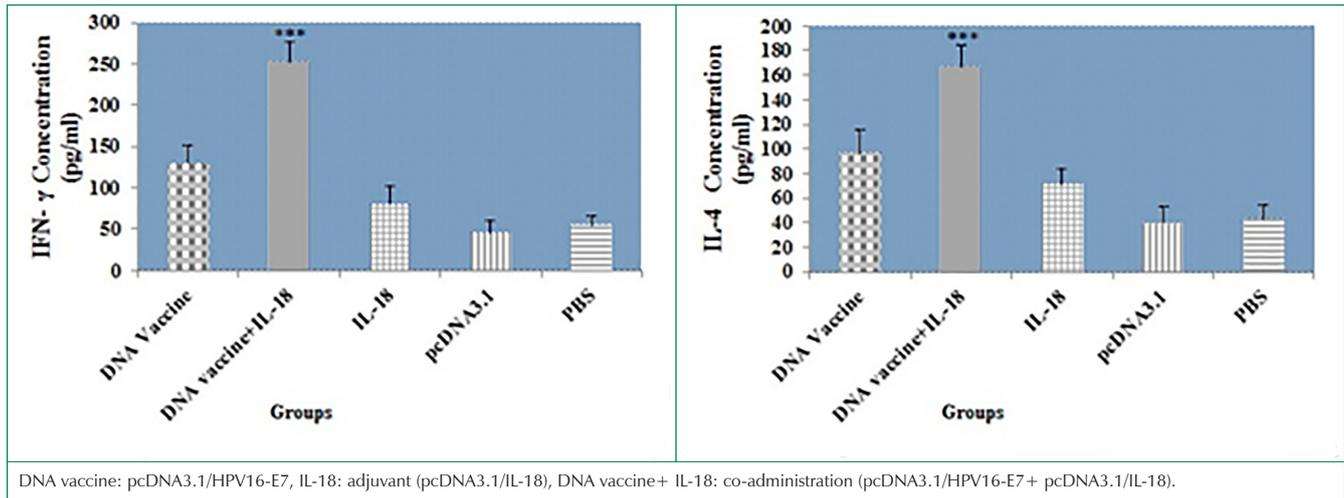
DNA vaccine: pcDNA3.1/HPV16-E7, IL-18: adjuvant (pcDNA3.1/IL-18), DNA vaccine+ IL-18: co-administration (pcDNA3.1/HPV16-E7+ pcDNA3.1/IL-18).

Figure 4. Results of the IFN-γ (A) and IL-4 (B) production by the splenocytes of immunized mice in comparison with pcDNA3.1 and PBS groups as the negative control groups. Co-administration significantly induced IFN-γ (A) and IL-4 (B) secretion more than the control groups (p<0.001).
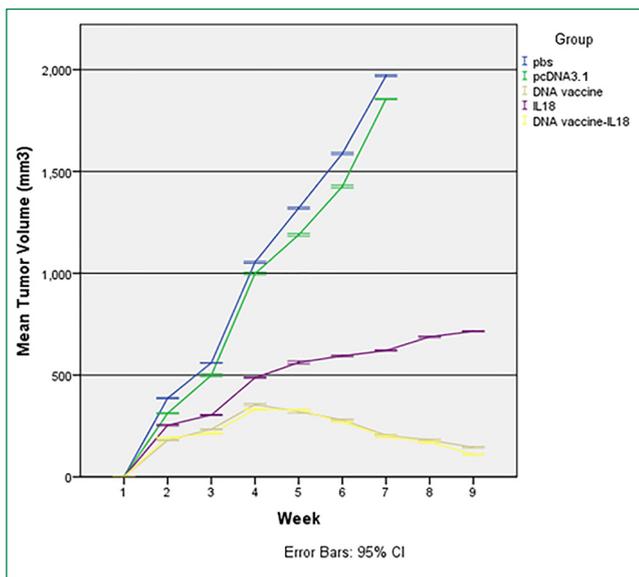

Figure 5. Tumor size of each group studied in a nine-week period. Reduction of the tumor size in the co-administration and the DNA vaccine groups was significantly more pronounced than in control and adjuvant groups (p<0.001), but there is no statistically significant difference between the co-administration and the DNA vaccine groups (p=0.15).

against the TC-1 tumor transplantation in C57BL/6 mice. For this, mice were subcutaneously vaccinated with a dose of 100μg of expression vector twice at a seven-day interval. After the tumor challenge, tumor growth was monitored. The analysis of results showed that reduction of the tumor size in the co-administration and the DNA vaccine groups was significantly more

pronounced than in the control and genetic adjuvant groups (p<0.001), but there was no statistically significant difference between two mentioned (DNA vaccine and co-administration) groups (p=0.15). This data showed that in the tumor size assay, there was not any synergism between DNA vaccine and genetic adjuvant. In addition, there were statistically significant differences between the genetic adjuvant group with PBS (p=0.03) and pcDNA3.1 (p=0.039) as the control groups. The tumor size changes of groups are shown in **Figure 5**.

