Immune Responses and Therapeutic Antitumor Effects of an Experimental DNA Vaccine Encoding Human Papillomavirus Type 16 Oncoproteins Genetically Fused to Herpesvirus Glycoprotein D

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Recombinant adenovirus or DNA vaccines encoding herpes simplex virus type 1 (HSV-1) glycoprotein D (gD) genetically fused to human papillomavirus type 16 (HPV-16) oncoproteins (E5, E6, and E7) induce antigen-specific CD8+ T-cell responses and confer preventive resistance to transplantable murine tumor cells (TC-1 cells). In the present report, we characterized some previously uncovered aspects concerning the induction of CD8+ T-cell responses and the therapeutic anticancer effects achieved in C57BL/6 mice immunized with pgD-E7E6E5 previously challenged with TC-1 cells. Concerning the characterization of the immune responses elicited in mice vaccinated with pgD-E7E6E5, we determined the effect of the CD4+ T-cell requirement, longevity, and dose-dependent activation on the E7-specific CD8+ T-cell responses. In addition, we determined the priming/boosting properties of pgD-E7E6E5 when used in combination with a recombinant serotype 68 adenovirus (AdC68) vector encoding the same chimeric antigen. Mice challenged with TC-1 cells and then immunized with three doses of pgD-E7E6E5 elicited CD8+ T-cell responses, measured by intracellular gamma interferon (IFN-γ) and CD107a accumulation, to the three HPV-16 oncoproteins and displayed in vivo antigen-specific lytic activity, as demonstrated with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled target cells pulsed with oligopeptides corresponding to the H-2Dd-restricted immunodominant epitopes of the E7, E6, or E5 oncoprotein. Up to 70% of the mice challenged with 5 × 105 TC-1 cells and immunized with pgD-E7E6E5 controlled tumor development even after 3 days of tumor cell challenge. In addition, coadministration of pgD-E7E6E5 with DNA vectors encoding pGM-CSF or interleukin-12 (IL-12) enhanced the therapeutic antitumor effects for all mice challenged with TC-1 cells. In conclusion, the present results expand our previous knowledge on the immune modulation properties of the pgD-E7E6E5 vector and demonstrate, for the first time, the strong antitumor effects of the DNA vaccine, raising promising perspectives regarding the development of immunotherapeutic reagents for the control of HPV-16-associated tumors.

Cancers pose unique challenges to therapeutic vaccines. Tumor-associated antigens are often self-antigens to which the patient is tolerant. In the case of virus-associated tumors, the viral oncoproteins commonly lack high-avidity T-cell epitopes and thus can evade immune surveillance. Cancer patients frequently show immunological abnormalities, such as T-cell anergy, peripheral and central tolerance, regulatory T cell (Treg)-mediated immunosuppression, and functional T-cell exhaustion (9, 31, 36). Therapeutic cancer vaccines, unlike prophylactic vaccines, thus need to be formulated not only to induce T-cell responses but also to overcome immunological unresponsiveness to tumor antigens.

Cervical cancer is the second most common cause of cancer death in women, claiming approximately 400,000 to 500,000 lives each year worldwide (32). Cervical cancer affects ~1% of all women and is the most common cause of cancer death in women under the age of 50. Virtually all cases of cervical cancers are associated with human papillomavirus (HPV) infections (2, 37). Prevalence of sexually transmitted infections with oncogenic genotypes of HPV varies from 20 to 80% of sexually active adults depending on the study population, with HPV type 16 (HPV-16) representing the most epidemiological relevant oncogenic virus type (2, 37). Two vaccines that express the major capsid protein-inducing serotype-specific HPV neutralizing antibodies have recently become available for preventive vaccination (14). While these vaccines can prevent virus infections with the corresponding HPV genotypes, they cannot affect viral clearance in already infected women or inhibit the development of HPV-associated malignancies. In contrast, therapeutic vaccines targeting HPV oncoproteins, mainly E6 and E7, which are expressed by all transformed epithelial cells, can activate antigen-specific cytotoxic CD8+ T-cell responses and eradicate infected cells before or after the malignant transformation event (18, 21, 27). In contrast to the conventional vaccines, such oncoprotein-based anti-HPV vaccine formulations may be used under either preventive or therapeutic conditions regarding the establishment and uncontrolled growth of the cancer cells under both experimental or clinical conditions (18).

