RESEARCH ARTICLE

CD8⁺ T cells, NK cells and IFN- γ are important for control of tumor with downregulated MHC class I expression by DNA vaccination

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One of the major hurdles facing cancer immunotherapy is that cancers may downregulate expression of MHC class I molecules. The development of a suitable tumor model with downregulated MHC class I expression is critical for designing vaccines and immunotherapeutic strategies to control such tumors. We developed an E7-expressing murine tumor model with downregulated MHC class I expression, TC-1 P3 (A15). Using this model, we tested DNA and vaccinia vaccines for their ability to control tumors with downregulated MHC class I expression. We found that vaccination with DNA encoding E7 linked to Mycobacterial heat shock protein 70 (HSP70) generated a significant antitumor effect against TC-1 P3 (A15), while vaccination with E7/HSP70 vaccinia did not generate an appreciable

antitumor effect. Lymphocyte depletion experiments revealed that both CD8⁺ T cells and NK cells were essential for the antitumor effect generated by E7/HSP70 DNA against TC-1 P3 (A15). Furthermore, tumor protection experiments using IFN- γ knockout mice revealed that IFN- γ was essential for the antitumor effect generated by E7/HSP70 DNA against TC-1 P3 (A15). Our results demonstrate that vaccination with E7/HSP70 DNA results in a significant antitumor effect against a neoplasm with downregulated MHC class I expression and the importance of CD8⁺ T cells, NK cells, and IFN- γ in generating this antitumor effect. Gene Therapy (2003) 10, 1311–1320. doi:10.1038/sj.gt.3301982

Keywords: DNA vaccine; cancer immunotherapy; HPV; immune evasion

Introduction

Antigen-specific DNA vaccines represent a promising immunotherapeutic approach to enhance T-cell-mediated immune responses against tumors (for review, see Chen and Wu¹). Activated CD8⁺ cytotoxic T cells specifically recognize MHC-I/peptide complexes on tumor cells, resulting in tumor-specific lysis. One concern about the efficacy of immunotherapy is that tumors can evade immune responses through various mechanisms, including downregulation of MHC class I molecules.² A number of human cancer has been shown to downregulate MHC class I expression, including melanoma,³ lung cancer,⁴ prostate cancer,⁵ breast cancer,⁶ ovarian cancer,⁷ colon cancer,⁷ and cervical cancer.⁸ By downregulating MHC class I expression, tumor cells avoid killing by antigen-specific CD8⁺ T cells. A suitable preclinical tumor model with downregulated MHC class I expression is needed to develop vaccines and immunotherapeutic strategies for the control of such tumors.⁹

We have previously demonstrated that Vac-Sig/E7/LAMP-1, a recombinant vaccinia vaccine encoding a signal peptide linked to human papillomavirus type 16 (HPV-16) E7 and the transmembrane and the cytoplasmic domains of the lysosome-associated membrane protein 1 (LAMP-1), enhances MHC class II presentation,¹⁰ improves CD4⁺ and CD8⁺ T-cell activity,¹¹ and generates an antitumor effect against E7-expressing tumors (TC-1) in vaccinated mice.¹² However, approximately 20% of mice treated with Vac-Sig/E7/LAMP-1 eventually develop tumors.¹² We took advantage of this breakthrough tumor to create the TC-1 P3 tumor cell line. We subjected TC-1, an MHC class I-positive E7-expressing tumor model, to three cycles of immunoselection with Vac-Sig/E7/LAMP-1. As a result, TC-1 P3 cells resisted immunotherapy by Vac-Sig/E7/LAMP-1 and demonstrated downregulation of MHC class I expression. Thus, TC-1 P3 represents a suitable tumor model for developing vaccines and immunotherapeutic strategies to control tumors with downregulated MHC class I expression.

It is now clear that effective vaccines should target antigen to professional antigen-presenting cells (APCs) to activate antigen-specific CD4⁺ and CD8⁺ T cells. Intradermal administration of DNA via a gene gun represents an efficient way to deliver DNA vaccines into

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Received 3 September 2002; accepted 11 December 2002

professional APCs *in vivo*. The gene gun approach enables delivery of DNA vaccines into epidermal Langerhans cells, which move into the draining lymph nodes to further activate T cells.¹³ Thus, this delivery method allows for direct targeting of genes of interest into professional APCs *in vivo*. We have successfully used this system to test several intracellular targeting strategies that enhance MHC class I and class II presentation and have generated tumor immunity and therapy.^{11,14–16} Among these strategies, DNA vaccine encoding *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) linked to HPV-16 E7 antigen demonstrated the remarkable ability to activate E7-specific CD8⁺ T-cell-mediated immune responses and antitumor effects.¹⁴

