SPANDIDOS PUBLICATIONS

Human HSP70 and modified HPV16 E7 fusion DNA vaccine induces enhanced specific CD8+ T cell responses and anti-tumor effects

JINBAO ZONG, QINGLIN PENG, QINGYONG WANG, TING ZHANG, DONGSHENG FAN and XUEMEI XU

Department of Biophysics and Structural Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing 100005, P.R. China

Received April 28, 2009; Accepted July 1, 2009

DOI: 10.3892/or_00000522

Abstract. Cervical cancer is one of the most common cancers in women worldwide and persistent infection with human papilloma virus (HPV)s is considered to be the major risk factor. Millions of women are currently infected with high risk genotypes. Therefore, it is imperative to develop therapeutic vaccines to eliminate established infection or HPV-related disease. In the current study, we generated two potential therapeutic HPV DNA vaccines, SigmE7/MtHSP70 and SigmE7/HuHSP70, using human and mycobacterium tuberculosis HSP70 linked, respectively, to HPV16 mE7 and the signal peptide gene of human CD33. Our comparative evaluation of these two vaccines found that vaccination with the SigmE7/HuHSP70 DNA vaccine induced a stronger E7-specific CD8+ T cell immune response and resulted in a more significant therapeutic effect against E7 expressing tumor cells. Our study may serve as an important foundation and significant reference for future clinical applications.

Introduction

Cervical cancer is the second leading cause of cancer-related deaths among women worldwide. Approximately 500,000 women develop the disease and 270,000 women, of whom 80% are in developing countries, die of their disease annually. Viral molecular epidemiology studies indicated that development of cervical cancer is associated with persistent infection of high risk human papilloma virus (HPV). A total of 15 genotypes of high risk HPV have been discovered (1), of which HPV16 is the most common high risk type, accounting for more than half (53.5%) of all cervical cancers (2). In some areas, rates of positive HPV16 infection in cervical cancer could reach up to 79.6% (3). Since development of cervical cancer is closely associated with persistent virus infection, we can anticipate prevention or elimination of cervical cancer through induction of effective specific immune responses to the virus. Although the newly licensed preventive HPV vaccines (Gardasil and Cervarix), have both a remarkable safety profile and clinical efficacy against the HPV genotypes from which they were derived (4,5), they are not effective in the elimination of pre-existing infection and HPV-related disease. The choice of target antigen is extremely important for designing therapeutic vaccines. Since the HPV-encoded E7 is highly expressed and essential for viral transformation in cancer cells and severe atypical hyperplasia cells, it has become an attractive target for the development of HPV therapeutic vaccines (6,7).

DNA vaccines generate effective cytotoxic T lymphocyte (CTL) and antibody responses by involvement of antigen presenting cells (APC) that stimulate CD4+ and CD8+ T cells. DNA vaccines can be easily prepared on a large scale with high purity and stability (8,9) and can also be repeatedly given to the same patient safely and effectively. These features make DNA vaccines a potentially attractive approach for HPV therapeutic vaccine development. While early DNA vaccines were weakly immunogenic, researchers are currently emphasizing the use of versatile immunostimulatory molecules in the newer therapeutic DNA vaccines. HSP70 is one member of the heat shock protein family that has been shown to act as a potent adjuvant to enhance antigen-specific tumor immunity and innate immunity. The use of HSP70 in the context of DNA vaccines represents a promising approach for enhancing antigen-specific T cell-mediated immune responses as well as humoral responses for DNA vaccine development. Immunological functions of HSP70 can be categorized into three major areas; i) Chaperoning properties. HSP70 can bind to tumor antigen peptides and enter the antigen processing and presentation pathway (10,11). ii)

Correspondence to: Dr Xuemei Xu, Department of Biophysics and Structural Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing 100005, P.R. China
E-mail: xuemeixu@vip.sina.com

Abbreviations: HPV, human papilloma virus; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; HSP, heat shock protein; APC, antigen presentation cell; DC, dendritic cell; mE7, modified and optimized HPV16 E7 gene; MtHSP70, mycobacterium tuberculosis heat shock protein 70; HuHSP70, human heat shock protein 70; LCMV, lymphocytic choriomeningitis virus; TCR, T cell receptor; TLR, toll-like receptor

Key words: heat shock protein 70, human papilloma virus 16, E7 gene, DNA vaccine, cervical cancer

Cross-priming abilities. Through binding to endocytic receptors on APC, HSP70 can participate in the MHC-I pathway and facilitate cross-presentation of associated antigens and induction of antigen-specific CTL responses (12). Several endocytic receptors, such as CD91 and LOX1 have been identified to be involved in HSP70-mediated cross-priming (13). iii) Pro-inflammatory activities. HSP70 can induce activation and maturation of dendritic cells (DC) by binding to the receptors of CD40, TLR-2, TLR-4 on the DC; subsequent activities are characterized by up-regulation of MHC class II, CD86 and CD83 expression, and secretion of pro-inflammatory cytokines, such as IL-1ß, IL-6 and IFN-α (14). Thus, HSP70 not only activates specific immunity but also innate immunity. The addition of a signal peptide to HSP70 may prompt the release of HSP70 out of cells and further enhance the antigen cross-priming ability of APC and specific $CD8^+$ T cell response (15,16).

Immunogenicity and safety are two key factors that should be considered and balanced when developing an effective vaccine. Many studies of DNA vaccines employ the mycobacterium tuberculosis derived HSP70 (MtHSP70) because it provides a strong 'danger signal' in the form of specific microbial epitopes to stimulate an immune response. Although MtHSP70 in the DNA vaccine formulation is effective as an adjuvant, the host response against the microbial epitopes may be harmful. For example, studies in mice suggested that MtHSP70 may induce autoimmune-mediated intestine inflammation (17-19). Although HSP70 from different species all show relatively strong immuno-stimulating activities in the present literature, no other study has been reported to our knowledge that compares the effectiveness of DNA vaccines containing HSP70 derived from mycobacterium tuberculosis and humans. Such a comparative study could provide useful information for choosing a safe and appropriate vaccine for human use.

In a previous study from our lab, we deleted the transformation activity and enhanced the immunogenicity of the HPV16 mE7 gene with a combination of gene shuffling, site-directed mutagenesis and codon optimization methods. In order to further strengthen the immunogenicity of HPV16 therapeutic DNA vaccines based on mE7, here we constructed and compared the immune enhancing activities of the SigmE7/MtHSP70 DNA vaccine and SigmE7/HuHSP70 DNA vaccine with signal peptide gene of human CD33.

