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Enhanced anti-tumor effect of a gene gun-delivered DNA vaccine encoding the human papillomavirus type 16 oncoproteins genetically fused to the herpes simplex virus glycoprotein D

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Enhanced anti-tumor effect of a gene gun-delivered DNA vaccine encoding the human papillomavirus type 16 oncoproteins genetically fused to the herpes simplex virus glycoprotein D

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Abstract

Anti-cancer DNA vaccines have attracted growing interest as a simple and non-invasive method for both the treatment and prevention of tumors induced by human papillomaviruses. Nonetheless, the low immunogenicity of parenterally administered vaccines, particularly regarding the activation of cytotoxic $CD8^+$ T cell responses, suggests that further improvements in both vaccine composition and administration routes are still required. In the present study, we report the immune responses and anti-tumor effects of a DNA vaccine (pgD-E7E6E5) expressing three proteins (E7, E6, and E5) of the human papillomavirus type 16 genetically fused to the glycoprotein D of the human herpes simplex virus type 1, which was administered to mice by the intradermal (*id*) route using a gene gun. A single *id* dose of pgD-E7E6E5 (2 µg/dose) induced a strong activation of E7-specific interferon-γ (INF-γ)-producing $CD8^+$ T cells and full prophylactic anti-tumor effects in the vaccinated mice. Three vaccine doses inhibited tumor growth in 70% of the mice with established tumors. In addition, a single vaccine dose consisting of the co-administration of pgD-E7E6E5 and the vector encoding interleukin-12 or granulocyte-macrophage colony-stimulating factor further enhanced the therapeutic anti-tumor effects and conferred protection to 60 and 50% of the vaccinated mice, respectively. In conclusion, *id* administration of pgD-E7E6E5 significantly enhanced the immunogenicity and anti-tumor effects of the DNA vaccine, representing a promising administration route for future clinical trials.

Key words: Gene gun; DNA vaccine; HPV-16; Anti-cancer vaccine

Introduction

Cervical cancer is the second leading cause of cancer deaths among women worldwide (1). Human papillomaviruses (HPV) are associated with virtually all cervical cancer cases. The genome of the human papillomavirus type 16 (HPV-16), the most cancer-prone HPV type, is found in at least 50% of the detected HPV-associated malignancies (2). Currently, two prophylactic anti-HPV vaccines based on virus-like particles (VLPs) are available: Gardasil (VLPs containing the L1 protein from the HPV types 6, 11, 16, and 18) and Cervarix (VLPs containing the L1 protein from the HPV types 16 and 18). Although these vaccines have been shown to be very effective in the generation of neutralizing antibodies, they cannot control existing HPV infections or HPV-associated cellular lesions. Thus, searching for other therapeutic anti-tumor vaccines is a priority that may have an immediate impact on the incidence of HPV-associated tumors.

The control of HPV-associated tumors requires an efficient induction of cellular immune responses, mostly based on antigen-specific $CD8^+$ T cells. The HPV-16 E6 and E7 oncoproteins, constitutively expressed in cervical tumor cells, are the main target antigens for anti-tumor therapeutic vaccines (3). Recently, DNA vaccines have emerged as a promising approach for inducing effective anti-cancer immunity. Although DNA vaccines may induce strong cellular and humoral responses in murine hosts, the specific immune responses observed in subjects in different clinical trials were usually meager (4). To date, various strategies to improve the immunogenicity of DNA vaccines have been tested, including alternative delivery methods and immunization routes. For instance, the intradermal (*id*) administration route has been shown to be more efficient than the intramuscular (*im*) administration route for DNA

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vaccines in terms of the DNA amount required to achieve a similar antigen-specific immune response (5).