In the current study, we compared the ability of Vac-Sig/E7/LAMP-1, Vac-E7/HSP70, and E7/HSP70 DNA vaccines to generate an antitumor effect against TC-1 and TC-1 P3 (A15). In addition, we evaluated the number of E7-specific CD8⁺ T cell precursors and the avidity of E7-specific CD8⁺ T cells generated by vaccination with E7/HSP70 DNA and Vac-E7/HSP70. We also used *in vivo* antibody depletion to determine the subset of lymphocytes that were essential for the antitumor effect generated by E7/HSP70 DNA against TC-1 or TC-1 P3 (A15). Furthermore, we performed a tumor protection experiment using IFN- γ knockout (KO) mice to determine if IFN- γ was also important in the antitumor effect mediated by E7/HSP70 DNA against TC-1 P3 (A15). Our results demonstrate that the E7/HSP70 DNA vaccine can generate a potent antitumor effect against an E7-expressing tumor model with downregulated MHC class

I expression and that CD8⁺ T cells, NK cells, and IFN- γ play a significant role in the antitumor effects mediated by the E7/HSP70 DNA vaccine.

Results

Immunoselection of TC-1 tumor cells leads to downregulation of MHC class I expression

The TC-1 P3 (A15) tumor cell line was generated as described in Materials and methods. Downregulation of MHC class I expression has been described as a means of immune evasion initiated by certain tumors, a process that reduces antigen presentation to CD8⁺ T cells. To assay for MHC class I expression, TC-1 (P0) cells and TC-1 P3 clones were harvested and prepared for flow cytometry analysis. B16 melanoma cells were used as the negative control. Our data indicated that TC-1 P3 exhibited downregulated MHC class I expression compared to TC-1 (Figure 1a). In addition, we found that TC-1 and TC-1 P3 were MHC class II-negative (data not shown) and exhibited a similar level of E7 expressions (Figure 1b). Flow cytometry analysis was also performed to characterize MHC class I and E7 expression of the TC-1 P3 (A15) clone. Our data indicated that the TC-1 P3 (A15) exhibited distinct downregulation of MHC class I expression relative to TC-1 (Figure 1a) and that the TC-1 P3 (A15) clone expressed the E7 antigen at a level slightly higher than TC-1 (Figure 1b).

Figure 1 Flow cytometry analysis to demonstrate MHC class I and E7 expressions of TC-1, TC-1 P3, and TC-1 P3 (A15) clones. Flow cytometry analysis was performed to characterize MHC class I and E7 expressions on TC-1 (P0) tumor cells, TC-1 P3 tumor cells, and TC-1 P3 (A15) tumor cell clone. (a) The figures depict MHC class I expression of parental TC-1 (P0), TC-1 P3 tumor cells, and TC-1 P3 (A15) tumor cell clone. TC-1 cells are MHC class I positive, while TC-1 P3 and TC-1 P3 (A15) tumor cell clone exhibit downregulated MHC class I expression. A representative P3 clone, TC-1 P3 (A15), was expanded for further studies. Note: The clear histogram represents MHC class I expression of TC-1(P0), while the filled histogram represents MHC class I expression of TC-1 P3 or TC-1 P3 (A15). Staining without primary antibody was used as a negative control. (b) The figures depict E7 expression of parental TC-1 (P0) and TC-1 P3 (A15) tumor cell clone. B16 was used as a negative control. Both the TC-1 and the TC-1 P3 (A15) tumor cells are E7 positive, while B16 does not express E7. Note: The clear histogram represents negative control (no E7 expression), while the filled histogram represents E7 expression of B16, TC-1, or TC-1 P3 (A15).

E7/HSP70 DNA is more effective than Vac-E7/HSP70 or Vac-Sig/E7/LAMP-1 for generating an antitumor effect against TC-1 P3 (A15)

Since most human cancers demonstrate downregulation of MHC class I molecules, TC-1 P3 (A15) may potentially represent a suitable tumor model for testing vaccine strategies that can control tumors with downregulated MHC class I. We have previously linked *M. tuberculosis* HSP70 to E7 in a DNA vaccine and found that it enhanced E7-specific CD8⁺ T-cell immune responses and generated a potent antitumor effect against TC-1.¹⁴ We vaccinated C57BL/6 mice with Vac-Sig/E7/LAMP-1, Vac-E7/HSP70, E7/HSP70 DNA and challenged them with TC-1 or TC-1 P3 (A15) tumor cells to determine whether these vaccines could control tumors with MHC class I downregulation. We found that vaccination with E7/HSP70 DNA or Vac-E7/HSP70 protected 100% of mice up to 90 days after TC-1 tumor challenge. Meanwhile, Vac-Sig/E7/LAMP-1 protected only 80% of mice and all unvaccinated mice developed tumors by day 10 after TC-1 tumor challenge (Figure 2a). We then investigated the antitumor effect generated by each of these vaccines following TC-1 P3 (A15) tumor challenge. Vaccination of mice with E7/HSP70 DNA generated the most potent protection against TC-1 P3 (A15) tumors with 80% of mice remaining free of tumors up to 90 days after tumor challenge (Figure 2b). Naïve mice and mice vaccinated with Vac-Sig/E7/LAMP-1 or Vac-E7/HSP70 all developed tumors within 21 days of tumor challenge. Our results indicated that the TC-1 P3 (A15) tumor cell line is more difficult to control than TC-1 tumor cells. In addition, we found that E7/HSP70 DNA was a more effective vaccine than Vac-E7/HSP70 or Vac-Sig/E7/LAMP-1 for generating an antitumor effect against an E7-expressing tumor cell line with downregulated MHC class I expression. This observation is consistent with our finding in a previous study that DNA vaccines can