Although DNA vaccines have been intensively studied as a promising immunization strategy for the control of HPV-associated tumors, vectors expressing HPV-16 E7 or E6 oncoprotein alone have shown low antigen-specific CD8+ T-cell acti-
vation and lack of protective antitumor effects in mice (5, 21, 30). Improved activation of antigen-specific CD8+ T-cell responses by anti-HPV DNA vaccines were achieved after genetic fusion of the E7 or E6 oncoprotein with different carrier proteins carrying cell targeting signals or mediators of immune responses (8–19). The focus of our DNA vaccines targeting HPV-induced cancers has been the augmentation of adaptive immune responses through the blockade of an immune inhibitory pathway based on the expression of hybrid proteins genetically fused with glycoprotein D (gD) of herpes simplex virus (HSV) (24,25). HSV gD binds the herpes virus entry mediator (HVEM) and competes for the same binding site as the B- and T-lymphocyte attenuator (BTLA). BTLA provides inhibitory signals to T and B cells upon binding to HVEM (7, 38). Blockade of the HVEM-BTLA pathway during activation of an adaptive immune response has been associated with increased immune responses, particularly E7-specific CD8+ T-cell responses, to the antigen encoded by the DNA vaccine (25).

We reported previously that expression of HPV-16 E5, E6, and E7 oncoproteins within HSV type 1 (HSV-1) gD, either by an adenovirus vector or a DNA vaccine, induces a potent CD8+ T-cell response that confines protective prevention to mice challenged with transplantable E6- and E7-expressing TC-1 cells (25). Herein we characterized previously unknown aspects of the antigen-specific immune responses elicited in mice immunized with the DNA vaccine and report, for the first time, the therapeutic antitumor effects of the pgD-E7E6E5 vector in mice. The results indicate that the DNA vaccine encoding chimeric oncoproteins genetically fused to the HSV-1 gD protein represents a promising approach for the therapeutic control of HPV-associated tumors.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice at 6 to 8 weeks of age were used for these studies. Animals were supplied by the Animal Breeding Center of the Biomedical Sciences Institute of the University of São Paulo and Charles River Laboratories and housed at the Microbiology Department of the University of São Paulo or at the Animal Facility of The Wistar Institute, respectively. All procedures involving the handling and sacrifice of animals were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals at the Biomedical Sciences Institute of the University of São Paulo or at The Wistar Institute.

Cell lines. The TC-1 cell line was kindly provided by T. C. Wu, Johns Hopkins University, Baltimore, MD. The cells had been transfected with a triple promoter retroviral vector carrying v-HA-eGFP together with E6 and E7 carrying genes of HPV-16 (26). TC-1 cells were propagated on HEK 293 cells and purified by CsCl gradient centrifugation.

DNA vaccines. The generation of the DNA vaccines encoding the in tandem-fused HPV-16 E7, E6, and E5 oncoproteins (pETE6E5) or the three oncoproteins genetically fused after amino acid 244 of the HSV-1 gD protein (pgD-E7E6E5) has been described previously (25). The correct in-frame cloning of E7, E6, and E5 encoding genes was confirmed by nucleotide sequencing. The DNA vaccine (pgD) encoding the complete nonfused HSV-1 gD has been described previously (24).

Adenovirus vectors. Vectors expressing either pETE6E5 or pgD-E7E6E5 were constructed as described. pETE6E5 and pgD-E7E6E5 chimeric genes were subcloned into an E1-deleted molecular clones of chimpanzee-derived adenovirus 68 (AdC68) as described previously (13). Recombinant Ad vectors were rescued and propagated on HEK 293 cells and purified by CsCl gradient centrifugation.

