RESEARCH ARTICLE

Recombinant DNA vaccines protect against tumors that are resistant to recombinant vaccinia vaccines containing the same gene

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Antigen-specific cancer immunotherapy involves the delivery of tumor-associated antigen to the host for the generation of tumor-specific immune responses and antitumor effects. We hypothesized that different delivery systems may influence the pattern of antigen-specific immune response and the outcome of antitumor effect. We therefore evaluated recombinant vaccinia virus and naked DNA for the generation of antigen-specific immune responses and antitumor effects. We previously found that recombinant vaccinia and naked DNA vaccines containing the chimeric Sig/E7/LAMP-1 gene were capable of controlling the growth of HPV-16 E7-expressing tumor cells (TC-1). In this study, we performed a head-to-head comparison of optimized delivery of Sig/E7/LAMP-1 vaccinia and DNA vaccines using dose-escalating tumor challenge. At a dose of 1×10^6 TC-1 cells per mouse, Sig/E7/LAMP-1 DNA provided 100% protection against subcutaneous growth of tumors, while Vac-Sig/E7/LAMP-1 protected only 40% of the mice. Furthermore, Sig/E7/LAMP-1 DNA vaccines are capable of protect-

ing against challenge with a more stringent subclone of TC-1 (TC-1 P2) established from TC-1 tumors that survived initial Sig/E7/LAMP-1 vaccinia vaccination. Immunological assays revealed that both vaccines induced comparable levels of CD8⁺ T cell precursors and anti-E7 antibody titers. Interestingly, Sig/E7/LAMP-1 vaccinia induced both E7-specific IFN- γ - and IL4-secreting CD4⁺ T cell precursors while Sig/E7/LAMP-1 DNA induced only E7-specific IFN- γ -secreting CD4⁺ T cell precursors. We also found that IL-4 knockout C57BL/6 mice vaccinated with Sig/E7/LAMP-1 vaccinia exhibited a more potent antitumor effect than vaccinated wild-type C57BL/6 mice in our tumor protection experiments. These results suggest that IL-4 may play a detrimental role in the antitumor effect mediated by vaccinia vaccines. Our findings suggested that DNA vaccines may provide better tumor protection than vaccinia vaccines employing the same gene, which may have implications in the future design of antigen-specific cancer immunotherapy. Gene Therapy (2001) 8, 128–138.

Keywords: vaccinia vaccine; DNA vaccine; HPV-16; E7; cancer vaccine

Introduction

The development of cancer vaccines has continued to show progress in recent years.¹ Immunotherapy has been shown to successfully control tumor growth in many murine tumor model systems. In addition, several phase I/II clinical trials using these vaccine strategies have yielded encouraging results in patients (for review, see Ref. 2). Tumor-specific antigens, when efficiently presented by antigen-presenting cells (APCs) to CD8⁺ cytotoxic T lymphocytes (CTLs) and CD4⁺ helper T (Th) cells, are capable of inducing potent T cell-mediated immunity – the most crucial component of antitumor immunity. The field of cancer immunotherapy is therefore moving towards the development of antigen-specific cancer vaccines, particularly those which enhance both

CD4⁺ and CD8⁺ T cell-mediated immune responses against tumors.

In an attempt to enhance antigen presentation to CD4⁺ T cells, we have previously linked the sorting signals of the lysosome-associated membrane protein-1 (LAMP-1) to the human papillomavirus-16 (HPV-16) E7 antigen, creating the Sig/E7/LAMP-1 chimera. This specific linkage led to the targeting of HPV-16 E7 to the endosomal and lysosomal compartments and enhanced MHC class II presentation of E7 to CD4⁺ T cells.³ Furthermore, we demonstrated that this strategy was not only capable of activating CD4⁺ T helper cells, it also led to enhancement of E7-specific CD8⁺ cytotoxic T cell activity.

The LAMP-1 targeting strategy was further investigated *in vivo* using vaccinia in an E7-expressing tumor model, TC-1. Our results indicated that intraperitoneal vaccination of Sig/E7/LAMP-1-containing recombinant vaccinia virus (Vac-Sig/E7/LAMP-1) can generate strong antitumor immunity against TC-1 inoculated subcutaneously.⁴ Subsequently, we have tested the Vac-Sig/E7/LAMP-1 vaccine in a liver tumor⁵ and lung metastasis model.⁶ Our results indicated that this vaccine was capable of preventing and treating E7-expressing tumors.

More recently, we have extended the LAMP-1 targeting strategy to naked DNA vaccines. We found that the Sig/E7/LAMP-1 DNA vaccine administered via gene gun provided potent protection against the subcutaneous growth of TC-1 cells and eradicated established TC-1 tumors.⁶ We also demonstrated a similarly potent anti-tumor immunity against TC-1 tumor metastases in the liver and lungs.⁵

Since different vector delivery systems may influence the pattern of antigen-specific immune response and the outcome of antitumor effect, it is important to discover which of these systems may lead to the best antitumor effect. While vaccinia vaccines have the advantage of efficient delivery of genes of interest to target cells, repeat vaccination with this delivery system may be inhibited due to antibodies generated against vaccinia or other tolerance effects. On the other hand, DNA vaccines are safe, can be repeatedly administered, and can be expressed over an extended period of time to enhance immunologic memory. The concern about DNA vaccines is their limited potency, an issue which has been addressed using the Sig/E7/LAMP-1 strategy. To determine if the choice of vector delivery system for vaccination may influence vaccine potency, we performed a head-to-head comparison of different vector delivery systems (naked DNA *versus* vaccinia) containing the same gene (Sig/E7/LAMP-1) for their ability to generate E7-specific immune responses and antitumor immunity against dose-escalating challenge with TC-1 tumor cells and also against challenge using a more stringent tumor model, TC-1 P2.