protect against tumors that are resistant to vaccinia vaccines containing the same gene.¹⁷

Vac-E7/HSP70 or E7/HSP70 DNA generates a comparable number of E7-specific CD8⁺ T-cell precursors in vaccinated mice

To determine if the difference in antitumor effect generated by Vac-E7/HSP70 and E7/HSP70 DNA was related to the number of E7-specific CD8⁺ T-cell precursors generated by each vaccine, we investigated vaccinated mice for the generation of E7-specific CD8⁺ T-cell precursors using intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 3, we found that mice vaccinated with Vac-E7/HSP70 and E7/HSP70 DNA generated a comparable number of E7-specific IFN- γ ⁺ CD8⁺ T-cell precursors (325 ± 20 and 315 ± 18 per 3.5×10^5 splenocytes, respectively). Thus, there was no significant difference in the number of E7-specific IFN- γ ⁺ CD8⁺ T-cell precursors generated by vaccination with Vac-E7/HSP70 or E7/HSP70 DNA.

Vaccination with E7/HSP70 DNA generates higher avidity E7-specific CD8⁺ T cells than vaccination with Vac-E7/HSP70

Studies have revealed that high-avidity T cells generate stronger antigen-specific antitumor effects than low-avidity T cells.^{18,19} Characterization of antigen-specific T-cell avidity can be achieved using soluble peptide-MHC tetramers.²⁰ We therefore determined the avidity of E7-specific CD8⁺ T cells generated by vaccination with either Vac-E7/HSP70 or E7/HSP70 DNA using E7 (aa 49–57) peptide-H2-D^b tetramer as described in Materials and methods. Splenocytes were isolated from mice and incubated with graded dilutions of E7 (aa 49–57) peptide/H2-D^b tetramer followed by anti-CD8

Figure 2 In vivo tumor protection experiments using TC-1 or TC-1 P3 (A15) tumor cells in mice vaccinated with various vaccines. C57BL/6 mice were immunized with DNA or vaccinia vaccines as described in Materials and methods. (a) Mice were challenged with 5×10^4 TC-1 cells 1 week after immunization with Vac-Sig/E7/LAMP-1, Vac-E7/HSP70, or E7/HSP70 DNA and monitored for evidence of tumor growth by visual inspection and palpation twice a week. (b) Mice were challenged with 5×10^4 TC-1 P3 (A15) cells subcutaneously 1 week after immunization with Vac-Sig/E7/LAMP-1, Vac-E7/HSP70, or E7/HSP70 DNA. Note: While E7/HSP70 DNA generated 80% protection against TC-1 P3 (A15) tumor challenge, none of the other vaccines generated protection against TC-1 P3 (A15) tumor challenge.

Figure 3 Flow cytometry analysis to determine the number of E7-specific CD8⁺ T-cell precursors generated by vaccination with Vac-E7/HSP70 or E7/HSP70 DNA. Splenocytes from mice vaccinated with Vac-E7/HSP70 or E7/HSP70 DNA were incubated in the presence or absence of the E7 peptide (aa 49–57) containing the MHC class I epitope and stained for CD8 marker and IFN- γ as described in Materials and methods to enumerate E7-specific CD8⁺ T cell precursors. (a) Representative data from flow cytometry analysis. (b) Histogram demonstrating the number of E7-specific CD8⁺ T-cell precursors in vaccinated mice. Results are from one representative experiment of two performed.

staining and flow cytometry analysis. We used 200 nM E7 (aa 49–57) peptide-H2-D^b tetramer as the starting concentration. Figure 4a provides representative data of the flow cytometry analysis using E7 (aa 49–57) peptide/H2-D^b tetramer. The number of tetramer-bound E7-specific CD8⁺ T cells is indicated in the upper right corner. Figure 4b depicts the percentage of the number of tetramer-positive CD8⁺ T cells at each dilution relative to the number of the tetramer-positive T cells in the undiluted sample (maximum response). With increasing dilutions of E7 (aa 49–57) peptide/H2-D^b tetramer, E7-specific CD8⁺ T cells from mice vaccinated with E7/HSP70 DNA exhibited a higher degree of tetramer binding than E7-specific CD8⁺ T cells from mice vaccinated with Vac-E7/HSP70. These data indicated that vaccination with E7/HSP70 DNA generated higher avidity E7-specific CD8⁺ T cells than vaccination with Vac-E7/HSP70.