Materials and methods

Plasmid DNA constructs and preparation. SigmE7 containing SigCD33 and *mE7* gene was amplified by overlap PCR with the primers 5'-CGAGTCGTGCGGCCGCCACCATGCCGC TGCTGCTACTGCTGCCCCTGCTGTGGGCAG-3' (P1) (*Not*I site underlined), 5'-CTG CCCCTGCTGTGGGCAGG GGCCCTGGCTATGATGGATCTGCTCATGGGCAC-3' (P2) and 5'-GCTCTAGAGCGGTAGTCTCGGGCTGCAG-3' (P3) (*Xba*I underlined). A DNA fragment encoding HuHSP70 was obtained from pMSHsp70, a gift from Dr Morimoto of Northwestern University (Evanston, IL, USA) with the primers 5'-GCTCTAGACATGGCCAAAGCCGCGGCG ATC-3' (*Xba*I underlined) and 5'-CGGGATCCCTAATCTA CCTCCTCAATGGTG-3' (*Bam*HI underlined). We digested SigmE7 with *Not*I and *Xba*I and ligated it to *Not*I/*Xba*I-digested pVR1012 to generate pVR1012-SigmE7. To generate pVR1012-SigmE7/MtHSP70, MtHSP70 digested with *Xba*I and *Bam*HI from pVR1012-mE7/MtHSP70 (constructed by our lab) was ligated into *Xba*I/*Bam*HI-digested pVR1012-SigmE7. To obtain pVR1012-SigmE7/HuHSP70, HuHSP70 digested with *Xba*I and *Bam*HI was ligated into *Xba*I/*Bam*HI-digested pVR1012-SigmE7/MtHSP70. The integrity of the DNA plasmids from each preparation were verified by electrophoresis on a 1% agarose gel. DNA concentrations were determined by absorbance measured at 260 nm. All constructs were validated by restriction enzyme digestion and DNA sequencing.

Mice and tumor cell line. Six- to eight-week-old female C57BL/6 mice were purchased from the Institute of Zoology Chinese Academy Sciences and were maintained in the animal facility of the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

TC-1 cells were generated by co-transfection of primary pulmonary epithelial cells from C57BL/6 mice with HPV16 E6 and E7 and activated c-Ha-ras oncogenes. The cells were grown in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM non-essential amino acids and 0.4 mg/ml G418 at 37˚C with 5% CO_2.

Transfections and Western blot analysis. COS-7 cells were grown to ~70% confluence in 6-well plates and transfected with 1 μg plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Supernatants were collected 48 h later and cells were lysed in 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40 and protease inhibitors. Protein concentration was determined using BCA™ protein assay kit (Pierce Inc., Rockford, IL, USA). Cell lysates (200 μg of total protein) and supernatants (200 μl) were denatured by heating at 100˚C for 5 min, loaded on a 10% SDS-PAGE gel, separated under reducing conditions and transferred to a polyvinylidene difluoride membrane (NEN Life Science, Boston, MA, USA) according to the manufacturer's instructions. Filters were blocked overnight with 4% BSA and incubated with polyclonal rabbit anti-human HPV16 E7 antibody (1:2,000; a gift from F.C. Zhang, Xinjiang University, Xinjiang, China) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG Ab (1:10,000; Zhongshan Goldenbridge Biotechnology, Beijing, China). Blots were developed by incubation in enhanced chemiluminescence reagent (ECL kit; Pierce Inc., Rockford, IL, USA) and exposed to film.

DNA vaccinations. DNA for injection and *in vitro* transfection of COS-7 cells was prepared with EndoFree Plasmid Purification Kits from Qiagen Inc, (Valencia, CA, USA), resuspended in endotoxin-free normal saline at a final concentration of 1 μg/μl and stored at -20˚C until used for injection. To target DNA uptake by muscle cells, mice were injected i.m. into the M. quadriceps with 50 μl bupivacaine hydrochloride (5 mg/ml) 24 h before injection i.m. at the same

 50 μg of DNA on each side. These injections were after 1 week.

ELISPOT assay. BD ELISPOT Plates (BD PharMingen, San Diego, CA, USA) were coated with 5 μg/ml rat anti-mouse IFN-γ antibody in 100 μl of PBS. After overnight incubation at 4˚C, the wells were washed and blocked with RPMI-1640 culture medium containing 10% fetal bovine serum. Different concentrations of freshly isolated spleen cells from each vaccinated mice group, from $1x10^6$ to $1.25x10^5$/well, were added to the wells along with 50 IU/ml IL-2 and 1 μg/ml E7-specific MHC class I CTL epitope ($H\text{-}2D^b$, amino acids 49-57, RAHYNIVTF). After culture at 37˚C for 24 h, the plate was washed and then followed by incubation with 2.5 μg/ml biotinylated IFN-γ antibody in 100 μl in PBS containing 10% FCS at 4˚C overnight. After washing, avidin-HRP in 100 μl of PBS was added and incubated for 1 h at room temperature. After washing five times, spots were developed by adding 100 μl AEC solution. The spots were counted using an ELISPOT Reader System.

Intracytoplasmic cytokine staining and flow cytometric analysis. To detect E7-specific $CD8^+$ T cell precursors and E7-specific $CD4^+$ T-helper cell responses, plenocytes from vaccinated groups of mice were incubated either with the MHC class I E7 peptide (amino acids 49-57) or the MHC class II E7 peptide (amino acids 30-67, NDSSEEEDEDGP AGQAEPDRAHT NIVTFC). Golgistop (BD PharMingen) was added 6 h before harvesting the cells from the culture. Cells were then washed once in staining buffer and labeled with FITC-conjugated rat anti-mouse CD8 or CD4 antibodies (BD PharMingen). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (PharMingen). PE-conjugated anti-IFN-γ or anti-IL-4 antibodies and the FITC-conjugated rat $IgG_{2a,k}$ or PE-conjugated at IgG_1 isotype control antibody were all purchased from PharMingen. Analyses was performed on a Beckman Coulter EPICS XL (Beckman Coulter Inc., Fullerton, CA, USA).

Anti-E7 ELISA. The anti-HPV16 E7 antibodies in the sera were identified by ELISA. Each well of a microwell plate was coated with 100 μl of 1 μg/ml bacteria-derived HPV16 E7 proteins and incubated at 4˚C overnight. The wells were then blocked with PBS containing 5% BSA. Sera were prepared from the mice on day 10 post-immunization, serially diluted in PBS added to the ELISA wells and incubated on RT for 2 h. After washing with PBS-T containing 0.05% Tween-20, the plate was incubated with 1:3000 dilution of a HRP-conjugated goat anti-mouse IgG antibody (Zhongshan Goldenbridge Biotechnology, Beijing, China) at room temperature for 1 h. The plate was washed five times, developed with O-phenylenediamine away from light at 37˚C for 15 min and stopped with 50 μl of 2 M H_2SO_4. The ELISA plate was read with a standard ELISA reader at 490 nm.

In vivo tumor protection experiments. For the tumor protection experiment, C57BL/6 mice (seven per group) were vaccinated via i.m. injections with 1 μg of pVR1012-SigmE7/MtHSP70, pVR1012-SigmE7/HuHSP70, or 100 μl NS, as described above. One week later, the mice were boosted with the same regimen as the first vaccination. One week after the last vaccination, mice were challenged s.c. with $7.5xl0^4$ TC-1 tumor cells per mouse in the right flank and then monitored twice a week for tumor growth.

