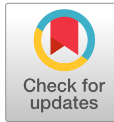
Research Communication



Small Heat Shock Proteins B1 and B6: Which One is the Most Effective Adjuvant in Therapeutic HPV Vaccine?

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Abstract

Therapeutic human papillomaviruse (HPV) vaccines have the potential to inhibit the tumor growth by targeting HPV E6 and E7 oncoproteins. Among different vaccine strategies, DNA and protein-based approaches are the most effective candidates for stimulation of the immune responses against HPV infections. Our study was designed to assess the efficacy of small heat shock proteins B1 (Hsp27) and B6 (Hsp20) as an adjuvant accompanied by HPV16 E7 and hPP10-E7 antigens in tumor mouse model. A major key for successful DNA and protein transfer into cells is the development of delivery systems with high efficiency and low cytotoxicity. Herein, we used hPP10 and MPG cell penetrating peptides (CPPs) for protein and DNA delivery *in vivo*, respectively. Our data indicated that the combination of Hsp27 with the recombinant hPP10-E7 protein in homologous protein/protein (hPP10-E7 + Hsp27) and heterologous DNA/protein (pcDNA-E7 + MPG/ hPP10-E7 + Hsp27) significantly enhanced the E7-specific T cell responses. Indeed, these regimens induced high levels of IgG2a, IFN-γ and IL-2 directed toward Th1 responses and also Granzyme B secretion as compared to other immunization strategies, and also displayed complete protection more than 60 days after treatment. These data suggest that the use of Hsp27 as an adjuvant and MPG and hPP10 as a gene and protein carrier would represent promising applications for improvement of HPV therapeutic vaccines. © 2018 IUBMB Life, 00(00):1–10, 2018

Keywords: HPV; E7; therapeutic vaccine; cell penetrating peptide; adjuvant; heat shock protein

INTRODUCTION

The most common high-risk types of human papillomaviruses (HPVs) are the HPV-16 and HPV-18, respectively, associated with pre-cancers and cancers in various anogenital sites (1–4). The E7 oncoprotein is considered as a promising vaccine candidate in immunotherapy of HPV16-associated cervical cancers. To develop an effective HPV vaccine, the best tumor antigens are usually combined with adjuvants or delivery systems to achieve positive clinical results (5). As known, heat shock proteins (HSPs) are a family of highly conserved intracellular chaperone proteins which are important mediators of tumor immunity. Indeed, HSP-based vaccines can activate tumor-specific immunity, trigger the proliferation of cancer-specific CD8^{+} T cells, and inhibit tumor growth (6). Previous studies demonstrated HSP-mediated stimulation of innate, cell-mediated, and humoral immune responses (7, 8). Among HSPs, small HSPs have a conservative α-crystallin domain (~90 amino acid residues) and show many functions such as protein folding, transportation, proteostasis, and immunity. The studies indicated that some small HSPs are expressed in all human tissues including HspB1 (Hsp27), HspB5 (αB-crystallin), HspB6 (Hsp20), and HspB8. Small HSPs are also capable of delivering antigens to major histocompatibility complexes for stimulation of adaptive immunity (9). The 27-kDa HSP (Hsp27) has a potent ability

Additional Supporting Information may be found in the online version of this article.

Abbreviations: CPP, cell penetrating peptide; HPV, human papillomavirus; HSP, heat shock protein; MHC, major histocompatibility complexes

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Volume 00, Number 00, Month 2018, Pages 1–10

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Received 26 March 2018; Accepted 6 June 2018
DOI 10.1002/iub.1892
Published online 00 Month 2018 in Wiley Online Library (wileyonlinelibrary.com)

to increase cell survival in response to a wide range of cellular challenges (10, 11). In one report, the overexpression of Hsp27 in a breast cancer cell line increased its susceptibility to cytolysis by γδ T cells (12). Moreover, Hsp20 contains α-crystallin domain known as a ligand for toll-like receptor 2/4 located on dendritic, macrophage, mast, monocyte, microglia, neutrophil, and T-cells (13). It is interesting that depending on the dose of Hsp-based vaccine, either immunosuppressive or immunostimulatory activities can be elicited (8).

Among a variety of delivery systems, cell penetrating peptides (CPPs) were recommended as a family of peptides with the ability to cross biological membranes and deliver different cargoes (e.g., DNA, siRNA, peptide, and protein into cells, (14)). For instance, MPG and hPP10 CPPs have been designed to deliver DNA and protein, respectively. The hPP10 is a CPP derived from human cell nucleoproteins and also one fragment of lysine specific demethylase polypeptide sequence (KDM4A protein) which has the ability to carry proteins in a covalent fashion into the cells (15). The short amphipathic peptide MPG (27 amino acids) is composed of three domains: an N-terminal hydrophobic domain, a hydrophilic lysine-rich domain (KKKRKV), and a spacer domain (WSQP) involved in effective delivery of nucleic acids *in vitro* and *in vivo* (16, 17). Except the use of adjuvants and delivery systems, heterologous prime-boost vaccination strategy is another way to develop both humoral and cellular immunity of therapeutic vaccines against a specific antigen (18, 19). In this study, we evaluated the efficiency of Hsp27 (or HspB1) and Hsp20 (or HspB6) as an adjuvant associated with hPP10 and MPG CPPs as a delivery system for development of a therapeutic vaccine against HPV infections. Herein, three vaccine modalities including homologous DNA, homologous protein, and heterologous DNA/protein were studied to stimulate HPV E7 antigen-specific immunity.

MATERIALS AND METHODS

Peptides

The MPG (27 residues: GALFLGFLGAAGSTM-GAWSQPKKKRKV) and hPP10 (KIPLPRFKLKCIFCKKRRKR) peptides synthesized by Biomatik Corporation (Cambridge, Canada).