Our group has developed different DNA vaccine vectors encoding the HPV-16 oncoproteins genetically fused with the herpes simplex virus type 1 (HSV-1) gD protein (6,7). The *im* administration of such DNA vaccines has shown enhanced preventive and therapeutic anti-tumor effects in mice implanted with tumor cells expressing the HPV-16 E7 and E6 oncoproteins. Recently, we reported the development of a DNA vaccine vector (pgD-E7E6E5) encoding three HPV-16 oncoproteins (E7, E6, and E5) with enhanced anticancer effects relative to the previously reported vaccines based on one (E7) or two (E7 and E6) oncoproteins (7). This newly developed vaccine conferred up to 70% therapeutic anti-tumor protection in mice with established tumor implants after the *im* administration of three vaccine doses (100 µg DNA/dose). In the present study, we evaluated the anti-tumor effects of the pgD-E7E6E5 vector delivered by *id* administration using a gene gun. The results showed that the *id* administration route significantly enhanced the activation of antigen-specific $CD8^+$ T cell responses and the preventive and therapeutic anti-tumor effects of the DNA vaccine.

Material and Methods

Mice

Female C57BL/6 mice at 6 to 8 weeks of age were supplied by the Animal Breeding Center of the Biomedical Sciences Institute of the University of São Paulo and were housed at the Parasitology Department of the University of São Paulo. All animal-related procedures were performed according to approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals of the Biomedical Sciences Institute, University of São Paulo.

Cell lines

The TC-1 cell line was kindly provided by Dr. T.C. Wu (John Hopkins University, Baltimore, MD, USA). These cells were transformed with v-HA-ras and the E6 and E7 genes of HPV-16 (8). The TC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM non-essential amino acids, 10 mM HEPES buffer, 50 U/mL penicillin/streptomycin, and 10% fetal bovine serum (FBS) and were kept at 37°C at 5% CO_2. Before inoculation, the TC-1 cells were harvested by trypsinization, washed twice, and suspended in serum-free media at 5 x 10^6 cells/mL.

DNA vaccines

The preparation of the DNA vaccines encoding the in tandem fused HPV-16 E7, E6, and E5 oncoproteins (pE7E6E5) or the three oncoproteins genetically fused after the amino acid 244 of the HSV-1 pgD protein (pgD-E7E6E5) has been described (7). The correct in-frame cloning of E7, E6, and E5 encoding genes was confirmed by DNA sequencing. The DNA vaccine (pgD) encoding the complete non-fused HSV-1 gD has been described (6).

Immunization and tumor cell challenge

Groups of five to ten mice were vaccinated with the DNA vaccines by *id* administration using a gene gun, through which DNA-coated gold particles (1 µg DNA/bullet) were delivered to the shaved abdominal region using a helium-driven device (Biomics, Brazil) with 400 psi charge pressure; each dose contained 2 µg DNA. Alternatively, vaccinations were performed by *im* administration; each dose contained 100 µg DNA, divided into two 50-µL aliquots and delivered into the tibialis anterior muscle of each hind limb. For the tumor protection experiments, mice were challenged subcutaneously (*sc*) with 5 x 10^5 TC-1 cells suspended in 100 µL serum-free medium; the cells were injected into the left rear flank of the mice 2 weeks after the vaccination. To determine the effect of post-challenge vaccination, mice were vaccinated on the same day 8 h after being challenged with 5 x 10^5 TC-1 cells. One or two additional vaccine doses were applied to the animals at weekly intervals thereafter. For the post-challenge experiments with the co-administration of plasmids expressing cytokines, mice were immunized with three doses, each containing 1 µg DNA of the vaccine vectors admixed to 1 µg DNA of the plasmid expressing interleukin-12 (IL-12) or granulocyte-macrophage colony-stimulating factor (GM-CSF). Tumor growth was monitored by visual inspection and palpation three times a week after the challenge. Mice were scored as tumor-bearing when tumors reached a size of approximately 1 to 2 mm in diameter. Mice were euthanized once tumors exceeded a diameter of 1 cm and became necrotic or burdensome to the animals. Tumor growth was otherwise followed for a period of 60 days after the challenge.