Immunization and tumor cell challenge. Groups of 5 to 10 mice were vaccinated intramuscularly (i.m.) with the DNA vaccines. Each dose contained 100 μg of DNA divided in two 50-μl aliquots and applied into the tibialis anterior muscle of each hind limb. Mice were i.m. immunized with 5 × 10⁸ virus particles (VP) of AdC68 vectors diluted in 100 μl of phosphate-buffered saline (PBS) into the tibialis anterior muscle of each hind limb. Mice were challenged subcutaneously (s.c.) with 5 × 10⁶ TC-1 cells suspended in 100 μl of serum-free media and injected into the left rear flank of the animals 2 weeks after the last vaccination. To perform the long-term protection assay, mice were immunized with one dose of the vaccine vectors and challenged 100 days later. To determine the effect of postchallenge vaccination, mice were vaccinated on the same day 8 h after challenge with 5 × 10⁶ TC-1 cells. One or two additional vaccine doses were given to the animals in weekly intervals thereafter. The postchallenge protocol was also performed with the three doses of immunization protocol starting at the following different time points: 0 (8 h later), 3, 5, 7 or 10 days after challenge. For the postchallenge experiments with coadministration of plasmids expressing cytokines, mice were immunized with 100 μg of DNA of the vaccine vectors admixed with 100 μg of DNA of the plasmid-expressing cytokine divided in two 50-μl aliquots. Tumor growth was monitored by visual inspection and palpation three times a week after challenge. Animals 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 or 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 gamma interferon (IFN-γ) staining was performed using blood samples or spleen cells treated for 5 min on ice with Ack lysing buffer (BioSource International, Camarillo, CA) to rupture red blood cells and then centrifuged at 1,000 × g for 5 min. Peripheral blood mononuclear cells (PBMC) or splenocytes were treated again with the lysis buffer, centrifuged, and suspended in DMEM. Cells were cultured at a concentration of 10⁶ cells/well for 5 h at 37°C in a 96-well round-bottom microtiter plate in 200 μl of DMEM supplemented with 2% FBS and 10–6 M 2-mercaptoethanol. Brefeldin A (GolgiPlug; BD Bioscience, San Jose, CA) was added at 1 μl/ml (24, 25). The E7-specific RAYNIVTF peptide, E6-specific VYDFAFRDL peptide, E5-specific VCLIRPLL peptide, or the V3 control peptide, delineated from the sequence of the envelope protein of HPV-1 clade B (VBEDEGCTLNSGF) (4, 12, 30, 24), were used as stimuli at concentrations of 3 μg/ml. After being washed, cells were incubated for 30 min at 4°C with 100 μg of a 1:100 dilution of a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to mouse CD8α (BD Bioscience, San Jose, CA). Cells were washed once with PBS, followed by permeabilization with Cytofix/Cytoperm (BD Bioscience, San Jose, CA) for 20 min at 4°C. Washed cells were treated again with the lysis buffer and stained with a 1:100 dilution of a phycoerythrin (PE)-labeled monoclonal antibody to mouse IFN-γ (BD Bioscience, San Jose, CA). After being washed, cells were suspended in PBS and examined by two-color flow cytometry using Epics Elite XL (Beckman Coulter, Fullerton, CA) or FACScalibur (BD Bioscience, San Jose, CA). Images were analyzed using the FlowJo software. The percentage of IFN-γ–stained, CD8α–stained positive cells was determined. Expression of the CD107a degranulation marker was evaluated as previously described (8). Spleen cells of vaccinated mice were stimulated with the E5-, E6-, or E7-specific peptide in the presence of brefeldin A (GolgiPlug; BD Bioscience, San Jose, CA), monensin (GolgiStop; BD Bioscience, San Jose, CA), and PE-conjugated monoclonal antibody to mouse CD8α (BD Bioscience, San Jose, CA) and incubated in the same buffer for 30 min at 4°C with 50 μg of a 1:100 dilution of a phycoerythrin (PE)-labeled monoclonal antibody to mouse IFN-γ (BD Bioscience, San Jose, CA). After being washed, cells were suspended in PBS and examined by two-color flow cytometry using Epics Elite XL (Beckman Coulter, Fullerton, CA) or FACScalibur (BD Bioscience, San Jose, CA). Images were analyzed using the FlowJo software. The percentage of IFN-γ–stained, CD8α–stained positive cell population was determined. Expression of the CD107a degranulation marker was evaluated as previously described (8). Spleen cells of vaccinated mice were stimulated with the E5-, E6-, or E7-specific peptide in the presence of brefeldin A (GolgiPlug; BD Bioscience, San Jose, CA), monensin (GolgiStop; BD Bioscience, San Jose, CA), and PE-conjugated monoclonal antibody to mouse CD8α (BD Bioscience, San Jose, CA) and incubated in the same buffer for 30 min at 4°C with 50 μg of a 1:100 dilution of a phycoerythrin (PE)-labeled monoclonal antibody to mouse IFN-γ (BD Bioscience, San Jose, CA). After being washed, cells were suspended in PBS and examined by two-color flow cytometry using Epics Elite XL (Beckman Coulter, Fullerton, CA) or FACScalibur (BD Bioscience, San Jose, CA). Images were analyzed using the FlowJo software. The percentage of IFN-γ–stained, CD8α–stained positive cell population was determined.
In the present study, we investigated the activation of E7-specific CD8$^+$ T-cell responses and induction of antitumor preventive effects in mice vaccinated with one or two pgD-E7E6E5 doses administrated via the i.m. route. Individual (A) and pooled (B) CD8$^+$ T-cell responses in mice immunized with one or two doses of pgD, pE7E6E5, or pgD-E7E6E5 delivered via the i.m. route (100 μg/dose). The level of E7-specific CD8$^+$ T cells was determined 2 weeks after the last vaccine dose. Detection of IFN-γ-producing E7-specific CD8$^+$ T cells was carried out with PBMC stimulated with the MHC-I-restricted E7 peptide (49RAHYNIVTF57) and stained for CD8 (with FITC) and intracellular IFN-γ (with PE).