DISCUSSION

The identification of appropriate tumor-specific antigens for cancer vaccine development is a priority aim in cancer research [21]. HPV type 16 is one of the main carcinogenic types of high-risk papillomaviruses, with 50% of cervical cancer cases. Viral proteins E6 and E7 are expressed in cancerous cells and contribute to disease progression and carcinogenesis. Therefore, these proteins are the best candidate targets for the development of cervical cancer vaccine [22].

Our study showed that DNA vaccine encoding E7 HPV-16 protein has the potential to induce a high level of cellular and humoral immunity against the TC-1 tumor. The current results also showed that in co-administration that inhibited tumor growth and activated cellular and humoral immunity immunities. In parallel with our results, Šmahel et al. [23] demonstrated that an HPV 16 E7 mutant vaccine reduced tumor volume of mice against TC-1 tumor challenge.

In another study, vaccinated mice with DNA vaccine expressing the modified variant with a mutation in the pRb-binding motif of the HPV-16 E7 oncoprotein showed significantly higher cellular immunity and tumor protection than wild-typeE7 DNA vaccine [24]. Fazeli et al. [25] also revealed that the association of the E7 gene with two E6 and L1 genes had a significant role in reducing tumor size in the C57BL/6 mouse model. In addition, a survey by Soleimanjahi et al. [26], reported that the designed DNA vaccine that significantly increased antitumor immunity in the experimental groups compared with the control groups [26]. All of these studies were consistent with the results of our study.

One of the cytokines most studied in cancer is IL-18, which is involved in the induction of Th1 immune responses and known as IFN-γ inducing factor (IGIF) [27]. In the study, a statistical significance was seen in high levels of IFN-γ and IL-4 in co-administration than other groups. Due to the dual function of this IL within different cancer contexts, *in vivo* studies have shown limited efficiency on cervical cancer. Lee et al. [28] evaluated the E6 and E7 effects on the IL-18 expression in NK and PBMC cells. They showed that expression of the viral oncogenes inhibited the induction of the IFN-γ expression through IL-18 activity [28]. A study by Zhu et al. [29] showed that DNA vaccine adjuvant with IL-18 increased the secretion of IFN-g, which was consistent with the results of our study [29]. The obtained data in this study showed that co-administration of IL-18 with E7 DNA vaccine had a small synergistic effect on splenocyte proliferation and decreased tumor size but this synergy was not statistically significant. The IL-18 as a genetic adjuvant functional effect, in the single group and in a combination group with the DNA vaccine, compared with the control groups, was significantly different, indicating the therapeutic effect of IL-18 in HPV-associated tumors.

A previous survey by Cho et al. [30] on the cell lines expressing the HPV-16 oncoproteins (CaSki, Hela, SiHa) and the non-contaminated immortalized C-33A cell, revealed that the E6 oncoprotein can inhibit the IL-18 function. Another study, by Kang et al. [31] was conducted on the SiHa cell line (derived from human cervical cancer) and the effect of mitomycin as an inducer of apoptosis. The findings of that study showed that mitomycin inhibited the growth of this cell line by inhibiting expression of IL-18 [31].

Perhaps one of the most important reasons for differences in the results of various studies is the complexity of *in vivo* conditions relative to *in vitro* conditions. As mentioned that this cytokine has a reversible effect on some cancers, but it should be noted that the use of IL-18 in some cancers such as lymphoma, melanoma, and renal cancers is in different phases of the clinical trial [32, 33]. Our findings showed that IL-18 had a little synergistic effect on the DNA vaccine used, which was not statistically significant. However, considering the inhibitory effect of this adjuvant on tumor growth in the IL-18 group by reducing the size and increasing the secretion of IFN-γ and IL-4 as cellular and humoral arms of the immune system, the therapeutic effect of this cytokine in the cervical cancer mice model cannot be ignored.

In conclusion, our study showed that IL-18, as a genetic adjuvant, had no significant synergistic effects with the DNA vaccine. However, IL-18 alone had a significant effect on the tumor by reducing its size, compared to control groups. Since studies showed that IL-12 has an appropriate synergy with IL-18 [34, 35], it is suggested that in subsequent studies these two synergies be evaluated with the DNA vaccine.

NOTES

Conflicts of interest disclosure

The authors declare no competing interests relevant to the content of this study.

Authors' contributions

All the authors declare to have made substantial contributions to the conception, or design, or acquisition, or analysis, or interpretation of data; and drafting the work or revising it critically for important intellectual content; and to approve the version to be published.

Availability of data and responsibility for the results

All the authors declare to have had full access to the available data and they assume full responsibility for the integrity of these results.

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