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Modified DCs and MSCs with HPV E7 antigen and small Hsps: Which one is the most potent strategy for eradication of tumors?

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ABSTRACT

Immunotherapy with DCs as antigen-presenting vehicles have already improved patients' outcome against a variety of tumors. Moreover, MSCs were recently used to develop anti-cancer therapeutic or anti-microbial prophylactic vaccines. The current study evaluated immune responses and anti-tumor effects generated by DCs and MSCs derived from mouse bone marrow which were modified with small heat shock proteins 27 and 20 (sHsp27 and sHsp20) and also E7 oncoprotein in tumor mouse model. Two vaccination strategies were utilized including homologous DC or MSC prime/ DC or MSC boost, and heterologous MSC or DC prime/ protein boost vaccinations. Our data revealed that DCs pulsed with E7 + Hsp27 and/or E7 + Hsp20 in homologous and heterologous prime/ boost vaccinations could stimulate high levels of IgG2a, IgG2b, IFN-γ and IL-10 directed toward Th1 responses. Moreover, these regimens induced an increased level of Granzyme B, and displayed complete protection more than 60 days after treatment. On the other hand, MSCs transfected with E7 + Hsp27 DNA in homologous and heterologous prime/ boost vaccinations could significantly enhance the E7-specific T-cell responses and suppress tumor growth in mice. However, MSCs transfected with E7 + Hsp20 DNA did not induce a complete protection against TC-1 tumor compared to DCs pulsed with E7 + Hsp20 protein complexes. These results indicated that DC- and MSC-based vaccinations with specific modalities will be a useful approach for immunotherapy and protection against HPV-associated cancers.

1. Introduction

Novel therapeutic strategies are urgently needed to decrease the risk of cancer recurrence. Human papillomavirus (HPV) infections especially types 16 and 18 indicate the most important risk factor for development of cervical cancer (Bellone et al., 2007). Moreover, HPV E7 and E6 oncoproteins are considered as specific targets for immunotherapy of cervical cancer. As known, dendritic cells (DCs) as strong antigen-presenting cells which prime *in vitro* and *in vivo* T-cell activities, have attracted a special interest in immunotherapy of several human malignancies (Bellone et al., 2007). DCs could either be loaded with target-specific peptide/protein antigens or transduced to express antigens *ex vivo* (Chabeda et al., 2018). A study showed that E7 antigen-loaded DCs could elicit a specific T cell response *in vitro* (Nonn et al., 2003). Moreover, combination treatments were suggested as a key strategy to treat HPV lesions (Chabeda et al., 2018; Ma et al., 2017). However, the goal of recent designs for DC-based vaccines is to induce strong tumor-specific CTL responses in patients with cancer (Zhou et al., 2002). Indeed, DCs could act as natural and effective adjuvants. The studies indicated that heat shock proteins (Hsps) are molecular chaperones that bind tumor antigens and mediate their uptake into antigen presenting cells (APCs) such as DCs. The Hsp-antigen complexes are directed toward the MHC class I or II pathways leading to the activation of T cells (Murshid et al., 2012). Moreover, small Hsps (sHsps) could play a major role in stem cell biology. Indeed, the levels of specific sHsps in stem cells changed during their differentiation suggesting their potential roles for therapeutic purposes. For instance, circulating sHsp27 in the plasma showed immunomodulatory and anti-

Abbreviations: APC, antigen presenting cell; DC, dendritic cell; MSC, mesenchymal stem cell; HPV, human papillomavirus; Hsp, heat shock protein; sHsp, small heat shock protein; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; KLH, keyhole limpet hemocyanin; r, recombinant; PIDC, pre-immune DC; GFP, green fluorescent protein; UTMD, microbubble destruction; MI, mechanic index; TGE, transient gene expression; EGE, extended gene expression; VLP, virus-like particles

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inflammatory functions. Moreover, sHsp20 as a potential therapeutic agent indicated anti-platelet aggregation (Bakthisaran et al., 2015). Another study showed that treatment with DCs modified with Hsp70 was safe and feasible in patients with HCV-related hepatocellular carcinoma (HCC) (Maeda et al., 2015).