Generation of DNA and Protein Constructs in Bacterial Systems

The recombinant HPV16 E7 protein (rE7) was expressed in *Escherichia coli* M15 strain and purified by affinity chromatography on Ni-NTA resin under denaturing conditions as previously reported (20). Moreover, the HPV16 E7 gene was fused to the nucleotide sequence of hPP10 (NM_014663.2) at the *Bam*HI/*Hind*III restriction site in the pET28a (+) vector. The pET-hPP10 was previously synthesized by Biomatik Corporation (Cambridge, Canada). In the next step, the *E. coli* Rosetta strain was transformed with the recombinant pET-hPP10-E7 plasmid. The hPP10-E7 protein was expressed by 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 3 h after induction and purified by affinity chromatography under denaturing conditions according to the manufacturer's instructions (Qiagen). Contrarily, the full length of *Mus musculus* Hsp20 gene (Accession No: NM_001012401) and Hsp27 gene (Accession No: NM_013560) were synthesized in a prokaryotic expression vector (pET-Hsp20, pET-Hsp27, Biomatik Company, Canada). The *E. coli* Rosetta strain was transformed by the recombinant pET-Hsp20 or pET-Hsp27 plasmids. The expression of proteins was induced by 1 mM IPTG at 4 h after induction and purified by affinity chromatography using a Ni-NTA agarose column under native conditions according to the manufacturer's instructions (Qiagen). Then, the purified proteins were dialyzed against PBS1×. The endotoxin contamination was <0.5 EU per milligram (mg) protein as monitored by LAL assay (QCL-1000, Lonza). Finally, their concentrations were measured by Bradford protein assay kit (Thermo Fisher Scientific) and NanoDrop spectrophotometry, and stored at −70 °C, until used.

Contrarily, the endotoxin-free plasmid DNA expressing HPV16 E7 protein (pcDNA-E7, 20) was prepared by an Endo-free plasmid Mega kit (Qiagen) according to the manufacturer's instructions.

Preparation of pcDNA-E7/MPG Complex

The MPG solution was added to 1 μg of pcDNA-E7 at an *N/P* ratio of 10:1 (peptide: DNA) in PBS (pH = 7.4), and incubated for 30 min at room temperature as previously reported (21). The condensation between MPG and DNA was assessed by gel retardation assay, and the size and morphology of the MPG/E7 DNA nanoparticles were analyzed with a scanning electron microscope (SEM) (KYKY-EM3200 model, China).

Mice Immunization

Inbred C57BL/6 female mice, 5–7 week old, were obtained from the breeding stocks maintained at the Pasteur Institute of Iran. Twenty-two groups of seven mice were selected. Mice were immunized on days 0, 14, and 28 with 50 μg of the E7 DNA construct and 10 μg of the recombinant HPV16 E7 and hPP10-E7 proteins in PBS1×. The MPG/pcDNA-E7 complex was used at an *N/P* ratio of 10:1. The recombinant proteins were emulsified with Montanide ISA720 (M720) at the ratio of 70:30 (*v/v*, oil: aqueous phase) and Freund's adjuvant at the ratio of 50:50 (*v/v*, oil:aqueous phase). Moreover, the recombinant proteins were combined with 10 μg of the recombinant Hsp27 and Hsp20 as an adjuvant.

Protection Against Tumor Growth

Inbred C57BL/6 female mice were subcutaneously injected in the right footpad with a variety of regimens three times with a 2-week interval as indicated in Table 1. Two weeks after third vaccination, mice were subcutaneously challenged in the right flank with 1×10^5 TC-1 tumor cells, and then monitored for tumor growth and the percentage of tumor-free mice (i.e., survival rates) by palpation twice a week. At each time point, tumor volume was calculated using the formula: $V = (a^2b)/2$.

Assessment of Therapeutic Efficiency

To determine the therapeutic efficacy, six groups were selected with respect to the results of mice protection (before section). Herein, 1×10^5 TC-1 tumor cells were subcutaneously inoculated in the right flank of four mice in each group. One week after TC-1 inoculation, C57BL/6 mice were injected with

TABLE 1 *Immunization program in preventive study*

Groups	Vaccine modality	Priming	Booster 1 (2 weeks after priming)	Booster 2 (2 weeks after booster 1)	Challenge with TC-1 (2 weeks after booster 2)
G1	DNA/DNA/DNA	pcDNA-E7 + MPG	pcDNA-E7 + MPG	pcDNA-E7 + MPG	TC-1
G2	Protein/protein/protein	E7 protein + Freund's adjuvant	E7 protein + Freund's adjuvant	E7 protein + Freund's adjuvant	TC-1
G3	Protein/protein/protein	E7 protein + Montanide	E7 protein + Montanide	E7 protein + Montanide	TC-1
G4	Protein/protein/protein	E7 protein + Hsp27	E7 protein + Hsp27	E7 protein + Hsp27	TC-1
G5	Protein/protein/protein	E7 protein + Hsp20	E7 protein + Hsp20	E7 protein + Hsp20	TC-1
G6	Protein/protein/protein	hPP10-E7	hPP10-E7	hPP10-E7	TC-1
G7	Protein/Protein/Protein	hPP10-E7 + Hsp27	hPP10-E7 + Hsp27	hPP10-E7 + Hsp27	TC-1
G8	Protein/protein/protein	hPP10-E7 + Hsp20	hPP10-E7 + Hsp20	hPP10-E7 + Hsp20	TC-1
G9	DNA/protein/protein	pcDNA-E7 + MPG	E7 protein + Freund's adjuvant	E7 protein + Freund's adjuvant	TC-1
G10	DNA/protein/protein	pcDNA-E7 + MPG	E7 protein + Montanide	E7 protein + Montanide	TC-1
G11	DNA/protein/protein	pcDNA-E7 + MPG	E7 protein + Hsp27	E7 protein + Hsp27	TC-1
G12	DNA/protein/protein	pcDNA-E7 + MPG	E7 protein + Hsp20	E7 protein + Hsp20	TC-1
G13	DNA/protein/protein	pcDNA-E7 + MPG	hPP10-E7	hPP10-E7	TC-1
G14	DNA/protein/protein	pcDNA-E7 + MPG	hPP10-E7 + Hsp27	hPP10-E7 + Hsp27	TC-1
G15	DNA/protein/protein	pcDNA-E7 + MPG	hPP10-E7 + Hsp20	hPP10-E7 + Hsp20	TC-1
G16	Peptide/peptide/peptide (control)	MPG	MPG	MPG	TC-1
G17	Protein/protein/protein (control)	Hsp27	Hsp27	Hsp27	TC-1
G18	Protein/protein/protein (control)	Hsp20	Hsp20	Hsp20	TC-1
G19	Empty vector (control)	pcDNA 3.1	pcDNA 3.1	pcDNA 3.1	TC-1
G20	Adjuvant	Freund's adjuvant	Freund's adjuvant	Freund's adjuvant	TC-1
G21	Adjuvant	Montanide	Montanide	Montanide	TC-1
G22	PBS (control)	PBS	PBS	PBS	TC-1