CA). The percentages of target-specific cell killing were calculated as previously described (8).

**Statistical analyses.** All data expressed as means ± standard deviations (SD) are representative of results from at least two independent experiments. Student's t tests or analyses of variance (ANOVA) were employed whenever individual data points were compared.

**RESULTS**

Activation of E7-specific CD8$^+$ T-cell responses and protection from TC-1 cell challenge in mice immunized with pgD-E7E6E5. In the present study, we investigated the activation of E7-specific CD8$^+$ T-cell responses and induction of antitumor preventive effects in mice vaccinated with one or two doses of the pgD-E7E6E5 vector. Approximately 50% of the mice immunized with one dose and all animals vaccinated with two doses of pgD-E7E6E5 developed significant amounts of E7-specific IFN-γ-producing CD8$^+$ T cells (Fig. 1A and B). Mice immunized with pgD or pE7E6E5 did not develop any detectable IFN-γ$^+$ CD8$^+$ T-cell responses. Despite the different magnitude of the E7-specific CD8$^+$ T-cell responses, all animals immunized with either one or two pgD-E7E6E5 doses were protected from a subsequent challenge with TC-1 cells (25; data not shown). In vivo depletion of CD8$^+$ T cells, but not CD4$^+$ T cells, abrogated protection conferred by pgD-E7E6E5, suggesting that CD4$^+$ T helper cells do not play a relevant role in the induction of protective effects in vaccinated mice (data not shown). To determine the longevity of the pgD-E7E6E5-induced protective response, mice vaccinated with one dose of pgD-E7E6E5 were analyzed for E7-specific CD8$^+$ T-cell activation and preventive antitumor responses for 6 months. Mice were immunized with either pgD or pE7E6E5 as a control. Frequencies of E7-specific CD8$^+$ T cells in mice immunized with pgD-E7E6E5 reached a peak 14 days after vaccination and then gradually declined (Fig. 2A). By day 100, when the E7-specific CD8$^+$ T cells were barely detectable, mice were challenged with TC-1 cells. The frequencies of E7-specific CD8$^+$ T cells increased sharply to levels similar to those seen at the initial response 21 days after challenge (Fig. 2B). After peaking on day 121, the numbers of E7-specific CD8$^+$ T cells contracted rapidly and remained at low levels until the end of the observation period (Fig. 2A). Mice challenged 100 days after pgD-E7E6E5 administration showed only a partial protection (50%) from tumor development (Fig. 2C). No protection was recorded in mice immunized with pgD or pE7E6E5.