Results

Vaccination with Sig/E7/LAMP-1 DNA generates a stronger antitumor effect compared with vaccination with Sig/E7/LAMP-1 vaccinia

We have previously demonstrated that Sig/E7/LAMP-1 vaccinia can generate a potent antitumor effect against a tumor challenge of 5×10^4 TC-1 cells per mouse.⁴ In addition, we have demonstrated that Sig/E7/LAMP-1 DNA is also capable of generating a potent antitumor effect against a tumor challenge of 5×10^4 TC-1 cells per mouse.^{5,6} In order to conduct a head-to-head comparison of Sig/E7/LAMP-1 DNA and Vac-Sig/E7/LAMP-1, it was necessary to first optimize the dose and vaccination regimen for each vaccine individually. We previously determined that a single vaccination with 1×10^7 p.f.u. per mouse Vac-Sig/E7/LAMP-1 generated the optimum antitumor effect.⁴ Furthermore, we found that single vaccination with Sig/E7/LAMP-1 vaccinia generated better E7-specific CD8⁺ T cell immune response than a prime-booster regimen of Sig/E7/LAMP-1 vaccinia.⁷ In recent studies with the Sig/E7/LAMP-1 naked DNA vaccine, we found that prime and booster of 2 μ g DNA generated the optimum antitumor effect.^{5,6} We also found that vaccination with Sig/E7/LAMP-1 DNA was more effective with a prime-booster regimen than with a single-shot.⁷ We therefore used these optimized vaccination regimens for our head-to-head comparison. We vaccinated mice with either Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 as described in Materials and methods. One week after vaccination, mice were challenged with 5×10^4 TC-1 cells per mouse subcutaneously in the right leg. As shown in Figure 1a, all of the unvaccinated con-

trol mice grew tumors within 3 weeks, whereas all of the mice vaccinated with either Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 remained tumor free 6 weeks after tumor challenge. In comparison, when mice were challenged with 2×10^5 TC-1 cells per mouse subcutaneously in the right leg, Sig/E7/LAMP-1 DNA generated 100% tumor protection for up to 55 days while Vac-Sig/E7/LAMP-1 generated 80% tumor protection after 10 days, falling to 60% after 21 days (Figure 1b). When the challenged tumor dose was increased to 1×10^6 TC-1 cells per mouse, Sig/E7/LAMP-1 DNA still provided 100% tumor protection against the subcutaneous growth of TC-1 tumor. In contrast, Sig/E7/LAMP-1 vaccinia vaccines could provide tumor protection in only 40% of the mice after 10 days (Figure 1c). These results indicated that Sig/E7/LAMP-1 DNA generated a more potent anti-tumor effect against TC-1 compared with Vac-Sig/E7/LAMP-1 in our tumor protection experiments.

Vaccination with either Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 and their therapeutic potential to eradicate established E7-expressing tumors in the lungs
To determine the therapeutic potential of Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 in treating TC-1 tumor metastases in the liver, mice were first challenged with 1×10^4 TC-1 per mouse via intravenous tail vein injection. Three days later, mice were vaccinated as described in Materials and methods. Mice were killed 28 days after tumor challenge. We determined that Sig/E7/LAMP-1 DNA and Vac-Sig/E7/LAMP-1 exhibited a significantly higher therapeutic effect compared with no treatment as assessed by counting the mean number of pulmonary nodules (Figure 2). Sig/E7/LAMP-1 DNA generated a slightly greater therapeutic effect compared with Vac-Sig/E7/LAMP-1, although this difference was not statistically significant. These results indicated that both Sig/E7/LAMP-1 DNA and Vac-Sig/E7/LAMP-1 can generate potent antitumor treatment in the lung metastasis model using TC-1 tumor cells.

Vaccination with Sig/E7/LAMP-1 DNA generates stronger protection against a more stringent tumor model

Our previous immunotherapy studies demonstrated that Vac-Sig/E7/LAMP-1 can effectively prevent and treat HPV-16 E7-expressing tumors (TC-1) in most cases. However, there is sporadic failure of this vaccine in up to 20% of incidents.⁴ Thus, we developed an experimental system to enrich and isolate TC-1 tumor cells that can evade immunotherapy with Vac-Sig/E7/LAMP-1, which can then be used to compare several features of such immunoresistant TC-1 cells with parental cells. As shown in Figure 3, we challenged E7-expressing TC-1 cells into Vac-Sig/E7/LAMP-1 vaccinated mice, and selected the immunoresistant variant that established tumors in these mice. This variant was isolated and then rechallenged into Vac-Sig/E7/LAMP-1 vaccinated mice and the immunoresistant tumor cells were selected. After two cycles of the same procedure, the isolated TC-1 variant was designated as TC-1 P2 and represents a more stringent tumor model.

