ACQUIRED DISEASES

RESEARCH ARTICLE

Intramuscular administration of E7-transfected dendritic cells generates the most potent E7-specific anti-tumor immunity

T-L Wang¹, M Ling¹, I-M Shih¹, T Pham¹, SI Pai², Z Lu², RJ Kurman^{1,3}, DM Pardoll^{1,2} and T-C Wu^{1,2,3,4}

Departments of ¹Pathology, ²Oncology, ³Gynecology and Obstetrics, and ⁴Molecular Microbiology and Immunology, The Johns Hopkins Medical Institution, Baltimore, MD, USA

Dendritic cells (DCs) are highly efficient antigen-presenting cells capable of priming both cytotoxic and helper T cells in vivo. Recent studies have demonstrated the potential use of DCs that are modified to carry tumor-specific antigens in cancer vaccines. However, the optimal administration route of DC-based vaccines to generate the greatest anti-tumor effect remains to be determined. This study is aimed at comparing the levels of immune responses and anti-tumor effect generated through different administration routes of DC-based vaccination. We chose the E7 gene product of human papillomavirus (HPV) as the model antigen and generated a stable DC line (designated as DC-E7) that constitutively

expresses the E7 gene. Among the three different routes of DC-E7 vaccine administration in a murine model, we found that intramuscular administration generated the greatest anti-tumor immunity compared with subcutaneous and intravenous routes of administration. Furthermore, intramuscular administration of DC-E7 elicited the highest levels of E7-specific antibody and greatest numbers of E7-specific CD4⁺ T helper and CD8⁺ T cell precursors. Our results indicate that the potency of DC-based vaccines depends on the specific route of administration and that intramuscular administration of E7-transfected DCs generates the most potent E7-specific anti-tumor immunity. Gene Therapy (2000) 7, 726–733.

Keywords: human papillomaviruses (HPV); E7; dendritic cell; vaccines; immunity; cervical cancer

Introduction

Antigen-specific cancer immunotherapy has recently emerged as a promising approach to control cancer because it is capable of directing specific immune responses against neoplastic cells while not attacking normal cells. Increasing evidence suggests that professional antigen presenting cells (APCs), particularly dendritic cells (DCs), are the central players for mediating cancer immunotherapy. DCs are potent professional APCs that are specialized to prime helper and killer T cells *in vivo* (for review see Refs 1–3). DCs can stimulate T cells due to their high expression levels of MHC class I and class II molecules, co-stimulatory molecules such as B7, and adhesion molecules including ICAM-1, ICAM-3 and LFA-3. To present antigens effectively, DCs perform a series of coordinated tasks.⁴ In the presence of maturation-inducing stimuli such as inflammatory cytokines or activation via CD40,⁵ DCs up-regulate the expression of adhesion and co-stimulatory molecules to become more potent and terminally differentiated stimulators of T cell-mediated immunity. DCs are equipped to capture antigens and present large numbers of immunogenic MHC-peptide complexes on their surface.^{6,7} DCs also

migrate to secondary lymphoid organs where they stimulate rare antigen-specific T cells.⁸ Thus, an effective vaccine most likely requires a strategy that targets tumor antigens to professional APCs, such as DCs, which have the potential to enhance both MHC class I and class II presentation of the tumor antigen to activate antigen-specific CD8⁺ and CD4⁺ T cells.

Vaccine strategies employing DCs generated *ex vivo* have been developed in recent years. The availability of large quantities of active DCs generated *ex vivo* has created the opportunity to test various vaccine strategies for immunotherapy. There are several vaccine strategies using DCs prepared with known tumor specific antigens such as HPV-16 E6/E7 or with undefined tumor antigens. Vaccine strategies using DCs generated *ex vivo* can be classified under the following: (1) DCs pulsed with peptides or proteins^{3,9–11}; (2) DCs transduced with genes encoding tumor specific antigens (TSAs) using naked DNA^{12,13} or viral vectors^{14–16}; and (3) DCs armed with the full antigenic spectrum of tumor cells (for tumors with uncharacterized tumor specific antigens).^{17–19} All of these strategies employing DC-based vaccines have demonstrated varying degrees of enhancement in immune responses and anti-tumor effects.

