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DNA vaccine encoding heat shock protein 60 co-linked to HPV16 E6 and E7 tumor antigens generates more potent immunotherapeutic effects than respective E6 or E7 tumor antigens

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Abstract

Objective. Vaccination based on tumor antigen is an attractive strategy for cancer prevention and therapy. Cervical cancer is highly associated with human papillomavirus, especially type 16. We developed DNA vaccines encoding heat shock protein 60 (HSP60) linked to HPV16 E6 or E7 to test if HSP60 chimeric DNA vaccines may generate strong E6 and/or E7-specific immune response and anti-tumor effects in vaccinated mice.

Methods. In vivo antitumor effects such as preventive, therapeutic, and antibody depletion experiments were performed. *In vitro* assays such as intracellular cytokine stainings, ELISA for Ab responses, and direct and cross-priming effects, were also performed.

Results. HSP60 chimeric DNA vaccines generated strong E6- or E7-specific immune responses and anti-tumor effects in vaccinated mice via direct and cross-priming effects. HSP60 was also linked with both E6 and E7 antigens and the HSP60/E6/E7 chimeric DNA vaccine generated more potent immunotherapeutic effects on E6- and E7-expressing tumors than HSP60/E6 or HSP60/E7 chimeric DNA vaccine alone.

Conclusion. Utilization of both E6 and E7 tumor antigens can advance the therapy of tumors associated with HPV-infections. The DNA vaccine encoding heat shock protein 60 co-linked to HPV16 E6 and E7 tumor antigens can generate more potent immunotherapeutic effects than E6 or E7 tumor antigens alone.

Keywords: DNA vaccine; Heat shock protein 60; HPV16; E7 antigen; E6 antigen

Introduction

DNA vaccines have become an attractive approach for generating antigen-specific cancer vaccine and immunotherapy. Naked plasmid DNA can be repeatedly administered and easily prepared in large scale with high purity, and are highly stable relative to proteins and other biological polymers [1]. In addition, intra-dermal administration of DNA vaccines using a gene gun represents an efficient means of targeting dendritic cells, the most potent professional antigen-presenting cells that are specialized to prime T helper and cytotoxic cells *in vivo* [2,3].

One of the concerns about DNA vaccines is their limited potency, because they do not have the intrinsic ability to amplify *in vivo* as viral vaccines do [1]. Several strategies have been applied to increase their potency. For example, targeting antigens for rapid intracellular degradation [4], directing antigens to APCs by fusion to ligands for APC receptors [5], or fusing antigens to a pathogen sequence, such as fragment C of tetanus toxin [6] have been made. Linkage of antigens to HSP may be another potential approach for increasing the potency of DNA vaccines. HSP-based protein vaccine can also be administered by fusing antigens to HSP [7–9]. Furthermore, immune response can be induced in mice with MHC that is either identical or different to the MHC of donor HSPs [10–12]. These

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investigations have made HSPs more attractive to use in cancer vaccine and immunotherapy.

More than 99% of cervical cancers contain HPV, particularly the high-risk HPV type such as 16 and 18 [11]. Two HPV oncoproteins, E6 and E7, are consistently expressed in HPV-associated cancer cells and are responsible for their malignant transformation. These two oncogenic proteins therefore represent ideal target antigens for developing vaccines and immunotherapeutic strategies against HPV-associated neoplasia. Numerous pre-clinical studies and some clinical studies have targeted the HPV16 oncogenic proteins E6 or E7 for vaccine development to control HPV-associated lesions [13–15].

In the past, most HPV researchers focused on E7, and thus an E7 immuno-dominant epitope and its associated immune responses have been well characterized [6]. The gene gun approach was previously used to test several strategies that are able to rout the human papilloma virus type 16 E7 model antigen and result in enhanced E7-specific $CD8^+$ T cell-mediated immune response and anti-tumor effects [16–19]. Since E6 represents another important target for potential vaccines to control HPV-associated lesions, it is also crucial to develop vaccines targeting E6.

We therefore developed a DNA vaccine encoding HSP60 linked to E6 and/or E7 to elucidate if DNA vaccines encoding HSP60 with two tumor antigens (E6 and E7) have the potential to prevent HPV infection and provide therapeutic effects on HPV-related cancers.

Materials and methods

Plasmid DNA constructs and preparation

For the generation of pcDNA3-HSP60, HSP60 was first amplified with PCR using human placenta cDNA as template and a set of primers, 5′-CCGGTCTAGAAGAAATGCTTCGGTTACCCACAG-3′ and 5′-CGCGGATCCACACTGCCTTGGGCTTCCTGTCA-3′. The amplified product was then cloned into the *Xba*I/*Bam*HI sites of the pcDNA3 vector (Invitrogen Corp., Carlsbad, California, USA). For the generation of pcDNA3-HSP60/E6, E6 was first amplified with PCR using DNA of the CaSki cell line as template and a set of primers, 5′-CGCGGATCCATGCACCAAAAGAGAACTGCAATGT-3′ and 5′-CCCAAGCTTTTACAGCTGGGTTTCTCTACGTGTTCT-3′. E6 was then cloned into the *Bam*HI/*Hin*dIII sites of pcDNA3-HSP60 to generate pcDNA3-HSP60/E6.

To generate pcDNA3-HSP60/E7, E7 was first amplified using DNA of the CaSki cell line as template and with a set of primers, 5′-CCGGGATCCATG-GAGATACACCTA -3′ and 5′-CCCAAGCTTTTGAGAACAGATGG -3′, and cloned into the *Bam*HI/*Hin*dIII sites of pcDNA3-HSP60 to generate pcDNA3-HSP60/E7. To generate pcDNA3-HSP60/E6/E7, E7 was cloned into the *Hin*dIII/*Hin*dIII sites of pcDNA3-HSP60/E6. Plasmid constructs were confirmed by DNA sequencing.

Cell line

TC-1

The TC-1 tumor cell line was generated as described previously [20]. On the day of tumor challenge, tumor cells were harvested by trypsinization, washed twice with 1× Hanks buffered salt solution (HBSS), and finally re-suspended in 1× HBSS to the designated concentration for injection.