Recently, the potent gene delivery was known as a major key for using mesenchymal stem cells (MSCs) in tissue engineering. Gene transfection was usually done by chemical or physical methods (*e.g.,* calcium phosphate, electroporation, lipofection). However, transfection efficiency of primary cells such as MSCs by non-viral systems was generally lower than that observed by viral infection methods (Haleem-Smith et al., 2005). Recently, the ability of modified MSCs to express and secrete antigens and induce immune responses have been attracted a special interest. In fact, MSCs were used to develop anti-cancer therapeutic or anti-microbial prophylactic vaccines (Tomchuck et al., 2012).

In this study, DCs and MSCs derived from mouse bone marrow were used to develop therapeutic vaccines against HPV-related cancers for the first time. The recent study evaluated immune responses and anti-tumor effects generated by DCs and MSCs modified with small heat shock proteins 27 and 20 (sHsp27 and sHsp20) and also a HPV-associated antigen (E7) in tumor mouse model.

2. Materials and methods

2.1. Production of the recombinant pEGFP-E7, pEGFP-Hsp20 and pEGFP-Hsp27 constructs

The eukaryotic expression vector harboring the full lengths of Mus musculus Hsp20 (pEGFP-Hsp20, Accession No: NM_001012401), Hsp27 (pEGFP-Hsp27, Accession No: NM_013560) and HPV16 E7 (pEGFP-E7, Accession No: K02718) as well as pEGFP-N1 as a positive control were prepared in large scale using DNA extraction Midi-kit (Qiagen) according to the manufacturer's instructions, and quantified by NanoDrop spectrophotometry (Basirnejad et al., 2018; Milani et al., 2017a, 2017b; Bolhassani et al., 2008). The presence of Hsp20, Hsp27, E7 genes was confirmed using digestion with restriction enzymes in pEGFP vector as previously reported by our group (Basirnejad et al., 2018; Milani et al., 2017a, 2017b; Bolhassani et al., 2008).

2.2. Generation of the recombinant HPV16 E7, mouse Hsp20 and mouse Hsp27 proteins

The recombinant HPV16 E7 protein (rE7) was expressed in *E. coli* M15 strain (Bolhassani et al., 2008) and the soluble fraction was purified by affinity chromatography using a Ni-NTA agarose column under native conditions according to the manufacturer's instructions (Qiagen). Moreover, the recombinant Hsp20 and Hsp27 proteins (rHsp20 and rHsp27) were expressed in *E. coli* Rosetta strain and purified by affinity chromatography under native conditions as previously reported by our group (Milani et al., 2017a, 2017b; Basirnejad and Bolhassani, 2018). The endotoxin contamination was less than 0.5 EU per milligram (mg) protein as monitored by LAL assay (QCL-1000, Lonza). The purified proteins were dialyzed, assessed by NanoDrop spectrophotometry and stored at −70 °C until used.

2.3. Preparation of dendritic cells (DCs)

For DC preparation, male C57BL/6 mice were provided by the animal center of Pasteur Institute of Iran. Mice were sacrificed and the long bone marrow extracted. After washing and lysis of red blood cells using ACK buffer, the cells were cultured in RPMI 1640 medium supplemented with FBS 10%, 20 ng/ml GM-CSF and 10 ng/ml IL-4. The culture medium and cytokines (GM-CSF and IL-4) were refreshed every two days. The cells were harvested on day 5. Then, the DCs were pulsed with rE7, rHsp20, rHsp27, rE7 + rHsp20 and rE7 + rHsp27 proteins (concentration: 10 μg/ mL) at 37 °C for 4 h, and cultured for another 48 h. Finally, DCs were harvested, washed and counted to determine the cell number and viability on day 7. The modified DCs (~ 1.0×10^6 cells) were used in the vaccination regimens. In addition, DCs were washed in FACS buffer (10% FBS and 0.2% Na Azide in PBS) and stained with conjugated antibodies for CD86, CD11c, CD83, CD40 and MHC class II markers (BD Pharmingen; Strome et al., 2002). FACS analysis was performed on a FACScan Flow Cytometer (Becton Dickinson). Furthermore, the cell staining was performed by Giemsa reagent.

2.4. Preparation of mesenchymal stem cells (MSCs)

For MSC preparation, male C57BL/6 mice were provided by the animal center of Pasteur Institute of Iran. MSCs were harvested from the bone marrow of femurs of these mice as previously reported (Pu et al., 2011). Briefly, bone marrow cells were flushed out with 20 mL complete DMEM containing 10% heat-inactivated FBS, 5 mg/mL glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were grown in a humidified atmosphere containing 5% CO_2. The medium was replaced 24 h later and refreshed every 3 days. The third passage of MSCs was adopted for transfection. The mesenchymal stem cells were identified by CD45 and CD90.2 surface markers using flow cytometry analysis.