**Priming-boosting effects of pgD-E7E6E5 on the CD8$^+$ T-cell responses.** The capability of pgD-E7E6E5 to prime and boost E7-specific CD8$^+$ T-cell responses in heterologous immunization regimens in mice submitted to immunization regimens employing a recombinant E1-deleted chimpanzee-derived serotype 68 adenovirus (AdC68) vector encoding the same target antigen, i.e., the three HPV-16 antigens genetically fused into gD, was measured. Mice primed with one dose of the recombinant adenovirus vector and subsequently boosted 3 months later with a single dose of pgD-E7E6E5 showed, 14 days later, a slight enhancement of E7-specific IFN-γ-producing CD8$^+$ T cells that gradually declined (Fig. 3). On the other hand, mice primed with the pgD-E7E6E5 vector showed a dramatic increase (47.4% of the total CD8$^+$ T-cell population) in the number of IFN-γ-producing CD8$^+$ T cells following a boost with a single dose of the recombinant adenovirus vector at 3 months after the priming with the DNA vaccine (Fig. 3). Both the priming effects of the DNA vaccine and the boosting effects of the adenovirus vector required fusion of the target antigens to the gD protein (Table 1). Collectively, these results indicate that the pgD-E7E6E5 vector has a remarkable priming effect on induced CD8$^+$ T-cell responses when used in a heterologous priming-boosting immunization regimen and further emphasize the strong adjuvant effects of the gD protein when encoded by a DNA vaccine vector as a chimeric protein genetically fused to the target antigen.

**Therapeutic antitumor effects of the pgD-E7E6E5 vaccine.** We also investigated the CD8$^+$ T-cell responses to the three HPV-16 oncoproteins under a scenario compatible with the therapeutic use of the pgD-E7E6E5 vector. Mice were challenged with TC-1 tumor cells and then immunized with three doses of the DNA vaccine, or control vectors, administered 1 week apart. Splenocytes were harvested 10 days after the last
vaccine dose, stimulated with peptides from E5, E6, or E7, and stained for detection of intracellular IFN-γ and CD107a, a degranulation marker of activated antigen-specific CD8+ T cells (1). Mice immunized with the pgD-E7E6E5 DNA vaccine showed increased frequencies of E6- and E7-specific IFN-γ-producing CD8+ T cells with regard to those of mice immunized with pE7E6E5 (Fig. 4A). E5-specific CD8+ T-cell responses were also detected in mice immunized with pgD-E7E6E5 but did not reach a statistically significant difference compared to those of mice immunized with pE7E6E5. Detection of the CD107a marker further demonstrated that immunization with pgD-E7E6E5 resulted in positive responses to all three HPV-16 oncoproteins in vaccinated mice, with regard to animals immunized with pE7E6E5 (Fig. 4B). These results indicated that mice therapeutically treated with pgD-E7E6E5 manage to circumvent the immune-suppressive conditions induced by the tumor growth and develop significant CD8+ T-cell responses against all three HPV-16 oncoproteins encoded by the DNA vaccine.

To further demonstrate that the CD8+ T-cell responses developed in mice therapeutically treated with the DNA vaccine show in vivo cytolytic activity to cells expressing the HPV-16 oncoproteins, we conducted experiments in which CFSE-stained cells were pulsed with E5-, E6-, or E7-derived peptides representing the immunodominant H-2Dα-restricted epitopes of each oncoprotein. As in the previously described experiments, mice were challenged with TC-1 cells, vaccinated at weekly intervals with 3 doses of pgD, pE7E6E5, or pgD-E7E6E5 vector, and injected with CFSE-labeled target cells 14 days after the last vaccine dose. Peptide-pulsed cells were brightly labeled with the dye, while control cells were labeled with a 10-fold-lower dose of CFSE. Splenocytes were harvested 24 h later from individual mice and tested for loss of the brightly stained cells as a measure for the specific CD8+ T-cell-mediated lysis (Fig. 4C and D).