Different subsets of lymphocytes are important for the antitumor effect against TC-1 or TC-1 P3 (A15) tumor cells mediated by E7/HSP70 DNA vaccine

To determine the subset of lymphocytes that are important for the antitumor effect generated by E7/HSP70 DNA against TC-1 or TC-1 P3 (A15) tumor cells, we performed *in vivo* antibody depletion experiments. CD4, CD8, and NK1.1 depletions next initiated 1 week prior to TC-1 or TC-1 P3 (A15) tumor challenge in E7/HSP70 DNA-vaccinated mice. As shown in Figure 5a, all CD8-depleted mice grew tumors within 10 days of TC-1

tumor challenge. All undepleted mice and mice depleted of CD4 or NK cells remained tumor-free up to 90 days after tumor challenge. In comparison, in an experiment using mice challenged with TC-1 P3 (A15), all CD8-depleted mice and 80% of NK-depleted mice developed tumors (Figure 5b). Meanwhile, 80% of undepleted mice and CD4-depleted mice remained tumor free at day 90. These results indicated that while CD8⁺ T cells were essential for the antitumor effect generated by the E7/HSP70 DNA vaccine against TC-1, both CD8⁺ T cells and NK cells were important for the antitumor effect mediated by E7/HSP70 DNA against TC-1 P3 (A15).

IFN- γ upregulates MHC class I expression on tumor cells and is required for protection against TC-1 P3 (A15) tumor challenge mediated by E7/HSP70 DNA

IFN- γ has been shown to upregulate the expression of MHC class I molecules.²¹ To determine whether IFN- γ stimulation can alter MHC class I expression on TC-1 and TC-1 P3 (A15) tumor cell lines, we examined the MHC class I expression of tumor cells after 12, 24, 48, 72, 96, and 120 h of IFN- γ stimulation or without IFN- γ stimulation. Cells were prepared for flow cytometry analysis. We found that while IFN- γ stimulation led to only a slight increase in MHC class I expression on the surface of TC-1 tumor cells, IFN- γ stimulation led to a significant increase in the level of MHC class I expression on TC-1 P3 (A15) tumor cells as early as 12 h after IFN- γ stimulation (comparable to the level on TC-1) (Figure 6a and b). The peak level of MHC class I expression on TC-1 P3 (A15) cells was reached by 48 h after IFN- γ stimulation. These results suggested that MHC class I expression on TC-1 P3 (A15) tumor cells could be upregulated with IFN- γ stimulation.

To determine if IFN- γ was required for tumor protection generated by E7/HSP70 DNA against TC-1 P3 (A15), normal C57BL/6 mice or IFN- γ KO C57BL/6 mice were immunized with E7/HSP70 DNA as described in Materials and methods. Naive mice were used as a negative control. Our data demonstrated that vaccination with E7/HSP70 DNA protected 80% of mice against the formation of TC-1 P3 (A15) tumors up to 90 days after tumor challenge (Figure 6c). In contrast, all IFN- γ KO C57BL/6 mice vaccinated with E7/HSP70 developed tumors within 10 days of tumor challenge. These results demonstrated that IFN- γ was an essential component of the protective antitumor effect generated by E7/HSP70 DNA against TC-1 P3 (A15) tumor challenge.

Discussion

Effective control of cancer requires strategies to overcome immune evasion by tumors. As a model of immune evasion, we generated the TC-1 P3 (A15) tumor cell line, which both evades immunotherapy and downregulates MHC class I expression. Our results demonstrated that vaccination with E7/HSP70 DNA was able to generate high-avidity E7-specific CD8⁺ T cells and a potent antitumor effect against TC-1 P3 (A15) tumors. We also found that IFN- γ could enhance MHC class I expression on TC-1 P3 (A15) tumor cells and that the antitumor effect generated by E7/HSP70 DNA was CD8 T cell-, NK cell-, and IFN- γ -dependent. These findings suggest that

Figure 4 Flow cytometry analysis to determine the avidity of E7-specific CD8⁺ T cells generated by vaccination with Vac-E7/HSP70 or E7/HSP70 DNA. To determine the avidity of E7-specific CD8⁺ T cells generated following vaccination with E7/HSP70 DNA or Vac-E7/HSP70, splenocytes from mice were incubated with different concentrations of E7 (aa 49-57) peptide/H2-D^b tetramer (200, 2, 1, 0.25 nM) and stained for CD8 marker as described in Materials and methods. We used 200 nM E7 (aa 49-57) peptide-H2-D^b tetramer as the starting concentration. (a) Representative data from flow cytometry analysis. (b) Number of tetramer-positive CD8⁺ T cells at each concentration as a percentage of the maximum number of tetramer-positive T cells (undiluted sample).

