Gene gun-mediated DNA vaccination induces antitumor immunity against human papillomavirus type 16 E7-expressing murine tumor metastases in the liver and lungs

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DNA vaccination has emerged as an attractive approach for tumor immunotherapy. The aim of this study was to evaluate the potency of DNA vaccines in preventing and treating the liver and lung metastases of a human papillomavirus-16 (HPV-16) E7-expressing murine tumor (TC-1). We used the gene gun method to vaccinate C57BL/6 mice intradermally with DNA vaccines containing the HPV-16 E7 gene, the E7 gene linked to the sorting signals of the lysosome-associated membrane protein-1 (Sig/E7/LAMP-1), or the 'empty' plasmid vector. The *in vivo* antitumor immunity was analyzed in both tumor prevention and tumor regression experiments. In addition, cytotoxic T lymphocyte (CTL) assays, enzyme-linked immunospot assay and enzyme-linked immunoabsorbent assay were used to

assess the E7-specific T cell-mediated and humoral immunity. Mice vaccinated with Sig/E7/LAMP-1 DNA generated the strongest E7-specific CTL activities, the highest numbers of E7-specific CD8⁺ cell precursors and the highest titers of E7-specific antibodies. While both E7 DNA and Sig/E7/LAMP-1 DNA generated potent antitumor immunity in the liver and lung metastases models, the Sig/E7/LAMP-1 DNA was more potent under stringent conditions. DNA vaccination with E7-expressing plasmids was effective in controlling liver and lung metastases of an E7-expressing murine tumor. Our data suggest that antigen-specific DNA vaccination can potentially be applied to control liver and lung metastases of tumors with defined tumor-specific antigens.

Keywords: DNA vaccine; metastasis; human papillomaviruses; LAMP-1; antitumor; E7

Introduction

Metastasis is a common cause of death in many cancer patients. Conventional therapies, such as surgery, chemotherapy and radiotherapy, have limited success in controlling cancer metastases. The ideal cancer therapy should act systemically in addition to its local antitumor effect. In this regard, immunotherapy is especially attractive because it has the potency to eradicate systemic tumors in multiple sites in the body and the specificity to discriminate between neoplastic and non-neoplastic cells.¹

Tumor-specific antigens, when efficiently presented by antigen presenting cells (APCs) to both CD8⁺ cytotoxic T lymphocytes (CTLs) and CD4⁺ helper T cells, are capable of inducing potent T cell-mediated immunity – the most crucial component of antitumor immunity. There are several additional advantages of using an antigen-specific cancer vaccine. It is less likely to generate nonspecific

autoimmunity, more flexible in deciding the amount of antigen administered and the methods of antigen presentation to the immune system, more reproducible from patient to patient and has the potential to correlate clinical outcome to a specific immune response.² Therefore, antigen-specific cancer immunotherapy represents a desirable approach for controlling tumors.

In the past few years, several reports have shown that antigen-specific cancer vaccines, in the form of cell-based vaccines, protein/peptide-based vaccines and viral vector-based vaccines, are effective for the treatment of metastases.¹ However, production of the autologous tumor cell-based vaccines is labor intensive and technically challenging.³ The application of peptide-based vaccines is limited by major histocompatibility complex (MHC) restriction and the necessity of defining the CTL epitopes. The disadvantages of viral vector-based vaccines include unnecessary immune responses to vectors and other potential virus-associated toxicity. In contrast, naked plasmid DNA is safe, low immunogenically and can be repeatedly administered. DNA vaccines can be easily prepared on a large scale with high purity and are highly stable relative to proteins and other biologic polymers (for review, see Refs 4–6).

Figure 1 Schematic diagram of wild-type E7 and Sig/E7/LAMP-1 DNA constructs. The wild-type HPV-16 E7 gene and the chimeric Sig/E7/LAMP-1 gene were cloned into the BamHI cloning sites of the pCMVneoBam expression vector downstream of the cytomegalovirus promoter as described in Materials and methods.

We chose human papillomavirus-16 (HPV-16) E7 as a model antigen for vaccine development because HPVs, particularly HPV-16, are associated with most cervical cancers. The HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and are co-expressed in most HPV-containing cervical cancers. Therefore, vaccines or immunotherapies targeting E7 and/or E6 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies.