**FIG. 3.** Priming/boosting effects on E7-specific CD8+ T-cell responses in mice submitted to a heterologous prime-boost immunization regimen using pgD-E7E6E5 and a recombinant adenovirus vector encoding the same antigen. Groups of mice (n = 5) were primed with pgD-E7E6E5 and subsequently boosted with the recombinant adenovirus vector (AdC68gD-E7E6E5) 3 months later (ø). The same procedure was repeated in mice immunized with AdC68gD-E7E6E5 and subsequently boosted with pgD-E7E6E5 (●). Detection of IFN-γ-producing E7-specific CD8+ T cells was carried out with pooled PBMC stimulated with the MHC-I-restricted E7 peptide (69RAHYNIVTF37) and stained for CD8 (with FITC) and intracellular IFN-γ (with PE).

**TABLE 1.** Flow cytometry analyses

| Prime  | % of IFN-γ+ CD8+ cells over total CD8+ cells |
|--------|---------------------------------------------|
|        | No boost | AdC68E7E6E5 | AdC68gD-E7E6E5 |
| None   | <0.1     | >0.1        | 21.9           |
| pE7E6E5| >0.1     | >0.1        | 21.2           |
| pgD-E7E6E5| 0.3      | 2.5        | 47.4 |

*Analyses of IFN-γ-secreting E7-specific CD8+ T-cell precursors in mice submitted to heterologous prime-boost immunizations using the pE7E6E5 or pgD-E7E6E5 DNA vector and the AdC68E7E6E5 or AdC68gD-E7E6E5 adenovirus vector.
served in mice immunized with the pgD or pE7E6E5 vector. In contrast, mice immunized with pgD-E7E6E5 clearly showed a reduction in the number of peptide-pulsed cells, particularly those labeled with peptides derived from the E6 or E7 peptides. All pgD-E7E6E5-immunized animals had reduced numbers of E7-pulsed target cells, 3 out of 4 animals showed a reduction in the number of target cells pulsed with the E6-specific peptide, and only 1 animal developed a specific lysis response to E5-pulsed cells (Fig. 4D). Collectively, these results demonstrate that post-challenge-vaccinated mice developed functionally active CD8\(^+\) T-cell responses to the HPV-16 oncoproteins.

We next measured the therapeutic antitumor effects of pgD-E7E6E5 in vaccinated mice previously inoculated with TC-1 cells. Mice were challenged with TC-1 cells and treated on the same day with one dose of pgD-E7E6E5 or the control vectors. Other groups received a second dose or a third dose of each DNA vaccine given at weekly intervals. As shown in Fig. 5, one dose of the pgD-E7E6E5 vector failed to prevent tumor progression. However, administration of a second dose and a third dose conferred only 50% and 70% protection, respectively, to mice immunized with pgD-E7E6E5 (Fig. 5A). Monitoring of the E7-specific CD8\(^+\) T-cell responses in mice immunized with pgD-E7E6E5 showed that a single dose of the DNA vaccine failed to induce a statistically significant CD8\(^+\) T-cell response. Nonetheless, mice treated with two or three doses of pgD-E7E6E5 showed a significant increase in the number of E7-specific CD8\(^+\) T cells, which reached maximum values 20 days after the tumor challenge (Fig. 5B). The CD8\(^+\) T-cell responses quickly declined and reached baseline values 1 week later. Animals receiving one or two doses of the pgD or pE7E6E5 vectors did not develop measurable E7-specific CD8\(^+\) T-cell responses (data not shown).
We also measured the therapeutic antitumor effects of the DNA vaccine in animals challenged up to 10 days after the first vaccine dose. As shown in Fig. 5C, mice treated with three pgD-E7E6E5 doses up to 3 days after the TC-1 cell challenge showed 70% protection to tumor development. When the interval between the tumor challenge and vaccine treatment was increased to 5 or 7 days, the antitumor protection effect was observed in only 50% and 25% of the vaccinated mice, respectively. No antitumor effect in mice vaccinated 10 days after the TC-1 challenge was observed.