Figure 5 In vivo antibody depletion of subsets of lymphocytes following E7/HSP70 DNA vaccination and TC-1 or TC-1 P3 (A15) tumor challenge. (a) Mice were immunized with E7/HSP70 DNA and challenged with 5×10^4 TC-1 cells/mouse subcutaneously. CD4, CD8, and NK1.1 depletions were initiated 1 week prior to tumor challenge. Note: All mice depleted of CD8⁺ T cells grew tumors within 10 days of tumor challenge, while all undepleted mice and all mice depleted of CD4⁺ T cells or NK cells remained tumor free up to 90 days after tumor challenge. (b) Mice were immunized with E7/HSP70 DNA and challenged with 5×10^4 TC-1 P3 (A15) cells/mouse subcutaneously. CD4, CD8, and NK1.1 depletions were initiated 1 week prior to tumor challenge. Note: All mice depleted of CD8⁺ T cells grew tumors within 17 days of tumor challenge. In total, 80% of undepleted mice and mice depleted of CD4⁺ T cells were tumor free at day 90, while only 20% of mice depleted of NK cells were tumor free at day 90. Data from in vivo antibody depletions of subsets of lymphocytes are from one representative experiment of two performed.

Figure 6 The role of IFN- γ on MHC class I expression and antitumor effect against TC-1 P3 (A15). Flow cytometry analysis was performed to determine MHC class I expression on (a) TC-1 and (b) TC-1 P3 (A15) tumor cell lines after IFN- γ stimulation (1 μ g/ml). Tumor cells were examined without IFN- γ stimulation or after 12, 24, 48, 72, 96, and 120 h with IFN- γ stimulation. Note: IFN- γ stimulation led to upregulation of MHC class I expression on the surface of TC-1 tumor cells as early as 12 h after stimulation. Meanwhile, IFN- γ stimulation caused a shift in TC-1 P3 (A15) tumor cells from lack of MHC class I expression to a level comparable to that of TC-1 24 h after stimulation. (c) Tumor protection experiment. Normal C57BL/6 mice or IFN- γ KO C57BL/6 mice were immunized with E7/HSP70 DNA as described in Materials and methods. Mice were challenged with 5×10^4 TC-1 P3 (A15) cells subcutaneously 1 week after immunization and monitored as described in Figure 2a. Note: E7/HSP70 DNA generated strong protection against TC-1 P3 (A15) tumor challenge (80% tumor-free mix) in C57BL/6 mice. In contrast, E7/HSP70 DNA did not generate a protective antitumor effect against TC-1 P3 (A15) in IFN- γ KO C57BL/6 mice. Results are from one representative experiment of two performed.

E7/HSP70 DNA may mediate a variety of processes to eradicate E7-expressing tumor cells with downregulated MHC class I expression, including generation of high-avidity E7-specific CD8⁺ T cells, recruitment of different effector cells, and cytokine-induced modification of tumor cells.

Our study demonstrated that vaccination with E7/HSP70 DNA generated a more potent antitumor effect than vaccination with E7/HSP70 vaccinia, even though there was no significant difference in the number of E7-specific CD8⁺ T-cell precursors generated by each vaccine. Although there were no observable quantitative differences in the T-cell response, qualitative attributes of E7-specific CD8⁺ T cells may have contributed to the difference in the observed antitumor effect. We found that vaccination with E7/HSP70 DNA generated higher avidity E7-specific CD8⁺ T cells than vaccination with E7/HSP70 vaccinia (Figure 4b). Recent studies have shown that T-cell-mediated immunity evolves through

selective expansion of T cells with higher TCR affinity for antigen.^{22,23} This so-called functional avidity maturation may occur through cross-linking of receptors,²⁴ activation-induced cholesterol enrichment of membranes,²⁴ or changes in signal transduction.^{22,25} Studies have revealed that higher avidity T cells generate stronger antigen-specific antiviral^{26,27} or antitumor^{9,18,19} effects. Derby *et al*²⁷ observed that following viral infection, high-avidity CTL mediate earlier antigen recognition because of higher sensitivity to antigen than low-avidity T cells and initiate antigen-specific lysis more rapidly than low-avidity T cells. Thus, high-avidity CTL generated by vaccination with E7/HSP70 DNA may contribute to the killing of tumors with low MHC class I expression.