293 D^bK^b cells

293 D^bK^b cells (a human embryonic kidney 293 cell line expressing the D^b and K^b (293 D^bK^b), two C57BL/6 mouse MHC class I molecules) [21] were kindly provided by Dr. TC Wu, Johns Hopkins Medical Institutes, Baltimore, MD.

Transfection of 293 D^bK^b cells

293 D^bK^b were tansfected with various DNA, such as E6, E7, E6/E7, HSP60/E6, HSP60/E7, or HSP60/E6/E7 using LipofectAMINE reagent (Life Technologies) according to the manufacturer's instructions. The 293 D^bK^b cells were collected 48 h after transfection for further experiments.

Immunoblotting

Transfected 293 D^bK^b cells with various DNA constructs were lysed in immuno-precipitation assay buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP40, 10% glycerol, 1 mg/ml BSA, 20 mM Tris, pH 8.0, and 2 mM orthovanadate and analyzed as previously described [22]. Briefly, 50 μg of cell lysates was resolved on a sodium dodecyl sulfate (SDS)-containing 12% polyacrylamide gel, transferred to polyvinylidene difluoride nylon membranes (Millipore, Bedford, Mass.), and probed with antibodies specific to E6 (Abcam, Cambridge, UK) or E7 (Zymed, San Francisco, CA) or β-actin (Chemicon International, Temecula, CA). The membrane was then probed with either horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibody.

The specific bands were visualized by an ECL (enhanced chemiluminescence) Western blot system (Amersham, Buckinghamshire, England). As shown in Fig. 1, the E6 (MW 18 kDa), E7 (MW 18 kDa), HSP60/E6 (MW 78 kDa), HPS60/E7 (MW 78 kDa), and HSP60/E6/E7 (MW 96 kDa) proteins showed similar expression levels.

Mice

Six to 8-week-old female C57BL/6J mice were purchased and kept in the animal facility of the School of Medicine, National Taiwan University. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

DNA vaccination

Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination were performed using a helium-driven gene gun according to a protocol described previously with some modifications [17,23]. Control plasmid (no insert), E6, E7, E6/E7, E6 mixed with E7, HSP60, HSP60 mixed with E6, HSP60 mixed with E7, HSP60/E6, HSP60/E7, HSP60/E6 mixed HSP60/E7, and HSP60/E6/E7-coated gold particles were delivered to the shaved abdominal region of mice using a low pressure-accelerated gene gun (BioWare Technologies Co. Ltd, Taipei, Taiwan) with a 50 psi discharge pressure of helium [23].

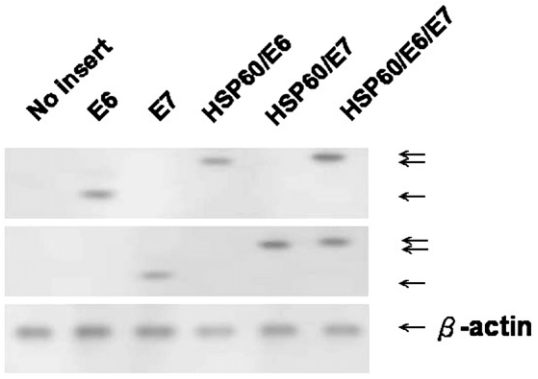

Fig. 1. The protein expression levels of respective constructs in transfected 293 D^bK^b cells. 293 D^bK^b cells were transfected with respective DNA. The E6 or E7 protein expression levels in various constructs are shown as follows: E6 (MW 18 kDa), E7 (MW 18 kDa), HSP60/E6 (MW 78 kDa), HPS60/E7 (MW 78 kDa), and HSP60/E6/E7 (MW 96 kDa).

Intracellular cytokine staining and flow cytometry analysis

Mice were immunized with 2 μg of the various DNA vaccines and received a booster with the same regimen 1 week later. Splenocytes were harvested 1 week after the last vaccination. Before intracellular cytokine staining, pooled splenocytes from each vaccination group were incubated for 16 h with either 1 μg/ml of E6 peptide (aa 50–57) [13] or E7 peptide (aa 49–57) [24], containing an MHC class I epitope for detecting E6- or E7-specific $CD8^+$ T cell precursors, or 50 μg/ml of E6 or E7 protein (kindly provided by Dr. CW Liao, Animal Technology Institute Taiwan, Miaoli, Taiwan) for detecting E6- or E7-specific $CD4^+$ T cell precursors. Cell surface marker staining of CD4 or CD8 and intracellular cytokine staining for IFN-γ, as well as flow cytometric analysis, were performed using conditions described previously [17,25].

Cytotoxic T lymphocyte assay using transfected 293 D^bK^b cells as target cells

Cytotoxic T lymphocyte (CTL) assays were performed by quantitative measurements of lactate dehydrogenase (LDH) using CytoTox96 non-radioactive cytotoxicity assay kits (Promega Corp., Madison, Wisconsin, USA) according to the manufacturer's protocol. Various DNA-transfected 293 D^bK^b cells served as target cells while a D^b-restricted E6- or E7-specific $CD8^+$ T cell line (provided by Dr. TC Wu, Johns Hopkins Medical Institutes, Baltimore, MD) was used as effector cells. Untransfected 293 D^bK^b cells were used as a negative control. The 293 D^bK^b cells were collected 40–44 h after transfection.

CTL assays were performed with effector cells and target cells mixed together at various effector/target (E/T) ratios, 1:1, 5:1, 15:1, and 45:1, in a final volume of 200 μl as described previously [16]. After a 5-h 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: $100\times(A-B)/(C-D)$, 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 dendritic cells pulsed with lysates of transfected 293 D^bK^b cells as target cells

CTL assays were performed using bone marrow-derived dendritic cells (DCs) pulsed with cell lysates as target cells and D^b-restricted E6- or E7-specific $CD8^+$ T cells as effector cells using the protocol described previously [26]. DCs were generated by culture of bone marrow cells in the presence of GM-CSF as described previously [27]. 293 D^bK^b cells were first transfected with various DNA constructs via lipofectamine. The protein concentration of lysates was determined using the BioRad protein assay (BioRad Laboratories Inc.) according to the vendor's protocol [16]. DCs were pulsed with different concentrations of cell lysates of various DNA-transfected 293 D^bK^b 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 ratio of 9:1 using E7-specific T cells mixed with prepared DCs in a final volume of 200 μl. Cytolysis was determined by quantitative measurements of LDH as described earlier.