Since down-regulation of MHC class I molecules or loss of E7 expression may influence the susceptibility of TC-1 P2 to killing by E7-specific T cells, we examined MHC class I expression level using flow cytometry analy-

sis and E7 expression level using Western blot analysis, comparing TC-1 P2 cells with TC-1 cells. As shown in Figure 4a, the level of MHC class I expression was similar for TC-1 P2 cells and original TC-1 cells. To determine the expression level of HPV-16 E7 in TC-1 P2 and original

Figure 1 In vivo tumor protection experiment using a dose-escalating tumor challenge. C57BL/6 mice either received no vaccination or were immunized with Sig/E7/LAMP-1 DNA via gene gun or immunized with Vac-Sig/E7/LAMP-1 intraperitoneally as described in Materials and methods. Three different tumor doses were given 1 week after immunization. (a) In the first experiment, mice were challenged with 5×10^4 TC-1 cells per mouse subcutaneously. (b) In the second experiment, mice were challenged with 2×10^5 TC-1 cells per mouse subcutaneously. (c) In the third experiment, mice were challenged with 1×10^6 TC-1 cells per mouse subcutaneously. In all of these protection experiments, mice were monitored for evidence of tumor growth by visual inspection and palpation twice a week.

Figure 2 E7-expressing DNA vaccines protect mice against the growth of TC-1 tumors in the lungs. C57BL/6 mice were immunized with Sig/E7/LAMP-1 DNA via gene gun or immunized with Vac-Sig/E7/LAMP-1 intraperitoneally as described in Materials and methods. One week after the second vaccination, mice (five per group) were challenged with TC-1 tumor cells via intravenous tail vein injection at a dose of 1×10^4 cells per mouse. Mice were monitored twice a week and were killed at day 28 after tumor challenge. The mean pulmonary nodule numbers ($\pm$ s.e.) shown here are from one representative experiment of two performed.

TC-1 cells, we performed a Western blot analysis and found that the expression level of E7 protein in TC-1 P2 was similar to that of the parent TC-1 (Figure 4b). These results indicated that the TC-1 P2 cells did not down-regulate the MHC-I molecule or lose expression of E7.

To determine whether Sig/E7/LAMP-1 DNA could generate antitumor immunity against TC-1 P2 tumor cells, C57BL/6 mice were vaccinated with Sig/E7/LAMP-1 DNA via gene gun. Mice receiving Vac-Sig/E7/LAMP-1 or no vaccination were used as controls. The mice were then challenged with 5×10^4 TC-1 P2 cells per mouse subcutaneously in the right leg. As shown in Figure 5, Sig/E7/LAMP-1 DNA vaccines generated 60% tumor protection up to 3 months. Although Vac-Sig/E7/LAMP-1 generated 33.3% tumor protection after 1 month, the tumor protection rate fell to 6.7% after 3 months. In contrast, all the unvaccinated mice grew tumors after 3 weeks. These data indicated that the Sig/E7/LAMP-1 DNA vaccine is capable of generating antitumor effects against TC-1 P2 tumor cells that are resistant to Vac-Sig/E7/LAMP-1 vaccine.

Figure 3 Schematic diagram depicting the generation of a more stringent tumor model, TC-1 P2. C57BL/6 mice were first immunized with Vac-Sig/E7/LAMP-1 and then challenged 1 week later with TC-1. About 20% of mice grew tumors. The outgrown TC-1 tumors were explanted, digested with collagenase, and expanded in vitro. The expanded vaccinia vaccine-resistant cell line was named TC-1 P1. The TC-1 P1 tumor cells were challenged to another set of mice previously vaccinated with Vac-Sig/E7/LAMP-1. About 40–60% of the mice grew tumors. The outgrown TC-1 P1 tumors were explanted, digested with collagenase, and expanded in vitro. The expanded vaccinia vaccine-resistant cell line was named TC-1 P2.

Vaccination with Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 generates comparable E7-specific CD8⁺ T cell immunity

To compare the E7-specific CD8⁺ T cell-mediated immune responses induced by Vac-Sig/E7/LAMP-1 and Sig/E7/LAMP-1 DNA, we performed an enzyme-linked immunospot (ELISPOT) assay and intracellular cytokine staining. ELISPOT assays provide a quantitative assessment of the number of activated antigen-specific CD8⁺ T cells.⁸ As shown in Figure 6a, IFN- γ -secreting spot-forming cells observed in mice vaccinated with Vac-Sig/E7/LAMP-1 (around 80/10⁶ splenocytes) appear to be slightly higher than in mice vaccinated with Sig/E7/LAMP-1 DNA (around 60/10⁶ splenocytes), although this difference is not statistically significant. The control (unvaccinated mice) and assays without E7 peptide demonstrated minimal background spots (<5/10⁶ splenocytes). Similarly, E7-specific CD8⁺ T cell precursors can also be determined by flow cytometry analysis using double staining for CD8 and intracellular IFN- γ . As shown in Figure 6b using flow cytometry analysis, mice vaccinated with Vac-Sig/E7/LAMP-1 generated similar numbers of E7-specific CD8⁺ T cell precursors (around 273/10⁶ splenocytes) compared with those obtained from mice vaccinated with Sig/E7/LAMP-1 DNA. Analysis of control mice (unvaccinated) did not reveal a significant number of E7-specific CD8⁺ T cell precursors. These results are consistent with data obtained in previous studies using CTL assays, demonstrating that Vac-Sig/E7/LAMP-1 generated CTL activity comparable to Sig/E7/LAMP-1 DNA.^{3,5}