We have previously linked the sorting signals of the lysosome-associated membrane protein-1 (LAMP-1) to the HPV-16 E7 antigen, creating a chimera, Sig/E7/LAMP-1 (see Figure 1). We found that mice vaccinated with Sig/E7/LAMP-1-containing recombinant vaccinia virus can generate strong antitumor immunity against HPV-16 E7-expressing tumors in subcutaneous,⁷ lung metastasis⁸ and hepatic metastasis models (Chen *et al*, unpublished data). However, immune responses to the vaccinia vaccine vector can limit the effectiveness of re-vaccination with vaccinia virus.⁹ The use of naked DNA vaccines can circumvent this problem.

We used liver and lung metastasis models in the current study because besides regional lymph nodes, the liver and lungs are the most common sites of distant metastases of cervical cancers.¹⁰ In addition, when developing clinical applications for the treatment of common solid malignancies, demonstrating efficacy of therapy within visceral sites such as the liver or lung is important.¹¹ Therefore, the aim of this study was to test whether DNA vaccines encoding HPV-16 E7 could induce protective immunity against HPV-16 E7-expressing murine tumor metastases in the liver or lungs.

Results

Vaccination with Sig/E7/LAMP-1 DNA generates potent E7-specific CD8⁺ T cell-mediated immune response

CD8⁺ T lymphocytes are one of the most crucial components of the antitumor effect.¹² CTL assay and enzyme linked immunospot (ELISPOT) assays were used to determine the E7-specific T cell-mediated immunity generated by DNA vaccine. For the standard chromium

Figure 2 CTL response in C57BL/6 mice immunized with various recombinant DNA vaccines. C57BL/6 mice were immunized with empty plasmid, E7 DNA or Sig/E7/LAMP-1 DNA via gene gun. Splenocytes were harvested 2 weeks after the last DNA vaccination and stimulated *in vitro* with E7-specific H-2D^b CTL epitope and interleukin-2 for 6 days. A standard 4 h chromium release assay was performed against MC57G (H-2^b) cells pulsed with E7 CTL peptide. Target cell-specific lysis was determined as described in Materials and methods. Results shown here are from one representative experiment of two performed.

Figure 3 E7-specific CD8⁺ T cell precursors in C57BL/6 mice immunized with various recombinant DNA vaccines using ELISPOT assays. C57BL/6 mice were immunized with empty plasmid, E7 DNA or Sig/E7/LAMP-1 DNA via gene gun. Splenocytes were harvested 2 weeks after the last DNA vaccination. The number of IFN- γ producing E7-specific CD8⁺ T cell precursors was determined using the ELISPOT assay (see text for the detailed method). (a) The spot numbers were the mean of triplicates $\pm$ s.e. in each vaccinated group. (b) Representative ELISPOT pictures in each vaccinated group. Results shown here are from one representative experiment of two performed.

release cytotoxicity assay, mice vaccinated with Sig/E7/LAMP-1 DNA generated the highest E7-specific CTL activity, while mice vaccinated with E7 DNA generated only slightly higher E7-specific CTL activity than the mice vaccinated with the empty plasmid (Figure 2). The ELISPOT assay is a sensitive function assay for IFN- γ production by individual cell and can be applied to quantify antigen-specific CD8⁺ T cell precursors.¹³ We used the ELISPOT assay to determine the precursor frequencies of E7-specific CD8⁺ T cells in each vaccination group. Around 120 E7-specific CD8⁺ T cells were detected per 10⁶ splenocytes derived from the Sig/E7/LAMP-1 DNA-vaccinated mice, but only 10–20/10⁶ in E7 DNA-vaccinated mice. We did not detect E7-specific IFN- γ secreting CD8⁺ T cells in control of empty plasmid-vaccinated mice (Figure 3). Our results from both CTL assay and ELISPOT

assay indicated that Sig/E7/LAMP-1 DNA-vaccinated mice generated the highest CD8⁺ T cell-mediated immunity.