pcDNA-E7 + MPG prime/pcDNA-E7 + MPG boost, rE7 + Montanide prime/rE7 + Montanide boost, hPP10-E7 + Hsp27 prime/hPP10-E7 + Hsp27 boost, pcDNA-E7 + MPG prime/rE7 + Montanide boost, and pcDNA-E7 + MPG prime/hPP10-E7 + Hsp27 boost, and Phosphate-buffered saline (PBS) as indicated in Table 2. Two booster doses were given to the mice 2 weeks after the first therapeutic dose. Tumor growth was monitored twice a week by inspection and palpation.

Monitoring Humoral Immune Responses

The mice in preventive study were bled from retro-orbital at 2 weeks after the second booster injection. Then, the sera were pooled for each group. The production of goat anti-mouse IgG1, IgG2a, IgG2b, and total IgG antibodies (diluted 1:10,000 in 1% BSA/PBS-Tween, Sigma) was assessed by indirect ELISA. Moreover, mice sera were diluted 1:100 in 1% BSA/PBS-Tween. These ratios of sera and antibodies were obtained after titration. The coated antigens were the recombinant E7 protein (rE7, 5 μg/mL), MPG and hPP10 peptides (10 μg/mL).

Cytokine Assay

Three mice from each group in preventive study were sacrificed randomly before TC-1 challenge. The spleens were removed and the red blood cell-depleted pooled splenocytes (2×10^6 cells/mL) were cultured in U-bottomed, 96-well plates (Costar, Cambridge, MA) for 72 h in the presence of 10 μg/mL of rE7 protein, 10 μg/mL of MPG or hPP10 peptides, RPMI 5% as negative control and 5 μg/mL of concanavalin A (ConA) as positive control in complete culture medium. Simultaneously, the lymph nodes as well as the spleens of the mice were harvested and the lymph node derived lymphocytes were subsequently stimulated *in vitro* with rE7, MPG, hPP10, and ConA. The supernatants were harvested and frozen at −70 °C, until the samples were analyzed. Production of IFN-γ, IL-5, and IL-2 was measured with the sandwich-based ELISA method using a DuoSet ELISA system (R&D Systems) according to the manufacturer's instructions. All data are represented as mean ± SD for each set of samples.

Granzyme B Assay

The P815 target cells (T) were seeded in triplicate into U-bottomed, 96-well plates (2×10^4 cells/well) incubated with E7

TABLE 2 *Immunization program in therapeutic study*

Groups	Vaccine modality	Challenge with TC-1	Priming (1 week after challenge)	Booster 1 (2 weeks after priming)	Booster 2 (2 weeks after booster 1)
G1	DNA/DNA/DNA	TC-1	pcDNA-E7 + MPG	pcDNA-E7 + MPG	pcDNA-E7 + MPG
G2	Protein/protein/protein	TC-1	E7 protein + Montanide	E7 protein + Montanide	E7 protein + Montanide
G3	Protein/protein/protein	TC-1	hPP10-E7 + Hsp27	hPP10-E7 + Hsp27	hPP10-E7 + Hsp27
G4	DNA/protein/protein	TC-1	pcDNA-E7 + MPG	E7 protein + Montanide	E7 protein + Montanide
G5	DNA/protein/protein	TC-1	pcDNA-E7 + MPG	hPP10-E7 + Hsp27	hPP10-E7 + Hsp27
G6	PBS (control)	TC-1	PBS	PBS	PBS

antigen (~30 μg/mL) for 24 h. The splenocytes (Effector cells: E) previously provided for cytokine assay were counted using trypan blue and added to the target cells at E:T ratio of 100:1 in which maximal release of Granzyme B (GrB) was observed. The target and effector cells were co-cultured in complete RPMI-1640 supplemented with 10% heat-inactivated Fetal calf serum (FCS) at 37 °C and 5% CO_2 under humidified conditions. The wells containing effector cells were considered for measurement of possible spontaneous release of Granzyme B. After 6 h incubation, microplates were centrifuged at 250×*g* for 5 min at 4 °C and the supernatants were harvested. The concentration of GrB in these samples was measured by ELISA (eBioscience) according to the manufacturer's instruction.

Statistical Analysis

The differences between the control and test groups in immunological studies were assessed using one-way ANONA (Graphpad Prism, GraphPad Software). Survival rate or the percentage of tumor-free mice was evaluated using the log-rank (Mantel-Cox) test. The value of $P < 0.05$ was considered statistically significant.