In an attempt to improve the therapeutic effects generated by the DNA vaccine, we combined the pgD-E7E6E5 vector with plasmids encoding interleukin-12 (IL-12) or granulocyte-macrophage colony-stimulating factor (GM-CSF), known to exert adjuvant effects on immune responses induced by DNA vaccines or synthetic peptides (10, 23, 35). Mice were challenged with TC-1 cells and immunized with 3 doses of pgD-E7E6E5 or pgD-E7E6E5 mixed with 100 μg of pcDNA3.1 (empty vector), pIL-12 encoding murine IL-12, or pGM-CSF encoding murine GM-CSF. As shown in Fig. 6, mice that received pgD-E7E6E5 combined with pIL-12 or with pGM-CSF increased the antitumor response to 100%. Control groups immunized with pgD-E7E6E5 and pcDNA3.1 did not show further enhancement of the therapeutic protection observed in mice immunized only with pgD-E7E6E5. Mice immunized with pcDNA3.1, pIL-12, or pGM-CSF, in combination or not with pgD-E7E6E5, did not show any antitumor protection (data not shown).

**DISCUSSION**

Our previous results demonstrated that immunization with HPV-16 oncoproteins genetically fused to the HSV-1 gD protein, encoded by a naked DNA or adenovirus vector, can induce strong anti-E7 CD8\(^+\) T-cell responses and elicit preventive antitumor responses in the murine model (25). In the present study, we extend our previous evidences and demonstrated that the pgD-E7E6E5 vector induces specific CD8\(^+\) T-cell responses to all three HPV-16 oncoproteins in mice previously challenged with TC-1 cells. In addition, we showed that the mice vaccinated with pgD-E7E6E5 elicit therapeutic antitumor responses even several days after the challenge with the tumor cells. More relevantly, our results demonstrate that the therapeutic anticancer effects of pgD-E7E6E5 can be further improved following coadministration with DNA vectors encoding GM-CSF or IL-12. All together, the present report adds new data concerning the induction of immune responses in mice vaccinated with pgD-E7E6E5 and shows that, under experimental conditions, this DNA vaccine represents a promising therapeutic approach for the control of HPV-16-associated cancer.

The reduced immunogenicity of E7, particularly when encoded by DNA vaccines, has led to the use of genetically fused carrier proteins that improve both the processing and/or presentation of the major E7-specific CD8\(^+\) T-cell epitope or augments positive interactions between antigen-presenting cells and effector CD8\(^+\) T cells, which resulted in enhanced...
antitumor protection responses (5, 6, 17–19, 21–22, 24–27, 41). Our results demonstrate that immunization with one or two doses of the pgD-E7E6E5 vector resulted in full protection from subsequent tumor cell challenges, but only half of the mice vaccinated with a single dose of pgD-E7E6E5 developed measurable precursor E7-specific CD8⁺ T cells. This result suggests that even small numbers of antigen-specific CD8⁺ T cells may suffice for the control of tumor growth or that additional immune cells contribute to the eradication of the cancer cells. Another interesting observation concerning activation of T-cell responses elicited in mice immunized with the pgD-E7E6E5 was the observation that, in contrast to other previously reported vaccine formulations (16, 41), depletion of CD4⁺ T cells did not reduce the antitumor protective effects of pgD-E7E6E5, an indication that T helper cells are not required for the initial activation of antigen-specific effector CD8⁺ T cells. Nevertheless, additional experiments should be conducted in order to elucidate the contribution of CD4⁺ T cells on the induction and persistence of the antitumor responses elicited in mice immunized with the pgD-E7E6E5 vector.

No information regarding the kinetics and longevity of the E7-specific CD8⁺ T-cell responses as well as the antitumor protection elicited in mice vaccinated with pgD-E7E6E5 has been reported. Our results show that mice immunized with a single dose of pgD-E7E6E5 sustain measurable CD8⁺ T-cell responses for up to 100 days following vaccine administration. More relevantly, 50% of the mice immunized once with pgD-E7E6E5 survived the challenge with TC-1 cells at 100 days after vaccine administration. After challenge with the tumor cells, the number of E7-specific CD8⁺ T cells increased quickly but returned to background values in 2 weeks, a feature that may reflect the immunosuppressive environment generated by the growing tumor cells. Experiments addressing the specific role of the gD protein on the induction of memory responses and persistence of the antitumor protection effects in pgD-E7E6E5-vaccinated mice should contribute to further improvements of the protective immunity elicited in mice immunized with this DNA vaccine.