In vivo tumor treatment experiments. To test the ability of DNA vaccination to inhibit the growth of established tumors, C57BL/6 mice (seven per group) were s.c. challenged with $7.5xl0^4$ TC-1 tumor cells per mouse in the right flank. Three days after the challenge with TC-1 tumor cells, mice were given 1 μg of pVR1012-SigmE7/Mt/HSP70, pVR1012-SigmE7/HuHSP70, or 100 μl NS. One week later, these mice were boosted with the same regimen as the first vaccination. Mice were monitored twice a week for tumor growth.

Data analyses. ELISPOT and FACS data were analyzed using mean of two sample comparison of poisson distribution. ELISA data were analyzed using the Student's t-test and tumor incidence data were analyzed by the Fisher Exact Probabilities in a 2x2 table. Values of $P<0.05$ were considered statistically significant.

Results

Detection of fusion proteins secreted from DNA vaccine plasmids. After 48 h of transfection with pVR1012-SigmE7/MtHSP70 and pVR1012-SigmE7/HuHSP70, COS-7 cells and supernatants were harvested, lysed and analyzed by Western blot analysis with the polyclonal rabbit anti-human HPV16 E7 antibody. The relative molecular weights of the mE7/MtHSP70 and mE7/HuHSP70 fusion proteins expressed in the cell lysates and culture supernatants were confirmed to be 85 kDa and 100 kDa, respectively, according to their theoretical molecular weights (Fig. 1A and B). ß-actin was detected in cell lysates at all three groups as the internal loading control (Fig. 1A). The ratio of mE7/MtHSP70 or mE7/HuHSP70 vs. ß-actin was 1.12 and 1.26 in cell lysates as determined by gray scale scanning analysis from a gel imaging system (Fig. 1C). Using the same conditions, the ratio of mE7/MtHSP70 or mE7/HuHSP70 vs. ß-actin was 0.75 and 0.73 in culture supernatants (Fig. 1C). No specific target proteins were expressed in cell lysates and culture supernatants from mock pVR1012-transfected COS-7 cells. These results suggested that while these two constructs had the same expression level both in cell lysates and culture supernatants, the expression level of mE7/MtHSP70 and mE7/HuHSP70 proteins in cell lysates were higher than corresponding proteins in culture supernatants.

SigmE7/HuHSP70 fusion DNA vaccines induced higher levels of antigen-specific $CD8^+$ T cells than SigmE7/MtHSP70. Since CTLs have been known to play a critical role in tumor immunity, we examined E7-specific $CD8^+$ T cell precursor frequency after DNA vaccination by ELISPOT assays and intracellular cytokine stains. Based on the previous study in our lab, we have determined that mice vaccinated with empty pVR1012 vector vaccine and NS show no difference in ability to elicit anti-tumor immunity. Therefore, we chose to use only NS as a negative control. Two weeks after the second

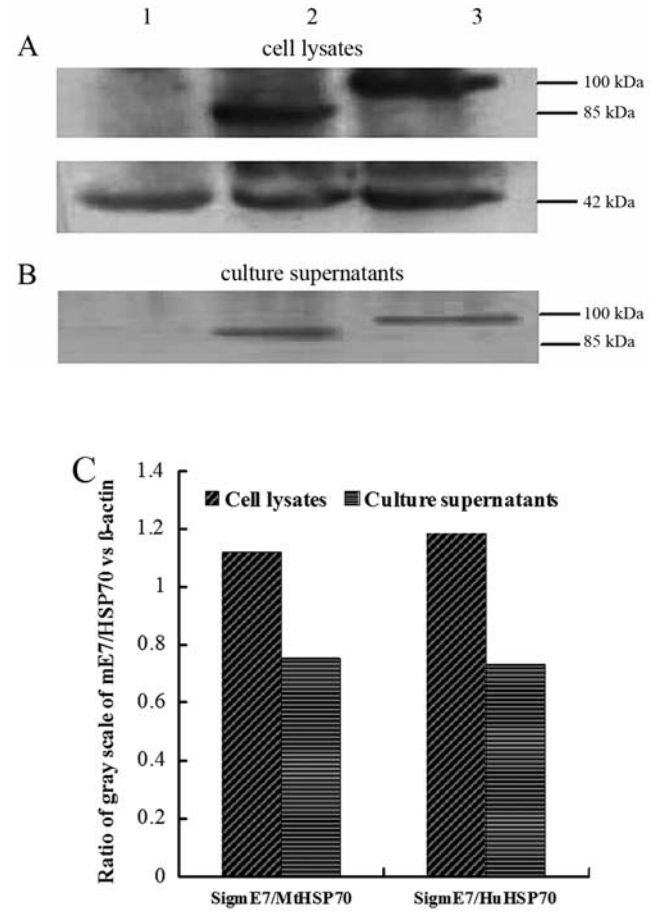

Figure 1. (A and B) Western blot analysis to determine the expression and secretion of mE7/HSP70 and the expression of ß-actin in cell lysates and culture supernatants. COS-7 cells were transiently transfected with each of the two expression vectors. Lane 1, pVR1012; Lane 2, pVR1012-SigmE7/MtHSP70; Lane 3, pVR1012-SigmE7/HuHSP70. Cell lysates and culture supernatants were blotted with a polyclonal rabbit anti-human HPV16 E7 antibody. The relative molecular weights of mE7/MtHSP70, mE7/HuHSP70 and ß-actin were ~85, 100 and 42 kDa, respectively. (C) Gray scale scanning analysis of ratio of mE7/MtHSP70 and mE7/HuHSP70 vs. ß-actin. The ratios of gray scale of mE7/MtHSP70 or and mE7/HuHSP70 vs. ß-actin was nearly identical both in cell lysates and culture supernatants.