In vivo tumor protection experiments

For the tumor protection experiments, mice (five per group) either received no vaccination or were immunized with 2 μg/mouse of various DNA vaccines with a gene gun. They were boosted with the same regimen as the first vaccination one week later. One week after the last vaccination, the mice were subcutaneously challenged with 5×10^4 of TC-1 cells/mouse in the right leg. They were monitored for evidence of tumor growth by palpation and inspection twice a week until they were sacrificed on day 60.

In vivo antibody depletion experiments

In vivo Ab depletions were performed as described previously [27,28]. Briefly, mice (five per group) were vaccinated with 2 μg/mouse of HSP60/E6 or HSP60/E7 DNA with a gene gun, boosted 1 week later, and challenged with 5×10^4 cells/mouse TC-1 tumor cells. Depletion was started 1 week before tumor challenge.

mAb GK1.5 was used for CD4 depletion [29], mAb 2.43 was used for CD8 depletion [30], and mAb PK136 was used for NK1.1 depletion [31]. Depletion was terminated on day 40 after tumor challenge. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week until they were sacrificed on day 60.

In vivo tumor treatment experiments

The therapeutic potential of each vaccine was assessed by performing an *in vivo* tumor treatment experiment using a previously described lung hematogenous spread model [16]. Two days after tumor challenge, mice received 16 μg/mouse of no insert DNA, E6 DNA, E7 DNA, E6 mixed with E7 DNA, HSP60 DNA, HSP60/E6 DNA, HSP60/E7 DNA, HSP60/E6 mixed with HSP60/E7 DNA, or HSP60/E6/E7 DNA by a gene gun, followed by a booster with the same regimen every 7 days for 4 weeks (a total of 64 μg DNA). Mice receiving no vaccination were used as negative control. The mice were sacrificed and the lungs were explanted on day 28. Pulmonary tumor nodules in each mouse were evaluated and counted by experimenters who were blinded to sample identity.

For the second *in vivo* tumor treatment experiment, mice were injected with 5×10^4 cells/mouse TC-1 tumor cells via the tail vein as described earlier. Seven days after tumor challenge, mice received various DNA vaccines such as HSP60/E6, HSP60/E7, HSP60/E6 mixed with HSP60/E7, and HSP60/E6/E7 as described earlier. The mice were sacrificed, and the lungs were explanted on day 28. The pulmonary tumors in each mouse were evaluated and counted as described earlier.

Statistical analysis

All data expressed as mean±SEM were representative of at least two different experiments. Data for intracellular cytokine staining with flow cytometry analysis and tumor treatment experiments were evaluated by analysis of variance (ANOVA). Comparisons between individual data points were made using Student's *t*-test. In the tumor protection experiment, the principal outcome of interest was the time to tumor development. The event time distributions for different mice were compared by the Kaplan and Meier and by log-rank analyses. A p value<0.05 was considered statistically significant.

Results

Vaccination with DNA encoding HSP60 linked to E6 alone, E7 alone, or both E6 and E7 significantly enhanced E6 and/or E7-specific $CD8^+$ T cell response

The representative figures of flow cytometric analysis are shown in Fig. 2A. As shown in Fig. 2B, vaccination with HSP60/E6 (140.0±14.1) or HSP60/E6/E7 (218.0±17.0) DNA generated higher frequencies of E6-specific IFN-γ-secreting $CD8^+$ T cell precursors when compared to mice vaccinated with E6 (6.5±2.1), E7 (4.0±1.4), E6/E7 (8.0±3.5), HSP60 mixed with E6 (12.5±2.8), or HSP60/E7 (39.0±5.7) DNA ($p<0.01$, one-way ANOVA). In addition, vaccination with HSP60/E7 (275.0±24.0), or HSP60/E6/E7 (987.5±27.6) DNA generated higher frequencies of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors when compared to mice vaccinated with E6 (7.5±1.4), E7 (7.0±2.8), E6/E7 (12.0±2.8), HSP60 mixed with E7 (14.5±2.1), or HSP60/E6 DNA (40.5±7.0) ($p<0.01$, one-way ANOVA) (Fig. 2C). We further evaluated whether HSP60 could enhance the E6, or E7-specific $CD4^+$ T cell response when linked to E6, or E7 in a DNA vaccine, respectively. As shown in