Intracellular cytokine staining

Intracellular interferon-γ (IFN-γ) staining was performed using blood samples treated for 5 min on ice with the ACK lysing buffer (BioSource International, USA) to rupture red blood cells and then centrifuged at 1000 *g* for 5 min. Peripheral blood mononuclear cells (PBMCs) were treated with the lysis buffer again, centrifuged and suspended in DMEM. PBMCs were cultured at the concentration of 10^6 cells/well for 5 h at 37°C in a 96-well round bottom microtiter plate in 200 µL DMEM supplemented with 10% FBS and 10^{-6} M β-mercaptoethanol. Brefeldin A (GolgiPlug; BD Bioscience, USA) was added at 1 µL/mL. The E7-specific RAHYNIVTF peptide, carrying the immunodominant epitope of E7 for mice of the $H\text{-}2^b$ haplotype (9), or the V3 control peptide, delineated from the sequence of the envelope protein of HIV-1 clade B (VVEDEGCTNLSGF), was used as a stimulus at a concentration of 3 µg/mL. After washing, the cells were incubated for 30 min at 4°C with 100 µL of a 1:100 dilution

of a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to mouse CD8a (BD Bioscience). The cells were washed once with PBS followed by permeabilization with Cytofix/Cytoperm (BD Bioscience) for 20 min at 4°C, washed twice with the Perm/Wash buffer (BD Bioscience) and incubated in the same buffer for 30 min at 4°C with 50 µL of a 1:100 dilution of a phycoerythrin (PE)-labeled monoclonal antibody to mouse IFN-γ (BD Bioscience). After washing, the cells were suspended in PBS and were examined by two-color flow cytometry using the FACSCalibur instrument (BD Bioscience). Data were analyzed using the FlowJo software. The percentages of $CD8^+$ cells positive for IFN-γ in all $CD8^+$ T cells were determined.

Statistical analyses

Data are reported as means ± SD and are representatives of at least two independent experiments. Student *t*-test or ANOVA was employed to compare individual data.

Results

Activation of E7-specific $CD8^+$ T cell responses and anti-tumor protective effects in mice immunized *id* with pgD-E7E6E5

Mice immunized *id* with one or two doses of pgD-E7E6E5 (2 µg/dose) developed significant numbers of E7-specific IFN-γ-producing $CD8^+$ T cells. Half the mice immunized with one dose of pgD-E7E6E5 developed E7-specific $CD8^+$ T cell responses, whereas two doses of the vaccine induced positive responses in all vaccinated mice (Figure 1A). Mouse groups immunized with the pgD vector or pE7E6E5 (not fused with the gD protein) did not develop any detectable E7-specific, IFN-γ-producing $CD8^+$ T cell responses.

