The generation and use of therapeutic human papillomavirus (HPV) DNA vaccines represent an appealing treatment method against HPV-associated cervical cancer owing to their safety and durability. Previously, we created a therapeutic HPV DNA vaccine candidate by linking the HPV16-E7 DNA sequence to calreticulin (CRT/E7), which we showed could generate significant E7-specific cytotoxic T lymphocyte (CTL)-mediated antitumor immune responses against HPV16 oncoproteins expressing murine tumor model TC-1. Here we assess the therapeutic efficacy of intravaginal immunization with pcDNA3-CRT/E7 followed by electroporation. In addition, we examined whether coadministration of DNA-encoding interleukin 2 (IL2) with the pcDNA3-CRT/E7 could improve the T-cell responses elicited by pcDNA3-CRT/E7. TC-1 tumor-bearing mice vaccinated intravaginally with both pcDNA3-CRT/E7 and IL2 DNA followed by electroporation induced stronger local antitumor CTL response in comparison to mice that received other treatment regimens. Additionally, we found that coadministration of IL2 DNA with pcDNA3-CRT/E7 modified the tumor microenvironment by decreasing the population of regulatory T cells and myeloid-derived suppressor cells relative to that of CTLs. Our data demonstrate the translational potential of local administration of IL2 and pcDNA3-CRT/E7 followed by electroporation in treating cervicovaginal tumors.

INTRODUCTION

The generation and use of therapeutic human papillomavirus (HPV) DNA vaccines represents an appealing treatment method against HPV-associated cervical cancer owing to their safety and stability. It has been discovered that the two oncoproteins encoded within the HPV genome, the early protein 6 (E6) and early protein 7 (E7), have vital roles in the tumorigenesis and upkeep of HPV-associated cervical cancer. As such, the E6 and E7 oncoproteins are believed to be optimal targets for the generation of immunotherapeutic strategies against HPV-associated cancer because they are tumor specific, constantly expressed and are foreign proteins; therefore, they do not possess any issues regarding immune tolerance. In our previous studies, we have designed and generated a potent therapeutic HPV DNA vaccine composed of a fusion construct of HPV16 E7 protein linked to calreticulin (CRT/E7), which we have shown to enhance the cross-presentation of the E7 antigen on to the major histocompatibility complex class I molecule. Furthermore, we previously demonstrated that immunization with CRT/E7 DNA resulted in a potent cytotoxic T lymphocyte (CTL) immune response, which translates into a potent antitumor effect against TC-1, a preclinical HPV E7+ tumor model. The promising data demonstrated in these preclinical studies have resulted in the evaluation of clinical grade CRT/E7 DNA vaccine in various clinical trials.

Even though therapeutic DNA vaccines are a potentially efficacious cancer therapy, their immunogenicity is often times unimpressive due to the limited expression of the DNA-encoded protein in vivo. Electroporation aids in DNA plasmid delivery by creating an electrical field at the vaccination location. This electrical field destabilizes the cell plasma membrane, inducing greater permeability and enabling more efficient passage of DNA plasmids into the target cells. Furthermore, electroporation produces adjuvant-like effects, which enhance the immune response. Previously, we have demonstrated that DNA immunization followed by electroporation elicited stronger antigen-specific CTL responses compared with intramuscular (IM) administration alone or gene gun vaccination. In addition, the use of therapeutic HPV DNA vaccine with electroporation was demonstrated to be safe, well tolerated and potentially effective in humans. These studies support the use of electroporation for the enhancement of DNA vaccine immunogenicity.

In addition to the method of therapeutic vaccination, the route of administration has also been identified to affect the location of optimal cell-mediated immune responses. It was previously demonstrated that local antitumor response is more important for tumor regression, compared with a systemic response only. Disease clearance has been correlated with localized immune responses at the lesion site, suggesting the significance of...
generating and targeting HPV-specific immune responses at the location of the lesion. Intravaginal immunization with HPV pseudoviruses was found to induce local CTL responses and antitumor effects against cervicovaginal tumors. We previously showed that immunization with a DNA vaccine, followed by a vaccinia virus vaccine, in the cervicovaginal region was able to stimulate an enhanced local, antigen-specific CTL response in TC-1 tumor-bearing mice when compared with mice that received IM vaccinations. Likewise, intravaginal DNA immunization followed by electroporation enhanced E7-specific CTLs both systemically and locally and prolonged the survival of tumor-bearing mice in comparison to mice that received IM immunization followed by electroporation.