2.5. Gene transfer into MSCs

The third passage of MSCs was cultured into the 12-well plate at concentration of 2.0×10^5 cells/per well for 24 h at 37 °C and 5% CO_2 conditions. In this study, the cells were divided into the following groups: a) the blank control group: MSCs with serum-free culture medium, b) the control group 1: MSCs transfected with pEGFP-N1, c) the control group 2: MSCs transfected with pEGFP-N1 along with heating, d) the test group 1: MSCs transfected with pEGFP-E7, e) the test group 2: MSCs transfected with pEGFP-E7 along with heating, f) the test group 3: MSCs transfected with pEGFP-Hsp27, g) the test group 4: MSCs transfected with pEGFP-Hsp27 along with heating, h) the test group 5: MSCs transfected with pEGFP-Hsp20, i) the test group 6: MSCs transfected with pEGFP-Hsp20 along with heating, j) the test group 7: MSCs transfected with pEGFP-Hsp20 + pEGFP-E7, k) the test group 8: MSCs transfected with pEGFP-Hsp20 + pEGFP-E7 along with heating, m) the test group 9: MSCs transfected with pEGFP-Hsp27 + pEGFP-E7, and n) the test group 10: MSCs transfected with pEGFP-Hsp27 + pEGFP-E7 along with heating. Two samples were used in each group. Before transfection, 4 μg/per well of the recombinant pEGFP-N1, pEGFP-E7, pEGFP-Hsp20, pEGFP-Hsp27, pEGFP-E7 + pEGFP-Hsp20, pEGFP-E7 + pEGFP-Hsp27 were mixed with 10 μL lipofectamin 2000 transfection reagent (Invitrogen, USA) for 15 min. Briefly, the cells were transfected with the recombinant plasmid/ lipofectamine complexes in serum-free media. Four hours after transfection, the tissue culture dish was covered with parafilm and fully incubated in a water bath at 42 °C for 2 h. After removal from the water bath, the transfection media without serum was replaced with fresh supplemented DMEM media. Controls were similarly transfected, but without any heat treatment. Cells were then incubated for 48 h at 37 °C and 5% CO_2. MSC transfection was performed for two times with 48 h intervals. MSCs were collected after transfection for 48 h. The efficiency of transfection was determined by flow cytometry (Partec). It should be mentioned that we also performed the transfection of MSCs with pEGFP-N1, pEGFP-Hsp20, pEGFP-Hsp27 and pEGFP-E7 using lipofection once, but the efficiency of transfection was low as mentioned in the results. Generally, the percentage of cells expressing GFP, Hsp20-GFP, Hsp27-GFP and E7-GFP was identified in the 500–530 nm emission wavelengths. The untransfected MSCs were considered as negative controls without fluorescent emission. Herein, the target gene (*e.g.,* E7, Hsp20 or Hsp27) was cloned into pEGFP-*N1* (*i.e.,* the N-terminal of EGFP gene) so that it is in

frame with the EGFP coding sequences, with no intervening in-frame stop codons. The inserted genes possess the initiating ATG codon. Thus, the expression of EGFP is an indicator of the gene expression which can be detected by flow cytometry due to the emission of green fluorescent light. On the other hand, western blotting was performed to represent the expression of GFP, E7-GFP, Hsp27-GFP and Hsp20-GFP proteins in the cells. The recombinant proteins were detectable using anti-GFP polyclonal antibody conjugated to horseradish peroxidase (1:5000 v/v, Abcam) and 3, 3′-diaminobenzidine (DAB) substrate. It should be mentioned that after achieving the highest expression of proteins in MSCs, the transfection method was performed again and the cell pellets were utilized to inject in mice.