One important issue of DC vaccination is the route of administration, which may play a key role in successful immunotherapy. The optimal route of DC-based vaccination for inducing a protective anti-tumor effect and potent immune responses has not been established. In

this study, we used the E7 gene of human papillomavirus (HPV) type 16 as the model antigen and used E7-expressing murine tumor cell line (TC-1) as the *in vivo* tumor model. We investigated three different routes of DCs administration (intramuscular, subcutaneous and intravenous) and compared the potency of E7-transfected DCs to generate E7-specific immune responses and anti-tumor effects through these different routes.

Results

Generation and characterization of E7-expressing dendritic cells

The immortalized dendritic cell line used in this study has been well-characterized and kindly provided by Dr Kenneth Rock at the University of Massachusetts Medical Center.²⁰ The dendritic cells were generated by transducing granulocyte-macrophage CSF into bone marrow cultures followed by supertransfection with myc and raf oncogenes. Electroporation was performed to introduce a pCMV plasmid with the E7 gene into an immortalized DC line.²¹ Limiting dilution was performed to isolate single cell clones that stably expressed the E7 gene (designated DC-E7). Using E7-specific primers, we performed RT-PCR in DC-E7, which amplified one major DNA product at the expected molecular weight of the E7 gene (Figure 1a). In contrast, RT-PCR with parental untransfected DCs revealed no such E7 expression. To examine if E7 protein was translated in DC-E7, Western blot analysis was performed with an E7-specific antibody. In DC-E7, a prominent band at 21 kDa corresponding to E7 protein was detected. In contrast, the band was absent in the parental DCs, indicating the absence of E7 protein expression (Figure 1b).

To determine if the DC-E7 still retained DC surface molecules that are important for T cell activation, immunostaining was performed using a panel of antibodies against MHC class I, class II, B7-1 and B7-2 costimulatory molecules, CD40, DEC-205, and Mac-1 followed by FACS analysis. The results revealed that the expression levels of these surface molecules in DC-E7 were similar to those in the parental DCs. Previously, Shen and Rock also found that the immortalized clones displayed typical dendritic cell morphology, and expressed dendritic cell-specific markers DEC-205 and

33D1, high levels of MHC-I and MHC-II molecules, and B7-1 and B7-2 costimulatory molecules.²⁰ Thus, there is no significant effect on the expression of cell surface markers after transfection of DCs with E7 (Figure 2).

DC-E7 but not parental DCs can present the E7 antigen to E7-specific CTLs *in vitro*

To assess whether E7-expressing DCs were capable of directly presenting the E7 antigen to T lymphocytes, DC-E7 was co-cultured *in vitro* with a MHC class I-restricted, E7-specific T cell line. After 24 h, IFN- γ -secreting T cells were detected using the ELISPOT assay. Figure 3a and b demonstrate a significant increase in IFN- γ secretion from activated T cells, indicating that the endogenously expressed E7 gene in DC-E7 was processed and presented in an MHC class I-restricted manner to cytotoxic T lymphocytes (CTL). In contrast, parental untransfected DCs failed to induce cytokine release from the E7-specific CTLs, indicating the lack of MHC class I presentation of the E7 antigen by untransfected DCs.

Vaccination with DC-E7 but not parental DCs protects against an E7-expressing tumor challenge

To determine if DC-E7 administration induced anti-tumor immunity, we vaccinated mice via the subcutaneous route of administration twice (1 week apart) with either parental DCs or DC-E7. Unvaccinated mice were used as a negative control. One week later, mice received subcutaneous tumor challenge at a dose of 10^4 TC-1 cells per mouse. TC-1 is an E7-expressing tumor cell line that is syngeneic to C57BL/6 (H-2^b) that we previously developed.²² We found that vaccination with parental control DCs did not induce significant delay of tumor growth. Two weeks after tumor cell challenge (day 30), all mice vaccinated with untransfected DCs (Figure 4a) and unvaccinated mice (data not shown) grew tumors within 2 weeks after tumor challenge. In contrast, vaccination of DC-E7 resulted in a significant delay of tumor growth and an increased tumor protection. We also performed survival analyses to monitor the percentage of surviving mice up until day 57 following tumor challenge. We found that 60% of mice vaccinated with DC-E7 survived at day 57 while none of the mice vaccinated with untransfected DCs or unvaccinated mice survived

Figure 1 Expression of E7 gene in transfected dendritic cells. Bone marrow-derived DCs were transfected using electroporation with a plasmid containing the E7 gene. (a) RT-PCR demonstrated the expression of the E7 gene in DC-E7. An amplified DNA band corresponding to the molecular weight of E7 was observed in DC-E7 (indicated with an arrow), but not in the parental DCs. Direct DNA sequencing confirmed the sequence of E7 in the PCR product. (b) Western blot analysis of lysates from parental DCs and DC-E7 demonstrated a protein band for DC-E7 (indicated with arrow) corresponding to the expected molecular weight of E7 protein.