Vaccination with Sig/E7/LAMP-1 DNA generates the highest T helper-type 1 cytokine responses

To determine the cytokine profiles generated by E7 DNA and Sig/E7/LAMP-1 DNA, splenocytes from vaccinated mice were stimulated *in vitro* with E7 protein for 72 h. The supernatants were harvested and assayed for the presence of IFN- γ and IL-4. As shown in Figure 4, the highest IFN- γ level was found in the mice vaccinated with Sig/E7/LAMP-1 DNA. Vaccination with E7 DNA generated the second highest IFN- γ release. IL-4 could not be detected in the supernatant of any vaccinated group. The detection limit was 5 pg/ml as determined from a linear standard curve. These results suggested

Figure 4 Cytokine secretion profiles of splenocytes in C57BL/6 mice immunized with various recombinant DNA vaccines. C57BL/6 mice were immunized with empty plasmid, E7 DNA or Sig/E7/LAMP-1 DNA via gene gun. Splenocytes (4×10^6) were harvested 2 weeks after the last DNA vaccination and were cultured with 10 μ g/ml E7 protein in a 24-well tissue culture plate for 72 h. The supernatants were harvested and assayed for specific cytokines using ELISA kit (Endogen). Results shown here are from one representative experiment of two performed.

that vaccination with Sig/E7/LAMP-1 DNA or E7 DNA vaccine generated T helper-type I cytokine profiles.

Vaccination with Sig/E7/LAMP-1 DNA generates the highest E7-specific antibody titers

The anti-HPV-16 E7 antibodies in the sera of the vaccinated mice were determined by a direct enzyme-linked immunoabsorbent assay (ELISA) at 1, 2 and 4 weeks after the last vaccination. The anti-HPV-16 E7 antibodies could be detected in the sera of Sig/E7/LAMP-1 DNA-vaccinated mice at both 2 and 4 weeks after the last vaccination. No anti-E7 antibodies were detected in the sera of mice vaccinated with E7 DNA or the empty plasmid (Figure 5).

Vaccination with both Sig/E7/LAMP-1 DNA and wild-type E7 DNA protects mice against TC-1 tumor metastases in the liver and lungs

To determine whether vaccination with E7-expressing DNA constructs protects mice against E7-expressing tumor metastases in the liver and lungs, mice were vaccinated with 2 μ g naked DNA per mouse via gene gun, and boosted with the same dose 1 week later. Mice were then challenged with 1×10^4 TC-1 per mouse (5 $\times$ minimal

Figure 5 E7-specific antibody response in C57BL/6 mice immunized with various recombinant DNA vaccines. C57BL/6 mice were immunized with empty plasmid, E7 DNA or Sig/E7/LAMP-1 DNA via gene gun. Serum samples were obtained from mice 2 weeks after the last vaccination. A direct ELISA assay was performed. Results shown here are from one representative experiment of two performed.

tumor dose) in the liver (liver metastasis model) or 5×10^4 TC-1 per mouse (10 $\times$ minimal tumor dose) via intravenous tail vein injection (lung metastasis model) 1 week after the last vaccination. The mice were killed 30 days following tumor challenge.

For the liver metastases model, we found that all of the mice that received either E7 DNA or Sig/E7/LAMP-1 DNA vaccination remained tumor-free in the liver. In contrast, all of the mice vaccinated with the empty plasmid and all of the naive mice developed hepatic tumors (Figure 6).

For the lung metastasis model, we counted pulmonary nodules to quantify tumor metastases in the lung. Mice vaccinated with either E7 DNA or Sig/E7/LAMP-1 DNA had the lowest mean number of pulmonary nodules (Figure 7). There was no statistical difference between E7 DNA and Sig/E7/LAMP-1 DNA. In our previous study,⁸ TC-1 tumors were detected only in the lungs after intravenous tail vein injection. Other solid organs including the brain, spleen, liver and kidneys remained free of tumors. Representative gross photographs of the lung tumors are shown in Figure 8.

Vaccination with both Sig/E7/LAMP-1 DNA and wild-type E7 DNA eradicates established E7-expressing tumors in the liver and lungs

To determine the therapeutic potential of E7-expressing DNA constructs in treating TC-1 tumor metastases in the liver and lungs, mice were first challenged with 1×10^4 TC-1 per mouse in the liver (liver metastases model) or 5×10^4 TC-1 per mouse via intravenous tail vein injection (lung metastasis model). The mice were then vaccinated with 2 μ g naked DNA via gene gun 7 days later and boosted with the same dose 1 week later. The mice were killed 30 days after tumor challenge.

For the liver metastases model, we found that 80% of the mice vaccinated with E7 DNA or Sig/E7/LAMP-1 DNA remained tumor-free. In contrast, all naive mice and mice vaccinated with the empty plasmid developed hepatic tumors (Figure 9).

