Cancer Immunotherapy Using a DNA Vaccine Encoding the Translocation Domain of a Bacterial Toxin Linked to a Tumor Antigen

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ABSTRACT

Certain domains of bacterial toxins have been shown to facilitate translocation from extracellular and vesicular compartments into the cytoplasm. This feature represents an opportunity to enhance class I presentation of exogenous antigen to CD8+ T cells. We investigated this notion by creating a novel fusion of the translocation domain (domain II) of *Pseudomonas aeruginosa* exotoxin A (ETA(dII)) with a model tumor antigen, human papillomavirus type 16 E7, in the context of a DNA vaccine. Our *in vitro* studies indicated that cells transfected with ETA(dII)/E7 DNA or dendritic cells pulsed with lysates containing ETA(dII)/E7 protein exhibited enhanced MHC class I presentation of E7 antigen. Vaccination of mice with ETA(dII)/E7 DNA generated a dramatic increase in the number of E7-specific CD8+ T cell precursors (~30-fold compared with wild-type E7 DNA) and converted a less effective DNA vaccine into one with significant potency against human papillomavirus type 16 E7-expressing murine tumors via a CD8-dependent pathway. These results indicate that fusion of the translocation domain of a bacterial toxin to an antigen may greatly enhance vaccine potency.

INTRODUCTION

CTLs are critical effectors of antitumor responses (reviewed in Refs. 1–3). Activated CTLs function directly as effector cells, providing antitumor immunity through the lysis of tumor cells or through the release of cytokines capable of interfering with the propagation of tumors. Furthermore, depletion of CD8+ CTL led to the loss of antitumor effects in several cancer vaccines (4, 5). Therefore, the enhancement of antigen presentation through the MHC class I pathway to CD8+ T cells has been a primary focus of cancer immunotherapy.

Recently, naked DNA vaccines have emerged as attractive approaches for vaccine development (reviewed in Refs. 6–11). DNA vaccines have been shown to generate long-term cell-mediated immunity (reviewed in Refs. 12). In addition, DNA vaccines are capable of generating CD8+ T cell responses in vaccinated humans (13). However, one of the limitations of these vaccines is their potency, because they do not have the intrinsic ability to amplify and spread *in vivo* as some replicating viral vaccine vectors do. Furthermore, some tumor antigens may be poorly immunogenic, such as HPV+ E7 (5). Therefore, strategies that enhance DNA vaccine potency may be applicable for the development of more effective cancer immunotherapy.

Increased understanding of the antigen presentation pathway creates the potential for designing novel strategies to enhance vaccine potency. One potential strategy to enhance the presentation of antigen through the MHC class I pathway to CD8+ T cells is the use of the translocation features of certain bacterial toxins such as ETA (reviewed in Refs. 14). ETA is one of several secreted bacterial toxins that are able to covalently modify particular proteins in mammalian cells through the translocation of bacterial toxin. Molecular characterization of ETA has revealed its three functional domains (15). Domain I is responsible for binding to a cell surface receptor (16), domain II is responsible for translocation to the cytosol (17–19), and domain III is responsible for the toxic capability of binding to ADP-ribosyl transferase (20). In particular, domain II of ETA has been used to engineer a chimeric multidomain protein to deliver DNA into the cytosol (21, 22).

The ability of domain II of ETA to facilitate translocation from the endosomal/lysosomal compartments to the cytoplasm suggests that it may lead to the enhancement of MHC class I presentation of exogenous antigen. We therefore engineered a DNA vaccine encoding ETA(dII) linked to a model antigen, which we hypothesized would enhance MHC class I presentation of antigen to CD8+ T cells and thereby enhance vaccine potency. We chose HPV-16 E7 as a model antigen for vaccine development because E7 is important in the induction and maintenance of cellular transformation and coexpressed in most HPV-containing cervical cancers and their precursor lesions (23). Therefore, vaccines targeting E7 provide an opportunity to prevent and treat HPV-associated cervical malignancies.

Our data indicated that vaccination with the chimeric ETA(dII)/E7 DNA vaccine enhanced MHC class I presentation of E7, leading to a dramatic increase in the number of E7-specific CD8+ T cell precursors. Furthermore, the ETA(dII)/E7 DNA vaccine generated potent antitumor effects against s.c. E7-expressing tumors and preestablished E7-expressing metastatic lung tumors. These results indicate that fusion of the translocation domain of ETA to an antigen may greatly enhance MHC class I presentation of antigen and represents a novel strategy to improve vaccine potency.