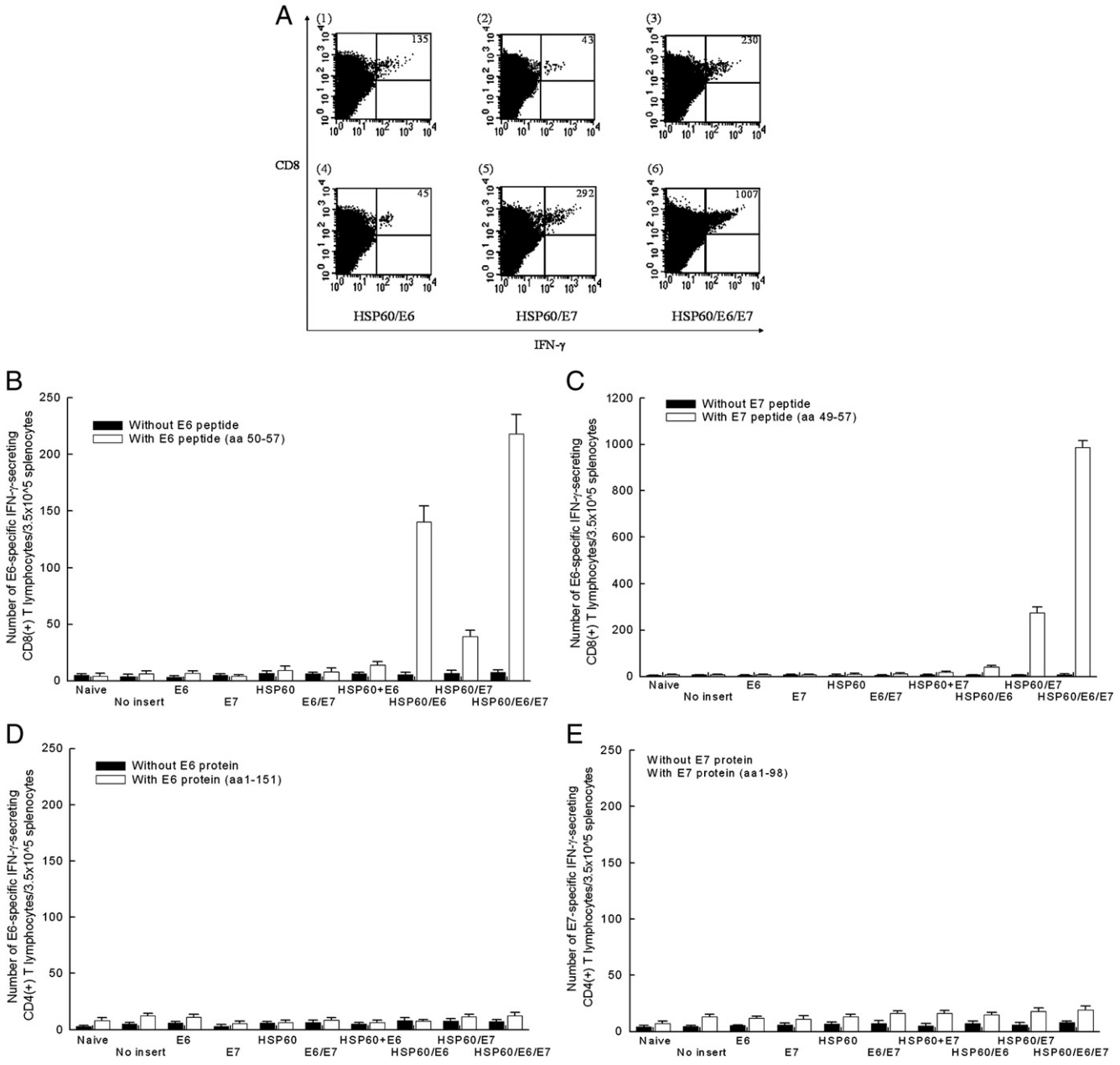

Fig. 2. Immunologic profiles of vaccinated mice using intracellular cytokine staining and flow cytometry analysis and ELISA. (A) Representative figures of flow cytometric analysis. (B) Bar graph depicting the number of E6-specific IFN-γ-secreting $CD8^+$ T cell precursors/3.5×10^5 splenocytes (mean±SEM). Mice vaccinated with HSP60/E6 or HSP60/E6/E7 DNA generated higher numbers of E6-specific IFN-γ-secreting $CD8^+$ T cell precursors than other vaccination groups ($p<0.01$, one-way ANOVA). (C) Bar graph depicting the number of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors/3.5×10^5 splenocytes (mean±SEM). Mice vaccinated with HSP60/E7 or HSP60/E6/E7 DNA generated higher numbers of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors than other vaccination groups ($p<0.01$, one-way ANOVA). (D) Bar graph depicting the number of E6-specific IFN-γ-secreting $CD4^+$ T cell precursors/3.5×10^5 splenocytes (mean±SEM). None of the vaccinated groups generated significantly higher number of E6-specific $CD4^+$ T cell precursors when compared with naive mice ($p>0.05$, one-way ANOVA). (E) Bar graph depicting the number of E7-specific IFN-γ-secreting $CD4^+$ T cell precursors/3.5×10^5 splenocytes (mean±SEM). None of the vaccinated groups generated significantly higher number of E7-specific $CD4^+$ T cell precursors when compared with naive mice ($p>0.05$, one-way ANOVA).

Figs. 2D and E, no increase in the number of E6- or E7-specific IFN-γ-secreting $CD4^+$ T cells in mice vaccinated with various DNA vaccines was observed.

Our data showed that physical linkage of HSP60 to E6 or E7 was required for enhancement of E6- or E7-specific $CD8^+$ T cell activity, since DNA encoding HSP mixed with E6 or E7 DNA did not generate enhancement of E6- or E7-specific $CD8^+$ T cell activity.

Enhanced presentation of E6, or E7, or E6 and E7 antigen through the MHC class I pathway in cells transfected with HSP60 linked with E6, or E7, or E6 and E7 DNA and in dendritic cells pulsed with various chimeric HSP60 proteins

We further explored potential explanations for the observed increase in E6 and/or E7-specific $CD8^+$ T cell precursors in mice vaccinated with HSP60/E6, HSP60/E7, or HSP60/E6/E7.