Vaccination with Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 generates comparable anti-E7 antibody titers

The anti-HPV-16 E7 antibodies in the sera of vaccinated mice were determined by a direct enzyme-linked immu-

Figure 4 Characterization of MHC class I and E7 expression in TC-1 P2 cells. (a) Flow cytometry analysis to demonstrate the relative expression of MHC class I molecules on TC-1 P2 and parental TC-1 cells. Note: the expression level of MHC-I molecules on TC-1 P2 cells was similar to that on parental TC-1. (b) Western blot analysis to demonstrate the relative expression of E7 protein in TC-1 P2 cells and parental TC-1 cells. Note: the E7 protein was detected in both TC-1 P2 and parental TC-1 cells, but not in the control dendritic cells (DC). The expression level of E7 protein was even higher in TC-1 P2 than in parental TC-1.

noabsorbent assay (ELISA) 2 weeks after the last vaccination. The anti-HPV 16 E7 antibodies could be detected in the sera of both Vac-Sig/E7/LAMP-1 and Sig/E7/LAMP-1 DNA vaccinated mice. No significant difference in E7 antibody titers was noted between these two groups (Figure 7). We also determined the isotype of E7-specific antibodies in the sera from these vaccinated mice. An IgG1 predominance was seen in Sig/E7/LAMP-1 DNA vaccinated mice, suggesting a Th2 response. In contrast, a mixed IgG1/IgG2a profile was seen in Vac-Sig/E7/LAMP-1 vaccinated mice, suggesting both a Th1 and Th2 response (data not shown).

Vaccination with Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 generates comparable E7-specific CD4⁺ Th1 type responses but different CD4⁺ Th2 type responses

To compare the E7-specific CD4⁺ T precursor cells and

Figure 5 *In vivo* tumor protection experiment using a more stringent tumor model, TC-1 P2. C57BL/6 mice were immunized as described in Figure 1. Mice were challenged with 5×10^4 TC-1 P2 cells per mouse subcutaneously 1 week after immunization. Mice were monitored for evidence of tumor growth by visual inspection and palpation twice a week. Data shown here are pooled results of two separate trials ($n = 15$ in each group).

the cytokine profiles generated by Vac-Sig/E7/LAMP-1 and Sig/E7/LAMP-1 DNA, we performed double staining for CD4 surface marker and intracellular IFN- γ or IL-4 on splenocytes from immunized mice followed by flow cytometry analysis. The splenocytes from immunized mice were cultured *in vitro* with E7 peptide (aa 30–67) overnight and were stained for both CD4 and intracellular IFN- γ . The E7 peptide (aa 30–67) contains a major T helper epitope in the E7 open reading frame protein of HPV-16.⁹ As shown in Figure 8a, mice vaccinated with Sig/E7/LAMP-1 DNA generated CD4⁺ IFN- γ ⁺ double-positive cells comparable to mice vaccinated with Vac-Sig/E7/LAMP-1. While no significant CD4⁺ IL-4⁺ double-positive cells were identified in mice vaccinated with Sig/E7/LAMP-1 DNA, significant CD4⁺ IL-4⁺ double-positive cells were identified in mice vaccinated with Vac-Sig/E7/LAMP-1 (Figure 8a). Analysis of control mice (unvaccinated) did not reveal a significant number of E7-specific CD4⁺ T cell precursors. The relative frequency of IFN- γ - or IL-4-secreting E7-specific CD4⁺ T cells was plotted in Figure 8b.

The combination of these tests demonstrated that while Vac-Sig/E7/LAMP-1 immunization generated comparable CD8⁺ E7-specific cellular immune responses and IFN- γ -secreting CD4⁺ T cell response as Sig/E7/LAMP-1 DNA, Vac-Sig/E7/LAMP-1 generated a greater IL-4-secreting CD4⁺ T cell response than Sig/E7/LAMP-1 DNA. These results indicated that vaccination with Vac-Sig/E7/LAMP-1 generates higher Th2 type CD4⁺ T cell responses.

IL-4 knockout mice vaccinated with Vac-Sig/E7/LAMP-1 generated a stronger antitumor effect than C57BL/6 mice vaccinated with Vac-Sig/E7/LAMP-1

In our results, we have observed that C57BL/6 mice vaccinated with Sig/E7/LAMP-1 DNA generated a better

antitumor effect than C57BL/6 mice vaccinated with Vac-Sig/E7/LAMP-1 (Figure 1). Furthermore, C57BL/6 mice vaccinated with Vac-Sig/E7/LAMP-1 generated higher levels of E7-specific IL-4-secreting CD4⁺ T cells (Figure 8). To investigate the potential relationship between IL-4 secretion and the antitumor effect generated by these vaccines, we performed a tumor protection experiment using IL-4 knockout mice. IL-4 knockout mice were vaccinated with Sig/E7/LAMP-1 DNA, Vac-Sig/E7/LAMP-1, or were unvaccinated as described in Materials and methods. One week after the last vaccination, mice were challenged with 1×10^6 TC-1 cells per mouse. As shown in Figure 9, 100% of IL-4 knockout mice vaccinated with either Sig/E7/LAMP-1 DNA or vaccinia remained tumor free up to 40 days after tumor challenge. In comparison, Sig/E7/LAMP-1 vaccinia could provide tumor protection in only 40% of the C57BL/6 mice after 10 days (Figure 1c). These results indicated that IL-4 knockout C57BL/6 mice vaccinated with Sig/E7/LAMP-1 vaccinia exhibited a more potent antitumor effect than vaccinated wild-type C57BL/6 mice in our tumor protection experiments.