Interleukin-2 (IL2) is a cytokine with numerous functions, including: stimulating T-cell proliferation, generating CTL and natural killer cells, and mediating B-cell proliferation and immunoglobulin synthesis. Previously, we have successfully targeted DNA-encoding IL2 to the tumor location to provoke robust therapeutic antitumor effects in tumor-bearing mice. Furthermore, IL2 was shown to strengthen the antigen-specific T-cell responses, specifically Th1 responses, generated by an HIV targeting DNA vaccine. IL2 is currently permitted to be used in patients with malignant melanoma and metastatic renal-cell carcinoma. Therefore, administration of DNA-encoding IL2 may further enhance the therapeutic response produced by the CRT/E7 DNA vaccine.

Here we investigated the use of DNA-encoding IL2 to enhance the CTL responses and antitumor effects elicited by pcDNA3-CRT/E7 immunization in the cervicovaginal tract via electroporation. We established that coadministration of DNA-encoding IL2 notably enhanced the HPV antigen-specific CTL responses and antitumor effects induced by intravaginal injection of pcDNA3-CRT/E7 vaccine followed by electroporation. Additionally, we showed that concomitant injection of IL2 DNA and pcDNA3-CRT/E7 with electroporation resulted in higher numbers of E7-specific, IFNγ+ CTLs in tumor-bearing mice. Moreover, coadministration of IL2 DNA with CRT/E7 DNA improved the ratio of immunocompetent cells relative to the amount of immunosuppressive cells, in terms of the ratio of CTLs to regulatory T (Treg) cells and the ratio of CTLs to myeloid-derived suppressor cells (MDSCs). Thus our study demonstrates that IL2 can enhance the E7-specific CTL responses and antitumor effects produced by pcDNA3-CRT/E7.
RESULTS

Coadministration of DNA-encoding IL2 significantly enhances the E7 antigen-specific CD8+ T-cell immune responses in the cervicovaginal tract induced by intravaginal injection of pcDNA3-CRT/E7

To assess the effect of pcDNA3-CRT/E7 vaccination combined with IL2 plasmid, we immunized C57BL/6 mice with various combinations of pcDNA3-CRT/E7 and pcDNA3-IL2 followed by electroporation. Mice were vaccinated submucosally in the cervicovaginal tracts with pcDNA3 empty vector, pcDNA3-IL2, pcDNA3-CRT/E7 or pcDNA3-IL2 together with pcDNA3-CRT/E7, each followed by electroporation. After 7 days, the mice were treated again with the same regimen. Splenocytes and cells from the cervicovaginal tract of treated mice were isolated and the presence of antigen-specific CD8+ T cells were analyzed using flow cytometry. The mice treated with pcDNA3-CRT/E7, both with or without IL2 DNA followed by electroporation, had a notably greater amount of E7-specific CD8+ T cells in comparison to those treated with IL2 DNA only or empty vector (Figures 1a–b). Furthermore, mice that were inoculated with pcDNA3-CRT/E7 combined with IL2 DNA, then electroporated, had more E7-specific CD8+ T cells in the cervicovaginal tract than mice inoculated with pcDNA3-CRT/E7 only (Figures 1c–d).

Coadministration of DNA-encoding IL2 significantly increases the antitumor effects elicited by intravaginal injection of pcDNA3-CRT/E7

Next we studied the antitumor effects elicited by the various DNA vaccines employed in this study, followed by electroporation. C57BL/6 mice were challenged submucosally in the cervicovaginal tract with $2 \times 10^4$ of TC-1-Luc cells and treated with different DNA plasmids 3 days after tumor challenge and then boosted 7 days later with the same regimen. Mice treated with pcDNA3-CRT/E7 only generated enhanced antitumor effects in comparison to mice treated with IL2 DNA or empty vector alone (Figures 2a–b). Additionally, mice treated with pcDNA3-CRT/E7 and IL2 DNA vaccines had significantly less luciferase activity compared with mice treated with pcDNA3-CRT/E7 only, suggesting more significant tumor control. Additionally, Figure 2c indicates that TC-1 tumor-bearing mice immunized with pcDNA3-CRT/E7 and IL2 DNA had prolonged survival in comparison to those only treated with pcDNA3-CRT/E7.