Figure 6 E7-expressing DNA vaccines protect mice against the growth of TC-1 tumors in the liver. C57BL/6 mice were immunized with empty plasmid, E7 DNA or Sig/E7/LAMP-1 DNA via gene gun. One week after the second vaccination, mice (five per group) were challenged with TC-1 tumor cells at a dose of 1×10^4 cells per mouse. Mice were monitored twice a week and were killed at day 30 after tumor challenge to check for the presence of liver tumors.

For the lung metastasis model, mice vaccinated with either E7 DNA or Sig/E7/LAMP-1 DNA had the lowest mean pulmonary nodule numbers (Figure 10). There was no statistical difference between E7 DNA and Sig/E7/LAMP-1 DNA.

These results showed that both E7 DNA and Sig/E7/LAMP-1 DNA could generate potent antitumor immunity in the liver and lung metastasis models of TC-1 tumor cells. However, there was no statistically significant difference in the antitumor effects between E7 DNA and Sig/E7/LAMP-1 DNA.

Sig/E7/LAMP-1 DNA is a more potent vaccine than wild-type E7 DNA

Both the humoral and cellular responses indicates that Sig/E7/LAMP-1 DNA may be a more potent DNA vaccine than E7 DNA. However, we cannot demonstrate the potency difference between Sig/E7/LAMP-1 DNA and E7 DNA in the *in vivo* antitumor effect by using the above vaccination and tumor doses. To test the difference between the antitumor potency generated by E7 DNA and that generated by Sig/E7/LAMP-1 DNA, we repeated the tumor protection and treatment experiments in the lung metastasis model under more stringent conditions. We decreased the DNA vaccine dose to 2 μ g per mouse (ie priming without further booster) and increased the tumor dose to 1×10^5 TC-1 per mouse. The mice were killed 21 days after tumor challenge. In the tumor protection experiment mice vaccinated with Sig/E7/LAMP-1 DNA had lower mean pulmonary nodule numbers com-

Figure 7 E7-expressing DNA vaccines protect mice against the growth of TC-1 tumors in the lungs. C57BL/6 mice were immunized with empty plasmid, E7 DNA or Sig/E7/LAMP-1 DNA via gene gun. One week after the second vaccination, mice (10 per group) were challenged with TC-1 tumor cells via intravenous tail vein injection at a dose of 5×10^4 cells per mouse. Mice were monitored twice a week and were killed at day 30 after tumor challenge. The mean pulmonary nodule numbers ($\pm$ s.e.) shown here are from one representative experiment of three performed.

pared with wild-type E7 DNA vaccination (Figure 11). In the tumor treatment experiments, there was no statistically significant difference in the mean pulmonary nodule numbers among Sig/E7/LAMP-1 DNA, E7 DNA-vaccinated mice and the control mice (data not shown). These results indicated that under more stringent conditions, Sig/E7/LAMP-1 DNA vaccination generated a better antitumor effect than wild-type E7 DNA.

Discussion

In this study we demonstrated that gene gun-mediated DNA vaccination with Sig/E7/LAMP-1 DNA generated the strongest E7-specific humoral and cellular immunity. Both Sig/E7/LAMP-1 DNA and E7 DNA could generate significant preventive and therapeutic effects in HPV-16 E7-expressing murine tumors metastasis in the liver or lungs. Furthermore, the Sig/E7/LAMP-1 DNA demonstrated a stronger antitumor effect than E7 DNA under more stringent conditions (priming without further booster). Our results demonstrated the therapeutic efficacy of DNA immunization for the treatment of cancer metastases. Although DNA vaccination with intramuscular inoculation^{14–22} or intradermal injection via gene gun^{23–25} have been shown to generate antitumor immunity for tumors inoculated subcutaneously, there are few reports of using DNA vaccines to treat cancer metastases.²⁴ Our study represents a good example of using

Figure 8 Representative gross pictures of the lung tumors in each vaccinated group. C57BL/6 mice were immunized with empty plasmid, E7 DNA or Sig/E7/LAMP-1 DNA via gene gun and were then intravenously injected with TC-1 tumor at 5×10^4 cells per mouse 7 days later. Mice were killed 30 days after tumor challenge. There are multiple grossly visible lung tumors in the control mice and mice vaccinated with empty plasmids (arrows). The rest of the lung tumors in each vaccinated group cannot be seen at the magnification in this figure.

antigen-specific DNA vaccine for the control of cancer metastases.