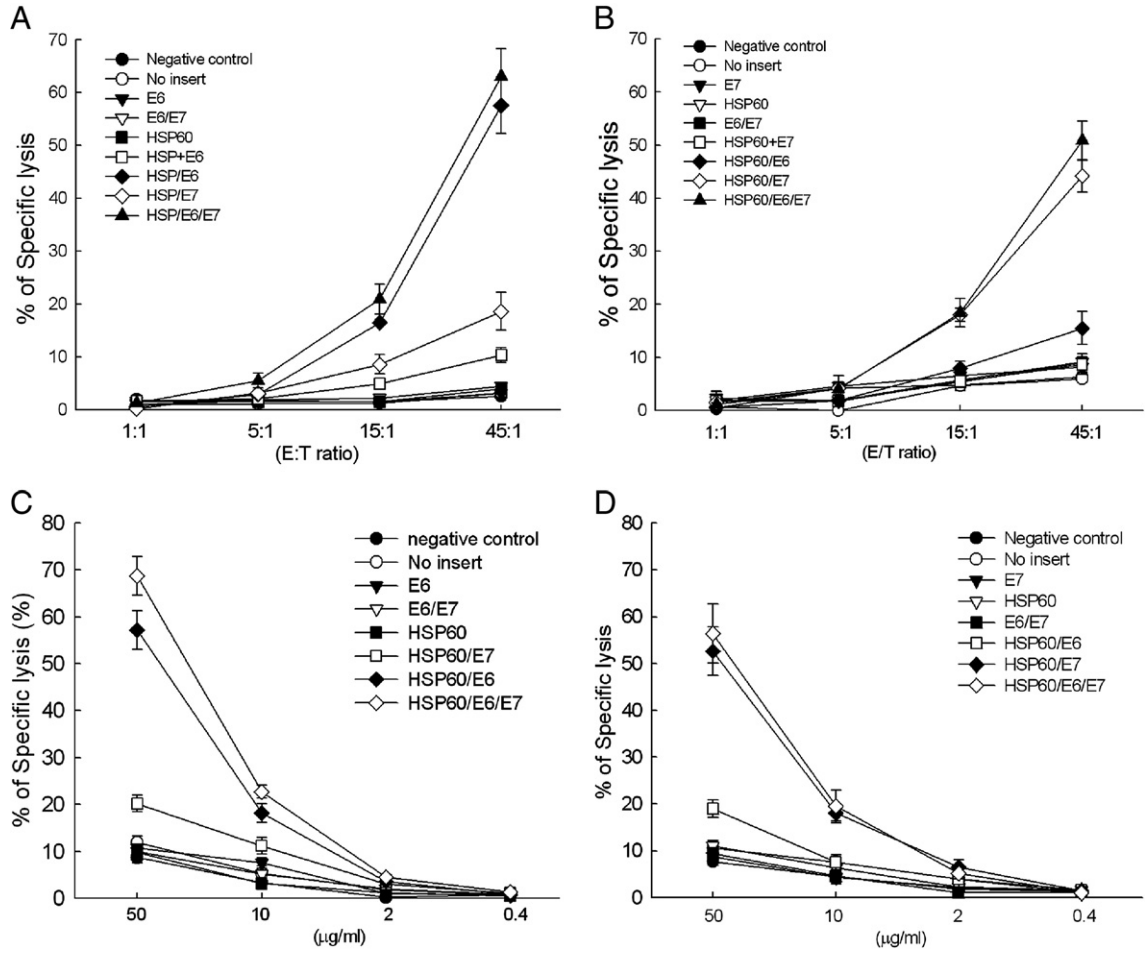

Fig. 3. CTL assays demonstrate enhanced presentation of E6 or E7 through the MHC class I pathway directly by 293 D^bK^b cells transfected with HSP60/E6, HSP60/E7, or HSP60/E6/E7 DNA constructs. (A) CTL assays with various E/T ratios (E/T = 1:1, 5:1, 15:1, 45:1) by using 293 D^bK^b cells transfected with various DNA constructs served as target cells and D^b-restricted E6-specific $CD8^+$ T cells as effector cells. (B) CTL assays with various E/T ratios (E/T = 1:1, 5:1, 15:1, 45:1) by using 293 D^bK^b cells transfected with various DNA constructs served as target cells and D^b-restricted E7-specific $CD8^+$ T cells as effector cells. CTL assays demonstrate enhanced presentation of E6, or E7 through the MHC class I pathway indirectly by bone marrow-derived DCs pulsed with cell lysates containing chimeric HSP60/E6, HSP60/E7, or HSP60/E6/E7 protein. (C) CTL assays at fixed E/T ratio (9:1) using bone marrow-derived DCs pulsed with different concentrations of cell lysates from various DNA-transfected 293 D^b cells, and D^b-restricted E6-specific $CD8^+$ T cells as effector cells. (D) CTL assays at fixed E/T ratio (9:1) using bone marrow-derived DCs pulsed with different concentrations of cell lysates from various DNA-transfected 293 D^bK^b cells and D^b-restricted E6-specific $CD8^+$ T cells as effector cells.

One was that there was direct enhancement of MHC class I presentation of E6 or E7 in cells expressing HSP60/E6, HSP60/E7, or HSP60/E6/E7. As shown in Fig. 3A, 293 D^bK^b cells transfected with HSP60/E6 (57.6±5.4%) and HSP60/E6/E7 (63.1±5.2%) DNA generated significantly higher percentages of specific lysis at 45:1 E/T ratios compared with cells transfected with E6 and HSP60 (10.3±1.4%), HSP60 (4.0±0.4%), E6/E7 (4.4±0.2%), or wild-type E6 (3.1±0.1%) DNA when the effector cells were the E6-specific $CD8^+$ T cell line ($p<0.001$, one-way ANOVA).

Similar phenomena were also observed in 293 D^bK^b cells transfected with HSP60/E7 (44.2±3.0%), and the HSP60/E6/E7 (50.9±3.6%) DNA also generated significantly higher percentages of specific lysis at 45:1 E/T ratios compared with cells transfected with E7 and HSP60 (8.7±1.4%), HSP60 (9.1±0.5%), E6/E7 (9.0±1.7%), or wild-type E7 (8.2±1.4%) DNA when the effector cells were changed to the E7-specific $CD8^+$ T cell line ($p<0.001$, one-way ANOVA) (Fig. 3B).

Another potential mechanism for the observed enhancement of E7-specific $CD8^+$ T cell immune responses *in vivo* was the so-called "cross-priming" effect [32], where the HSP70/E7 protein released from cells were taken up and processed by other antigen-presenting cells (APCs) via the MHC class I-restricted pathway [27]. As shown in Fig. 3C, DCs pulsed with lysates of 293 D^bK^b cells transfected with HSP60/E6 (57.2±4.0%) or HSP60/E6/E7 (68.7±4.1%) DNA generated a significantly higher percentage of specific lysis than DCs pulsed with lysates of 293 D^bK^b cells transfected with HSP60/E7 (20.2±1.8%) or the wide-type E6 (10.7±0.6%) DNA construct ($p<0.001$, one-way ANOVA), when the effector cells were the E6-specific $CD8^+$ T cell line.