Figure 2 Flow cytometry analyses to demonstrate the expression of DC-associated surface molecules in DC-E7 and parental DCs. Flow cytometry was performed on parental DCs and DC-E7 using antibodies against MHC class I and class II molecules, B7-1 and B7-2 co-stimulatory molecules, CD40, DEC-205, and Mac-1. We observed similar expression levels of these molecules in parental DCs and DC-E7. The shaded area represents the negative control, staining with only secondary antibodies.

beyond day 30 (Figure 4b). These data demonstrated the potency of vaccination of mice with DC-E7 compared with vaccination with untransfected DCs.

Vaccination with DC-E7 administered via intramuscular route generates the most potent anti-tumor effect

To address the issue of whether different routes of immunization elicit different potency in anti-tumor efficacy, a tumor growth protection assay was performed. Mice were immunized with DC-E7 through three different routes of administration including intramuscular, intravenous and subcutaneous. One week later, mice received subcutaneous tumor challenge at a lethal dose of 3×10^4 E7-expressing TC-1 cells per mouse. Tumor growth was monitored twice a week. Mice immunized through the intramuscular route generated the most effective protection against the tumor challenge, as shown by a significant delay in tumor formation (Figure 5a). Mice that were immunized subcutaneously demonstrated only moderate anti-tumor protection. In contrast, mice that immunized through the intravenous route demonstrated the least significant anti-tumor effect. We also performed survival analyses to monitor the percentage of surviving mice up until day 57 following tumor challenge. We found that 60% of mice receiving intramuscular immunization of DC-E7 survived at day 57. In contrast, only 20% of mice receiving subcutaneous immunization of DC-E7 survived at day 57 and none of the mice

receiving intravenous immunization survived beyond day 30 (Figure 5b).

DC-E7 vaccine administered via the intramuscular route of administration induces the most potent humoral and cell-mediated immune responses

To compare the humoral responses elicited through different routes of vaccine administration, the serum levels of anti-E7 antibody generated by each route were determined using an enzyme-linked immunosorbent assay (ELISA). Intramuscular immunization generated the highest anti-E7 antibody titers among the three different routes of DC-E7 immunization (Figure 6). This result indicated that the intramuscular route of immunization was more potent at eliciting humoral immune responses.

The enzyme-linked immunospot (ELISPOT) assay is a sensitive technique to detect and quantify cytokine production from individual T cells after antigen stimulation. To determine the frequency of antigen-specific CD8⁺ T cell precursors, splenocytes isolated from vaccinated mice were stimulated with an E7-specific CTL epitope (aa 49–57 of E7 protein).²³ Our results indicated that E7-specific T cells could be detected in mice using different routes of DC-E7 vaccination. Intramuscular immunization with DC-E7 induced the highest number of E7-specific CTL precursors (Figure 7). Subcutaneous administration elicited only a moderate number of IFN- γ -secreting spots, while intravenous vaccination elicited the

Figure 3 ELISPOT assays to demonstrate that DC-E7 can present E7 antigen through the MHC class I pathway. DC-E7 and parental DCs were co-cultured with the E7-specific CTLs overnight. Using serial dilutions of E7-specific CTLs, the ELISPOT assay was performed to detect IFN- γ -secreting T cells. (a) ELISPOT assay revealed a significantly higher number of spots for DC-E7 than for parental DCs. (b) Serial dilutions demonstrated that increasing numbers of E7-specific CTLs co-cultured with DC-E7 generated a proportionally increasing number of spots, while parental DCs did not. 3×10^5 parental DCs or DC-E7 were used as the antigen presenting cells. Data are expressed as the mean $\pm$ s.e.m. ($n = 3$). Statistical significance and error bars were generated using ANOVA, with a P value of 0.0001.

lowest number of spots. We also stimulated splenocytes extracted from vaccinated mice with an E7-specific T helper epitope (aa 30–67)²⁴ and assayed for IFN- γ secretion. Our results demonstrated that the number of IFN- γ -secreting E7-specific T helper cell precursors was highest following intramuscular immunization of DC-E7 (Figure 8). These results indicated that the DC-E7 generated CD4⁺ Th1-type immune responses. In contrast, we failed to detect measurable amounts of IL-4 responses using the ELISPOT assay in any of the vaccinated groups, indicating a lack of Th2 response to DC-E7 vaccination (data not shown). As negative controls, all ELISPOT assays were also performed in the absence of E7-specific peptide and we did not detect significant background spots in any of the vaccination groups (data not shown).