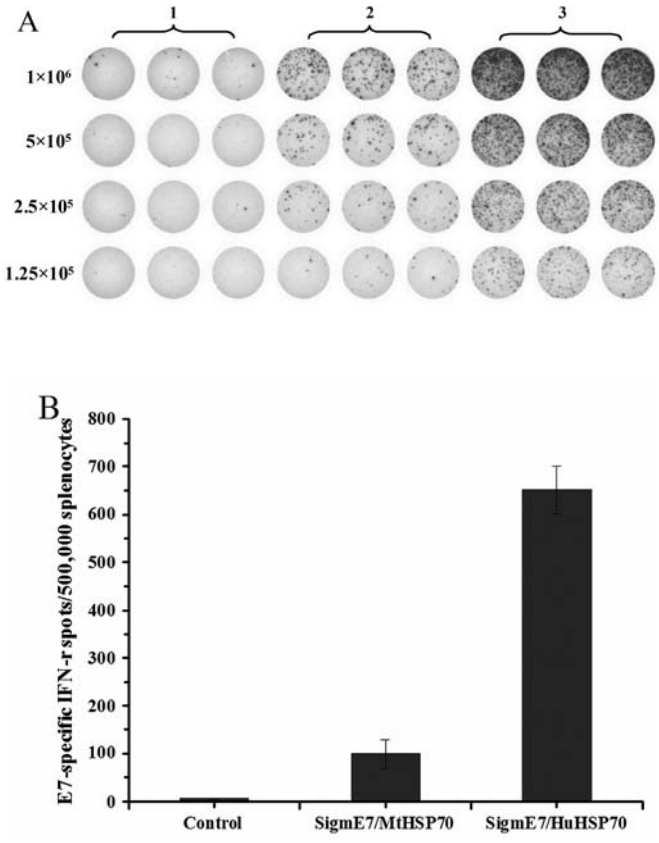

Figure 2. The quantity of E7-specific $CD8^+$ T cell secreting IFN-γ in the immunized mice spleen cells was tested by ELISPOT. (A) A spot picture of different numbers of spleen cells from each vaccinated mice group photographed by the ELISPOT Reader System. Different numbers of spleen cells are indicated; 1, control group, 2, SigmE7/MtHSP70 group, 3, SigmE7/MtHSP70 group. Each group was tested in triplicate. (B) Graph of E7-specific IFN-γ spots counted. The spot numbers were the mean of triplicates ± SE in each vaccinated group at $5x10^5$ cell number. Results shown here are E7-specific spot-forming cells from three groups and are from one representative experiment of two performed tests.

immunization, splenocytes were harvested and stimulated with E7-specific MHC class I CTL epitope. In ELISPOT assays, as shown in Fig. 2A and B, 660 IFN-γ spot-forming $CD8^+$ T cells specific for the E7 peptide were detected per $5x10^5$ splenocytes derived from the SigmE7/HuHSP70DNA vaccinated mice, compared to only 100 of the that derived from the SigmE7/MtHSP70 DNA-vaccinated mice. Therefore, the numbers of IFN-γ-producing $CD8^+$ T cells in the splenocytes from mice immunized with the SigmE7/HuHSP70 fusion DNA were greater than five times that from mice with the SigmE7/MtHSP70 fusion DNA ($P<0.01$). Results of the intracellular cytokine staining and flow cytometric analysis (Fig. 3A) correlated with the ELISPOT results presented in Fig. 2B. As shown in Fig. 3A, subtracting the background produced by the NS alone (280 cells/$3x10^5$ splenocytes), mice vaccinated with SigmE7/HuHSP70 DNA generated the highest number of E7-specific IFN-γ $CD8^+$ T cell precursors (~406 cells/$3x10^5$ splenocytes) by flow cytometric analysis, whereas mice vaccinated with SigmE7/MtHSP70 DNA generated only around 109 E7-specific IFN-γ $CD8^+$ T cell precursors per $3x10^5$ splenocytes ($P<0.01$). From the above results, we conclude that while HuHSP70 or MtHSP70 linked to mE7 could both induce the activation of antigen-specific $CD8^+$ T cells, the response to the fusion of mE7 with HuHSP70 was enhanced over that with MtHSP70.

Neither SigmE7/HuHSP70 nor SigmE7/MtHSP70 fusion DNA vaccines enhanced E7-specific $CD4^+$ T cell-mediated immune responses. To determine E7-specific $CD4^+$ T precursor cells and the cytokine profiles generated by SigmE7/HuHSP70 and SigmE7/MtHSP70 DNA vaccines, we performed double staining for the CD4 surface marker and the intracellular IFN-γ or IL-4 on splenocytes from immunized mice, followed by flow cytometric analysis. The splenocytes from immunized mice were cultured *in vitro* with the MHC class II E7 peptide (T-helper epitope, amino acids 30-67) overnight and stained for both CD4 and intracellular IFN-γ. The percentage of $CD4^+$ T cells secreting IFN-γ was analyzed using flow cytometry. As shown in Fig. 3B, mice vaccinated with SigmE7/HuHSP70 DNA generated a similar number of $CD4^+$/IFN-γ^+ double-positive cells compared to mice vaccinated with SigmE7/MtHSP70 or NS alone ($P>0.05$). We then analyzed E7-specific $CD4^+$ T cells secreting IL-4 in mice vaccinated with various