Coadministration of DNA-encoding IL2 enhances the production of tumor-infiltrating E7-specific interferon-γ (IFNγ)-secreting CD8+ T cells elicited by intravaginal injection of pcDNA3-CRT/E7

Next we examined the generation of E7-specific IFNγ-secreting CD8+ T cells following intravaginal injection of the various DNA vaccines. Tumor-bearing mice were vaccinated with the different DNA constructs and electroporated and then boosted 7 days later with the identical regimen. Cells from the spleen and the cervicovaginal tract were harvested and assessed by intracellular cytokine staining and flow cytometric analysis. Mice treated with pcDNA3-CRT/E7 alone or in combination with IL2 DNA followed by electroporation produced a larger amount of systemic E7-specific IFNγ+ CD8+ T cells compared with those treated with IL2 DNA only or empty vector (Figures 3a and b). Importantly, Figures 3c and d demonstrate that immunization with CRT/E7 DNA stimulates a significantly greater quantity of local E7-specific IFNγ+ CD8+ T cells in the cervical vaginal tract than immunization with IL2 DNA or empty vector alone. Furthermore, immunization with pcDNA3-CRT/E7 combined with IL2 DNA generates the most robust local IFNγ+ CD8+ T-cell response.

Coadministration of DNA-encoding IL2 increases the CD8+ T cell-to-Treg cell ratio among splenocytes generated by intravaginal injection of pcDNA3-CRT/E7 DNA

We then sought to gain a better understanding of the mechanism by which the pcDNA3-CRT/E7 and IL2 DNA vaccine enhances immune responses by examining its effect on the ratio of CD8+ T cells to Treg cells. TC-1 tumor-bearing mice were immunized and then boosted with the various DNA constructs shown in Figure 2. One week after vaccination boost, splenocytes and tumor cells were isolated and the lymphocytes expressing CD4 and CD25 were classified as Treg cells. Among the splenocytes of tumor-bearing mice, those administered both pcDNA3-CRT/E7 and IL2 DNA plasmids had the highest ratio of CD8+ T cells to Treg cells (Figure 4a). Furthermore, among cells located inside cervicovaginal tumor, mice administered pcDNA3-CRT/E7 had a greater ratio of CD8+ T cells to Treg cells in comparison to those treated with IL2 DNA or empty vector alone (Figure 4b). Of note, mice administered both pcDNA3-CRT/E7 and IL2 DNA plasmids had the greatest CD8+ T cell-to-Treg cell ratio. In summary, these results suggest that coadministration of IL2 DNA with pcDNA3-CRT/E7 DNA improves the ratio of immunocompetent cells relative to the number of immunosuppressive cells.

Coadministration of DNA-encoding IL2 increases the ratio of CD8+ T cells to MDSCs among cervicovaginal tumor cells generated by intravaginal injection of pcDNA3-CRT/E7 DNA

We then examined the effect of pcDNA3-CRT/E7 and IL2 DNA vaccination on the ratio of CD8+ T cells to MDSCs. Tumor-bearing mice received the same treatment regimen described above. One week after vaccination boost, splenocytes and tumor cells were isolated from the mice and cells expressing CD11b and Gr-1 were classified as MDSCs. Between the groups of treated mice, we did not observe a significant difference in the ratio of CD8+ T cells to MDSCs in the splenocytes of tumor-bearing mice (Figure 5a). However, we observed a greater ratio of CD8+ T cells to MDSCs in cervicovaginal tumor of mice treated with pcDNA3-CRT/E7 compared with those treated with IL2 DNA or empty vector alone (Figure 5b). Furthermore, of cells located in cervicovaginal tumor, mice immunized with pcDNA3-CRT/E7 and IL2 DNA plasmids had a greater ratio of CD8+ T cells to MDSCs compared with mice treated with pcDNA3-CRT/E7 only. These results provide further support that coadministration of IL2 DNA with pcDNA3-CRT/E7 followed by electroporation generates the highest amount of immunocompetent cells (CD8+ T cells) relative to the number of immunosuppressive cells in (MDSCs) the tumor microenvironment.