Discussion

Our results demonstrated that the Sig/E7/LAMP-1 DNA vaccine can generate antitumor immunity against a high dose of TC-1 tumor cells and against TC-1 P2 tumor cells that were resistant to the Vac-Sig/E7/LAMP-1 vaccine. Vac-Sig/E7/LAMP-1 generated E7-specific CD8⁺ T cell activity, CD4⁺ IFN- γ ⁺ T cell activity, and antibody activity comparable to that in Sig/E7/LAMP-1 DNA. However, the vaccinia vaccine also generated CD4⁺ IL-4⁺ T cell precursors and did not generate significant antitumor immunity against TC-1 P2 tumor cells. These data demonstrated that vaccines containing the same gene but

Figure 6 Comparison of CD8 $^+$ T cell-mediated immune responses using ELISPOT and intracellular cytokine staining with flow cytometry analysis. C57BL/6 mice were immunized with Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 vaccines. Splenocytes were harvested 12 days after vaccination. (a) The number of IFN- γ -producing E7-specific CD8 $^+$ T cell precursors was determined using the ELISPOT assay (see text for the detailed method). The spot numbers were the mean of triplicates $\pm$ s.e. in each vaccinated group. Mice vaccinated with Vac-Sig/E7/LAMP-1 generated higher IFN- γ $^+$ spot numbers. Unvaccinated mice were used as a control. Furthermore, ELISPOT assays performed without E7 peptide were also used as a control. Results shown here are E7-specific spot numbers (subtracting the spot numbers without adding the E7 CTL peptide). (b) Splenocytes were cultured *in vitro* with E7 peptide (aa 49–57) overnight and stained for both CD8 and intracellular IFN- γ . The number of IFN- γ secreting CD8 $^+$ T cell precursors was analyzed by flow cytometry. Mice vaccinated with Vac-Sig/E7/LAMP-1 generated higher IFN- γ -secreting E7-specific CD8 $^+$ T cells. The right upper corner indicates the numbers of CD8 $^+$ IFN- γ $^+$ double-positive T cells in 3×10^5 splenocytes. The data of ELISPOT and intracellular cytokine staining shown here are one representative experiment of two performed.

delivered via different vector delivery systems may generate different qualities of immune responses and antitumor effects.

We observed that Vac-Sig/E7/LAMP-1 generated a greater E7-specific IL-4-secreting CD4 $^+$ T cell response compared with Sig/E7/LAMP-1 DNA. Vac-Sig/E7/LAMP-1 also generated an E7-specific IFN- γ -secreting CD4 $^+$ T cell response comparable to that of Sig/E7/LAMP-1 DNA. A higher IL-4 response may have a negative influence on the antitumor immunity of Vac-Sig/E7/LAMP-1 because IL-4 produced by Th2 cells may suppress the generation of Th1 cells,¹⁰ which are important in supporting the activation of CTL.¹¹ Our *in vivo* tumor protection experiment using IL-4 knockout mice was consistent with this notion (Figure 9). Previous studies, however, have diverged as to the true effect of IL-4 on antitumor immune responses. Fu *et al*¹² showed that IL-4 could suppress the antitumor effect of T cells, while Hung *et al*¹³ showed that the antitumor immunity of cytokine-modified cancer cell vaccines was partially lost in IL-4 knockout mice. Schuler *et al*¹⁴ further demonstrated that IL-4 may help in the generation of Th1-associated CTL-mediated tumor immunity. It is possible that different forms of vaccination may induce different subsets and qualities of effector cells for the control of tumors. For example, while GM-CSF-transduced tumor cell-based vaccines may induce eosinophilic infiltration leading to

regression of tumors which relies on the function of IL-4,¹³ DNA or vaccinia vaccines may not rely on IL-4 for their antitumor effect. In fact, IL-4 may actually be detrimental to the antitumor effect generated by vaccinia. Thus, IL-4 may play a different role in different forms of vaccines.

It is also interesting to note that our flow cytometry analysis revealed a substantial population of IL-4-secreting, CD4-negative cells in the splenocytes of mice vaccinated with Vac-Sig/E7/LAMP-1. There are a variety of possible explanations for the expansion of such cells following vaccination with Vac-Sig/E7/LAMP-1. One possibility is that these CD4-/IL4 $^+$ cells are $\gamma\delta$ T cells. It has been suggested that $\gamma\delta$ T cells are involved in establishing primary immune responses.¹⁵ Previous studies have shown that $\gamma\delta$ T cells discriminate between different pathogens early in infection by producing cytokines associated with the appropriate T-helper response. For example, T cells bearing $\gamma\delta$ receptors from mice infected with *Nippostrongylus brasiliensis* have been shown to produce IL-4.¹⁵ Another possibility is that these CD4-/IL4 $^+$ cells are CD4 $^-$ /CD8 $^-$ NKT cells, a subset of IL-4 producing NKT cells.¹⁶ The presence of NKT cells has been postulated to support both Th1 and Th2 cell responses and affect the development of antigen-specific T-regulatory cells involved in peripheral tolerance.¹⁷ Further investigation of IL-4-secreting CD4 $^-$ splenocytes will facilitate the understanding of the mechanism of the antitumor

Figure 7 Comparison of E7-specific antibody response using ELISA. C57BL/6 mice were immunized with Sig/E7/LAMP-1 DNA (DNA) or Vac-Sig/E7/LAMP-1 vaccines (Vac). The mice were bled 2 weeks after the last vaccination. A direct ELISA assay was performed. Results shown here are from one representative experiment of two performed.

effect generated by Vac-Sig/E7/LAMP-1.