In addition, DCs pulsed with 50 μg/ml lysates of 293 D^bK^b cells transfected with HSP60/E7 (52.6±5.2%) or HSP60/E6/E7 (56.4±6.3%) DNA generated a significantly higher percentage of specific lysis than DCs pulsed with lysates of 293 D^bK^b cells transfected with HSP60/E6 (19.0±1.8%) or the wide-type E7

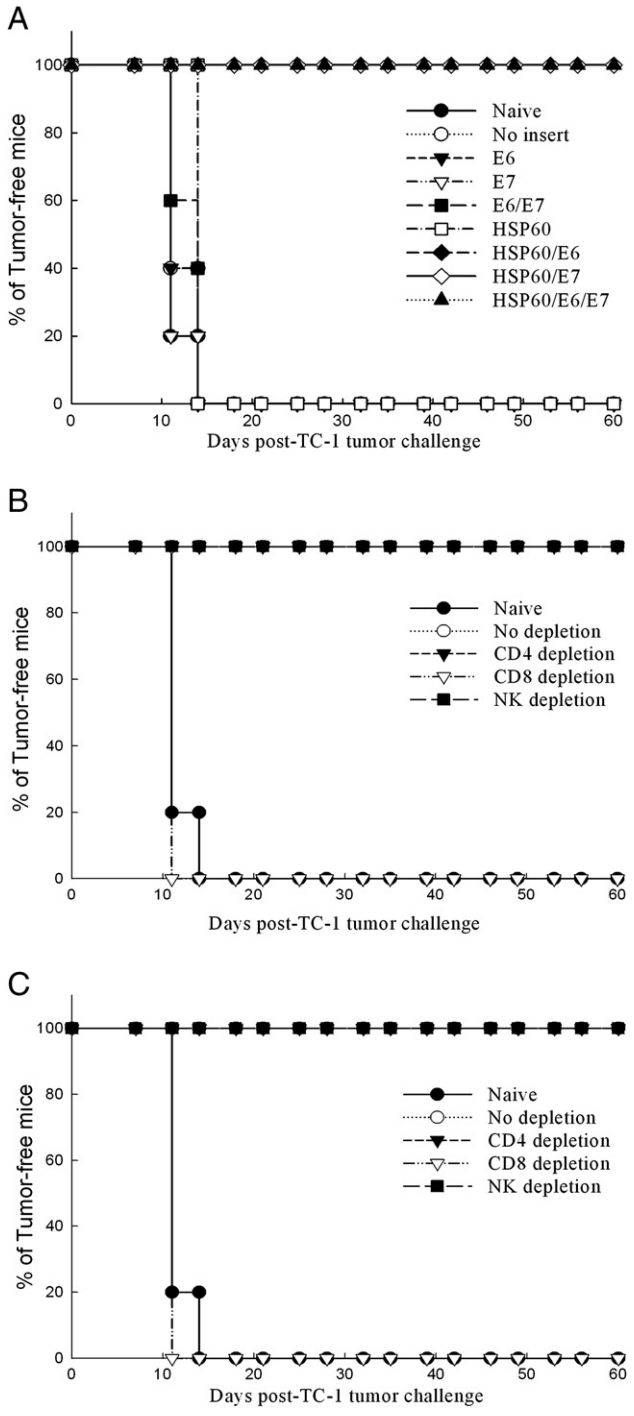

Fig. 4. *In vivo* tumor protection experiments in mice vaccinated with various protein vaccines and *in vivo* Ab depletion experiments in mice vaccinated with HSP60/E6 or HSP60/E7 DNA vaccines. (A) *In vivo* tumor protection experiments. 100% of mice receiving HSP60/E6, HSP60/E7, or HSP60/E6/E7 remained tumor-free 60 days after TC-1 challenge. (B) *In vivo* Ab depletion experiments of mice vaccinated with HSP60/E6 DNA vaccine. (C) *In vivo* Ab depletion experiments of mice vaccinated with HSP60/E7 DNA vaccine. All of the HSP60/E6 or HSP60/E7 vaccinated mice depleted with $CD8^+$ T lymphocytes, as well as naive mice, developed tumors within 14 days after TC-1 tumor challenge. All of the HSP60/E6 or HSP60/E7 vaccinated mice depleted of $CD4^+$ T lymphocytes or $NK1.1^+$ cells were tumor-free after 60 days of TC-1 tumor challenge.

(8.8±1.3%) DNA construct, when the effector cells were the E7-specific $CD8^+$ T cell line ($p<0.001$, one-way ANOVA) (Fig. 3D).

Our *in vitro* experiments revealed that HSP60/E6, HSP60/E7, and HSP60/E6/E7 chimeric molecules may enhance antigen-specific immunity via direct and/or cross-priming effects.

Vaccination with HSP60/E6, HSP60/E7, or HSP60/E6/E7 DNA enhanced tumor protection in mice challenged with an E6 and E7-expressing tumor cell line

To determine if the observed enhancement of E6- or E7-specific $CD8^+$ T cell response translated into a significant E6- or E7-specific protective anti-tumor effect, we performed an *in vivo* tumor protection experiment using a previously characterized E6- and E7-expressing tumor model, TC-1 [20]. As shown in Fig. 4A, 100% of mice receiving HSP60/E6, HSP60/E7, or HSP60/E6/E7 DNA vaccination, when challenged with TC-1 tumor cells, remained tumor-free 60 days after TC-1 challenge. In comparison, all mice vaccinated with the wild-type E6, E7, E6/E7, or HSP60 mixed with E6 or E7 DNA developed tumors within 14 days of challenge.

These results indicated that fusion of HSP60 to E6 and/or E7 antigens is required for anti-tumor immunity of E6 and E7-expressing TC-1 tumor cells.