2.6. in vivo vaccination vaccination

Inbred C57BL/6 female mice, 5–7 week old, were obtained from the breeding stocks maintained at the Pasteur Institute of Iran. All mice were maintained under specific pathogen-free conditions and all procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. For protection experiments against tumor growth, five mice were immunized in each group by different regimens three times with a 2-week interval as mentioned in **Tables 1 & 2**. The recombinant proteins were emulsified with Montanide ISA720 at the ratio of 70:30 (*v/v*, oil: aqueous phase). Two weeks after final vaccination, mice were subcutaneously challenged in the right flank with 1×10^5 TC-1 tumor cells (ATCC number: CRL-2785), and then monitored for tumor growth (Saleh et al., 2015). The percentage of tumor-free mice was assessed by palpation twice a week for 60 days. Mice were euthanized when tumor diameter exceeded more than 5% of body weight. For therapeutic experiments of the established tumors, seven groups were selected according to the results of preventive experiments (*i.e.,* G5 and G10 for MSCs: Table 1 and G4, G5, G9 & G10 for DCs: Table 2 as well as PBS). At first, three mice in each group were injected subcutaneously with 1×10^5 TC-1 tumor cells. Seven days after TC-1 injection, animals were treated with different vaccination regimens three times with a 2-week interval. Tumor growth was monitored twice a week for 60 days following the challenge.

2.7. Monitoring humoral immune responses

In preventive study, the mice were bled from retro-orbital at two weeks after the second booster. Then, the sera were pooled for each group. The production of goat anti-mouse IgG1, IgG2a, IgG2b, and total IgG antibodies (Sigma, Germany) was assessed by indirect ELISA (Bolhassani et al., 2018). Moreover, mice sera were diluted 1:100 in 1% BSA/PBS-Tween. The coated antigen was rE7 protein (5 μg/ mL).

2.8. Assessment of cytokines and Granzyme B

Two 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 5 μg/ml of rE7 protein, RPMI 5% as negative control and 5 μg/ml of concanavalin A (ConA) as positive control in complete culture medium. The supernatants were harvested to assess the levels of IFN-γ, IL-5 and IL-10 cytokines by the sandwich-based ELISA method using a DuoSet ELISA system (R&D Systems) according to the manufacturer's instructions. For assessment of Granzyme B, the P815 target cells (T) were seeded in triplicate into U-bottomed, 96-well plates (2×10^4 cells/ well) incubated with E7 antigen (˜ 30 μg/ ml) for 24 h. The splenocytes (Effector cells: E) were added to the target cells at E: T ratio of 100:1 in which maximal release of Granzyme B was observed. The target and effector cells were co-cultured in complete RPMI-1640 supplemented with 10% heat-inactivated FBS. The effector cells were individually considered to assay 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 to measure the concentration of Granzyme B by ELISA (eBioscience) according to the manufacturer's instruction.

2.9. Statistical analysis

The differences between the control and test samples or groups in transfection efficiency, and in immunological studies were assessed using the unpaired Student's *t*-test, and one-way ANONA (Graph-pad Prism, GraphPad Software), respectively. Survival rate or the percentage of tumor-free mice was evaluated using the log-rank (Mantel-Cox) test. Differences were considered statistically significant for $p < 0.05$. All the parameters were represented as mean ± standard deviation (SD) for each set of samples. Similar results were obtained in two independent experiments.

Table 1
Different protective vaccination strategies using MSCs and proteins in C57BL/6 mice.