Although only half the animals immunized with pgD-

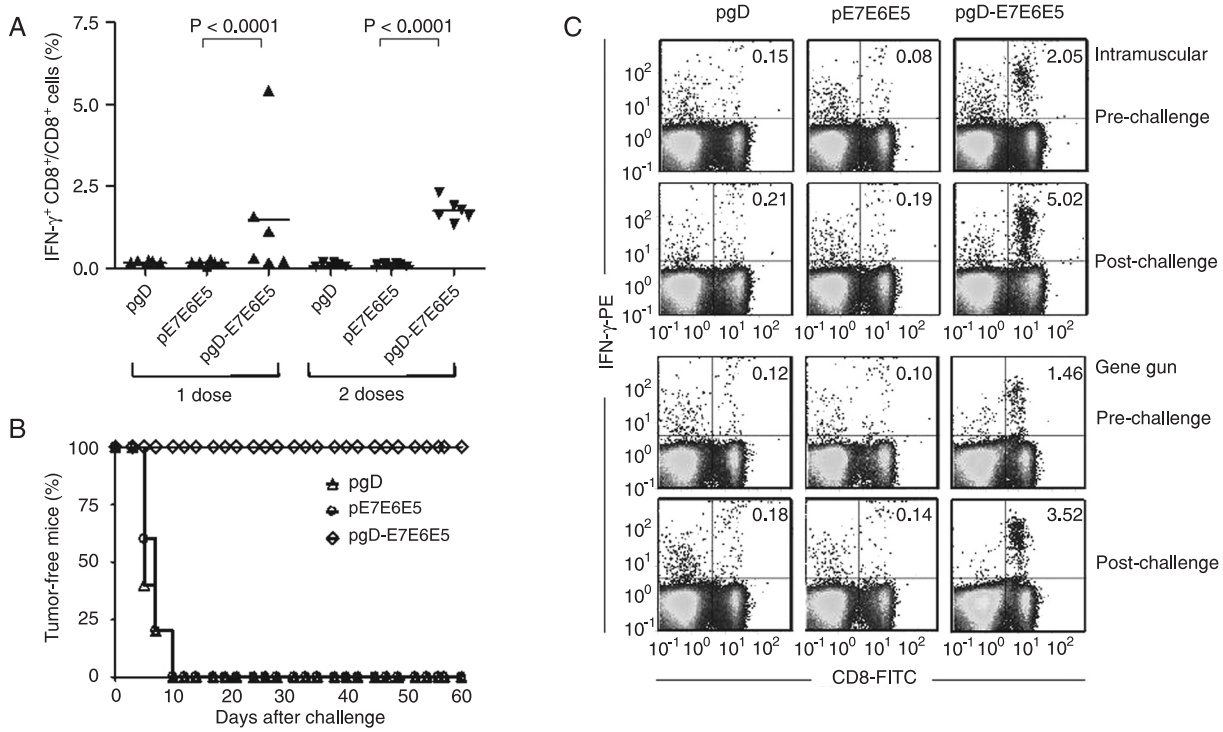

Figure 1. Induction of E7-specific, IFN-γ-producing $CD8^+$ T cell precursors and preventive anti-tumor effects in mice immunized *id* with the pgD-E7E6E5 vaccine. E7-specific $CD8^+$ T cells were detected with PBMCs incubated with the synthetic MHC class I-restricted E7 peptide (^{49}RAHYNIVTF^{57}) and stained for the CD8 marker (FITC) and accumulated intracellular IFN-γ (PE). *A*, Individual $CD8^+$ T cell responses in mice immunized with one or two doses of pgD, pE7E6E5 or pgD-E7E6E5 delivered with a gene gun (2 µg/dose). The number of E7-specific $CD8^+$ T cells was determined 2 weeks after the last vaccine dose. *B*, Mice immunized with one *id* dose of pgD-E7E6E5, pE7E6E5, or pgD were challenged with 5.10^5 TC-1 tumor cells 2 weeks after the last vaccine dose. Tumor growth was followed up to 60 days after inoculation of the TC-1 cells. *C*, Mice were immunized with one dose of pgD-E7E6E5, pE7E6E5, or pgD delivered via the *id* or *im* route, and the frequencies of E7-specific $CD8^+$ T cells were determined 2 weeks after the last immunization (pre-challenge) and 2 weeks after the TC-1 challenge (post-challenge) in pooled PBMCs. The numbers at the right upper corners represent the frequencies of E7-specific $CD8^+$ T cells as a percentage of IFN-γ-producing $CD8^+$ T cells of the total detected $CD8^+$ T cells. IFN-γ = interferon-γ; PE = phycoerythrin; FITC = fluorescein isothiocyanate; PBMCs = peripheral blood mononuclear cells. Statistically significant differences (P < 0.001) were noted with regard to mice immunized with pgD or pE7E6E5 control vectors (ANOVA and Turkey test).