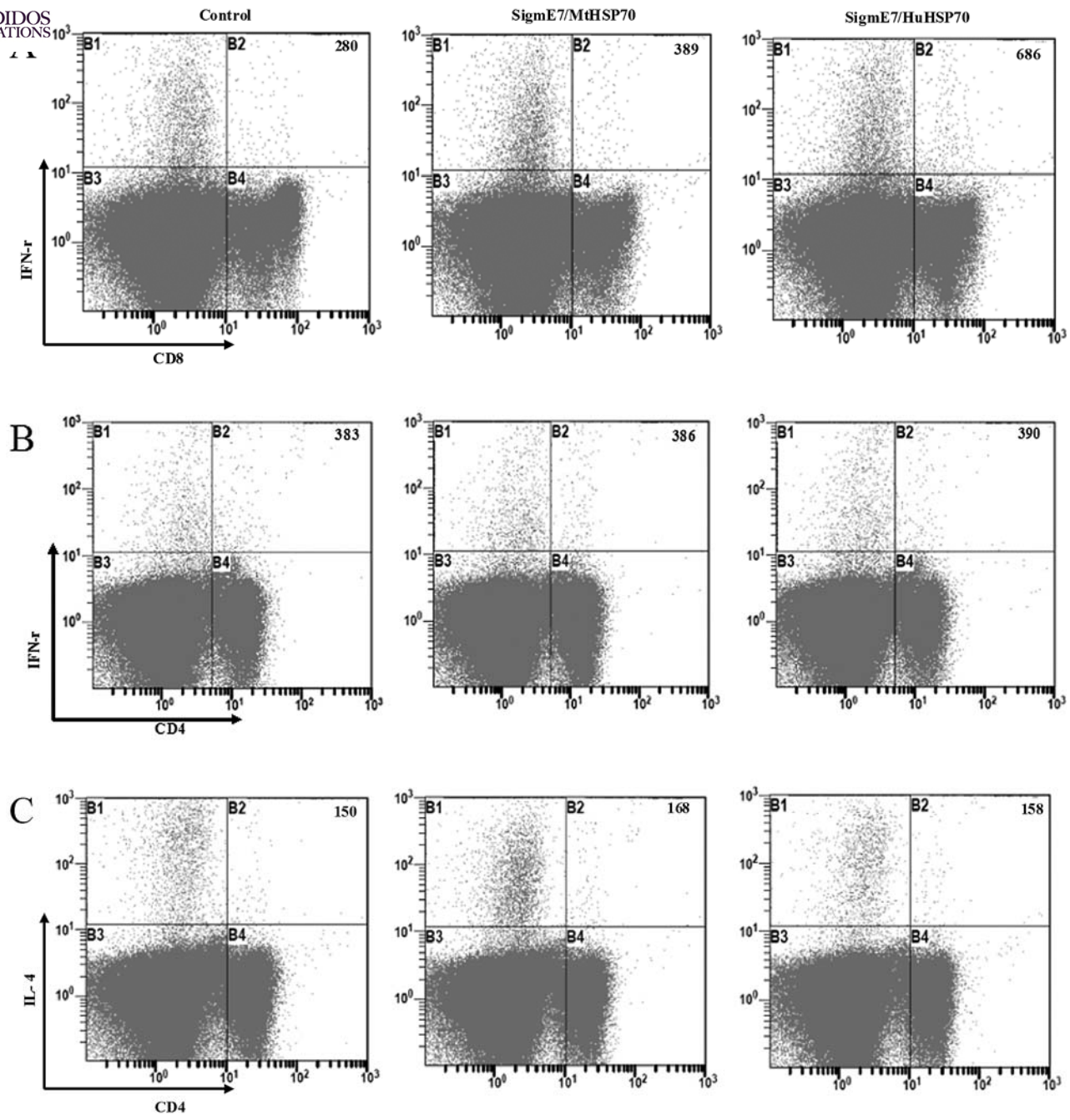

Figure 3. Flow cytometry analysis of IFN-γ-secreting E7-specific CD8$^+$ T cells, IFN-γ-secreting E7-specific Th1 cells and IL-4-secreting Th2 cell activation. (A) splenocytes from vaccinated mice were cultured *in vitro* with the E7 peptide (amino acids 49-57) overnight and were stained for both CD8 and intracellular IFN-γ. Mice vaccinated with SigmE7/HuHSP70 DNA generated the highest IFN-γ$^+$/CD8$^+$ double-positive T cells. (B and C) Splenocytes from vaccinated mice were cultured *in vitro* with the E7 peptide (amino acids 30-67) overnight and were stained for both CD4 and intracellular IFN-γ or IL-4. Mice vaccinated with SigmE7/MtHSP70 or SigmE7/HuHSP70 DNA generated comparable IFN-γ$^+$/CD4$^+$ and IL-4$^+$/CD4$^+$ double-positive cells when compared to mice vaccinated with NS. The numbers of double-positive T cells in $3x10^5$ splenocytes are indicated in the upper right corner (B2 region). Results shown here are from one representative experiment of three performed tests.

DNA vaccines. No significant levels of CD4$^+$/IL-4$^+$ double-positive cells could be identified in the mice that received SigmE7/HuHSP70, SigmE7/MtHSP70 and NS (Fig. 3C). From these two assays, we determined that both SigmE7/HuHSP70 and SigmE7/MtHSP70 do not activate E7-specific Th1 and Th2 cell responses.

Neither SigmE7/HuHSP70 nor SigmE7/MtHSP70 fusion DNA vaccines induced E7-specific antibodies. The quantity of anti-HPV16 E7 antibodies in the sera of the vaccinated mice was determined by ELISA 10 days after the last vaccination. No anti-E7-specific antibodies could be detected in the sera of mice of any vaccinated group (Fig. 4). This result suggested that the modifications of these two DNA vaccines did not enhance antibody responses.

SigmE7/HuHSP70 and SigmE7/MtHSP70 DNA vaccines prevented tumors in vivo. To determine whether vaccination with the SigmE7/HuHSP70 or SigmE7/MtHSP70 fusion DNA vaccines protects mice against E7-expressing tumors, the mice

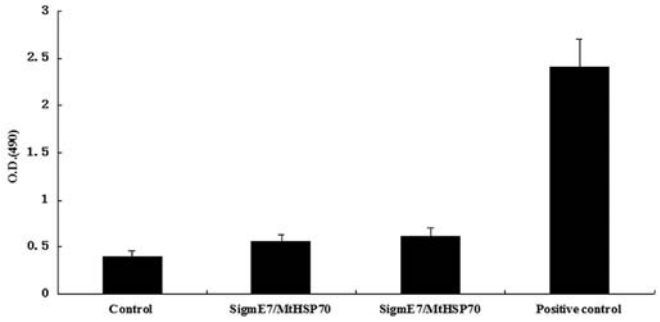

Figure 4. E7-specific antibody responses in C57BL/6 mice immunized with SigmE7/MtHSP70 and SigmE7/HuHSP70 DNA vaccines. C57BL/6 mice were immunized with NS-negative control and anti-E7 antibody-positive control. Serum samples were obtained from immunized mice 10 days after vaccination. The presence of the E7-specific antibody was detected by ELISA using serial dilution of sera. The results from the 1:20 dilution are presented showing the mean absorbance (A_{490} nm) ± SE.

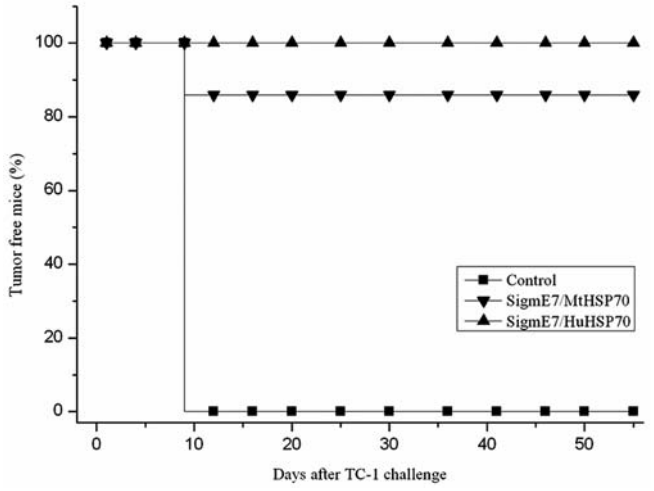

Figure 5. *In vivo* tumor protection experiment. Vaccination with both pVR1012-SigmE7/MtHSP70 and pVR1012-SigmE7/HuHSP70 DNA protected mice against growth of the HPV16 E7-expressing tumor line TC-1. C57BL/6 mice (seven mice per group) were immunized i.m. with two times with 100 μg pVR1012-SigmE7/MtHSP70 (1 μg/μl), pVR1012-SigmE7/HuHSP70 DNA (1 μg/μl) and 100 μl NS. At 1 week after the second vaccination, mice were challenged s.c. with 7.5xl0^4 TC-1 cells in the right flank per mouse. The mice were monitored for evidence of tumor growth twice a week. The results shown here are from a representative experiment of two performed.

were challenged s.c. in the right flank with 7.5x10^4 TC-1 cells 7 days after the last vaccination. For the mice receiving vaccination, 100% of those receiving SigmE7/HuHSP70 DNA vaccination remained tumor-free 55 days after the TC-1 challenge, whereas only 86% of mice receiving SigmE7/MtHSP70 DNA vaccination remained tumor-free. In contrast, all of the mice receiving NS developed a tumor growth on 12 days after the tumor challenge (Fig. 5). Taken together, these results indicated that both SigmE7/HuHSP70 and SigmE7/MtHSP70 fusion DNA could significantly enhance immunity against the growth of TC-1 tumors. A higher percentage of mice vaccinated with the SigmE7/HuHSP70 fusion DNA were tumor-free than mice vaccinated with SigmE7/MtHSP70 fusion DNA.