RESULTS

Generation of the Recombinant Proteins

Our data showed that the recombinant hPP10-E7, Hsp27, and Hsp20 proteins were generated in the Rosetta strain at 3–4 h after induction. The purified hPP10-E7, Hsp20, and Hsp27 proteins migrated as a clear band of ~25, ~20, and ~27 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). The recombinant proteins had a concentration range between 0.7 and 1.0 mg/mL.

Formation of MPG/ E7DNA Nanoparticles

The nanoparticles were formed by interaction of the negatively charged pcDNA-E7 plasmid with the cationic peptide (MPG) at an *N/P* ratio of 10:1 in agarose gel. SEM analysis of nanoparticles showed a spherical shape with a size of ~200 nm as previously reported (21).

Evaluation of Antibody Responses

The serum levels of total IgG and the related subclasses (IgG1, IgG2a, and IgG2b) were detected against the recombinant E7 protein (rE7), MPG, and hPP10 peptides in different groups. Our

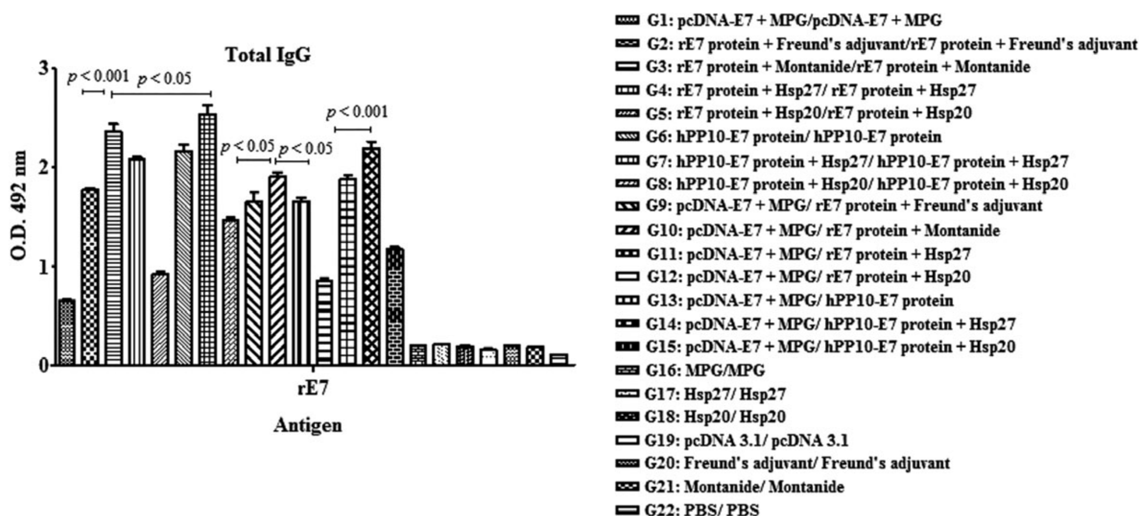

FIG 1 *Antibody response (total IgG; 1:10,000 dilution) against E7 antigen in different regimens: Mice sera were prepared from the whole blood samples of each group (n = 7) 2 weeks after the last immunization. All analyses were performed in duplicate for each sample. The results from the 1:100 sera dilutions are shown as mean absorbance at 492 nm ± SD. The results were shown for groups from left (G1) to right (G22).*

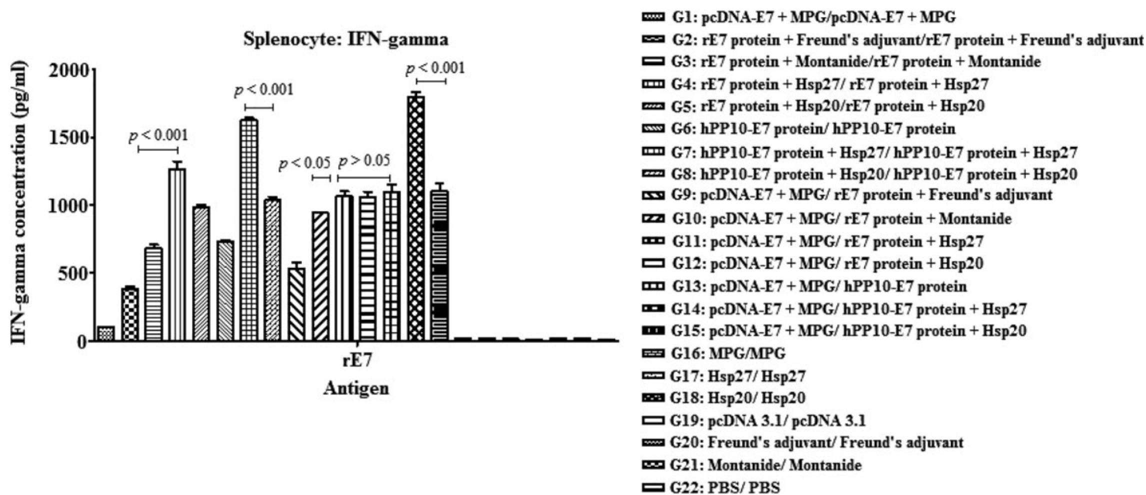

FIG 2 *IFN-γ level in immunized groups with various formulations: The cultures of pooled splenocytes were prepared from three mice in each group (*n *= 3 per group) and re-stimulated with rE7 protein* in vitro. *The level of IFN-γ was determined in the supernatant with ELISA as mean absorbance at 450 nm ± SD for each set of samples. All analyses were performed in duplicate for each sample.*