DISCUSSION

We showed that coadministration of DNA-encoding IL2 enhanced the therapeutic efficacy of pcDNA3-CRT/E7 administered intravaginally following by electroporation. Specifically, tumor-bearing mice immunized with IL2 DNA and pcDNA3-CRT/E7 produced more potent E7-specific CTL responses and antitumor effects in comparison to those treated with pcDNA3-CRT/E7 only. Additionally, IL2 DNA enhanced the amount of IFNγ-secreting E7-specific CD8+ T cells in the cervicovaginal tumors of mice. Finally, we demonstrated that DNA-encoding IL2 coadministered with pcDNA3-CRT/E7 improved the proportion of CD8+ T cells to immunosuppressive cells, including Treg cells and MDSCs in the tumor microenvironment. In summary, these results demonstrate that IL2 DNA can greatly enhance the therapeutic effects elicited by local immunization of the pcDNA3-CRT/E7 followed by electroporation.

Electroporation in the vaginal cavity of mice has previously been used to enhance DNA transfection through the vaginal wall.10,11 However, to date, there are no clinical trials investigating...
the therapeutic effects of local DNA vaccination followed by intravaginal electroporation. Several preclinical animal studies have documented tissue damage after electroporation; however, this damage has been minimal and animals recovered quickly. In one study, the degree of damage was shown to be dependent upon factors, such as the electric field intensity, and length of pulse. Thus, by using a lower field intensity, tissue damage was minimized. An additional study reported a correlation between increased muscle damage and increased pulse duration; however, after 2 weeks, muscle tissue appeared grossly normal, showing that damaged tissue had regenerated.

Before the current treatment strategy can become applicable in the clinic, a suitable electroporation device for intravaginal use that is tolerable for patients will need to be created. Current electroporation devices utilized in the clinic to enhance the immunogenicity of DNA vaccines are applied conjointly with IM vaccination. A recent Phase I clinical trial tested IM administration of pNGVL4a-CRT/E7(detox) DNA vaccine followed by electroporation using the Ichor Medical Systems TriGrid Delivery System in patients with head and neck cancer (NCT01493154). In addition, the TriGrid Delivery System was combined with GX-188E, another therapeutic HPV DNA vaccine, in patients with cervical intraepithelial neoplasia 3 lesions (NCT02411019). Furthermore, a Phase II trial was conducted in patients with cervical intraepithelial neoplasia 2/3 lesions to evaluate the efficacy of IM VGX-3100 DNA vaccination followed by electroporation using CELLECTRA Constant Current Device. Participating patients showed histopathological regression and clearance after vaccination of VGX-3100 followed by electroporation.

Although our study provides considerable incentive for the development of an electroporation device that can be safely administered to the cervicovaginal region in humans, we understand the potential concerns that may be raised regarding the clinical translation of intravaginal electroporation, including invasiveness and pain level. In a previous study, participating patients reported pain at the vaccine injection site after electroporation. However, this pain was believed to be caused by the high voltage (>200 V) of electroporation that was administered at the injection site after vaccination. In this study, we deliver electroporation at the injection site at a much lower voltage (72 V), which we believe would cause patients less pain at injection site during clinical trials. Furthermore, local anesthetic can be employed in clinical studies to minimize any discomfort produced by vaccination and electroporation. Electroporation has great potential to improve the efficacy of many therapeutic vaccines; however, we believe that electroporation technology must be improved before intravaginal electroporation can be administered in clinical trial. If improved, this may be an extremely effective vaccine delivery route that can expand upon the current electroporation technologies available.