Our *in vivo* antibody depletion experiments revealed that control of TC-1 and TC-1 P3 (A15) tumor cells required different subsets of lymphocytes (Figure 5a and b). We found that CD8⁺ T cells, but not CD4⁺ T cells or

NK cells, were essential to the antitumor effect generated by E7/HSP70 DNA vaccine against TC-1 tumor cells. In contrast, both CD8⁺ T cells and NK cells were essential for the E7/HSP70 DNA-mediated antitumor effect against TC-1 P3 (A15). These data are consistent with the finding that reduced MHC class I expression or impaired antigen processing prevents recognition of tumor cells by CTLs, but may target them for NK cell attack,²⁸ since NK cells are able to kill MHC-I-negative tumor cells.²⁹ Also, HSP70 has been shown to enhance proliferation and cytolytic activity of NK cells.³⁰ Thus, the generation of potent immune responses against tumors with downregulated MHC class I expression may require the activity of both CD8⁺ T cells and NK cells.

Our study demonstrated that IFN- γ was required for the generation of a potent antitumor effect against TC-1 P3 (A15). We found that the potency of the E7/HSP70 DNA vaccine in normal C57BL/6 mice was lost in IFN- γ KO C57BL/6 mice (Figure 6c). These results are in accordance with those from previous studies demonstrating the importance of IFN- γ for mediating a potent antitumor effect.^{31,32} Our results also indicated that vaccination with E7/HSP70 DNA enhanced the generation of IFN- γ -secreting E7-specific CD8⁺ T cells¹⁴ (also see Figure 3) and that IFN- γ may help overcome MHC class I downregulation and restore normal MHC class I expression (Figure 6a and b). These results are consistent with previous studies, which demonstrated that IFN- γ can increase MHC class I expression on tumor cells²¹ and restore MHC class I expression that was downregulated in oncogene-transformed cells.³³

Differences in cytokine production also provide an explanation for the differences observed in the antitumor effect mediated by vaccination with E7/HSP70 DNA or E7/HSP70 vaccinia. Plasmid DNA may contain immunostimulatory elements known as CpG islands, which have been shown to contribute to the induction of certain Th1 cytokines such as IFN- γ and IL-12 and enhance antigen-specific immunity.³⁴ CpG islands can also trigger maturation and activation of dendritic cells,³⁵ induce Th1 responses,³⁶ and induce NK cell lytic activity.³⁷ Meanwhile, vaccinia virus secretes soluble receptors for IFN- γ ^{38,39} or other cytokines^{40,41} that may counteract the immune response. Further studies would help elucidate the relative influence of these vaccine characteristics on the antitumor effect.

While MHC class I downregulation represents one mechanism of immune evasion,^{2,42} tumor cells can also undergo a variety of other mechanisms to evade host defenses. Tumors may downregulate other surface molecules, such as MHC class II⁴³ or costimulatory molecules,⁴⁴ to elude immune effector cells. Defects in the antigen-processing machinery of tumor cells play a role in the escape of tumors from recognition by T cells.⁴⁵ For example, deficiency in TAP allows tumors to evade immune surveillance.^{28,46} Another potential evasion method is expression of Fas ligand on tumor cells, which can induce apoptosis of T cells.^{47–49} Other mechanisms of immune evasion include altered expression of adhesion molecules⁵⁰ and dysregulation of cytokine expression (ie secretion of immunosuppressive cytokines).⁵¹ To obtain a better understanding of immune evasion, it will be important in the future

to assess the characteristics of TC-1 P3 tumor cells other than MHC class I downregulation that may be responsible for immune evasion, to develop other tumor cell lines that evade the immune system through the aforementioned mechanisms, and to design vaccine strategies to overcome these evasion mechanisms.

In summary, our results indicate that E7/HSP70 DNA can generate an impressive antitumor effect against HPV-16 E7-expressing murine tumors with downregulated MHC class I expression. We found that CD8⁺ T cells, NK cells, and IFN- γ played an important role in mediating this antitumor effect. Thus, the preclinical tumor model that we have generated (TC-1 P3) represents a suitable model for investigating mechanisms of immune evasion and developing immunotherapeutic strategies to target cancers that evade the immune system.