MATERIALS AND METHODS

Plasmid DNA Constructs and Preparation. The generation of pcDNA3-E7 has been described previously (5). For the generation of pcDNA3-ETA(dII), the pgW601 plasmid (Ref. 24; most kindly provided by Dr. Darrell R. Galloway, Ohio State University, Columbus, OH) was used as the template for amplification of ETA(dII). The DNA fragment containing ETA(dII) was generated using PCR with a set of primers: 5'-CGCGGAGATCTAGGCGGTGC-ATGCATTCCCGAGGCCC-3' and 5'-CGCGGATTCTTGCTGGTCGGCGC- GGGTGCTGAA-3'. The amplified DNA fragment was then cloned into the EcoRI site of pcDNA3 (Invitrogen, Carlsbad, CA). For the generation of pcDNA3-ETA(dII)/E7, the DNA fragment containing ETA(dII) DNA was cloned into the EcoRI site of pcDNA3-E7. For the generation of pcDNA3-GFP, the DNA fragment encoding the GFP was first amplified with PCR using pEGFPN1 DNA (Clontech, Palo Alto, CA) and a set of primers: 5'-ATCGCCTATTCGGAATTC-CAAACG-3' and 5'-GGGAAGCTTTAC-TTGACGAGC-3'. The amplified product was then cloned into the BamHI/HindIII cloning sites of pcDNA3. For the generation of pcDNA3- E7/GFP, E7 was subcloned from pcDNA3-E7 into the EcoRI/BamHI sites of pcDNA3.
pcDNA3-GFP. For the generation of pcDNA3-ETA(dII)/E7/GFP, the DNA fragment encoding ETA(dII) was amplified using pcDNA3-ETA(dII) DNA and a set of primers: 5′-GGGCTTACAGTCGCCCTGATCTTTC-GCGAGGC-3′ and 5′-CGGAGATTCTGCTGCTCTGGCCGGGTGT-GAA-3′. The amplified product was then cloned into the XbaI/EcoRI sites of pcDNA3/E7/GFP. The accuracy of all of the constructs was confirmed by DNA sequencing. DNA for vaccination was prepared using an endotoxin-free kit (Qiagen, Valencia, CA).

Western Blot Analysis. Twenty μg of DNA were transfected into 5 × 10⁶ 293 D⁹,K⁹ cells (25) using Lipofectamine 2000 (Life Technologies, Inc., Rockville, MD). Forty-eight h after transfection, medium from transfected culture was concentrated using a millipore filter (Millipore, Bedford, MA), and cells were lysed with protein extraction reagent (Pierce, Rockford, IL). Equal amounts of proteins (10 μg) were loaded and separated by SDS-PAGE using a 10% polyacrylamide gel. The gels were electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Blots were blocked with PBS/0.05% Tween 20 (TTBS) containing 5% nonfat milk for 2 h at room temperature. Membranes were probed with anti-GFP (Clontech, Palo Alto, CA) at 1:1000 dilution in TTBS containing 5% nonfat milk. Membranes were washed four times with TTBS and developed using enhanced Hyperfilm-enhanced chemiluminescence (Amersham, Piscataway, NJ) at 1:1000 dilution in TTBS containing 5% nonfat milk. Cells were collected 40–44 h after transfection. Transfected 293 D⁹,K⁹ cells (25) were used as target cells, whereas a D⁹-restricted E7-specific CD8⁺ T cell line (26) served as effector cells. Untransfected 293 D⁹,K⁹ cells were used as a negative control. Cytolysis was determined by quantitative measurements of LDH using CytoTox96 nonradioactive cytotoxicity assay kits (Promega, Madison, WI) according to the manufacturer’s protocol. CTL assays were performed with effector cells and targets cells (1 × 10⁶ per well) mixed together at various ratios (1:1, 3:1, 9:1, and 27:1) in a final volume of 200 μl. After a 5- incubation at 37°C, 50 μl of the cultured media was collected to assess the amount of LDH. The percentage of lysis was calculated from the following equation: 100 × (A – B)/(C – D), where A is the reading of experimental-effector signal value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, and D is the target spontaneous background signal value.