E7E6E5 developed significant anti-E7 $CD8^+$ T cell responses after a single *id* administration, all vaccinated mice developed full preventive protection against tumor growth after being implanted with TC-1 cells (Figure 1B). The same result was obtained in mice immunized with two *id* doses of pgD-E7E6E5 (data not shown). No anti-tumor protective effects were observed in mice immunized *id* with one or two doses of the pgD or pE7E6E5 vectors.

Two weeks after the challenge with TC-1 cells, the number of E7-specific, IFN-γ-producing $CD8^+$ T cells increased in mice immunized with one dose of pgD-E7E6E5 delivered either by the *id* (2 µg/dose) or *im* (100 µg/dose) route (Figure 1C). Collectively, these results demonstrated that the *id* route significantly enhanced the immunogenicity and anti-tumor effects of the pgD-E7E6E6 vaccine compared to the *im* route, which required 50-fold more DNA to induce similar immune responses.

Therapeutic anti-tumor effects of the *id* delivered pgD-E7E6E5 vector

We further investigated the therapeutic anti-tumor effects of pgD-E7E6E5 in mice with established tumors after *id* administration. One dose of the pgD-E7E6E5 vector did not halt tumor progression (Figure 2A). However, two or three doses conferred 30 and 70% protection to the mice with established tumors, respectively (Figure 2A). As indicated in Figure 2, mice therapeutically treated with one dose of pgD-E7E6E5 failed to mount a significant E7-specific $CD8^+$ T cell response in pooled PBMCs. However, mice treated with two or three doses of pgD-E7E6E5 showed a significant increase in the number of E7-specific $CD8^+$ T cells in a dose-dependent manner, reaching maximum values 20 days after TC-1 cell implantation (Figure 2B). As expected, mice immunized with one, two or three doses of the pgD or pE7E6E5 vectors did not develop anti-tumor or E7-specific $CD8^+$ T cell responses (data not shown).

Co-administration of the plasmid expressing IL-12 or GM-CSF enhanced the therapeutic anti-tumor effects of *im* delivered pgD-E7E6E5 (7). Similarly, mice immunized with a single dose of pgD-E7E6E5 and pIL-12 (Figure 3A) or pGM-CSF (Figure 3B; 2 µg/dose) using the gene gun developed 60 and 50% therapeutic protection against pre-implanted tumor cells, respectively. Under the same conditions, no anti-tumor protection was observed in mice immunized with a single dose of pgD-E7E6E5 (Figure 3). No significant anti-tumor protection was observed in mice immunized with the pIL-12 or pGM-CSF vector (Figure 3).

Discussion

The immunization route is a critical aspect to evaluate the efficacy of DNA vaccines aiming at clinical applications. In this study, we tested the *id* immunization route using a gene gun delivering a DNA vaccine expressing three HPV-16 oncoproteins. The results clearly showed that the *id* administration route required less DNA (compared to the *im* route) to achieve a similar antigen-specific $CD8^+$ T cell response and, in particular, to achieve prophylactic and therapeutic anti-tumor effects. Specifically, the present findings showed that a 50-fold reduction in the DNA amount injected by the *id* route preserved the same immunogenicity and anti-tumor effects observed in mice immunized by the *im* route (7). Considering that most DNA vaccines tested under clinical conditions showed lower immunogenicity compared to that under experimental conditions, the possibility of improving the performance of DNA vaccines by changing the administration route represents a significant improvement in the development of therapeutic vaccines targeting HPV-associated tumors.