Discussion

In this study, we demonstrated that the E7-transfected immortalized dendritic cells expressed MHC class I, class II, B7-1 and B7-2 costimulatory molecules, CD40, DEC-205, and Mac-1 dendritic cell markers. DC-E7 also

Figure 4 In vivo tumor protection and survival analysis in mice vaccinated with DC-E7 and DCs. (a) Groups of mice received two subcutaneous immunizations (one week apart) with 10^6 parental DCs (filled circle) or DC-E7 (open square). One week after the last immunization, mice received subcutaneous challenge with 10^4 E7-expressing tumor cells (TC-1) in the left flank. Afterwards, tumor growth was monitored twice a week. Data shown are from one experiment that is representative of three performed. Statistical significance was determined using the log-rank test, with a P value of 0.0116. (b) Survival analysis to monitor the percentage of surviving mice up until day 57 following tumor challenge. Statistical significance was determined using the log-rank test, with a P value of 0.0483.

presented the E7 gene product in the context of MHC class I molecules when DC-E7 was co-cultured *in vitro* with a CD8⁺ E7-specific T cell line. Vaccination with DC-E7 in mice was capable of mediating effective anti-tumor protection against an E7-expressing murine tumor cell (TC-1). We observed higher E7-specific antibody titers and higher numbers of E7-specific CD8⁺ CTL and CD4⁺ Th1 precursors in mice vaccinated with DC-E7 compared with mice vaccinated with untransfected DCs. We also observed higher titers of E7-specific antibodies, higher numbers of E7-specific CD8⁺ CTL and CD4⁺ Th1 precursors, and more potent anti-tumor effects in mice that received DC-E7 through the intramuscular route of administration compared with mice immunized via the subcutaneous or intravenous routes of administration.

Figure 5 In vivo tumor protection and survival analysis in mice vaccinated with DC-E7 through different routes of immunization. (a) Groups of mice received two immunizations (1 week apart) with 10^6 DC-E7 cells using different routes of administration: intramuscular (open square), subcutaneous (open diamond) and intravenous (filled circle). One week after the final immunization, mice were challenged with 3×10^4 E7-expressing tumor cells subcutaneously in the left flank. Afterwards, tumor growth was monitored twice a week. The results indicated that DC-E7 immunization via the intramuscular route yielded the most effective tumor protection. Data shown are from one experiment that is representative of three performed. Statistical significance was determined using the log-rank test, with a P value of 0.0103. (b) Survival analysis to monitor the percentage of surviving mice up until day 57 following tumor challenge. Statistical significance was determined using the log-rank test, with a P value of 0.0045.

This finding has potential implications for the clinical application of dendritic cell-based vaccines.

We chose HPV-16 E7 as a model antigen for vaccine development because human papillomaviruses, particularly HPV-16, are associated with most cervical cancers and their precursors. E7 is an oncogenic HPV protein that is expressed in most HPV-positive cervical carcinomas and plays an important role in the initiation and maintenance of cellular transformation. Accordingly, vaccines targeting the E7 protein appear to be a promising approach to prevent and/or treat HPV-associated cervical malignancies. The results obtained from our DC-E7 experiments warrant further investigation into the use of DC-based vaccines for the treatment of HPV-16 E7-expressing cervical cancers in a clinical setting.

Figure 6 E7-specific antibody responses in mice immunized with DC-E7 through different routes of administration. Mice received two immunizations (1 week apart) with DC-E7 using different routes of administration. Naïve mice were used as a negative control. Serum samples were obtained from immunized mice at day 7 after the second vaccination. E7-specific antibodies were detected by ELISA using serial dilution of sera from the mice. Naïve mice did not generate significant E7-specific antibodies. The results from 1:10, 1:40, 1:160 and 1:640 dilutions of sera are presented, with a mean absorbance (OD) at 450 nm $\pm$ s.e.m. Statistical significance and error bars were generated using ANOVA, with a P value of 0.0001.