One alternative method used to improve the efficacy of therapeutic HPV DNA vaccines that may also address the concerns of using electroporation includes administering the vaccine proximal to tumor-draining lymph nodes (TDLNs). It is believed that TDLNs have a unique role in tumor immunology and malignant disease progression on several levels. It has been reported that the local microenvironment in TDLNs has a major role in local immune response to tumors. One study investigated the effects of adjuvant therapy on the immunological crosstalk...
Figure 3. IL-2 combined with HPV DNA vaccine followed by electroporation generates significantly more tumor-infiltrating IFN-γ-secreting CD8+ T cells. Five-to-8-week-old female C57BL/6 mice were injected with $2 \times 10^4$ of TC-1-Luc cells submucosally. The mice were then immunized with the DNA constructs via intravaginal injection followed by electroporation on day 3. One week later, the mice were boosted with the same regimen. Seven days after the last vaccination, splenocytes and cervicovaginal cells were harvested and stimulated with HPV16 E7aa49–57 peptide in the presence of GolgiPlug, stained for surface CD8 and intracellular IFN-γ and analyzed by flow cytometric analysis. (a) Representative flow cytometric image for the amount of IFNγ+/CD8+ T cells among splenocytes. (b) Bar graph summary of flow cytometric analysis. (c) Representative flow cytometric image for the amount of IFNγ+/CD8+ T cells among cervicovaginal cells. (d) Summary of flow cytometric analysis. *P < 0.05; **P < 0.01; NS = not significant.

Figure 4. Administration of HPV DNA vaccine combined with IL-2 increases the ratio of CD8+ T cells to Treg cells in splenocytes and cervicovaginal tumor cells. The TC-1 tumor-bearing mice were treated as described in Figure 2. Briefly, 7 days after the last vaccination, splenocytes and tumor cells were isolated from the mice. To detect Treg cells, the lymphocytes were stained with anti-mouse CD4 and CD25 antibodies. (a) Bar graph summary of the ratio of CD8+ T cells/Treg cells among splenocytes. (b) Bar graph summary of the ratio of CD8+ T cells/Treg cells among tumor-infiltrating lymphocytes. *P < 0.05; **P < 0.01.
and release IL-2. One study found that a wide variety of tissues associated viral vector-mediated gene transfer to both encode addition, two previous studies used recombinant adenovirus to improve its tolerability. Coadministration of DNA-encoding IL-2 has not yet been clinically tested.

In order to reduce the toxicity and associated adverse effects of IL-2 therapy, a previous study combined IL-2 with a peptide vaccine, gp100, to treat patients with metastatic melanoma and found that gp100 improved response rates and progression-free survival. In addition, two previous studies used recombinant adenovirus to improve IL-2 treatment. These data suggest that the cervicovaginal tract may be an ideal location for vaccine administration to generate a more potent local CTL response. Other investigators have demonstrated that intravaginal immunization with HPV pseudoviruses produced a potent HPV-specific intracervical epithelial CD103+ CD8+ T-cell response by stimulating and retaining primed HPV-specific CD8+ T cells in the cervicovaginal tract. Moreover, it has been demonstrated that local vaccination with IL-2 and pcDNA3-CRT/E7 followed by electroporation elicited potent HPV antigen-specific immune responses, both locally and systemically. These data suggest that the cervicovaginal tract may be an ideal location for vaccine administration to generate a more potent local CTL response. Other investigators have demonstrated that intravaginal immunization with HPV pseudoviruses produced a potent HPV-specific intracervical epithelial CD103+ CD8+ T-cell response by stimulating and retaining primed HPV-specific CD8+ T cells in the cervicovaginal tract. Moreover, it has been demonstrated that local vaccination in the mucosa generated more potent CD8+ T-cell responses against mucosal TC-1 tumors in mice. The therapeutic effects of the coadministration of our DNA vaccine-encoding IL-2 were demonstrated through immune response of CTLs and a strong antitumor response against TC-1 tumors. In this system, these data suggest that CD4+CD25+ Tregs may have been as crucial to the immunotherapeutic effect of this vaccine as CD8+ T cells. However, it is believed that the generation and homing of effector memory T cells to the cervicovaginal tract epithelium may be crucial for the initiation of a potent HPV-specific immune response against cervical tumors. Future studies are necessary in order to determine the mechanism of this process.