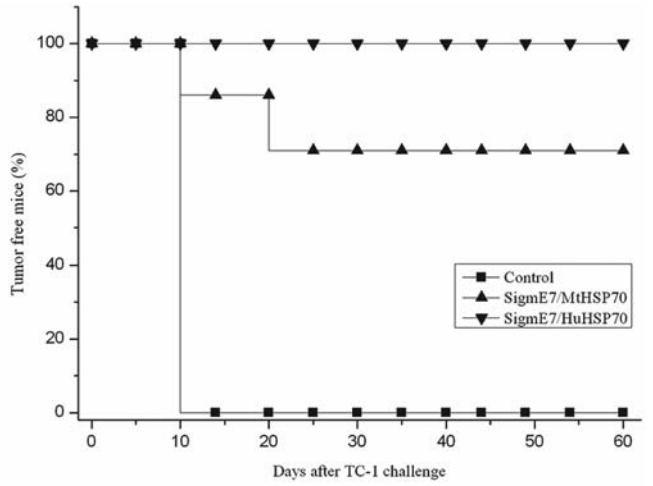

Figure 6. *In vivo* tumor treatment experiment. Vaccination with SigmE7/HuHSP70 DNA enhances the anti-tumor immunity more significantly than SigmE7/MtHSP70 DNA. Vaccination with SigmE7/HuHSP70 DNA eradicated pre-existing TC-1 tumor cells, 100% of mice keep tumor-free until 60 days after TC-1 challenge, while 71% of mice vaccinated with SigmE7/MtHSP70 keep tumor-free at the 60th day. Each mouse was initially challenged s.c. with 7.5xl0^4 TC-1 cells in the right flank and then was vaccinated i.m. with SigmE7/MtHSP70, SigmE7/HuHSP70 and NS on days 3 and 10. The mice were monitored for tumor growth twice a week. The results shown here are from a representative experiment of two performed.

SigmE7/HuHSP70 fusion DNA vaccines eradicated tumors in vivo. To test the therapeutic effect of the DNA vaccines in eradicating established TC-1 tumors, we first inoculated mice s.c. in the right flank with 7.5x10^4 TC-1 tumor cells and then vaccinated them i.m. on days 3 and 10. As shown in Fig. 6, the TC-1 tumor was eliminated from 100% of mice receiving the SigmE7/HuHSP70 fusion DNA vaccination. However, some of the mice receiving SigmE7/MtHSP70 fusion DNA vaccination developed a tumor growth on day 14 and 20, respectively after the tumor challenge and the TC-1 tumor was eliminated only from 71% of these mice. In contrast, all of the mice receiving NS developed a tumor growth on day 14 after the tumor challenge. There was a statistically significant difference in the therapeutic effect of TC-1 tumor between SigmE7/HuHSP70 and SigmE7/MtHSP70 DNA vaccinated group ($P<0.01$). In summary, these results showed that while vaccination with both SigmE7/HuHSP70 and SigmE7/MtHSP70 fusion DNA could eradicate previously inoculated E7-expressing tumors in mice, the SigmE7/HuHSP70 DNA resulted in eradication of all the established E7-expressing tumors and induced stronger anti-tumor activity *in vivo*.

Discussion

Results of this study showed that SigmE7/HuHSP70 and SigmE7/MtHSP70 can produce relatively strong CD8$^+$ T cell response and anti-tumor effects. CD4$^+$ T cells expressing IFN-γ or IL-4 and E7-specific antibody were not detected in the two vaccines tested in this study, indicating that Th1 and Th2 mediated cellular immunity and B cell mediated humoral immunity likely did not play primary roles in anti-tumor effect with these DNA vaccines. The above results also showed that

tumor effects of these two DNA vaccines mainly on E7-specific CD8+ T cell responses and may not depend on the assistance of CD4+ T cells. Currently, the mechanism of CD4+ T cell-independent CD8+ T cell function is not well defined. Studies by Garza *et al* and Kyburz *et al* showed naive T cells can be activated by specific peptides independent of CD4+ T cell (20,21).

In the study by Wang *et al* and Harmala *et al* (22,23), the transgenic mice which were absent of CD4+ T cells produced LCMVgp33-specific TCR expressing CD8+ T cells when transfected with LCMVgp33-specific TCR gene P14. Their study showed that TCR-Tg CD8+ T cell can be activated by LCMV without the assistance of CD4+ T cells. A possible explanation is that these cells can generate 'self-help' by secreting certain cytokines and produce immune responses independent of CD4+ T cells. Endogenous IL-2 also probably plays an important role in this process. It has been indicated in other studies that antigen-HSP70 fusion genes generated specific CD8+ T cells responses that can be independent of CD4+ T cell help (24,25). Possible mechanisms of HSP70 enhancement of CD4+ T cell-independent CD8+ T cell response is activation of DC to release proinflammatory cytokines and the intrinsic molecular chaperone function of HSP70. As a cytoplasmic protein, HSP70 plays an important role, not only in the process of protein folding, transport and degradation, but also in participating in directing more efficient antigen presentation to CD8+ T cells through the MHC-I pathway (26-28). Huang *et al* showed HSP70 might activate APC and promote their maturation of directly and indirectly through a mechanism similar to viruses. Activated APC can produce proinflammatory cytokines, which can activate CD8+ T cells efficiently independent of CD4+ T cell help (29). Liu *et al* (30) showed fusion proteins composed of HPV16 E7 and mycobacterium tuberculosis HSP65 can induce not only E7-specific CD8+ T cell responses, but also produce and maintain memory CD8+ T cell responses independently of CD4+ T cells. Additionally, Chen *et al* demonstrated that depletion of CD4+ T cell did not decrease the anti-tumor immunity generated by E7-HSP70 DNA vaccine in antibody depletion experiments *in vivo* (24).

Because of the relatively weak immunogenicity of DNA vaccines and because the number of precursor and activated CD4+ T cells are much less than those of CD8+ T cells, we could not detect them. Before antigen exposure, the frequency of naive T cells is typically 1 in 10^5 to 10^6 lymphocytes; after antigen exposure, the numbers of T cells specific for that antigen may increase to ~1 in 10 for CD8+ T cell and 1 in 100 to 1000 for CD4+ T cell. The numbers rapidly decline when antigen is eliminated and after the immune response subsides, the surviving memory cells specific for the antigen is ~1 in 10^4. Thus, the current technology used in this study may not reach this level of sensitivity. It has been suggested that, although E7-specific CD4+ T cells could not be detected in mice immunized by HPV16 E7/HSP70 DNA vaccine, it was possible that HSP70-specific CD4+ T cells may be produced to promote the activation and proliferation of CD8+ T cells (31,32).