 CTL Assay Using Transfected 293 D⁹,K⁹ Cells as Target Cells. A human embryonic kidney 293 cell line expressing the D⁹ and K⁹ (293 D⁹,K⁹), two C57BL/6 mouse MHC class I molecules, was kindly provided by Dr. James C. Yang (National Cancer Institute, NIH, Bethesda, MD). Twenty μg of pcDNA3 (no insert), ETA(dII), E7, or ETA(dII)/E7 DNA were transfected into 5 × 10⁶ 293 D⁹,K⁹ cells using Lipofectamine 2000 (Life Technologies, Inc., Rockville, MD). Cells were collected 40–44 h after transfection. Transfected 293 D⁹,K⁹ cells (25) were used as target cells, whereas a D⁹-restricted E7-specific CD8⁺ T cell line (26) served as effector cells. Untransfected 293 D⁹,K⁹ cells were used as a negative control. Cytolysis was determined by quantitative measurements of LDH using CytoTox96 nonradioactive cytotoxicity assay kits (Promega, Madison, WI) according to the manufacturer’s protocol. CTL assays were performed with effector cells and targets cells (1 × 10⁶ per well) mixed together at various ratios (1:1, 3:1, 9:1, and 27:1) in a final volume of 200 μl. After a 5- incubation at 37°C, 50 μl of the cultured media was collected to assess the amount of LDH. The percentage of lysis was calculated from the following equation: 100 × (A – B)/(C – D), where A is the reading of experimental-effector signal value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, and D is the target spontaneous background signal value.

 CTL Assay Using DCs Pulsed with Lysates of Transfected 293 D⁹,K⁹ Cells as Target Cells. CTL assays were performed with freshly isolated bone marrow-derived DCs pulsed with cell lysates as target cells and E7-specific CD8⁺ T cells as effector cells using a protocol similar to that described previously (27). The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad) according to vendor’s protocol. 293 D⁹,K⁹ cells were transfected as described earlier. Cell lysates from E7 or ETA(dII)/E7 DNA-transfected 293 D⁹,K⁹ cells were standardize for E7 protein concentration using an ELISA. DCs were prepared by pulsing them with different concentrations of cell lysates of various DNA-transfected 293 D⁹,K⁹ cells (50 μg/ml, 10 μg/ml, 2 μg/ml, and 0.4 μg/ml) in a final volume of 2 ml for 16–20 h. CTL assays were performed at a fixed E:T (9:1) ratio with 9 × 10⁶ E7-specific T cells mixed with 1 × 10⁶ prepared DCs in a final volume of 200 μl. Cytolysis was determined by quantitative measurements of LDH as described earlier.

DNA Vaccination. Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad) according to a previously described protocol (5). DNA-coated gold particles (1 μg DNA/bullet) were delivered to the shaved abdominal region of mice using a helium-driven gene gun (Bio-Rad) with a discharge pressure of 400 p.s.i.

Intracytoplasmic Cytokine Staining and Flow Cytometry Analysis. Cell surface marker staining of CD8 or CD4 and intracellular cytokine staining for IFN-γ and IL-4 as well as FACScan analysis were performed using conditions described previously (28). Before FACScan, splenocytes from naive or vaccinated groups of mice were incubated for 20 h with either 1 μg/ml of E7 peptide (aa 49–57) containing MHC class I epitope for detecting E7-specific CD8⁺ T cell precursors or 10 μg/ml of E7 peptide (aa 30–67) containing MHC class II peptide for detecting E7-specific CD4⁺ T cell precursors.

ELISA. For detection of HPV-16 E7-specific antibodies in the sera of vaccinated mice, we performed a direct ELISA with 1:100, 1:500, and 1:1000 dilutions of sera in 1× PBS using a previously described protocol (29). Briefly, sera was added to microwell plates coated with bacteria-derived HPV-16 E7 proteins with subsequent incubation with peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA).

In Vivo Tumor Protection Experiments. For the tumor protection experiment, mice (five per group) were vaccinated via gene gun with 2 μg of pcDNA3 without insert, ETA(dII) DNA, E7 DNA, ETA(dII) mixed with E7, or chimeric ETA(dII)/E7 DNA. One week later, the mice were boosted with the same regimen as that of the first vaccination. One week after the last vaccination, mice were s.c. challenged with 5 × 10⁶ cells/mouse TC-1 tumor cells (4) in the right leg and then monitored twice a week.