Our results indicated that there was no significant difference in the number of E7-specific CD8⁺ T cell precursors generated by vaccination with Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1. Although our assays detected similar numbers of E7-specific CD8⁺ T cells for both vaccines, there may be limitations in our assays for determining the actual quantity and quality of active E7-specific CD8⁺ T cells. It has been shown that vaccinia vaccines may interfere with the presentation of some CTL epitopes of certain antigens.¹⁸ It is therefore possible that CTLs generated by Sig/E7/LAMP-1 DNA were more heterogeneous, reactive to not only the dominant E7 epitope (aa 49–57)¹⁹ but also to various other E7 CTL epitopes. Consequently, using the E7 (aa 49–57) peptide to define E7-specific cellular immunity in the CTL assay and ELISPOT assay may underestimate the actual E7-specific CD8⁺ cellular immune responses generated by the Sig/E7/LAMP-1 DNA vaccine. Furthermore, if TC-1 P2 selectively lost presentation of the E7 (aa 49–57) epitope but retained the ability to present other E7 CTL epitopes, we would also observe a significant difference in the anti-tumor effect generated by the Sig/E7/LAMP-1 DNA vaccine *versus* the Vac-Sig/E7/LAMP-1 vaccine. A loss of CTL epitope presentation may also be caused by single amino acid substitution due to point mutations within the sequences encoding the CTL recognition epitopes.²⁰

We sequenced the E7 gene in TC-1 P2 cells, but found no such mutations (data not shown).

Another potential difference between these vaccines is the quality of CTLs. In particular, the avidity of CTLs generated by Sig/E7/LAMP-1 DNA may be different from that generated by Vac-Sig/E7/LAMP-1. It has been shown that CTLs cultured with exceedingly low-dose peptide generated high-avidity CTLs. In contrast, a higher concentration of peptides generated low-avidity CTLs. Alexander-Miller *et al*²¹ demonstrated that high-avidity CTLs are much more effective at viral clearance than low-avidity CTLs, despite the fact that both high-avidity and low-avidity CTLs can lyse virus-infected targets *in vitro*. This indicates that the *in vitro* CTL assay does not necessarily reflect the ability of *in vivo* viral clearance. This knowledge indicates that although Sig/E7/LAMP-1 DNA and Vac-Sig/E7/LAMP-1 generated comparable *in vitro* CD8⁺ T cell-mediated immune responses, the effectiveness of T cell responses may vary because the CTLs generated by each vaccine may possess varying degrees of avidity.

The different route of administration for Sig/E7/LAMP-1 DNA and Vac-Sig/E7/LAMP-1 vaccines may have influenced the induction of different immune responses. In genetic immunization, it has been suggested that gene gun-mediated DNA vaccination elicits Th2 responses. In contrast, intramuscular injection typically elicits Th1 responses. However, Barry *et al*²² showed that increasing the amount of plasmid delivered via gene gun can shift the Th2 response to Th1. In addition, vaccinia administered through different routes of administration have been shown to generate different types of immune responses in a herpes simplex virus model.²³ Therefore, the difference in antitumor immunity and T helper responses generated by Sig/E7/LAMP-1 DNA and Vac-Sig/E7/LAMP-1 vaccines may be due to the combined effects of different antigen doses and different routes of administration. It is interesting to note that the immune response generated by Sig/E7/LAMP-1 DNA was Th1-like, according to the intracellular cytokine staining (IFN- γ predominance). In contrast, the response was Th2-like according to the antibody isotype (IgG1 predominance). This discrepancy needs further characterization.

The differential effects of Sig/E7/LAMP-1 DNA *versus* Vac-Sig/E7/LAMP-1 on TC-1 P2 tumor cells may be due to differences in the activity of immune effectors other than CD4⁺ and CD8⁺ T cells. Hung *et al*¹³ demonstrated that both eosinophils and macrophages collaborate within the site of tumor challenge to cause tumor cell destruction via the production of both superoxide and nitric oxide. It would be interesting to discover whether such effectors were involved in the antitumor immunity generated by Vac-Sig/E7/LAMP-1 or Sig/E7/LAMP-1 DNA. Another immune effector type is the natural killer (NK) cell. The CpG motif in plasmid DNA has an immunostimulatory effect, possibly causing maturation of dendritic cells,²⁴ inducing Th1 responses²⁵ and NK cell lytic activity.²⁶ NK cells can kill MHC-I-negative tumor cells.²⁷ However, TC-1 P2 cells did not lose MHC class I expression, so the potential role of NK cells in mice vaccinated with Sig/E7/LAMP-1 DNA still remains to be determined.