results indicated that the level of total IgG in the sera of mice vaccinated with hPP10-E7 + Hsp27 in protein formulation (G7) was significantly higher than other groups ($P < 0.05$, Fig. 1). Moreover, Hsp27 as an adjuvant could significantly increase the secretion of total IgG, IgG1, IgG2a, and IgG2b as compared to Hsp20 in similar regimens (G4 *vs* G5; G7 *vs* G8; G11 *vs* G12; G14 *vs* G15; $P < 0.05$, Supporting Information 1). Generally, the highest levels of IgG2a and IgG2b were detected in the sera of mice immunized with homologous hPP10-E7 + Hsp27 (G7) and heterologous pcDNA-E7 + MPG/ hPP10-E7 + Hsp27 (G14) compared to other groups, respectively ($P < 0.05$). Indeed, the data indicated the mixture of IgG1, IgG2a, and IgG2b with high intensity toward IgG2a responses in groups 7 and 14 (G7 and G14). No significant anti-E7 antibody responses could be detected in the sera of control groups. Importantly, all test groups did not show any significant response against MPG and hPP10 peptides compared to control groups ($P > 0.05$; data not shown). Thus, the seroreactivities were completely E7 antigen-specific responses in C57BL/6 mice.

Secretion of Cytokines

The cytokine results for the pooled splenocytes of three mice in each group indicated that the level of E7-specific IFN-γ secretion in the groups vaccinated with rE7 + Hsp27 (G4), rhPP10-E7 + Hsp27 (G7), and pcDNA-E7 + MPG/ rhPP10-E7 + Hsp27 (G14) was significantly higher than that in other groups ($P < 0.05$, Fig. 2). All mice immunization with different modalities effectively enhanced the levels of IFN-γ as compared to control groups ($P < 0.05$). The data indicated that Hsp27 is more effective than Hsp20 as an adjuvant in stimulation of IFN-γ secretion. Among all the test groups, the group immunized with pcDNA-E7 + MPG (G1) showed a significant IL-5 response (~72 pg/mL) compared to other groups (~10–25 pg/mL, $P < 0.05$). However, the results obtained from the pooled splenocytes of three mice were similar to those from each mouse in one group indicating mice were inbred (data not shown). As known, the Th1 cellular immune response is highly characterized by IFN-γ responses. Our data also showed that the hPP10 CPP and Hsp27 adjuvant could significantly activate this response. The same results were obtained to secrete IFN-γ from the lymph node derived lymphocytes re-stimulated with rE7 protein in various groups. It was interesting that the group vaccinated with pcDNA-E7 + MPG/ hPP10-E7 + Hsp27 (G14) had the highest level of IFN-γ secretion in both splenic- and lymph node-derived lymphocytes (Supporting Information 2). Moreover, there was no significant difference for IL-5 secretion in all groups (~10–15 pg/mL, $P > 0.05$). The secretion of IL-2 in the splenocytes re-stimulated with rE7 protein was significantly higher in the groups vaccinated with rhPP10-E7 + Hsp27 (G7) and pcDNA-E7 + MPG/rhPP10-E7 + Hsp27 (G14) than that in other groups similar to IFN-γ secretion ($P < 0.05$, Supporting Information 2). However, the levels of IL-2 were significantly lower than IFN-γ in these groups ($P < 0.05$). There was no considerable difference for IL-2 secretion in the lymph node derived lymphocytes re-stimulated with rE7 protein in different groups (~15–20 pg/mL, $P > 0.05$). In addition, no significant difference was observed for IFN-γ, IL-5, and IL-2 production with respect to the MPG and hPP10 in groups immunized with different formulations and control groups ($P > 0.05$).

Granzyme B Secretion

Two weeks after the last immunization, the splenocytes from each immunized group were co-cultured with p815 target cells in *E*:*T* ratio of 100:1 for 6 h at 37 °C, and the supernatants were harvested. *Granzyme B* secretion in each sample was measured by ELISA (Fig. 3). The data showed that the groups immunized with pcDNA-E7 + MPG/rhPP10-E7 + Hsp27 (G14) and rhPP10-E7 + Hsp27/rhPP10-E7 + Hsp27 (G7) produced significantly higher concentrations of GrB than other groups, respectively ($P < 0.05$, Fig. 3).

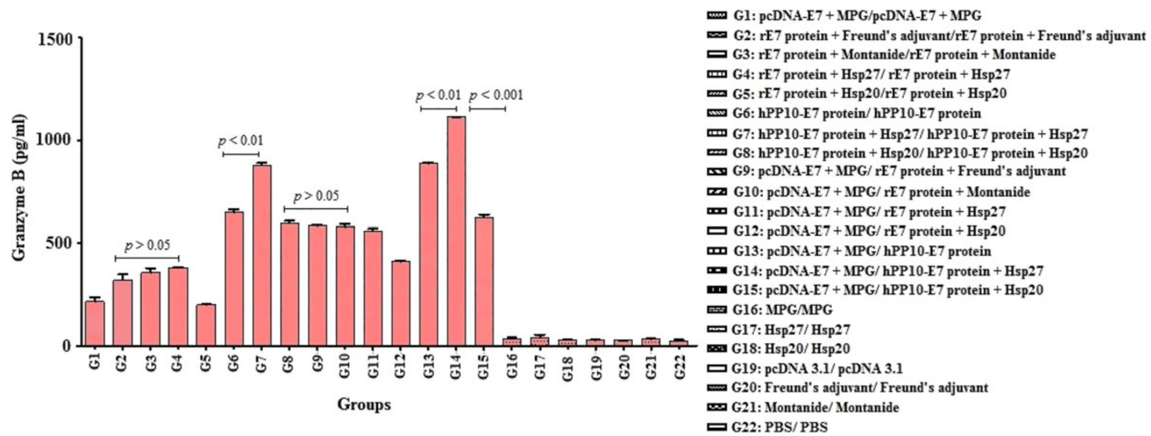

FIG 3 *GrB concentration measured by ELISA using pooled splenocytes from three mice in each group (n = 3 per group). All analyses were performed in triplicate for each sample. The results represent mean values calculated from triplicate samples as well as the standard deviation (SD) as error bars.*

Indeed, rhPP10-E7 could significantly enhance GrB secretion compared to rE7 in different regimens. However, Hsp20 was less effective than Hsp27 as an adjuvant in secretion of *Granzyme B* ($P < 0.05$).