Materials and methods

Generation of TC-1 P3 (A15) tumor cell line

The production and maintenance of TC-1 cells have been described previously.¹² Vac-Sig/E7/LAMP-1-vaccinated mice were challenged with TC-1 tumor cells. Vaccination with Vac-Sig/E7/LAMP-1 elicits E7-specific antitumor responses against HPV-16 E7-expressing tumors (TC-1), although the vaccine fails to prevent tumor formation in approximately 20% of the vaccinated mice.¹² The outgrown TC-1 tumors from Vac-Sig/E7/LAMP-1-vaccinated mice were explanted, cut into pieces of less than 1 mm in diameter, digested with collagenase at a concentration of 1 mg/ml in DMEM (GIBCO BRL, Rockville, MD, USA), and expanded *in vitro*. These expanded cell lines were called TC-1 P1. Vac-Sig/E7/LAMP-1-vaccinated mice were then challenged with TC-1 P1 tumor cells. Approximately 40% of vaccinated mice developed tumors (data not shown). The outgrown tumors from these vaccinated mice were then explanted and expanded *in vitro* to create the TC-1 P2 tumor cell line. Vac-Sig/E7/LAMP-1-vaccinated mice were then challenged with TC-1 P2 tumor cells. This time approximately 60–80% of vaccinated mice developed tumors (data not shown). The outgrown tumors from these vaccinated mice were further explanted and expanded *in vitro* to generate the TC-1 P3 tumor cell line.⁵⁰ TC-1 P3 clones were generated by limiting dilution. Among the TC-1 P3 clones, a representative clone with marked downregulation of MHC class I expression was isolated and expanded, creating the TC-1 P3 (A15) tumor cell line. Both TC-1 and TC-1 P3 (A15) cells were grown in RPMI 1640, supplemented with 10% (vol/vol) fetal bovine serum, 50 U/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 × Hanks buffered salt solution (HBSS) and finally resuspended in 1 × HBSS to the designated concentration for injection.

Mice

Female C57BL/6 mice, 6–8 weeks old, were purchased from the National Cancer Institute (Frederick, MD, USA)

and stored in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD, USA). IFN- γ KO mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Flow cytometry analysis to determine MHC class I, MHC class II, and E7 expression of TC-1 and TC-1 P3 (A15) tumor cells

TC-1 or TC-1 P3 (A15) tumor cells were harvested by trypsinization, washed, and resuspended in FACSCAN buffer. To detect MHC class I or MHC class II expression, anti-H-2K^b/H-2D^b (clone 28-8-6) or anti-I-A^b (clone 25-9-17) (PharMingen, San Diego, CA, USA) monoclonal antibody was used, respectively. In addition, to detect HPV-16 E7 expression, cells were fixed in 10% buffered formalin for 20 min followed by staining with 1 μ g/ml of E7-specific mouse-derived monoclonal Ab (Zymed, San Francisco, CA, USA). After incubation with primary antibody, cells were stained with FITC-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA). Staining without primary antibody was used as negative control. Analysis was done on a Becton-Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA, USA). In a separate experiment, MHC class I expression of TC-1 or TC-1 P3 (A15) tumor cells was assessed at various time points with IFN- γ stimulation. MHC class I expression was assessed by flow cytometry analysis as described above without IFN- γ stimulation or with IFN- γ stimulation for 12, 24, 48, 72, 96, or 120 h at a concentration of 1 μ g/ml.

Preparation of vaccinia and DNA constructs

Recombinant Sig/E7/LAMP-1 vaccinia was generated as described previously.¹⁰ E7/HSP70 vaccinia was generated using a similar method. Briefly, the E7/HSP70 DNA fragment was first isolated from pcDNA3-E7/HSP70¹⁴ and cloned into the *EcoRI*/*HindIII* sites of pGEM II vector. The E7/HSP70 DNA fragment was then isolated from pGEM II-E7/HSP70 and cloned into the *SphI*/*HindIII* sites of pSC11MCS2 vector. Recombinant vaccinia viruses were selected and expanded following the protocol of Earl and Moss.^{52–54} The E7 expression of recombinant E7/HSP70 vaccinia was characterized by Western blot analysis (data not shown). Vaccinia viral stocks were preserved at -70°C prior to vaccination. The preparation of pcDNA3-E7, pcDNA3-HSP70, and pcDNA3-E7/HSP70 DNA has also been described previously.¹⁴

Vaccination of mice

For Vac-Sig/E7/LAMP-1 or Vac-E7/HSP70 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 PFU/ml. C57BL/6 mice (five per group) were vaccinated with 10^7 PFU/mouse of Vac-Sig/E7/LAMP-1 or Vac-E7/HSP70 vaccinia intraperitoneally.

Gene gun-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad) according to

the manufacturer's protocol as described previously.¹¹ C57BL/6 mice (five per group) were immunized with 2 μ g of pcDNA3-E7 DNA, pcDNA3-HSP70 DNA, pcDNA3-E7/HSP70 DNA, or empty plasmid via a gene gun and then boosted 1 week later with the same regimen as the first vaccination.