DNA vaccination is an attractive approach to generate antigen-specific vaccines because it induces cellular as well as humoral immunity with high efficacy.²⁶ Even though the existing viral vectors are efficient in delivering DNA to targeted cells, DNA vaccines possess several advantages over these viral vectors. For example, immune recognition following adenoviral vector or vaccinia delivery inhibits repeat vaccination with the same delivery vector, while retroviral vectors are potentially toxic due to helper virus replication and insertional mutagenesis. In contrast, naked DNA vaccines have the clear advantages of purity, simplicity of preparation and stability. DNA-based vaccines can be prepared inexpensively and rapidly on a large scale. Furthermore, the CpG motif in plasmid DNA may cause simultaneous maturation of immature dendritic cells (DCs) and activation of mature DCs²⁷ and thus can preferentially induce the T helper 1 response,²⁸ which is important for the antitumor effect.

The exact mechanisms of DNA vaccines remain controversial in spite of the demonstrated antitumor immunity. Several studies have shown that bone marrow-derived APCs are the most important cells for presenting the antigen to the immune system in gene gun-mediated DNA vaccination.^{29,30} Following the gene gun delivery, the gene-transfected DCs travel to local lymph nodes, where the antigen-loaded DCs directly activate the naive T cells.^{29,31,32} This antigen presentation is probably not due to cross-presentation by untransfected DC of antigen released from or associated with transfected epidermal cells.³¹

As epidermal DCs are the professional APCs in gene gun-mediated DNA vaccination, several points need to be addressed about the superiority of the Sig/E7/LAMP-1

DNA over the E7 DNA. Several pieces of indirect evidence imply that this superiority may be related to the enhanced MHC-II presentation in DCs and the subsequent CD4⁺ T cell activation via the LAMP-1 targeting strategy. The enhanced MHC-II presentation by LAMP-1 has been demonstrated previously in the HPV-16 E7 antigen³³ and human immunodeficiency virus gp160 antigen models³⁴ using vaccinia virus as vectors. That Sig/E7/LAMP-1 DNA generated much higher E7-specific antibody titer than E7 DNA also indicated the help of CD4⁺ cells. Furthermore, intracellular cytokine staining showed that Sig/E7/LAMP-1 DNA generated higher numbers of IFN- γ -positive CD4⁺ T cells (Ji and Wang, personal communication). Alternative explanations for the enhanced potency of Sig/E7/LAMP-1 DNA include protein expression in the chimeric constructs and the enhanced access of antigens to the endoplasmic reticulum via the signal peptide.^{35,36}

Although the superiority of Sig/E7/LAMP-1 DNA over E7 DNA in the antitumor effect was demonstrated, it was relatively difficult to see this 'narrow' window. Interestingly, it is easier to demonstrate that Sig/E7/LAMP-1 is more potent than E7 using vaccinia virus as vectors in both immunological assays and antitumor effects.^{7,33} This may imply that the mechanisms of the antitumor effects generated by the same constructs in the form of vaccinia vectors and naked DNA are different. The favored mechanism of gene gun-mediated DNA vaccination is the direct priming of naive T cells by gene-transfected DCs.^{31,37} In contrast, E7-expressing recombinant vaccinia virus may use the alternative MHC-I pathway (cross-priming) for the initiation of CTL responses³⁸ in addition to the direct priming route. The enhanced CD4 presentation by LAMP-1 can help the induction of CD8⁺ CTL responses by cross-priming.³⁹ Thus, CD4⁺ cell-mediated help may be more important for cross-priming

Figure 9 E7-expressing DNA vaccines eradicate previously established TC-1 tumors in the liver. C57BL/6 mice (five per group) were injected intrahepatically with TC-1 tumor at 1×10^4 cells per mouse and were immunized with empty plasmid, E7 DNA or Sig/E7/LAMP-1 DNA via gene gun 7 days later. Mice were killed 30 days after tumor challenge to check for the presence of liver tumors.

than for direct priming. This hypothesis may partially explain why it is easy to demonstrate the superiority of Sig/E7/LAMP-1 over E7 in the antitumor effect if it is administered in the form of recombinant vaccinia, but not if it is administered in the form of naked DNA vaccine.