Hsp27 Accompanied by hPP10 and MPG CPPs Could Increase Mice Protection Against E7-Expressing Tumor Cells

For evaluation of vaccine potency, tumor growth was assessed in all groups. As shown in Fig. 4a, there was a significant decrease in tumor growth induced by the groups immunized with different formulations as compared to control groups (G16–G22). Indeed, all mice in control groups developed tumor growth on approximately 7–21 days. As indicated in Fig. 4b, immunization with pcDNA-E7 + MPG (G1), rhPP10-E7 (G7), and pcDNA-E7 + MPG/ hPP10-E7 + Hsp27 (G14) could protect all mice from tumor growth.

Treatment with pcDNA-E7 + MPG/hPP10-E7 + Hsp27 Effectively Suppressed the Tumor Growth in Mice

Mice with pre-established E7-expressing tumors were treated with five vaccine modalities (Table 2), 1 week after inoculation with TC-1 tumor cells. Tumor size and animal survival were monitored for 60 days following the challenge. As shown in Fig. 5a,b, mice in groups vaccinated with pcDNA-E7 + MPG (G1), rhPP10-E7 (G3), and pcDNA-E7 + MPG/ hPP10-E7 + Hsp27 (G5) displayed complete regression and remained tumor-free >60 days following treatment. Moreover, the group vaccinated with rE7 + Montanide (G2) and pcDNA-E7 + MPG/rE7 + Montanide (G4) demonstrated significantly the reduction of tumor growth following TC-1 challenge compared to control group (G6, $P < 0.001$, Fig. 1c). Indeed, the tumor growth of mice in G2 and G4 delayed until on day 49 and 56, respectively, and the tumor-free percentage of mice in these groups was 75% (G4) and 50% (G2) against 0% survival for G6 (control). Mice were euthanized when tumor diameter exceeded >5% of body weight.

DISCUSSION

Adjuvants and also delivery systems are often used to enhance the efficiency of vaccination strategies (22). Adjuvants should be stable with long-term life, biodegradable, cheap to produce, not induce immune responses against themselves, and promote an appropriate immune response against infections. However, the selection of adjuvant depends on some factors such as the antigen, the species to be vaccinated, the route of administration, and the likelihood of side-effects (23). Hence, adjuvant compounds are important to potentiate an effective immunity against the antigen of interest. Furthermore, determination of an antigen-delivery system with a special chemical composition is critical which type of immune response will develop such as isotypes of antibodies or cytokines (24). Our main objectives of this study include (i) to compare the potency of small HSPs B1 (Hsp27) and B6 (Hsp20) as an endogenous adjuvant associated with HPV16 E7 as antigenic model, (ii) to clarify hPP10 and MPG effects as a delivery system on the immune responses with and without covalent binding to E7, respectively, (iii) to investigate the results of immunity against E7 antigen using Freund's adjuvant and Montanide ISA720 as commercial adjuvants, and (iv) to evaluate the potency of three vaccination modalities including homologous DNA, homologous protein, and heterologous DNA/-protein. First, the recombinant E7, hPP10-E7, Hsp27, and Hsp20 proteins were expressed in *E. coli* as His-tagged protein and purified by affinity chromatography using Ni-NTA column. The plasmid harboring HPV16 E7 gene was prepared to form stable nanoparticles (~200 nm in size) with MPG peptide at an *N/P* ratio of 10:1. Next, the HPV16 E7 specific antibody and T-cell immune responses were evaluated in C57BL/6 mice immunized with different formulations. The results showed that all mice immunized with various vaccine regimens increased IgG titer and T-cell responses to the HPV-16 E7 as compared to control groups. The HPV16 E7-specific immunity in mice who received pcDNA-E7 + MPG/ rhPP10-E7 + Hsp27 and rhPP10-E7 + Hsp27/rhPP10-E7 + Hsp27 resulted in higher secretion of IFN-γ and IL-2 cytokines