In vivo tumor protection experiments

In the first set of experiments, C57BL/6 mice (five per group) were vaccinated with pcDNA3-E7/HSP70 DNA, Vac-Sig/E7/LAMP-1, or Vac-E7/HSP70 as described above. After 1 week of the last vaccination, mice were challenged with 5×10^4 TC-1 or TC-1 P3 (A15) tumor cells by subcutaneous injection in the right leg. In a second set of experiments, C57BL/6 mice (five per group) were vaccinated with pcDNA3-E7 DNA, pcDNA3-HSP70 DNA, pcDNA3-E7/HSP70 DNA, or empty plasmid (second experiment) as described above. At 1 week after the last vaccination, mice were challenged with 5×10^4 TC-1 P3 (A15) tumor cells subcutaneously in the right leg. In a third set of experiments, normal C57BL/6 mice (five per group) or IFN- γ KO C57BL/6 mice (five per group) were immunized with 2 μ g of E7/HSP70 DNA and then boosted 1 week later with the same regimen as the first vaccination. Mice were challenged with 5×10^4 TC-1 P3 (A15) tumor cells subcutaneously in the right leg 1 week after the last vaccination. For all experiments, naive mice received the same amount of TC-1 or TC-1 P3 (A15) cells to assess natural tumor growth control. Tumor growth was monitored by visual inspection and palpation twice weekly.

In vivo tumor treatment experiments

Preparation of tumor cells, DNA vaccines, and vaccinia vaccines was performed as described above. Mice (five per group) were challenged with 1×10^4 TC-1 P3 (A15) tumor cells/mouse intravenously via the tail vein using a previously described lung metastasis model.^{55,56} At 3 days after tumor challenge, mice were treated with 2 μ g of E7 DNA, HSP70 DNA, E7/HSP70 DNA, or empty plasmid. Mice were boosted with the same regimen as the first vaccination, 1 week later. Mice were killed at day 28 after tumor challenge. After removal of lungs, pulmonary tumor nodules on the surface of the lung were counted under a dissecting microscope. Pulmonary specimens without grossly visible tumors were fixed with 10% neutral-buffered formalin, embedded in paraffin and cut into 5 μ m sections from different levels, followed by hematoxylin and eosin staining for examination under a conventional light microscope.

Intracytoplasmic cytokine staining followed by flow cytometry analysis

Splenocytes from naive or vaccinated groups of mice were incubated in the presence or absence of E7 peptide (aa 49–57) containing the MHC class I epitope.⁵⁷ Cells were stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse CD8 antibody (PharMingen) and subjected to intracellular cytokine staining using the Cytotfix/Cytoperm kit according to the manufacturer's instructions (PharMingen, San Diego, CA, USA). Analysis was done using a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson

Immunocytometry System). A total of 3.5×10^5 splenocytes were analyzed and the number of CD8 and IFN- γ double-positive cells represented T-cell precursors.

Tetramer staining to determine avidity of E7-specific CD8⁺ T cells

PE-labeled E7 (aa 49–57) peptide-H2-D^b tetramer was provided by the Tetramer Core Facility at NIH. We used 200 nM E7 (aa 49–57) peptide-H2-D^b tetramer as the starting concentration. Briefly, splenocytes from naive or vaccinated groups of mice were incubated with PE-labeled E7 (aa 49–57) peptide-H2-D^b tetramer at various concentrations (20, 2, 1, 0.25 nM). Cells were then stained with monoclonal rat anti-mouse CD8 antibody (PharMingen, San Diego, CA, USA) and analyzed using a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System).

In vivo antibody depletion experiment

In vivo antibody depletions have been described previously.¹² Briefly, C57BL/6 mice (five per group) were vaccinated with 2 μ g of E7/HSP70 DNA via gene gun, boosted 1 week later, and challenged with 5×10^4 cells/mouse TC-1 or TC-1 P3 (A15) tumor cells subcutaneously 1 week after the last vaccination. Depletions were started 1 week prior to tumor challenge. MAb GK1.5 was used for CD4 depletion, MAb 2.43 was used for CD8 depletion, and MAb PK136 was used for NK1.1 depletion. The completeness of depletion was checked by flow cytometry analysis. For each time point of analysis, >99% depletion of the appropriate subset was achieved with normal levels of the other subsets. Depletion was terminated on day 90 after tumor challenge.

Acknowledgements

We thank Drs Robert Kurman, Drew Pardoll, and Hyam Levitsky for helpful discussions. We also thank Drs Richard Roden and Ralph Hruban for a critical review of the manuscript. We thank The Tetramer Core Facility at NIH for providing PE-labeled E7 (aa 49–57) peptide-H2-D^b tetramer. Dr Wen-Fang Cheng (RE88P005) is a recipient of the NHRI physician scientist fellowship award. Dr Cheng is supported by National Science Committee of Taiwan (NSC91-2341-B-002-369) and National Taiwan University Hospital (NTUH 91-F002). Ken-Yu Lin is a Howard Hughes predoctoral fellow.

This work was supported by NIH 5 PO1 34582-01, U19 CA72108-02, RO1 CA72631-01, RO1 CA83706-01, PO1A148203-01, and American Cancer Society.

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