This study showed that human HSP70 can produce stronger E7-specific CD8+ T cell responses and anti-tumor effects than mycobacterium tuberculosis HSP70. Some investigators have hypothesized that MtHSP70 derived from bacteria would have the intrinsic 'danger signal' function of heat shock protein. One might believe that MtHSP70 would generate stronger immune responses than HuHSP70, because it would also be considered an allogenous protein due to specific epitopes originating from bacteria. Signal receptors participating in 'danger signals' mainly include TLR2, TLR4, and CD40 (33). Innate immunity can be activated after combining these receptors and HSP70, resulting mainly in up-regulation of expression of pro-inflammatory factors and co-stimulatory molecules. Some studies have shown immuno-enhancement of HuHSP70 and MtHSP70 mainly depending on cross-presentation of peptide-specific MHC-I in HSP70/peptide compound, which is related to the molecular chaperone functions of HSP70 (34). Homologous proteins derived from different species have differences in amino acid sequence, which result in differences in recognition and conjugation ability. When HuHSP70 is introduced into the human body as a homologous protein, affinity to HSP70 receptors on DC may be higher than MtHSP70 from bacteria because of naturally increased affinity to the receptors than those of MtHSP70. Even very low concentrations of HuHSP70 protein that can efficiently bind with the HSP70 receptor would significantly improve the molecular chaperone functions to enhance antigen cross-presentation and subsequent CD8+ T cell activation. Studies in mice by Li *et al* showed that homologous HSP70 from mice generated greater anti-tumor effects than allogenous mycobacterium tuberculosis HSP70. They presumed that a possible reason is that production of HSP70 antibody generated by mycobacterium tuberculosis HSP70 prevented the APC from recognizing the fusion protein of antigen and MtHSP70 (35). Some studies have also shown that mycobacterium tuberculosis HSP70 may initiate the induction and proliferation of regulatory T cells (e.g. CD4+ CD25+ T cells) with immuno-suppressive functions which would weaken the antigen-specific immune response generated by a DNA vaccine (36,37).

Many researchers anticipate that HSP70 can be used as an adjuvant to enhance immunogenicity of tumor therapeutic DNA vaccines. However, the safety of these types of vaccines should be considered not only in terms related to routine DNA vaccines, but also in terms of the potential risk of the adjuvant itself in humans. A concern of DNA vaccines is that they can potentially integrate into the host genome, leading to inactivation of anti-oncogenes or activation of proto-oncogenes and accordingly result in malignant transformation of host cells. Fortunately, this kind of integration frequency is far lower than spontaneous mutation of host genes, and would not pose a real risk (38). HPV16 E7 is a viral transformed gene and its DNA vaccine can produce HPV16 E7 onco-proteins in hosts, which can inactivate the tumor suppressor protein pRB (39). Therefore, E7 is potentially carcinogenic. Studies have found that the transformation activity of E7 can be eliminated if of the amino acid locus in two zinc finger binding areas and the pRB binding area of E7 are mutated, or the E7 gene is cut or rearranged of E7 (40-43). In early studies from our group, the wild-type E7 gene was optimized by using many strategies, including gene cutting and rearrangement, codon optimization and amino acid site-specific mutagenesis to obtain the mE7 gene.

Although all HSP70s derived from different species could enhance the immunogenicity of DNA vaccines, concern still remains regarding the use of allogenous HSP70 for vaccine formulations in the clinic due to its potential toxicities. Because MtHSP70 is derived from bacteria, potential risk in future clinical applications remain. Amino acid structure, sequence and function of HSP70 are highly conserved between prokaryotes and eukaryotes. Homology of HSP70 is >70% in different biological species. Therefore, these HSP70 proteins may generate many common epitopes, which may evoke cross immune responses, even leading to autoimmune disease (44). Because MtHSP70 comes from bacteria, it will be recognized as an external antigen after entering the human body and induce the production of crossreactive T cells aimed directly at MtHSP70. Homology of MtHSP70 and HuHSP70 is relatively high and they have many common epitopes. Therefore, it is likely that cross-reactive T cells specific for MtHSP70 can also identify HuHSP70, potentially causing development of autoimmune disease. On the other hand, the likelihood of HuHSP70 causing autoimmune disease in humans is significantly decreased. Steinhoff *et al* (18) and Zugel and Kaufmann (19), showed that after being immunized by mycobacterium tuberculosis HSP70, autoimmune disease can be induced in mice, manifested mainly by autoimmune enteritis in the small intestine. A report by Sinha *et al* (45) showed that autoreactive T cells, that likely escaped from the thymic selective process, circulate in healthy people. Because these autoreactive cells are few in quantity or restricted by regulatory mechanisms, they usually remain dormant without harmful effects in the body. However, when receiving continuous stimulation with external antigens having high similarity epitopes (e.g. common epitopes of self HSP70 and HSP70 from bacteria) with a human self antigen, silent autoreactive T cells are likely to be activated. We conclude from the analysis above that using human HSP70 instead of mycobacterium tuberculosis HSP70 in a DNA vaccine can significantly decrease the risk of autoimmune disease resulting from activation of autoreactive T cells.

In summary, we tested a therapeutic HPV16 DNA vaccine that is capable of generating significantly high levels of antigen-specific anti-tumor effects with a high safety profile. Our observations may serve as an important foundation and significant reference for future clinical applications.

Acknowledgements

We gratefully acknowledge Professor Richard I. Morimoto (Northwest University, Evanston, IL, USA) for kindly providing the pMSHsp70 plasmid. This study was supported by a grant from the National Natural Science Foundation of China (No.30271355).