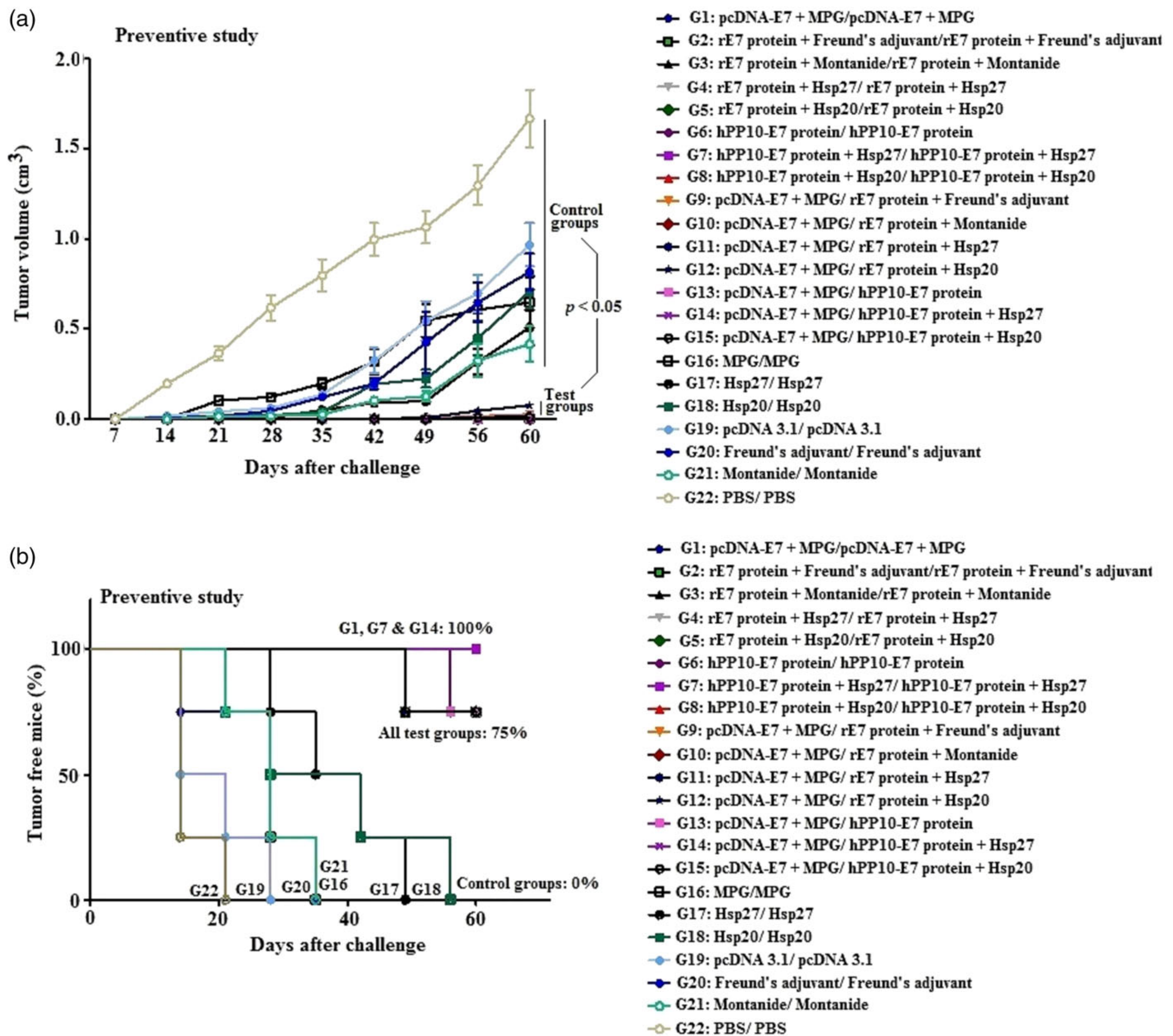

FIG 4 *Preventive studies against TC-1 tumor cells in mice immunized with different vaccine formulations: in preventive study, 22 groups of C57BL/6 mice were vaccinated three times with a 2-week interval. The mice were challenged with 1×10^5 TC-1 in the right flank 2 weeks after second booster. Tumor volumes were measured twice a week (a). Moreover, the percentage of tumor-free mice was determined over time in various groups (b).*

and antibody responses especially IgG2a than other groups. Regarding to our observations in protective and therapeutic studies, these regimens could completely confer protection against TC-1 tumor-challenged mice depending on stimulation of $CD4^+$ T cell-dominated Th1 responses as well as GrB secretion. Moreover, E7 DNA/MPG nanoparticles could completely eradicate TC-1 tumors *in vivo* at an *N/P* ratio of 10:1 as reported previously (21). Contrarily, the mixture of Montanide ISA720, Hsp20, and Freund's adjuvants with rE7 and rhPP10-E7 were able to cause the regression of TC-1 tumors *in vivo*, but these adjuvants could not increase the potency of antigen to eradicate TC-1 tumors completely as compared to Hsp27 adjuvant. A study indicated that low levels of Hsp27 expression in T lymphocytes, even after heat shock, may play a role in Cytotoxic T lymphocyte (CTL) resistance to granule-mediated lysis (12). Other data showed Hsp27 as a potent therapeutic target in breast cancer, bone metastasis and skeletal tumor growth (25). Indeed, HSP27 is particularly involved in protection from programmed cell death by inhibition of caspase-dependent apoptosis (26). Contrarily, over-expression of the small HSP Hsp27 was shown to inhibit the *in vitro* proliferation rate and to delay tumor development of a human melanoma cell line (A375) in nude mice (27). Recently, a study showed that the use of Hsp27 in protein regimen could improve HIV-1 Nef-specific B-cell and T-cell immune