Previous studies have shown that the administration

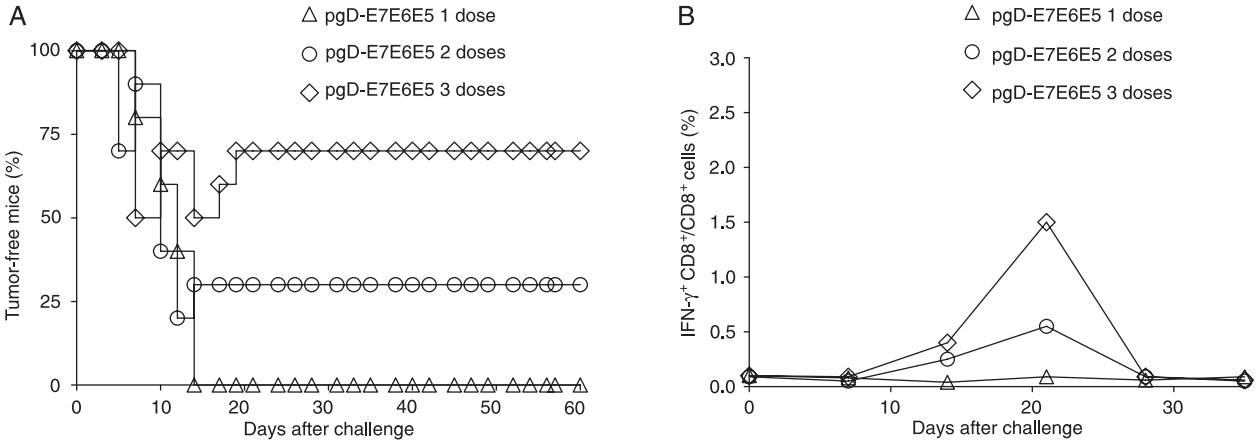

Figure 2. Therapeutic anti-tumor effects and E7-specific $CD8^+$ T cell responses in mice immunized *id* with pgD-E7E6E5. *A*, Therapeutic anti-tumor effects in mice previously inoculated with TC-1 cells and immunized with one, two or three doses of pgD-E7E6E5 administered with a gene gun. *B*, Detection of E7-specific $CD8^+$ T cells in pooled PBMCs from mice inoculated with TC-1 tumor cells and immunized with one, two or three doses of pgD-E7E6E5 delivered via the *id* route.

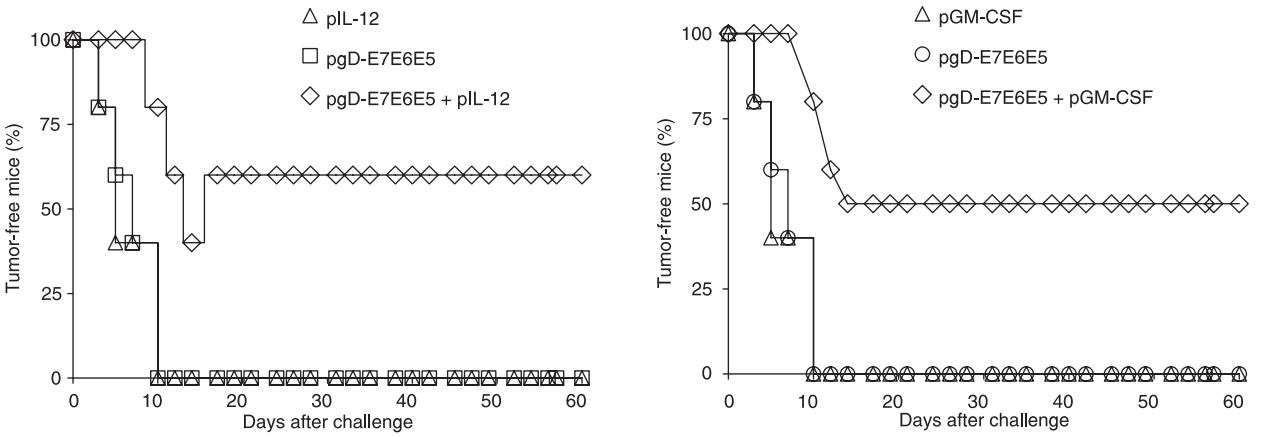

Figure 3. Co-administration of pgD-E7E6E5 and IL-12 or GM-CSF-encoding plasmids confers enhanced anti-tumor therapeutic effects. Mice were immunized *id* with a single dose of pgD-E7E6E5 admixed with pIL-12 (A) or pGM-CSF (B) vectors (1 µg/dose of each vector). Mice immunized with a single dose of the pgD-E7E6E5, pIL-12 or pGM-CSF are also indicated. The vaccines were administered 8 h after inoculation of 5 x 10^5 TC-1 cells. IL-12 = interleukin-12; GM-CSF = granulocyte-macrophage colony-stimulating factor.