In summary, the current study presents a treatment regimen for HPV-associated lesions consisting of pcDNA3-CRT/E7 and IL-2 DNA...
administered intravaginally followed by electroporation. We showed that this treatment regimen could produce an effective therapeutic antitumor immune response and render the tumor microenvironment more immunocompetent. Owing to the importance of local T-cell responses in clearing HPV-associated tumors, the current described approach represents a potential strategy for the control of HPV-associated lesions. Future studies are warranted to develop a clinically applicable electroporation device that is safe and tolerable for vaginal use.

MATERIALS AND METHODS

Mice
Five-to-eight-week-old female C57BL/6 mice were acquired from the National Cancer Institute and housed in the Johns Hopkins Oncology Center Animal Facility (Baltimore, MD, USA) under specific pathogen-free conditions. Only female mice were chosen for this study because cervical cancer only affects females. Mice were weighted before randomization for studies to further ensure the health of the animals and to control this variable. All animal procedures were performed in accordance with animal protocols approved by the Johns Hopkins Institutional Animal Care and Use Committee and in compliance with recommendations for the proper use and care of laboratory animals. The sample size for each experiment was five mice per group (n = 5). Based on previous experiments, this sample size was large enough to see a statistical significance between treatment groups. All mouse experiments were replicated in the laboratory two times.

Cells
The generation of luciferase-expressing TC-1 cells (TC-1-Luc) has been described previously.33 The cells were cultured in RPMI 1640 supplemented with 50 units ml⁻¹ of penicillin/streptomycin, 1 mM of sodium pyruvate, 10% of fetal bovine serum, 2 mM of L-glutamine and 2 mM of non-essential amino acids, at 37 °C with 5% CO₂. The TC-1 cell line has been tested and authenticated using the method described previously.38 Additionally, the TC-1 cell line is tested monthly for mycoplasma contamination.

Peptides, antibodies and HPV16 E7 tetramer
Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated anti-mouse CD8α (clone 53.6.7, catalog nos. 553031 and 553032, respectively) and FITC-conjugated anti-mouse IFN-γ (clone XMG1.2, catalog no. 554411) antibodies were acquired from BD Pharmingen (San Diego, CA, USA). Purified anti-mouse CD16/32 (Fc Block, catalog no. 553141) was purchased from BD Phamrigen. FITC-conjugated anti-mouse CD4 (catalog no. 11-0042-82), allophycocyanin (APC)-conjugated CD25 (catalog no. 17-0251-82), FITC-conjugated Gr-1 (catalog no. 11-5931-82) and PE-conjugated CD11b (catalog no. 12-0112-82) antibodies were acquired from eBioscience (San Diego, CA, USA). The H-2D⁺-restricted HPV16 peptide, E7aa49–57 (RAHYNIVTF), was synthesized by Macromolecular Resources (Willingboro, NJ, USA) with ≥ 80% purity. PE-conjugated HPV16 E7aa49–57 peptide-loaded H-2D⁺ tetramer was provided by NIH Tetramer Core Facility (Baltimore, MD, USA).

DNA vaccines
The generation of the pcDNA3-CRT/E7 DNA vaccine has been previously described.6 The construction of the pcDNA3-IL2 plasmid was cloned by our laboratory. The pcDNA3-luciferase DNA plasmid was kindly provided by Dr Hyam I. Levitsky (Johns Hopkins Medical Institutions, Baltimore, MD, USA).

DNA vaccination/electroporation
Mice were anesthetized prior to immunization. In all, 20 μg of DNA constructs were administered into the cervicovaginal tracts by submucosal injection and followed by electroporation with an ECM830 Square Wave Electroporation System (BTX, Harvard Apparatus Inc., Holliston, MA, USA). Electroporation was administered at the site of injection using a tweezertrode with one plate inside the vagina and the other on the ipsilateral vulva. Eight unipolar, electrical pulses of 72 V were delivered for 20 ms (pulse duration) at 200 ms intervals. The mice received the same treatment regimen 7 days later.