Figure 7 Demonstration of E7-specific CD8⁺ T cell precursors in mice immunized with DC-E7 through different routes of administration. Splenocytes were isolated from mice that received intramuscular, subcutaneous, or intravenous vaccination with DC-E7. Mice receiving parental untransfected DCs (open bars) were used as a negative control. Splenocytes were stimulated with an E7-specific CTL epitope (aa 49–57) to detect CD8⁺ T cells (filled bars). The ELISPOT assay revealed that intramuscular administration of DC-E7 generated the highest number of spots. Data are expressed as the average number of spots per 2.4×10^6 DC $\pm$ s.e.m. Statistical significance and error bars were generated using ANOVA, with a P value of 0.0001. Only a few spots could be detected in mice vaccinated with parental untransfected DCs. Results shown here are from one experiment that is representative of three performed.

An interesting finding in our study is that the potency of the DC-E7 vaccine was dependent on the route of DC-E7 administration. It has been postulated that different routes of DC-based vaccination may result in the differential distribution of DCs in the body. A clinical study performed on patients with metastatic carcinoembryonic antigen (CEA)-expressing cancer supports this hypoth-

Figure 8 Demonstration of IFN- γ secreting, E7-specific T helper cell precursors in mice immunized with DC-E7 through different routes of administration. Splenocytes were isolated from mice that received intramuscular, subcutaneous, or intravenous vaccination with DC-E7. Mice receiving parental untransfected DCs were used as a negative control (open bars). Splenocytes were stimulated with an E7-specific T helper epitope (aa 30–67) to detect IFN- γ -secreting E7-specific T helper cells (filled bars). ELISPOT assay revealed that intramuscular administration of DC-E7 generated the highest number of spots. Statistical significance and error bars were generated using ANOVA, with a *P* value of 0.0001. Only a few spots could be detected in mice vaccinated with parental untransfected DCs. Data are expressed as the average number of spots per 2.4×10^6 DC $\pm$ s.e.m. Results shown here are from one experiment that is representative of three performed.

esis. After the intravenous injection of CEA RNA-pulsed DCs, these cells first localized in the lungs, then redistributed to the liver, spleen, and bone marrow.²⁵ In contrast, intradermal injection of DCs resulted in rapid migration of DCs to the regional lymph nodes.²⁵ This finding has important implications since differential distribution of DCs has been correlated with anti-tumor effects. A recent study using a peptide-pulsed DC vaccine demonstrated that intravenous injection of DCs resulted in a less potent anti-tumor effect than subcutaneous administration of DCs.²⁶ Similarly, our findings indicated that subcutaneous administration generated more potent anti-tumor effects than intravenous administration (Figure 5).

Our results demonstrated that intramuscular administration generated the greatest humoral and cellular immune responses among three different routes of vaccine administration. The immune responses generated through intramuscular administration resulted in the strongest anti-tumor effect. Several hypotheses for this result can be made through histological observation of the site of intramuscular injection, where we observed significant inflammatory infiltration. A possible explanation for this observation is that tissue damage as the result of intramuscular injection may elicit 'danger signals' in the muscle, which may potentiate a local immune response, creating an environment suitable for direct presentation of E7 antigen by DC-E7 to CD4⁺ and CD8⁺ T cells. For example, inflammatory cytokines such as GM-CSF and TNF- α have been shown to induce maturation of DCs (for review, see Banchereau *et al*²⁷). Mature DCs are more potent at presenting antigens to T cells. Alternatively, following intramuscular immunization, DCs may migrate to sites in the body favorable for priming and activation of E7-specific humoral and cellular immune responses, thus contributing to a widespread immuno-

therapeutic effect. It would be interesting to define the precise mechanism of how an inflammatory response influences dendritic cell biology and distribution following intramuscular administration of DC-E7 in future studies.