$CD8^+$ T cells but not $CD4^+$ T cells or natural killer cells were essential for anti-tumor effect generated by HSP60/E6 or HSP60/E7 DNA

To determine the subset of lymphocytes important for the anti-tumor effect, we performed *in vivo* Ab depletion experiments [16]. As shown in Figs. 4B and C, depleted of $CD8^+$ T cells grew tumors within 15 days after tumor challenge in all naive mice and those vaccinated with HSP60/E6 or HSP60/E6 DNA vaccine. In contrast, all of the non-depleted mice and those depleted of $CD4^+$ T cells or NK1.1 cells remained tumor-free 60 days after tumor challenge.

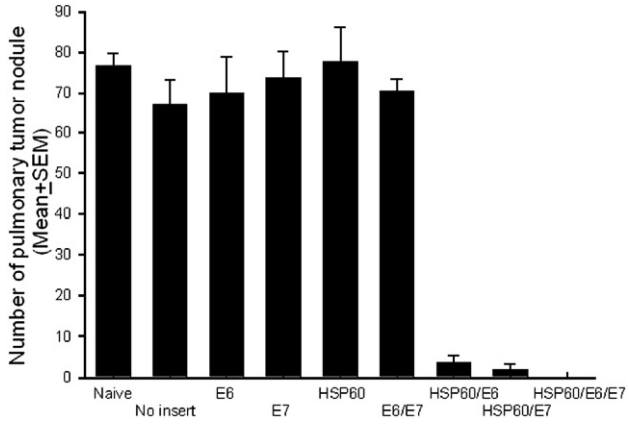

Fig. 5. *In vivo* tumor treatment experiments in mice at a high therapeutic dose. Mice treated with DNA encoding HSP60/E6, HSP60/E7, or HSP70/E6/E7 showed similar numbers of tumor nodules, all significantly lower than those in mice treated with DNA encoding E6, E7, or E6/E7. Data are expressed as mean number of pulmonary tumor nodules+SEM.

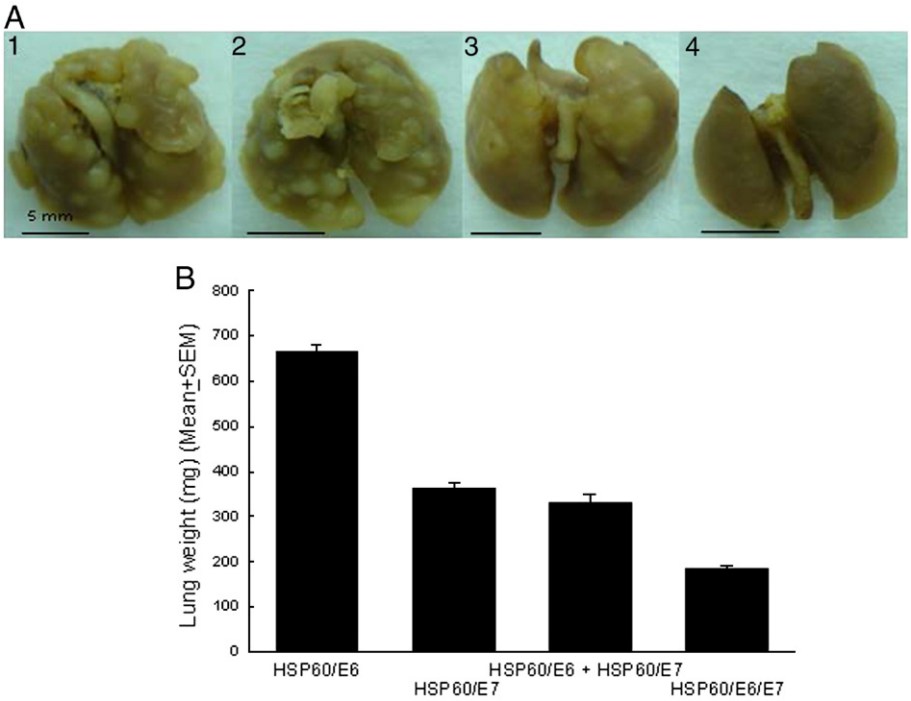

Fig. 6. *In vivo* tumor treatment experiments in mice with various therapeutic conditions. (A) Representative pulmonary tumor nodules in various DNA vaccinated groups. (1) HSP60/E6 group, (2) HSP60/E7 group, (3) HSP60/E6 mixed with HSp60/E7 group, (4) HSP60/E6/E7 group. (B) Mean lung weights in various DNA vaccinated groups. Mice treated with DNA encoding HSP60/E6/E7 showed significantly lower lung weights than those treated with either HSP60/E6 or HSP60/E7 DNA only. Mice vaccinated with HSP60/E7 DNA had lower lung weights than those vaccinated with HSP60/E6 DNA. Data are expressed as mean number of pulmonary tumor nodules ± SEM.

These results suggested that $CD8^+$ T cells are required for anti-tumor immunity generated by HSP60/E6 and HSP60/E7 DNA vaccines.

Treatment with HSP60/E6/E7 led to significant reduction of pulmonary tumor nodules

As shown in Fig. 5, mice treated with HSP60/E6 DNA (3.7 + 1.7), HSP60/E7 (1.8 ± 1.6), or HSP60/E6/E7 (0.0 ± 0.0) all exhibited significantly fewer pulmonary tumor nodules than mice treated with the wild-type E6 (70.0 ± 9.0), wild-type E7 (73.6 ± 6.7), or E6/E7 (70.4 ± 3.1) DNA vaccine ($p < 0.001$, one-way ANOVA), when started on treatment 2 days after tumor injection.

The representative figures of pulmonary tumor nodules treated with various DNA vaccines after 7 days of TC-1 tumor injection are shown in Fig. 6A. As shown in Fig. 6B, mice treated with HSP60/E6/E7 DNA led to significantly lower lung weights (185.0 + 7.1 mg) than those with HSP60/E6 mixed with HSP60/E7 (330.0 ± 20.3 mg), or HSP60/E7 (362.0 ± 13.8 mg), or HSP60/E6 DNA (665.2 ± 14.8 mg) (one-way ANOVA, $p < 0.05$), when started on treatment 7 days after tumor injection.