Splenocyte and cervicovaginal cell preparation
Vaginal tissues were surgically removed via sterile technique, placed in RPMI-1640 medium supplemented with 100 U ml⁻¹ of penicillin and 100 μg ml⁻¹ of streptomycin and then washed with phosphate-buffered saline (PBS). Next the tissues were minced into 1–2-mm pieces and placed in serum-free RPMI-1640 medium supplemented with 0.05 mg ml⁻¹ of collagenase I, 0.05 mg ml⁻¹ of collagenase IV, 0.25 mg ml⁻¹ of DNase I, 0.025 mg ml⁻¹ of hyaluronidase IV, 100 μg ml⁻¹ of streptomycin and 100 μg ml⁻¹ of penicillin. The minced tissue were incubated at 37 °C with periodic agitation for 1 h. The undigested tissue fragments in the solution were then filtered through a 70-μm nylon filter mesh. The individual cervicovaginal cells were then washed twice using PBS, and viable cells were determined by trypan blue dye exclusion. To prepare the splenocytes, the spleen was minced using a 70-μm nylon filter mesh. The red blood cells were lysed using ACK lysis buffer, and the cells were washed. Viable cells were determined by trypan blue dye exclusion.

Flow cytometric analysis
All in vitro experiments were replicated three times in the laboratory. To analyze the HPV16 E7-specific CTL responses using tetramers, splenocytes and cervicovaginal cells were washed once with fluorescence-activated cell sorter (FACS) wash buffer (PBS+0.5 bovine serum albumin). All cells were reincubated with BD Fc Block to avoid nonspecific antibody binding through the surface Fc receptor. The cells were then stained with FITC-conjugated anti-mouse CD8α (catalog no. 553031) from BD Pharmingen and PE-conjugated HPV16 E7aa49–57 peptide loaded H-2D⁺ E7 tetramer. Following the wash, the cells were acquired with a FACS Calibur flow cytometer (BD Biosciences, Mountain View, CA, USA) and analyzed using the FlowJo software (TreeStar Inc., Ashland, OR, USA).

To analyze the HPV16 E7-specific CTL responses using intracellular IFN-γ staining, cervicovaginal cells and splenocytes were stimulated overnight with 1 μg ml⁻¹ HPV16 E7aa49–57 peptide in the presence of 1 μl ml⁻¹ of GolgiPlug (BD Pharmingen). After stimulation, the cells were washed one time with FACS wash buffer and then stained with PE-conjugated anti-mouse CD8α antibody (catalog no. 553032) acquired from BD Pharmingen. After staining, the cells were permeabilized and then fixed using the Cytofix/Cytoperm Kit following the instructions provided by the manufacturer (BD Pharmingen). Intracellular IFN-γ staining was performed using FITC-conjugated anti-mouse IFN-γ antibody (catalog no. 554411) purchased from BD Pharmingen. After the wash, the cells were acquired with a FACS Calibur flow cytometer.

For the detection of Tregs, single-cell preparations from the mouse spleen and tumor were resuspended in FACS staining buffer and incubated with anti-CD4-FITC and anti-CD25-APC (catalog no. 17-0251-82), FITC-conjugated anti-mouse CD4 (catalog no. 11-0042-82) and PE-conjugated CD8α (catalog no. 553032) antibodies (catalog no. 553032) acquired from BD Pharmingen. Following the wash, the cells were acquired with a FACS Calibur flow cytometer.

In vivo tumor treatment experiments
Mice were challenged with 2 × 10⁶ of TC-1-Luc cells injected submucosally into the wall of the vagina. Three days following the tumor challenge, mice were immunized with 20 μg of DNA constructs as indicated. The mice received the same regimen again 7 days later. The growth of cervicovaginal tumor was monitored using bioluminescence via Xenogen IVIS 100 imager at the indicated times. The experiments were performed in a non-blind manner.

Statistical analysis
All data are expressed as means ± s.d., including error bars based on s.d. to account for any statistical uncertainty. Two-tailed Student’s t-test was used to compare the differences between individual data points. Non-parametric Mann-Whitney Y test was used to compare two different groups. The tumor treatment experiment results were evaluated using Kaplan-Meier analysis. P-values < 0.05 were considered significant.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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