In Vivo Tumor Treatment Experiments. Mice were i.v. challenged with 1 × 10⁶ cells/mouse TC-1 tumor cells via the tail vein on day 0. Three days after challenge with TC-1 tumor cells, mice were treated with 2 μg of pcDNA3 without insert, ETA(dII) DNA, E7 DNA, or chimeric ETA(dII)/E7 DNA via gene gun. One week later, these mice were boosted with the same regimen as the first vaccination. Mice were killed on day 25. The number of pulmonary metastatic nodules of each mouse was evaluated and counted by experimenters blinded to sample identity.

In Vivo Antibody Depletion Experiments. In vivo antibody depletions have been described previously (4). Briefly, mice were vaccinated with 2 μg ETA(dII)/E7 DNA via gene gun, boosted 1 week later, and challenged with 5 × 10⁶ cells/mouse TC-1 tumor cells s.c. Depletions were started 1 week before 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. Depletion was terminated on day 63 after tumor challenge.

RESULTS

Generation and Characterization of the ETA(dII)/E7 DNA Vaccine. A schematic diagram showing the domains of full-length ETA and the construct of chimeric ETA(dII)/E7 is presented in Fig. 1A. Chimeric ETA(dII)/E7 was created by linking ETA(dII) (aa 247–416) to the E7 protein. We performed a Western blot analysis to characterize protein expression in E7 and ETA(dII)/E7 DNA-transfected cells (Fig. 1B). Because we observed that Western blot analysis using available E7-specific mouse monoclonal antibodies generated significant background, we used green GFP linked to E7 as a tag for our assays. As shown in Fig. 1B, lysates from E7/GFP DNA-transfected 293 D⁹,K⁹ cells revealed a protein band with a size of approximately M₅,000 corresponding to E7/GFP protein in Lane 1. In comparison, lysates of ETA(dII)/E7 GFP DNA-transfected 293 D⁹,K⁹ cells showed a protein band with a size of approximately M₅,000 corresponding to ETA(dII)/E7 GFP protein in Lane 2. Our results indicated that E7 DNA-transfected cells exhibited comparable levels of E7 protein expression compared with ETA(dII)/E7 DNA-transfected cells. We also examined the secretion of E7/GFP protein in cells transfected with E7/GFP or ETA(dII)/E7/GFP DNA. As shown in Lanes 3 and 4 of Fig. 1B, E7/GFP protein was not detected in the culture medium of cells transfected with either E7/GFP or ETA(dII)/E7/GFP DNA. E7 secretion was detected from cells transfected with Sig/E7 DNA, which served as a positive control (data not shown).
Enhanced Presentation of E7 through the MHC Class I Pathway in Cells Transfected with ETA(dII)/E7 DNA. To demonstrate if the addition of the translocation domain of ETA to E7 can directly enhance MHC class I presentation of E7, we performed CTL assays to characterize the MHC class I presentation of E7 by 293 Db,Kb cells. As shown in Fig. 2B, DCs pulsed with lysates of 293 Db,Kb cells transfected with ETA(dII)/E7 DNA generated significantly higher percentages of specific lysis as compared with DCs pulsed with lysates of 293 D0,Kb cells transfected with the other DNA constructs and naïve DCs (P < 0.001). These results revealed that the fusion of ETA(dIII) to E7 may enhance MHC class I presentation of E7 via a cross-priming mechanism.

Significant Enhancement of E7-Specific CD8+ T Cell Precursors in Mice Vaccinated with ETA(dII)/E7 DNA. To determine whether mice vaccinated with various DNA vaccine constructs can generate E7-specific CD8+ T cell precursors, we performed intracellular cytokine staining for E7-specific CD8+ T cell precursors in spleens extracted from vaccinated mice (5). As shown in Fig. 3A, mice vaccinated with ETA(dII)/E7 DNA generated an ~30-fold increase in the number of E7-specific IFN-γ+ CD8+ T cell precursors (308/3 × 105 splenocytes) compared with mice vaccinated with E7 DNA (11/3 × 105 splenocytes; P < 0.01). These results also indicated that fusion of ETA(dII) to E7 was required for enhancement of E7-specific CD8+ T cell activity, because ETA(dIII) mixed to E7 (ETA(dIII) + E7 DNA) did not generate enhancement of CD8+ T cell activity. Furthermore, the linkage of irrelevant proteins (such as GFP and CTLA-4) to E7 did not generate enhancement of E7-specific CD8+ T cell activity (data not shown).

We found no significant difference in the number of E7-specific CD4+ IFN-γ+ T cells (Fig. 3B) or CD4+ IL-4+ T cells (data not shown) among each of the vaccination groups. Using an ELISA, we observed no significant enhancement of E7-specific antibody re-
responses in mice vaccinated with ETA(dII)/E7 DNA compared with the other vaccination groups (data not shown).