In this study, we observed no loss of MHC class I expression or E7 expression in TC-1 P2 tumor cells. One

Figure 8 Comparison of CD4⁺ T cell-mediated immune responses using intracellular cytokine staining with flow cytometry analysis. C57BL/6 mice were immunized with Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 vaccines. Splenocytes were harvested 12 days after vaccination. (a) Splenocytes were cultured *in vitro* with E7 peptide (aa 31–60) overnight and were stained for both CD4 and intracellular IFN- γ or IL-4. The number of IFN- γ or IL-4-secreting CD4⁺ T cell precursors was analyzed using flow cytometry. Splenocytes from unvaccinated mice were used as a control. While mice vaccinated with Vac-Sig/E7/LAMP-1 generated IFN- γ -secreting E7-specific CD4⁺ T cells comparable to mice vaccinated with Sig/E7/LAMP-1 DNA, mice vaccinated with Vac-Sig/E7/LAMP-1 generated higher IL-4, CD4 double-positive T cells. (b) The numbers of IFN- γ (filled bar) and IL-4-producing (open bar) antigen-specific CD4⁺ T cells were determined by flow cytometry. Columns are presented from one set of experiments. Similar results were observed in two sets of experiments.

possible mechanism for escape of TC-1 P2 cells from immune effector cells is the mutation of E7. We therefore sequenced PCR-amplified E7 protein and did not find any mutations (data not shown). Other potential mechanisms include expression of Fas ligand,²⁸ loss of TAP-1,^{29,30} decreased expression of some adhesion molecules,³¹

and/or secretion of some immunosuppressive substances.³² Further studies will help elucidate whether such characteristics are responsible for the immune evasion exhibited by TC-1 P2 cells.

In summary, our results indicated that DNA vaccines containing Sig/E7/LAMP-1 generate better tumor pro-

Figure 9 *In vivo* tumor protection experiment using IL-4 knockout mice. C57BL/6 IL-4 knockout mice (five per group) either received no vaccination or were immunized with Sig/E7/LAMP-1 DNA via gene gun or immunized with Vac-Sig/E7/LAMP-1 intraperitoneally as described in Materials and methods. Mice were challenged with 1×10^6 TC-1 cells per mouse subcutaneously. Mice were monitored for evidence of tumor growth by visual inspection and palpation twice a week.

tection than vaccinia vaccines containing the same construct, even using a more stringent tumor model. The discrepancy in the antitumor effect observed between Vac-Sig/E7/LAMP-1 and Sig/E7/LAMP-1 DNA may be attributed to higher IL-4 cytokine production (leading to Th1 suppression), or other possibilities, such as limited recognition of different CTL epitopes, production of CTLs with differing degrees of avidity, and varying production of other immune effector cells against TC-1 P2. The efficacy of antitumor immunity generated by active vaccination is probably influenced by an interaction between the immune effectors and tumor cells that involves some combination of the mechanisms proposed in this study. Continued investigation of these mechanisms will facilitate the design of better antigen-specific cancer immunotherapy in the future.

Materials and methods

Murine tumor cell lines

TC-1: The production and maintenance of TC-1 cells has been described previously.⁴ In brief, HPV-16 E6, E7 and *ras* oncogene were used to transform primary C57BL/6 mice lung epithelial cells. This tumorigenic cell line was named TC-1.

TC-1 P1: Vac-Sig/E7/LAMP-1 vaccinated mice were challenged with TC-1 tumor cells. The Vac-Sig/E7/LAMP-1 failed to protect the growth of TC-1 tumors in 20% of the vaccinated mice. The outgrown TC-1 tumors from the Vac-Sig/E7/LAMP-1 vaccinated mice were explanted, cut into pieces, digested with collagenase, and expanded *in vitro*. These expanded cell lines were called TC-1 P1.

TC-1 P2: Vac-Sig/E7/LAMP-1 vaccinated mice were challenged with TC-1 P1 tumor cells. The outgrown TC-

1 P1 tumors from the Vac-Sig/E7/LAMP-1 vaccinated mice were explanted, cut into pieces, digested with collagenase, and expanded *in vitro*. These expanded cell lines were called TC-1 P2.

TC-1, TC-1 P1, and TC-1 P2 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, tumor cells were harvested by trypsinization, washed twice with 1× Hank's buffered salt solution and finally resuspended in 1× Hank's buffered salt solution to the designated concentration for injection.

MHC-I expression of TC-1 P2 by flow cytometry analysis

TC-1 P2 or TC-1 tumor cells were harvested by trypsinization, washed, and resuspended in FACScan buffer. Anti-H-2K^b/H-2D^b monoclonal antibody (clone 28-8-6; PharMingen, San Diego, CA, USA) was added and incubated for 30 min on ice. After washing with FACS buffer twice, FITC-conjugated goat anti-mouse antibody (Southern Biotechnology, Birmingham, AL, USA) was added and incubated for 20 min on ice. The samples were resuspended in FACScan buffer. Analysis was done on a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA, USA).