Heterologous prime-boost immunization regimens represent a powerful approach to increase cellular immune responses in mammalian species and have been successfully used in the development of improved vaccines for the control of viral and parasite infections (40). In the present study, we demonstrated that priming with pgD-E7E6E5 and subsequent boosting with an adenovirus vector encoding the same target antigen dramatically increased the number of activated E7-specific CD8⁺ T cells in vaccinated mice. The priming effects conferred by the pgD-E7E6E5 vector were dependent on the fusion of the target antigen with the HSV-1 gD protein, suggesting that blockade of the BTLA-mediated coinhibitory pathway contributes to a more efficient activation of antigen-presenting cells and the subsequent positive interaction with CD8⁺ T cells (25).

The recent report of a successful anticancer vaccine trial in patients suffering with cervical cancer and vulvar intraepithelial neoplasia indicates that the therapeutic antitumor vaccines are feasible and represent a real possibility in the near future (20, 39). Our data showed that the pgD-E7E6E5 vector induces not only full protective antitumor responses but also, and most relevantly, antitumor therapeutic effects that may reach up to 100% of mice with previously established tumors derived from a high-load TC-1 cell inoculum. Our data demonstrate that a DNA vaccine encoding the three HPV-16 oncoproteins induces CD8⁺ T-cell precursors to the immunodominant H-2D⁺-restricted epitopes of the E7, E6, and E5 proteins. The pgD-E7E6E5 vector also induced activated CD8⁺ T-cell responses, as indicated by detection of CD107a, a degranulation marker of activated CD8⁺ T cells (1). In addition, functional in vivo assays showed that mice therapeutically vaccinated with the pgD-E7E6E5 vector developed active cytolytic CD8⁺ T cells targeting both HPV-16 E6 and E7 oncoproteins, which are expressed by the TC-1 cells. The lower E5-specific CD8⁺ T-cell responses were expected, since this antigen is not expressed by the TC-1 cells and would not, therefore, be boosted after immunization with the DNA vaccine. The higher therapeutic antitumor effects of mice immunized with pgD-E7E6E5 with regard to previously tested DNA vectors encoding E7, E6, or both E6 and E7 may be explained, at least in part, by the more efficient activation of CD8⁺ T cells by antigen-presenting cells based on the blockade of the BTLA-mediated coinhibitory signals. As shown previously (25), insertion of larger peptides at the C-terminal part of the gD protein may result in stronger binding to the HVEM receptor and more efficient blockade of the BTLA-binding site.

Three doses of pgD-E7E6E5 partially controlled the growth of tumor cells inoculated in mice up to 5 days before the initial vaccine dose. Noticeably, the protection levels conferred by the vaccine, which ranged from 70% (mice treated up to 3 days after inoculation of the TC-1 cells) to 25% (mice treated 7 days after inoculation of the TC-1 cells), were determined under restrictive conditions (5 + 10⁵ cells/mouse) of TC-1 cells that grow into solid tumors in approximately 1 week and reach large sizes (more than 1.5 cm) within 2 weeks. Under less restrictive conditions (5 × 10⁴ cells/mouse), the therapeutic effects of the DNA vaccine can be significantly higher, reaching 100% of the treated animals (our unpublished observations). In addition, our results show that coadministration of IL-12- or GM-CSF-encoding plasmid further enhanced the antitumor protective effects even following challenges with larger TC-1 cell loads. Both IL-12 and GM-CSF have a long record of successful application in cancer treatment and, although acting through different mechanisms, promote more efficient activation of cytotoxic T cells (10, 11, 23, 28, 33, 35). Indeed, the therapeutic efficacy of these cytokines for the control of different cancer types have also been demonstrated under clinical conditions (15, 29, 34). Based on such evidences, it is possible that the combined use of pgD-E7E6E5 and cytokine-encoding plasmids represents a powerful new tool to induce tumor-protective responses under experimental conditions.

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