Vaccination with ETA(dII)/E7 Fusion DNA Enhances Protection of Mice against the Growth of E7-Expressing Tumors. To determine whether the observed enhancement in E7-specific CD8+ T cell-mediated immunity translated to a significant E7-specific antitumor effect, we performed an in vivo tumor protection experiment using a previously characterized E7-expressing tumor model, TC-1 (4). As shown in Fig. 4A, 100% of those receiving ETA(dII)/E7 DNA vaccination remained tumor-free 56 days after TC-1 challenge, whereas all of the other groups of mice developed tumor growth within 15 days after tumor challenge. These results also indicated that fusion of ETA(dII) to E7 was required for antitumor immunity, because ETA(dII) mixed with E7 (ETA(dII)+E7 DNA) did not generate enhancement of antitumor immunity. Furthermore, the linkage of irrelevant proteins (such as GFP and CTLA-4) to E7 did not enhance tumor protection (data not shown).

Treatment with ETA(dII)/E7 Fusion DNA Eradicates Established E7-Expressing Tumors in the Lungs. To determine the therapeutic potential of chimeric ETA(dII)/E7 DNA in treating TC-1 tumor metastases in the lungs, an in vivo tumor treatment experiment was performed using a lung metastasis model (31). As shown in Fig. 4B, mice vaccinated with ETA(dII)/E7 DNA revealed the lowest mean number of pulmonary nodules (1.6 ± 1.1) compared with mice vaccinated with wild-type E7 DNA (77.6 ± 22.1), or ETA(dII) DNA (73.4 ± 14.6; one-way ANOVA; P < 0.001). These data indicated that mice treated with ETA(dII)/E7 could control established E7-expressing tumors in the lungs.

CD8+ T Cells but not CD4+ T cells or NK cells Are Essential for the Antitumor Effect Generated by Chimeric ETA(dII)/E7 DNA. To determine the subset of lymphocytes that are important for the rejection of E7-positive tumor cells, we performed in vivo antibody depletion experiments. As shown in Fig. 4C, all naïve mice and all of the mice depleted of CD8+ T cells grew tumors within 14 days after tumor challenge. In contrast, all of the nondepleted mice and all of the mice depleted of CD4+ T cells or NK1.1 cells remained tumor-free for 60 days after tumor challenge. These results suggest that CD8+ T cells are essential for the antitumor immunity generated by the ETA(dII)/E7 DNA vaccine.

DISCUSSION

In this study, we demonstrated that direct linkage of ETA(dII) to E7 can dramatically enhance the potency of HPV-16 E7-containing DNA vaccines. A DNA vaccine encoding ETA(dII) fused to HPV-16 E7 elicited strong E7-specific CD8+ T cell-mediated immunity and generated significant CD8+ T cell-dependent preventive effects against HPV-16 E7-expressing murine tumors. Furthermore, the chimeric
ETA(dII)/E7 DNA vaccine was capable of controlling lethal E7-expressing metastatic lung tumors.

Our vaccine represents one successful example of using the translocation domain of a bacterial toxin for the development of gene therapy. Previous studies have used the translocation domain of ETA linked to a DNA-binding protein to facilitate the entry of DNA into the cytosol (21, 22). Truncated forms of this chimeric protein lacking the translocation domain failed to facilitate efficient DNA transfer (21). Therefore, these studies indicated that domain II may serve as a useful tool to introduce exogenous protein into the cytosol, although the precise mechanism of such translocation remains unclear.

One of the potential explanations that may account for the observed enhancement of E7-specific CD8+ T-cell activity in mice vaccinated with ETA(dII)/E7 DNA may be the enhanced MHC class I presentation of E7 in cells expressing this chimeric protein. Indeed, in our in vitro assays, we found that cells transfected with ETA(dII)/E7 DNA presented E7 through the MHC class I pathway more efficiently than cells transfected with wild-type E7 DNA (Fig. 2A). Because ballistic DNA delivery can introduce DNA directly into dermal professional APCs, ETA(dII)/E7 DNA-transfected APCs may directly enhance the presentation of E7 through MHC class I pathway to CD8+ T cells and contribute to the generation of E7-specific CD8+ T cell precursors in vivo.