Western blot analysis of E7 protein in tumor samples

Proteins were extracted from TC-1 or TC-1 P2 cells with M-PERTM Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). The protein concentration was determined by BSA assay (Bio-Rad, Hercules, CA, USA). Fifty µg protein was separated on 4–20% linear gradient Precast Tris-HCl protein gel (Life Technology, Rockville, MD, USA) and then transferred on to nitrocellulose membrane (Bio-Rad). After blocking, the membrane was hybridized with 1:1000 diluted anti-E7 monoclonal antibody (Zymed, San Francisco, CA, USA). Antibody binding was detected using a peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham, Piscataway, NJ, USA) and chemiluminescence (ECL⁺ detection kit; Amersham).

Preparation of Vac-Sig/E7/LAMP-1 and Sig/E7/LAMP-1 DNA constructs

A large batch of viral stock of Vac-Sig/E7/LAMP-1 was generated as described previously.³ These viral stocks, after being proven to be able to generate antitumor immunity against TC-1,⁴ were preserved at -70°C before vaccination. The preparation of Sig/E7/LAMP-1 DNA vaccine has also been described previously.^{5,6}

Mice

Six- to 8-week-old female C57BL/6 mice from the National Cancer Institute (Frederick, MD, USA) were purchased and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD, USA). C57BL/6 IL-4 knockout mice were kindly provided by Dr Ephraim Fuchs at the Johns Hopkins University. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Vaccination

Previous studies in our laboratory have demonstrated that gene gun immunization of DNA vaccines is the most effective route of administration for the control of tumors (Wu, personal communication). Our studies have also indicated that intraperitoneal vaccination is the most effective route of administration for vaccinia vaccines.

For Vac-Sig/E7/LAMP-1 vaccination, the virus was thawed, sonicated in liquid phase for 30 s, trypsinized with trypsin/EDTA in 37°C water bath for 30 min, and diluted with minimal essential medium containing 2.5% fetal bovine serum to the final concentration of 1×10^8 p.f.u./ml. Each mouse was vaccinated with 10^7 p.f.u. of Vac-Sig/E7/LAMP-1 (0.1 ml of the diluted vaccine) intraperitoneally.

For DNA vaccination, gene gun-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad) according to the protocol provided by the manufacturer as described previously.^{5,6} C57BL/6 mice were immunized with 2 µg Sig/E7/LAMP-1 via gene gun and then boosted 1 week later with the same regimen as the first vaccination.

In vivo tumor protection experiments

C57BL/6 mice and IL-4 knockout mice were vaccinated with Sig/E7/LAMP-1 DNA or Vac-Sig/E7/LAMP-1 as described above. One week after vaccination, C57BL/6 mice were challenged with TC-1 or TC-1 P2 tumor cells at a range of doses (5×10^4 , 2×10^5 , or 1×10^6 cells per mouse) and IL-4 knockout mice were challenged with TC-1 at 1×10^6 cells per mouse by subcutaneous injection in the right leg. In addition, unvaccinated mice received the same amount of TC-1 or TC-1 P2 cells for natural tumor growth control. Tumor growth was monitored by visual inspection and palpation twice weekly.

In vivo tumor regression experiments

Preparation of tumor cells and DNA and vaccinia vaccines was performed as described above. The mice were challenged with TC-1 tumor cells first: 1×10^4 TC-1 cells per mouse for the lung metastasis model^{5,33} (five mice per group) intravenously in the tail vein. Three days after challenge with TC-1 tumor cells, mice were vaccinated with Sig/E7/LAMP-1 DNA (2 µg per mouse) or Vac-Sig/E7/LAMP-1 (1×10^7 p.f.u. per mouse). One week later, the mice receiving DNA were boosted with the same regimen as the first vaccination. Mice were monitored twice a week and were killed at day 28 after tumor challenge. Lungs were removed after mice were killed. The pulmonary metastatic nodules were counted under dissecting microscope. All pulmonary specimens without gross visible tumors were then fixed with 10% neutral buffered formalin, embedded in paraffin and cut into 5 µm sections from different levels. After hematoxylin and eosin staining, the slides were examined using a conventional microscope.

ELISPOT assay

In brief, 96-well filtration plates (Millipore, Bedford, MA, USA) were coated with rat anti-mouse IFN-γ antibody (PharMingen). Fresh isolated spleen cells from each vaccinated mice were added to each well with or without 1 µg/ml E7 aa 49–57 MHC-I peptide¹⁹ and incubated at 37°C for 24 h. After culture, the plate was incubated with biotinylated IFN-γ antibody (PharMingen). The spots

were developed by adding streptavidin-alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA, USA) and BCIP/NBT solution (Boehringer Mannheim, Indianapolis, IN, USA). The spot numbers were counted by computer-assisted quantification as described previously.⁵

Intracytoplasmic cytokine staining

In brief, splenocytes from naive or vaccinated groups of mice were incubated in the presence or absence of the E7 peptide (aa 49–57) that contains the MHC class I epitope¹⁹ or E7 peptide (aa 30–67) that contains the MHC class II peptide.⁹ Cells were stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse CD8 or CD4 antibody (PharMingen) and subjected to intracellular cytokine staining using the Cytotfix/Cytoperm kit according to the manufacturer's instructions (PharMingen). Analysis was performed on the same number of splenocytes (1×10^6) in each flow cytometry analysis using a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System).

ELISA

The anti-HPV-16 E7 antibodies in the sera were determined by a direct ELISA as described previously.^{3,5} The sera were prepared from the mice on day 14 after immunization.

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