References

1. Clifford GM, Smith JS, Plummer M, Munoz N and Franceschi S: Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br J Cancer 88: 63-73, 2003.
2. Muñoz N, Bosch FX, Castellsagué X, *et al*: Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer 111: 278-285, 2004.
3. Wu Y, Chen Y, Li L, Yu G, Zhang Y and He Y: Associations of high-risk HPV types and viral load with cervical cancer in China. J Clin Virol 35: 264-269, 2006.
4. Siddiqui MA and Perry CM: Human papillomavirus quadrivalent (types 6, 11, 16, 18) recombinant vaccine (Gardasil): profile report. BioDrugs 20: 313-316, 2006.
5. Crosbie EJ and Kitchener HC: Cervarix - a bivalent L1 virus-like particle vaccine for prevention of human papillomavirus type 16- and 18-associated cervical cancer. Expert Opin Biol Ther 7: 391-396, 2007.
6. Hausen H: Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2: 342-350, 2002.
7. Zehbe I, Wilander E, Delius H and Tommasino M: Human papillomavirus 16 E6 variants are more prevalent in invasive cervical carcinoma than the prototype. Cancer Res 58: 829-833, 1998.
8. Gurunathan S, Klinman DM and Seder RA: DNA vaccines: immunology, application, and optimization. Annu Rev Immunol 18: 927-974, 2000.
9. Donnelly JJ, Ulmer JB, Shiver JW and Liu MA: DNA vaccines. Annu Rev Immunol 15: 617-648, 1997.
10. Li Z, Menoret A and Srivastava P: Roles of heat-shock proteins in antigen presentation and cross-presentation. Curr Opin Immunol 14: 45-51, 2002.
11. Srivastava P: Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. Annu Rev Immunol 20: 395-425, 2002.
12. Asea A, Kraeft SK, Kurt-Jones EA, *et al*: HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med 6: 435-442, 2000.
13. Singh-Jasuja H, Scherer HU, Hilf N, Arnold-Schild D, Rammensee HG, Toes RE and Schild H: The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. Eur J Immunol 30: 2211-2215, 2000.
14. Kuppner MC, Gastpar R, Gelwer S, Nössner E, Ochmann O, Scharner A and Issels RD: The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. Eur J Immunol 31: 1602-1609, 2001.
15. Massa C, Guiducci C, Arioli I, Parenza M, Colombo MP and Melani C: Enhanced efficacy of tumor cell vaccines transfected with secretable hsp70. Cancer Res 64: 1502-1508, 2004.
16. Hauser H, Shen L, Gu QL, Krueger S and Chen SY: Secretory heat-shock protein as a dendritic cell-targeting molecule: a new strategy to enhance the potency of genetic vaccines. Gene Ther 11: 924-932, 2004.
17. Raska M and Weigl E: Heat shock proteins in autoimmune diseases. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 149: 243-249, 2005.
18. Steinhoff U, Brinkmann V, Klemm U, *et al*: Autoimmune intestinal pathology induced by hsp60-specific CD8 T cells. Immunity 11: 349-358,1999.
19. Zugel U and Kaufmann SHE: Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev 12: 19-39, 1999.
20. Garza KM, Chan SM, Suri R, Nguyen LT, Odermatt B, Schoenberger SP and Ohashi PS: Role of antigen-presenting cells in mediating tolerance and autoimmunity. J Exp Med 191: 2021, 2000.
21. Kyburz D, Aichele P, Speiser DE, Hengartner H, Zinkernagel RM and Pircher H: T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. Eur J Immunol 23: 1956, 1993.
22. Wang B, Norbury CC, Greenwood R, Bennink JR, Yewdell JW and Frelinger JA: Multiple paths for activation of naive $CD8^+$ T cells: CD4-independent help. J Immunol 167: 1283-1289, 2001.
23. Harmala LA, Ingulli EG, Curtsinger JM, *et al*: The adjuvant effects of Mycobacterium tuberculosis heat shock protein 70 result from the rapid and prolonged activation of antigen-specific $CD8^+$ T cells in vivo. J Immunol 169: 5622-5629, 2002.
24. Chen CH, Wang TL, Hung CF, Yang Y, Young RA, Pardoll DM and Wu TC: Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. Cancer Res 60: 1035-1042, 2000.

F, Hung CF, Cheng WF, He L, Slater LA, Ling M and C: Enhancement of suicidal DNA vaccine potency by linking Mycobacterium tuberculosis heat shock protein 70 to an antigen. Gene Ther 8: 376-383, 2001.
26. Singh-Jasuja H, Hilf N, Scherer HU, Arnold-Schild D, Rammensee HG, Toes RE and Schild H: The heat shock protein gp96: a receptor-targeted cross-priming carrier and activator of dendritic cells. Cell Stress Chaperones 5: 462-470, 2000.
27. Binder RJ, Han DK and Srivastava PK: CD91: a receptor for heat shock protein gp96. Nat Immunol 1: 151-155, 2000.
28. Delneste Y, Magistrelli G, Gauchat J, *et al*: Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. Immunity 17: 353-362, 2002.
29. Huang Q, Richmond JF, Suzue K, Eisen HN and Young RA: In vivo cytotoxic T lymphocyte elicitation by mycobacterial heat shock protein 70 fusion proteins maps to a discrete domain and is CD4 (+) T-cell independent. J Exp Med 191: 403-408, 2000.
30. Liu H, Wu BH, Rowse GJ and Emtage PC: Induction of CD4-independent E7-specific $CD8^+$ memory response by heat shock fusion protein. Clin Vaccine Immunol 14: 1013-1023, 2007.
31. Barrios C, Lussow AR, Van Embden J, *et al*: The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guérin priming. Eur J Immunol 22: 1365-1372, 1992.
32. Suzue K and Young RA: Adjuvant-free hsp70 fusion protein system elicits humoral and cellular immune responses to HIV-1 p24. J Immunol 156: 873-876, 1996.
33. Calderwood SK, Theriault JR and Gong J: Message in a bottle: role of the 70-kDa heat shock protein family in anti-tumor immunity. Eur J Immunol 35: 2518-2527, 2005.
34. Tobian AA, Harding CV and Canaday DH: Mycobacterium tuberculosis heat shock fusion protein enhances class I MHC cross-processing and - presentation by B lymphocytes. J Immunol 174: 5209-5214, 2005.
35. Li Y, Subjeck J, Yang G, Repasky E and Wang XY: Generation of anti-tumor immunity using mammalian heat shock protein 70 DNA vaccines for cancer immunotherapy. Vaccine 24: 5360-5370, 2006.
36. Van Eden W, Koets A, van Kooten P, Prakken B and van der Zee R: Immunopotentiating heat shock proteins: negotiators between innate danger and control of autoimmunity. Vaccine 21: 897-901, 2003.
37. Rook GA, Martinelli R and Brunet LR: Innate immune responses to mycobacteria and the downregulation of atopic responses. Curr Opin Allergy Clin Immunol 3: 337-342, 2003.
38. Nichols WW, Ledwith BJ, Manam SV and Troilo PJ: Potential DNA vaccine integration into host cell genome. Ann NY Acad Sci 772: 30-39, 1995.
39. Lukas J, Müller H, Bartkova J, *et al*: DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1. J Cell Biol 125: 625-638, 1994.
40. Osen W, Peiler T, Ohlschlager P, *et al*: A DNA vaccine based on a shuffled E7 oncogene of the human papillomavirus type 16 (HPV 16) induces E7-specific cytotoxic T cells but lacks transforming activity. Vaccine 19: 4276-4286, 2001.
41. Shi W, Bu P, Liu J, Polack A, Fisher S and Qiao L: Human papillomavirus type 16 E7 DNA vaccine: mutation in the open reading frame of E7 enhances specific cytotoxic T-lymphocyte induction and antitumor activity. J Virol 73: 7877-7881, 1999.
42. Smahel M, Sima P, Ludvikova V and Vonka V: Modified HPV16 E7 genes as DNA vaccine against E7-containing oncogenic cells. Virology 281: 231-238, 2001.
43. Liu WJ, Gao F, Zhao KN, Zhao W, Fernando GJ, Thomas R and Frazer IH: Codon modified human papillomavirus type 16 E7 DNA vaccine enhances cytotoxic T-lymphocyte induction and anti-tumour activity. Virology 301: 43-52, 2002.
44. Young RA: Stress proteins and immunology. Annu Rev Immunol 8: 401-420, 1990.
45. Sinha AA, Lopez M and McDevitt HO: Autoimmune diseases: the failure of self tolerance. Science 248: 1380-1388, 1990.