route may have a significant impact, both quantitatively and qualitatively, on the immune responses elicited in vaccinated mice. In particular, DNA vaccines administered by the *id* route have been reported to elicit Th2-biased immune responses, whereas administration by the *im* route preferentially induces a Th1-biased immune response (10-15). In contrast to other DNA vaccines, our results demonstrated that the *id* administration of the pgD-E7E6E5 vector did not change the pattern of immune response elicited in vaccinated mice when compared to the *im* administration. These results indicate that the immunization route should be evaluated for each DNA vaccine construct regarding the activation of specific immune responses to the encoded antigen because features other than the administration route might affect the immunogenicity of the DNA, such as the encoded antigen itself, the animal's genetic background and the vector backbone.

A comparative study conducted by Trimble et al. (16) used needle *im*, biojector and gene gun immunization of a DNA vaccine expressing E7 from HPV-16 fused to *Mycobacterium tuberculosis* heat shock protein 70. The authors observed that gene gun immunization induced the highest number of antigen-specific $CD8^+$ T cells and slightly better anti-tumor effects against TC-1 tumors. Our study showed similar results. Although no statistically significant differences in the numbers of E7-specific IFN-γ-producing $CD8^+$ T cells or in anti-tumor effects were detected in mice submitted to the two immunization routes, the amount of DNA used in the gene gun immunization was 50-fold less. Taken together, these results indicate that the *id* administration route can significantly improve the performance of DNA vaccines encoding HPV-16 oncoproteins.

The better performance of DNA vaccines delivered with a gene gun for inducing cell-mediated immunity can be attributed to the cell types involved in antigen processing and presentation. The *id* route preferentially favors the stimulation of epidermal keratinocytes as well as professional antigen-presenting cells (APCs) such as Langerhans cells (17,18). The higher numbers of activated APCs in epidermal keratinocytes relative to those in muscular tissue, where cross-priming prevails, probably at least partially contribute to the enhanced activation of major histocompatibility complex class I-restricted cytotoxic T lymphocytes (19-21).

Cytokines or chemokines simultaneously delivered with DNA vaccines as plasmids or purified proteins have been shown to increase Ag-induced immune responses or to alter the Th1:Th2 balance (22-28). Significantly higher therapeutic anti-tumor protection levels were observed after *id* immunization with a single dose of pgD-E7E6E5 co-administered with the plasmid expressing IL-12 or GM-CSF. Treatment with IL-12 DNA has been shown to enhance antigen-specific cell-mediated immunity and to promote anti-tumor activity in different animal models (28,29). The ability of IL-12 to augment antigen-specific immunity is related to the induction of a Th1-biased immune response, leading to the enhanced activation of cytotoxic T lymphocyte responses (30-32). GM-CSF has been successfully used to increase the immune responses to antigens encoded by DNA vaccines (33,34). GM-CSF has been reported to initiate the proliferation, differentiation, and activation of macrophages, neutrophils, and various APCs (35-39).

The present study demonstrates that the *id* delivered pgD-E7E6E5 vector can generate strong antigen-specific $CD8^+$ T cell responses in vaccinated mice and confer enhanced anti-tumor protection with much lower DNA loads when compared to the *im* delivered DNA vaccine. These results will contribute to the design of therapeutic DNA vaccines against HPV-associated tumors for clinical applications.

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