In this study, we have abided by a 30-day time-frame for *in vivo* tumor protection and treatment experiments in the lung/liver metastasis model. Similarly to a previous study,⁸ we have used this time-frame to obtain results since information about metastatic tumors is unobtainable from the exterior, requiring mice to be killed at a given deadline. In the future, it would be interesting to expand the 30-day time-frame in order to determine the length of protective immunity generated by E7-specific DNA vaccines. Furthermore, in order to determine if repeated DNA vaccination is able to control established tumor nodules, we would first determine if a higher dosage of TC-1 or increased time between tumor challenge and initial vaccination would generate established tumor nodules before DNA administration. We could then proceed to use different regimens of DNA vaccination, varying the booster dosage or the booster frequency in order to identify the optimal regimen for DNA administration. The information generated would provide helpful insights into the long-term control of tumor growth by DNA vaccines.

In summary, our results indicate that the E7-expressing DNA vaccine is an effective vaccine for preventing and treating HPV-16 E7-expressing tumor metastases in the liver or lungs. Both E7 DNA and Sig/E7/LAMP-1 DNA

Figure 10 E7-expressing DNA vaccines eradicate previously established TC-1 tumors in the lungs. C57BL/6 mice (10 per group) were challenged with TC-1 tumor cells via intravenous tail vein injection at a dose of 5×10^4 cells per mouse. One week after the tumor challenge, mice were immunized with empty plasmid, E7 DNA or Sig/E7/LAMP-1 DNA via gene gun. Mice were monitored twice a week and were killed at day 30 after tumor challenge. The mean pulmonary nodule numbers ($\pm$ s.e.) shown here are from one representative experiment of three performed.

have potent antitumor effects, however, Sig/E7/LAMP-1 DNA generates more potent tumor protection than E7 DNA when tested under more stringent conditions. In addition, Sig/E7/LAMP-1 DNA generates the strongest E7-specific CTL activity, the highest numbers of E7-specific CD8⁺ T cell precursors and the highest titer of anti-E7 antibody. Although our tumor metastasis models were designed for HPV-16 E7 antigen-containing tumors, similar strategies can potentially be applied to other cancer systems with known tumor-specific antigens. As more tumor-specific antigens are characterized, using recent advances in molecular immunology, DNA vaccination will become more widely applicable for the treatment of cancer metastases.

Materials and methods

Plasmid DNA constructs and preparation

The Sig/E7/LAMP-1 chimeric gene was generated as described earlier.³³ The chimeric gene and the HPV-16 E7 gene were cloned into the unique *Bam*HI and *Eco*RI cloning sites of the pCMV neo expression vector downstream of the cytomegalovirus promoter.³³ The schematic diagrams of the wild-type E7 and Sig/E7/LAMP-1 constructs are shown in Figure 1. The plasmid DNAs were transfected into subcloning efficient DH5 α TM cells (Life Technologies, Rockville, MD, USA). DNAs were then amplified and purified by double CsCl purification (BioServe Biotechnologies, Laurel, MD, USA). The integrity of plasmid DNA and the absence of *Escherichia coli*

Figure 11 Sig/E7/LAMP-1 DNA generates more potent protective effect against the growth of TC-1 tumors in the lungs. C57BL/6 mice (10 per group) were immunized with E7 DNA or Sig/E7/LAMP-1 DNA via gene gun. In contrast to the previous set of experiments, the mice were vaccinated once without further booster. One week after the vaccination, mice were challenged with TC-1 tumor cells via intravenous tail vein injection at a dose of 1×10^5 cells per mouse. Mice were monitored twice a week and were killed at day 21 after tumor challenge. Mice vaccinated with Sig/E7/LAMP-1 DNA had significantly lower mean pulmonary nodule numbers than mice vaccinated with E7 DNA.

DNA or RNA were checked in each preparation using 1% agarose gel electrophoresis. DNA concentration was determined by the optical density measured at 260nm. The presence of inserted Sig/E7/LAMP-1 and E7 fragments was confirmed by restriction enzyme digestion and gel electrophoresis.