Our findings indicated that intramuscular administration of DC-E7 not only generated substantial E7-specific CD8⁺ T cell responses, but also induced significant CD4⁺ T cell responses. Since E7 is a cytoplasmic protein, it is expected that E7-transfected DCs can present E7 antigen in the context of MHC class I molecules and induce an E7-specific CD8⁺ T cell response *in vivo*. A more intriguing finding was our observation of E7-specific CD4⁺ T cell responses induced by DC-E7, suggesting *in vivo* presentation of E7 via the MHC class II pathway. Direct presentation of E7 through the MHC class II pathway by DC-E7 is a possibility; however, this would be highly unlikely considering the fact that E7 is a cytoplasmic/nuclear protein. In general, cytoplasmic proteins are presented through the MHC class I pathway.²⁸ Alternatively, the E7 antigen may be released by DC-E7 caused by CTL-mediated apoptosis or lysis, allowing for the re-uptake of E7 protein by other antigen presenting cells and processing of E7 via the MHC class II processing pathway. Thus, increased uptake of E7 protein/peptide released by DC-E7 may contribute to the enhancement of humoral and cell-mediated responses via intramuscular administration of DC-E7.

In conclusion, DCs transfected with the E7 DNA can effectively evoke a systemic immune response and protect against a lethal E7-expressing tumor challenge. We found that intramuscular administration is the most potent and efficient route of DC-based vaccination. Our results provide insight into the role of different routes of administration in mediating immune activation and tumor protection, which may be applicable to the future development of DC cell-based vaccines.

Materials and methods

Cell lines

The immortalized dendritic cell line used in this study has been kindly provided by Dr Kenneth Rock.²⁰ Dendritic cells were grown in CTL medium containing 10% fetal calf serum, penicillin/streptomycin (50 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (2 mM), 2-mercaptoethanol (1 mM), and 0.4 mg/ml of G418 and grown at 37°C, 5% CO₂. TC-1 is an E7-expressing tumor cell line that is syngeneic to C57BL/6 (H-2^b) that we previously developed.²² The TC-1 tumor cell line was grown in complete medium containing 10% fetal calf serum, penicillin/streptomycin (50 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), and non-essential amino acids (2 mM), grown at 37°C, 5% CO₂.

Construction of the DC-E7 vaccine

DCs expressing the E7 gene were generated by transfecting a pCMV plasmid containing the E7 gene into dendritic cells using electroporation (BioRad, Hercules, CA, USA) at 220 V, 2850 μ F. E7-expressing transfectants were selected in G418 (800 μ g/ml). Stable clones were established using limiting dilution.

RT-PCR

Total RNAs from parental DCs and E7-transfected DCs were extracted and purified with acid-guanidium and phenol-chloroform. Reverse transcription (RT) was performed with 2 μ g total RNA in 20 μ l buffer containing 50 mM Tris-HCl (pH 7.4), 60 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 U/ml RNase inhibitor, 0.5 mM of each dNTP, 500 pmole of random hexamer, and 200 U of M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA). The RT reaction was run at 42°C for 50 min. PCR was run with 2 μ l of the cDNA products from the RT reaction. The PCR buffer contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.4 mM dNTP, 0.2 μ M 5' and 3' E7 primers, and 2.5 U of Amplitaq (Perkin Elmer Cetus, Norwalk, CT, USA). PCR was performed on a programmable thermocycler (Perkin Elmer) with the following parameters: 94°C 4 min, 35 cycles of 94°C 1 min, 50°C 1 min, 72°C 2 min, then extended at 72°C 10 min.

Western blot analysis

DCs were lysed in RIPA buffer (0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate in PBS) and separated by a standard 4–20% gradient SDS-PAGE. Fractionated proteins were blotted on to a PVDF membrane, incubated with the primary antibody against the HPV-16 E7 protein (Zymed, South San Francisco, CA, USA) for 2 h at room temperature, washed, and incubated for 2 h with 1:1000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. The blot was washed and detected using chemiluminescence (ECL kit; Amersham, Arlington Heights, IL, USA).

E7-specific T cell line

Six-week-old female C57BL/6 (H-2^b) mice were immunized using intraperitoneal injection of 10⁷ p.f.u. of Sig/E7/LAMP-1 vaccinia virus. Splenocytes were harvested at day 8 after immunization. Autologous, irradiated splenocytes pulsed with 1 μ M E7 peptide (amino acid 49–57) were used as stimulators. Recombinant human IL-2 was added to the culture at a final concentration of 30 U/ml.

Tumor protection experiment and survival analysis for DC-E7 versus untransfected DCs

Mice received subcutaneous vaccination with either parental DCs or DC-E7 (10⁶ cells per mouse) twice (1 week apart). Unvaccinated mice were used as a negative control. Seven days after the final vaccination, mice were challenged with a lethal dose (10⁴ cells per mouse) of E7-expressing tumor cells (TC-1) subcutaneously in the left flank. Mice were monitored for tumor growth and survival twice a week. Each set of experiments was repeated at least twice.