Another important mechanism that may contribute to the enhanced E7-specific CD8+ T cell immune responses in vivo is the so-called cross-priming effect of ETA(dII)/E7 protein, whereby release of ETA(dII)/E7 antigen can lead to uptake and processing by other APCs via the MHC class I-restricted pathway (30). Although Western blot analysis on the supernatant of transfected cells failed to detect secretion of ETA(dII)/E7 from ETA(dII)/E7-DNA transfected cells, we could not rule out the possibility that ETA(dII)/E7 protein was eventually released from ETA(dII)/E7 DNA-transfected cells after lysis. Our results suggested that the linkage of ETA(dII) to E7 may lead to enhanced stimulation of E7-specific CD8+ T cells via a cross-priming mechanism. One previous study found that exogenous ETA (domains I and II) chimerically linked to influenza A protein or nucleoprotein resulted in MHC class I processing and presentation of the antigen to CTLs (32). However, our data suggested that linkage to domain II of ETA alone is sufficient for delivering exogenous antigen into the MHC class I presentation pathway.

Another important factor for the enhancement of antigen-specific CD8+ T cell activity and the antitumor effect generated by chimeric ETA(dII)/E7 DNA may be the biology of professional APCs at the vaccination sites. It is now clear that bacterial DNA can contain immunostimulatory elements such as CpG islands (33, 34), which have been shown to cause simultaneous maturation and activation of murine DCs (35). Furthermore, CpG islands may elicit the secretion of IL-12 and IFN-γ (34, 36), which are related to the T-helper-1-like inflammatory response and important for the antitumor effect. However, it is not clear whether vaccination with chimeric ETA(dII)/E7 DNA leads to greater maturation of DCs or secretion of Th1-related cytokines compared with vaccination with the other DNA constructs. Thus, it would be interesting to investigate further whether chimeric ETA(dII)/E7 DNA influences the biology of DCs or the cytokine profile.

The success of the ETA(dII)/E7 DNA vaccine and the importance demonstrated by the ETA domain II component warrants consideration of strategies that use the translocation domains of other bacterial toxins to enhance vaccine potency. The translocation domains for several bacterial toxins have been reported in previous studies, including diphtheria toxin (37, 38), clostridial neurotoxins such as tetanus neurotoxins and botulinum neurotoxins (39, 40), anthrax toxin lethal factor (41, 42), shiga toxin (43), *Escherichia coli* heat-labile toxin (44), yersinia cytotoxins (YopE and YopH; Ref. 45), listeria toxin (listeriolysin O; Ref. 46), and pertussis adenylate cyclase toxin (47). The understanding of these translocation domains may allow such molecules to be incorporated into vaccine designs similar to the one we describe in this study.

ETA(dII)/E7 generates potent E7-specific CD8+ T cell responses through enhanced MHC class I presentation, and the antitumor effect was completely CD4-independent. Interestingly, these features resembled those observed in a previously described chimeric DNA vaccine using *Mycobacterium tuberculosis* heat shock protein 70 linked to the E7 antigen (5). In the future, it would be important to perform a head-to-head comparison to determine which vaccine is more suitable for clinical application. Furthermore, continued investigation of the mechanisms that account for the efficient antigen presentation of these vaccines would help in the development of better vaccines.

Although the ETA(dII)/E7 targets antigen to the MHC class I presentation pathway for the enhancement of CD8+ T cell activity, other constructs that target antigen to MHC class II presentation pathways may provide enhanced CD4+ T cell responses. This realization raises the notion of coadministration of vaccines that directly enhance MHC class I- and class II-restricted pathways. Previously we had developed a chimeric Sig/E7/LAMP-1 DNA vaccine that uses the LAMP-1 endosomal/lysosomal targeting signal for enhancing the MHC class II presentation pathway of E7 (28). The ETA(dII)/E7 vaccine described here in conjunction with a MHC class II-targeting vaccine such as Sig/E7/LAMP-1 may activate multiple arms of the immune system in a synergistic fashion, leading to significantly enhanced CD4+ and CD8+ T-cell responses and potent antitumor effects.

In summary, our findings illustrate the promise of enhancing vaccine potency by the linkage of ETA(dII) to antigen, leading to enhanced antigen-specific CD8+ T-cell activity and potent antitumor effects in vivo. Because a majority of cervical cancers express HPV E7, our vaccine can potentially be used for the prevention and treatment of HPV-associated tumors. Furthermore, the strategy of using the translocation property of bacterial toxins may be promising in future vaccine development for the control of cancers and infectious diseases.

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