DNA vaccination

Gene gun particle-mediated DNA vaccination was done using a helium-driven gene gun (Bio-Rad, Hercules, CA, USA) according to the protocol provided by the manufacturer. Briefly, DNA-coated gold particles were prepared by combining 25 mg of 1.6 μ m gold microcarriers (Bio-rad) and 100 μ l of 0.05 M spermidine (Sigma, St Louis, MO, USA). Plasmid DNA (50 μ g) and 1.0 M CaCl_2 (100 μ l) were added sequentially to the microcarriers while mixing by vortex. This mixture was allowed to precipitate at room temperature for 10 min. The microcarrier/DNA suspension was then centrifuged (16 000 g for 5 s) and washed three times in fresh absolute ethanol before resuspending in 3 ml of polyvinylpyrrolidone (0.1 mg/ml) (Bio-rad) in absolute ethanol. The solution was then loaded into tubing and allowed to settle for 4 min. The ethanol was gently removed and the microcarrier/DNA suspension was evenly attached to the inside surface of the tubing by rotating the tube. The tube was then dried by 0.4 liter per minute flowing nitrogen gas. The dried tubing coated with microcarrier/DNA was then cut to 0.5-inch cartridges and stored in a capped dry bottle at 4°C. As a result, each cartridge contained

1 μ g of plasmid DNA and 0.5 mg of gold. The DNA-coated gold particles (1 μ g DNA per bullet) were delivered to the shaved abdominal region of the mice using a helium-driven gene gun (Bio-rad) with a discharge pressure of 400 p.s.i.

ELISA

The anti-HPV-16 E7 antibodies in the sera were determined by a direct ELISA as previously described.³³ A 96-microwell plate was coated with 100 μ l of 10 μ g/ml bacteria-derived HPV-16 E7 protein at 4°C overnight and then the wells were blocked with phosphate-buffered saline (PBS) containing 20% fetal bovine serum. Sera were prepared from the mice on day 14 after immunization, serially diluted in PBS, added to the ELISA wells, and incubated at 37°C for 2 h. After washing with PBS containing 0.05% Tween-20, the plate was incubated with 1/2000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA, USA) at room temperature for 1 h. The plate was washed six times, developed with TMB (Pierce, Rockford, IL, USA), and stopped with 1 M H_2SO_4 . The ELISA plate was read with a standard ELISA reader at 450 nm.

CTL assay

CTL assay was done in a standard ^{51}Cr release assay as described before.³³ Briefly, mouse spleen cells from each group were harvested at day 14 after vaccination. The splenocytes were cultured for 6 days in the presence of 1 μ g/ml E7-specific H-2D^b CTL epitope (E7 49-57, RAHYNIVTF)⁴⁰ and 20 units/ml murine interleukin-2. A standard 4 h chromium release assay was performed against MC57G (H-2^b) cells pulsed with E7 peptide (aa 49-57) and labeled with 0.2 mCi of $\text{Na}_2^{51}\text{CrO}_4$ (Amersham, Piscataway, NJ, USA). The mean percent specific lysis of triplicate wells was calculated as: $\text{specific lysis} = [(\text{c.p.m. experimental release} - \text{c.p.m. spontaneous release}) / (\text{c.p.m. maximum release} - \text{c.p.m. spontaneous release})] \times 100$.

ELISPOT assay

The ELISPOT assay described by Miyahira *et al*⁴¹ and Murali-Krishna *et al*¹³ was modified to detect HPV-16 E7-specific CD8⁺ T cells. The ELISPOT assay was used to determine the number of antigen-specific T cell precursors based on the secretion of IFN- γ by individual activated T cells. Therefore, it was important to incubate the plates with anti-mouse IFN- γ antibody in order to capture the IFN- γ secretion by these activated T cells. The 96-well filtration plates (Millipore, Bedford, MA, USA) were coated with 10 μ g/ml rat anti-mouse IFN- γ antibody (clone R4-6A2; Pharmingen, San Diego, CA, USA) in 50 μ l of PBS. After overnight incubation at 4°C, the wells were washed and blocked with culture medium containing 10% fetal bovine serum. Serial dilutions of fresh isolated spleen cells from each vaccinated mouse group, starting from 1×10^6 per well, were added to the well along with 15 U/ml interleukin-2. Cells were incubated at 37°C for 24 h either with or without 1 μ g/ml E7-specific H-2D^b CTL epitope (E7, aa 49-57). After culture, the plate was washed and followed by incubation with 5 μ g/ml biotinylated IFN- γ antibody (clone XMG1.2; Pharmingen) in 50 μ l PBS at 4°C overnight. After six washes, 1.25 μ g/ml avidin-alkaline phosphatase (Sigma, St Louis, MO, USA) in 50 μ l PBS was added and the plate

was incubated for 2 h at room temperature. After washing, spots were developed by adding 50 μ l BCIP/NBT solution (Boehringer Mannheim, Indianapolis, IN, USA), and incubating at room temperature for 1 h. The images of the spots in each well were captured by CCD video camera and the quantification of spot numbers was performed on a Macintosh computer using the NIH Image program 1.6 (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>) according to the method used by McCutcheon *et al.*⁴²