Tumor protection experiment and survival analysis for different routes of administration

Mice received intramuscular, subcutaneous, or intravenous vaccination with DC-E7 (10⁶ cells per mouse) twice (1 week apart). Mice receiving subcutaneous injection of untransfected DCs were used as a negative control. Seven days after the final vaccination, mice were challenged with a lethal dose (3 $\times$ 10⁴ cells per mouse) of E7-expressing tumor cells (TC-1) subcutaneously in the left flank. Mice were monitored for tumor growth and

survival twice a week. Each set of experiments was repeated at least twice.

Flow cytometry

Flow cytometry analysis was performed to evaluate the expression of DC-associated markers in the immortalized DC line. The markers included MHC class I, class II, B7-1 and B7-2 co-stimulatory molecules, CD40, DEC-205, and Mac-1. DCs were incubated with antibodies for 30 min at 4°C, washed, and incubated for 20 min with FITC-conjugated goat anti-mouse or anti-rat IgG (Jackson Laboratories, Bar Harbor, ME, USA). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). Anti-mouse H2-K^b/H2-D^b (28–8-6), I-A^b (25-9-17), anti-CD40 (3/23), and anti-Mac-1 (CD11b, M1/70) antibodies were purchased from Pharmingen, San Diego, CA, USA. The DEC-205-specific mouse monoclonal antibody, NLDC145,²⁹ was kindly provided by Dr Elizabeth Jaffee at the Johns Hopkins University. Both anti-CD80 and anti-CD86 antibodies were purchased from Caltag Laboratory, Burlingame, CA, USA.

Enzyme-linked immunosorbent assay (ELISA)

An ELISA was used to measure levels of anti-E7 IgG in mouse sera. Mice were immunized with DCs twice (10⁶ cells per mouse) at 1-week intervals. Seven days after the final vaccination, blood was drawn from the tail vein. The E7 protein (10 μ g/ml) was coated on to an immunoplate. Serum samples were diluted in PBS containing Tween-20 (0.1%) and 0.5% BSA. Peroxidase-labeled rat anti-mouse IgG Ab was used as the detecting antibody (Jackson ImmunoResearch, West Grove, PA, USA). We performed the ELISA using 1:10, 1:40, 1:160, and 1:640 dilutions of sera. Sera from naive mice were used to evaluate the background absorption value.

Enzyme-linked immunospot (ELISPOT) assay

Mice were immunized with DC-E7 (10⁶ cells per mouse) twice (1 week apart). Seven days after the final vaccination, splenocytes were harvested from the mice. An ELISPOT assay was performed to detect HPV-16 E7-specific CD8⁺ and CD4⁺ T cells.³⁰ A 96-well filtration plate (Millipore, Bedford, MA, USA) was coated with either anti-IFN- γ antibody (Pharmingen) or anti-IL-4 antibody (Endogen, Woburn, MA, USA). The wells were washed with PBS and blocked with culture medium for 1 h. Splenocytes from vaccinated mice were added to the wells at a density of 2 $\times$ 10⁵ cells per well. Cells were incubated at 37°C either with or without 1 μ g/ml E7-specific H-2D^b CTL epitope (amino acid 49–57)²³ or E7 peptide containing the T helper epitope (amino acid 30–67).²⁴ After 24 h, the plate was washed with PBS containing 0.2% Tween-20 (PBST) and then incubated with the biotinylated IFN- γ antibody (Pharmingen) or the IL-4 antibody (Endogen). After washing with PBST, avidin-alkaline phosphatase (Sigma, St Louis, MO, USA) was added and the plate was incubated for 2 h at room temperature. Spots were developed by adding 100 μ l of BCIP/NBT solution (Sigma) and incubating at room temperature for 10–20 min. Images of the cytokine spots were captured by a CCD video camera and the quantification of spot numbers was analyzed on a Macintosh computer using the NIH Image program v 1.6.

Statistical analyses

The statistical significance of the tumor protection and survival experiments was determined using the log-rank test. The statistical significance and the error bars for the ELISA and ELISPOT assays were determined using the analysis of variance (ANOVA) test, which determines the mean differences between each group. *P* values less than 0.05 were considered statistically significant.

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