These data indicated that HSP60, when linked with either E6 or E7 tumor antigens, could generate more potent anti-tumor effects than wild-type E6 or E7 DNA vaccine in a lung hematogeous spread therapeutic model. Moreover, HSP60, when linked with E6 and E7 antigens together, could generate more potent anti-tumor effects than HSP60 linked with either E6 or E7 tumor antigen, or HSP60/E7 mixed with HSP60/E7.

Discussion

Mice vaccinated with HSP60/E6 or HSP60/E7 DNA enhanced E6- or E7-specific $CD8^+$ T cell responses. One possible mechanism for the enhancement of $CD8^+$ T cell responses was the effect of HSP60 in inducing direct-priming mechanism. Our *in vitro* data also revealed that HSP60 chimeric DNA vaccine might enhance $CD8^+$ T cell responses via this mechanism. Ballistic DNA delivery might introduce chimeric HSP60/E6 or HSP60/E7 DNA directly into the dermal precursors or professional APCs to enhance the antigen processing and presenting processes [33]. It was also shown that direct-priming of $CD8^+$ T cells by gene-transfected dendritic cells was the key event in gene gun-mediated DNA immunization [34]. We posit that HSP60/E6 or HSP60/E7 DNA vaccine was transfected into the DCs via the gene gun delivery system to directly enhance E6- or E7-specific T cell immunity.

Another possible mechanism for the enhancement of $CD8^+$ T cell responses by the chimeric HSP60 DNA vaccine was the "cross-priming" of HSP/peptide complexes, where the HSP led exogenous proteins to the MHC-I restricted antigen presentation pathway. We demonstrated that cross-priming might be one of possible mechanisms for the chimeric HSP60 DNA vaccine. HSP60/E6 and HSP60/E7 might be released from other cell types, such as keratinocytes (which were also transfected by gene gun vaccination) and then these chimeric HSP60/E6 or HSP60/E7 proteins were released exogenously to be taken up and processed by other APCs via the MHC class I-restricted pathway [35,36].

HSP complexes taken up by professional APCs are supposed to play an important role in introducing HSP-associated

peptides into the MHC-I antigen presentation pathway [37]. These mechanisms may provide an explanation for the observed enhancement of E6- or E7-specific T cell immunity. CD14 [38] and Toll-like receptors (TLR) 2 and 4 [39,40] are involved in HSP60-mediated cell activation. As regards HSP60, studies have shown that CD14, TLR2, and TLR4 are involved in signal transduction in macrophages [38,41]. It is interesting to evaluate the *in vivo* mechanism of HSP60/E7 and HSP60/E6 DNA vaccine using TLR2 or TLR4 deficient mice in the future.

Heat-shock proteins of the HSP60 family are molecular chaperones that enhance immune responses. HSP60 can transport antigens and after internalization, mediates antigen-specific cytotoxic T cell response [36,42] as observed in this study. HSP60 protein has also been identified in many infectious agents as an immuno-dominant antigen with a protective effect. Immunization of laboratory animals by selected HSP60, HSP70, or HSP90 isolated from several pathogens induces protective host immunity and significantly reduce the clinical manifestation of infection [43,44].

Self HSP60 protein and its derived peptide as carriers in a conjugated vaccine have been shown to protect against lethal *Streptococcus pneumoniae* [45,46]. Milan *et al.* reported that naked HSP60 DNA provides better protective effects than recombinant HSP60 protein [43]. Recently, naked HSP60 DNA has also been utilized to control adjuvant arthritis [47]. Naked human HSP60 DNA vaccine can also be used to inhibit insulitis and diabetes in the NOD mice by vaccination with a DNA construct encoding human HSP60 [48]. Mice, either unprimed or primed with *M. Tuberculosis var. bovis* (Bacillus Calmette-Guerin, BCG), produce a high and long-lasting titer of anti-peptide antibodies when immunized with repetitive malaria synthetic peptide (NANP) conjugated to mycobacterial HSP60 [49]. Although this carrier effect is associated with the risk of immune responses against self HSPs, it still provides a novel approach for the development of vaccines by a fusion of peptides or antigens to HSP60.

The $293K^bD^b$ cells are always used as the antigen-presenting cells in various immunologic assays. Ideally, immunologic assays that evaluate HPV-related vaccines are better used in actual HPV-generated tumor cells. However, it is impossible to create HPV-generated tumor cells in the animal system. Hence, we tried to use $293K^bD^b$ cells to test the immunogenicity of various HSP60 chimeric DNA vaccines. Although it is not relevant to actual HPV-generated tumor cells, 293 cells previously transfected and selected to stably express murine class I MHC molecules H2-K^b and H2-D^b (designated $293K^bD^b$) are good alternative target cells in evaluating the efficacy of DNA vaccine in the animal model.

DNA vaccine encoding heat shock protein 60 co-linked to HPV16 E6 and E7 tumor antigens generates more potent immunotherapeutic effects than E6 or E7 tumor antigens alone. Both E6 and E7 can be utilized as target antigens of cancer vaccine and immunotherapy. The fusion of HSP60 family to E6 or E7 enhances E6- or E7-specific $CD8^+$ T cell-mediated immune responses. In the past, most HPV researchers focused on E7 [50]. Since E6 represents another important target for potential vaccines to control HPV-associated lesions, it is crucial to develop vaccines targeting E6. To combine E6 and E7 as target tumor antigens is important in aiding the future development of HPV vaccines. In addition, the ability of HSP60 to enhance immune responses when linked to two different antigens in DNA vaccines also suggests that HSP60 may be broadly applicable as a strategy to enhance DNA vaccines encoding a variety of antigens. This possibility will be explored further in future DNA vaccination studies, in which HSP60 will be linked to other viral or tumor-specific antigens.

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