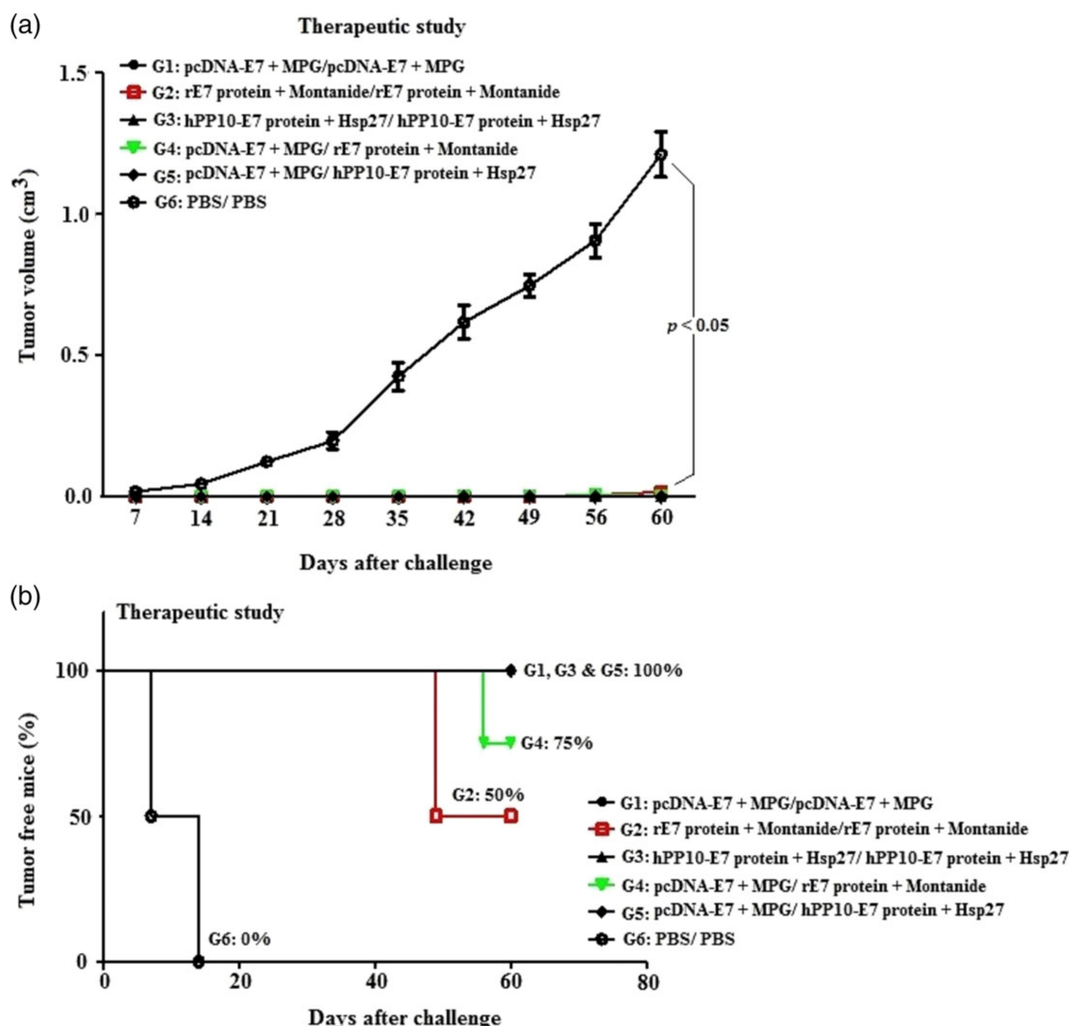

FIG 5 *Therapeutic studies against TC-1 tumor cells in mice immunized with different vaccine formulations: In therapeutic study, 1 week after TC-1 inoculation, six groups of C57BL/6 mice were injected with various regimens. Tumor growth was monitored twice a week by inspection and palpation (a). The percentage of tumor-free mice was determined in different groups (b).*

responses (28). Ortiz *et al.* reported that DNA fragment containing B and T cell epitopes of the N-terminal region of Hsp20 with other *Babesia bovis* antigens elicited high levels of specific IgG antibodies (29). We also showed that the combination of mouse Hsp20 with viral antigen could increase E7-specific antibody responses. Other study indicated that both N-terminal (aa 1-105) and C-terminal (aa 48-177) regions of *Babesia bovis* Hsp20 were immunogenic for the majority of cattle, stimulating strong proliferation and IFN-γ production (30).

Furthermore, a study showed that the purified hPP10-Apoptin fusion protein could significantly decrease the tumor growth in two doses of 50 and 100 μg compared to PBS control group (15). The findings of this study showed that human origin peptide hPP10 fused to HPV16 E7 could importantly reduce the tumor growth and protect mice against TC-1 tumor in lower dose (~10 μg/mL). Indeed, the optimal conditions must be individually determined for each cargo according to cell type and CPP (31, 32). Currently, several preclinical and clinical trials are ongoing that utilize CPPs as a therapeutic carrier such as AZX100 (for keloid scarring, Phase II), KAI-9803 (for myocardial infarction, Phase II), RT001 (for wrinkling skin, Phase II), XG-102 (for hearing loss, Phase II), and DTS-108 (for cancer, Preclinical study, (33, 34). Our data showed that the group immunized with the heterologous pcDNA-E7+ MPG prime/ rhPP10-E7 + Hsp27 boost has the highest levels of IFN-γ, IL-2 and Granzyme B and induce complete protective and therapeutic effects. Shafer-Weaver *et al.* indicated that the release of Granzyme B by cytolytic lymphocytes upon effector-target interaction may be a more specific indicator of CTL and Natural killer (NK) cytotoxic ability than IFN-γ secretion (35). Lin *et al.* reported that the secretion of Granzyme B by human memory CD4 T cells is less strictly regulated compared to memory CD8 T cells (36). Contrarily, heterologous prime-boost immunization protocols using various gene expression systems have proven to be successful approaches for protection against different disorders in preclinical and even clinical trials (18, 37). In this study,

the efficiency of heterologous DNA/protein vaccination was proved using effective adjuvants and delivery systems, as well. As know, IL-2 cytokine is another signaling molecule that has been observed to play a central role in the proliferation and differentiation of T-cells in the immune system. A study indicated the significant percentages of CD4+ and CD8+ cells producing IFN-γ, TNF-α, and IL-2 after administration of plasmid DNA encoding E6E7 suggesting that the vaccine was successful in inducing a potent immune response (38). Our data also confirmed that the IL-2 secretion in groups vaccinated with hPP10-E7 + Hsp27/hPP10-E7 + Hsp27 and pcDNA-E7 + MPG/hPP10-E7 + Hsp27 is significantly higher than other groups similar to IFN-γ and GrB secretion.

CONCLUSION

Generally, our data indicated that E7 DNA + MPG prime/hPP10-E7 + Hsp27 boost-based vaccine drives T-cell responses toward a Th1-type and could completely confer protection against TC-1 tumor-challenged mice (i.e., 100% tumor free mice). Moreover, the potency of Hsp27 along with rhPP10-E7 in stimulation of suitable immune responses and complete protection was proved as compared to other adjuvants such as Freund's adjuvant, Montanide and Hsp20. Hsp27 and Hsp20 did not show any adverse effects (e.g., inflammation) in the vaccinated mice. However, further studies are required to determine its mechanism of action and also protective effects in large animals.

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