ELISA for cytokines

Splenocytes (4×10^6) were harvested 2 weeks after the last vaccination and cultured with 10 μ g/ml E7 protein in a total volume of 2 ml of RPMI 1640, supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids in a 24-well tissue culture plate for 72 h. The supernatants were harvested and assayed for the presence of IFN- γ and IL-4 using ELISA kits (Endogen, Woburn, MA, USA) according to the manufacturer's protocol.

Murine tumor cell line

The production and maintenance of TC-1 cells has been described previously.⁷ Briefly, HPV-16 E6, E7 and *ras* oncogene were used to transform primary C57BL/6 mice lung epithelial cells. The cells were grown in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids and 0.4 mg/ml G418 at 37°C with 5% CO₂. On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed twice with 1 $\times$ Hank's buffered salt solution and finally resuspended in 1 $\times$ Hank's buffered salt solution to the designated concentration for injection.

Mice and tumor cell inoculation

Mice used in this study were purchased as 6- to 8-week old male C56BL/5 mice from the National Cancer Institute (Frederick, MD, USA) and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD, USA). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. For the hepatic metastasis model, the mice were anesthetized with intraperitoneal injection of 0.1 ml solution containing ketamine hydrochloride (25 mg/ml), xylazine (2.5 mg/ml), and 14.25% ethyl alcohol diluted 1:3 in 0.9% NaCl solution. A small midline laparotomy incision was made and the left lobe of the liver was exteriorized. TC-1 cells in 10 μ l Hank's buffered salt solution were injected with a 30-gauge needle directly into the liver parenchyma. The localized injection was confirmed by blanching of the liver parenchyma. Gentle compression for 60 s was used to minimize cellular extravasation and promote hemostasis. The liver was then placed back into the peritoneal cavity, and the incision was closed with metal wound clips. For the lung metastasis model, the TC-1 cells in 100 μ l HBSS were injected into each mouse's tail vein.

In vivo tumor protection experiments

For the tumor protection experiment, mice were vaccinated via gene gun with 2 μ g of either Sig/E7/LAMP-1

DNA, E7 DNA, or the control plasmid without insert. One week later, the mice were boosted with the same regimen as in the first vaccination. In total, each mouse received 4 μ g of plasmid DNA. One week after the second vaccination, mice were challenged with TC-1 tumor cells at a dose of 1×10^4 cells per mouse for the hepatic metastasis model (five mice per group) and 5×10^4 TC-1 cells per mouse for the lung metastasis model (10 mice per group). Mice were monitored twice a week and were killed at day 30 after tumor challenge.

In vivo tumor regression experiments

Tumor cells for injection and the DNA vaccines for immunization were prepared as described above. The mice were challenged with TC-1 tumor cells first: 1×10^4 cells per mouse for the hepatic metastasis model (five mice per group), and 5×10^4 TC-1 cells per mouse for the lung metastasis model (10 mice per group for both tumor doses). Seven days after challenge with TC-1 tumor cells, mice were given 2 μ g of Sig/E7/LAMP-1 DNA, E7 DNA or control plasmid via gene gun. One week later, these mice were boosted with the same regimen as the first vaccination. In total, each mouse received 4 μ g of plasmid DNA. Mice were monitored twice a week and were killed at day 30 after tumor challenge.

Tumor assessment and histologic studies

For the hepatic metastasis model, the livers were removed after killing the mice and were examined in the fresh state by inspection and palpation for the presence of tumor. The tumor size was measured in animals in which tumor was found. For the lung metastasis model, the lungs were removed after killing the mice. The pulmonary metastasis nodules were counted under dissecting microscope. All hepatic specimens and those pulmonary specimens without gross visible tumors were then fixed with 10% neutral buffered formalin, embedded in paraffin and cut into 5 μ m sections from different levels. After hematoxylin and eosin staining, the slides were examined using a conventional microscope.

Statistical analysis

Student's *t* test was used to compare the ELISPOT numbers and mean pulmonary nodules among different groups. *P* values (two-tailed) less than 0.05 were considered statistically significant.

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