Group	Modality	First injection	Second injection	Third injection
G1	MSC/MSC/MSC	MSC (E7 DNA) (2×10^5 cells)	MSC (E7 DNA) (2×10^5 cells)	MSC (E7 DNA) (2×10^5 cells)
G2	MSC/MSC/MSC	MSC (Hsp20 DNA) (2×10^5 cells)	MSC (Hsp20 DNA) (2×10^5 cells)	MSC (Hsp20 DNA) (2×10^5 cells)
G3	MSC/MSC/MSC	MSC (Hsp27 DNA) (2×10^5 cells)	MSC (Hsp27 DNA) (2×10^5 cells)	MSC (Hsp27 DNA) (2×10^5 cells)
G4	MSC/MSC/MSC	MSC (E7 + Hsp20 DNA) (2×10^5 cells)	MSC (E7 + Hsp20 DNA) (2×10^5 cells)	MSC (E7 + Hsp20 DNA) (2×10^5 cells)
G5	MSC/MSC/MSC	MSC (E7 + Hsp27 DNA) (2×10^5 cells)	MSC (E7 + Hsp27 DNA) (2×10^5 cells)	MSC (E7 + Hsp27 DNA) (2×10^5 cells)
G6	MSC/protein/protein	MSC (E7 DNA) (2×10^5 cells)	E7 protein + Montanide (10 μg)	E7 protein + Montanide (10 μg)
G7	MSC/protein/protein	MSC (Hsp20 DNA) (2×10^5 cells)	Hsp20 protein + Montanide (10 μg)	Hsp20 protein + Montanide (10 μg)
G8	MSC/protein/protein	MSC (Hsp27 DNA) (2×10^5 cells)	Hsp27 protein + Montanide (10 μg)	Hsp27 protein + Montanide (10 μg)
G9	MSC/protein/protein	MSC (E7 + Hsp20 DNA) (2×10^5 cells)	E7 + Hsp20 protein + Montanide (5 μg + 5 μg)	E7 + Hsp20 protein + Montanide (5 μg + 5 μg)
G10	MSC/protein/protein	MSC (E7 + Hsp27 DNA) (2×10^5 cells)	E7 + Hsp27 protein + Montanide (5 μg + 5 μg)	E7 + Hsp27 protein + Montanide (5 μg + 5 μg)
G11	Protein/ Protein/ Protein	E7 protein + Montanide	E7 protein + Montanide	E7 protein + Montanide
G12	Protein/ Protein/ Protein	Hsp20 + Montanide	Hsp20 + Montanide	Hsp20 + Montanide
G13	Protein/ Protein/ Protein	Hsp27 + Montanide	Hsp27 + Montanide	Hsp27 + Montanide
G14	Protein/ Protein/ Protein	E7 + Hsp20 protein + Montanide	E7 + Hsp20 protein + Montanide	E7 + Hsp20 protein + Montanide
G15	Protein/ Protein/ Protein	E7 + Hsp27 protein + Montanide	E7 + Hsp27 protein + Montanide	E7 + Hsp27 protein + Montanide
G16	Control	PBS	PBS	PBS
G17	Control	MSC	MSC	MSC

Table 2
Different protective vaccination strategies using DCs in C57BL/6 mice.

Group	Modality	First injection	Second injection	Third injection
G1	DC/DC/DC	DC (E7 protein) (1×10^6 cells)	DC (E7 protein) (1×10^6 cells)	DC (E7 protein) (1×10^6 cells)
G2	DC/DC/DC	DC (Hsp20 protein) (1×10^6 cells)	DC (Hsp20 protein) (1×10^6 cells)	DC (Hsp20 protein) (1×10^6 cells)
G3	DC/DC/DC	DC (Hsp27 protein) (1×10^6 cells)	DC (Hsp27 protein) (1×10^6 cells)	DC (Hsp27 protein) (1×10^6 cells)
G4	DC/DC/DC	DC (E7 + Hsp20 protein) (1×10^6 cells)	DC (E7 + Hsp20 protein) (1×10^6 cells)	DC (E7 + Hsp20 protein) (1×10^6 cells)
G5	DC/DC/DC	DC (E7 + Hsp27 protein) (1×10^6 cells)	DC (E7 + Hsp27 protein) (1×10^6 cells)	DC (E7 + Hsp27 protein) (1×10^6 cells)
G6	DC/protein/protein	DC (E7 protein) (1×10^6 cells)	E7 protein + Montanide (10 μg)	E7 protein + Montanide (10 μg)
G7	DC/protein/protein	DC (Hsp20 protein) (1×10^6 cells)	Hsp20 protein + Montanide (10 μg)	Hsp20 protein + Montanide (10 μg)
G8	DC/protein/protein	DC (Hsp27 protein) (1×10^6 cells)	Hsp27 protein + Montanide (10 μg)	Hsp27 protein + Montanide (10 μg)
G9	DC/protein/protein	DC (E7 + Hsp20 protein) (1×10^6 cells)	E7 + Hsp20 protein + Montanide (5 μg + 5 μg)	E7 + Hsp20 protein + Montanide (5 μg + 5 μg)
G10	DC/protein/protein	DC (E7 + Hsp27 protein) (1×10^6 cells)	E7 + Hsp27 protein + Montanide (5 μg + 5 μg)	E7 + Hsp27 protein + Montanide (5 μg + 5 μg)
G11	Control	PBS	PBS	PBS
G12	Control	DC	DC	DC

3. Results

3.1. Generation of the recombinant plasmids and proteins

Our data showed that the purified HPV16 E7, Hsp20 and Hsp27 proteins migrated as clear bands of ~ 23 kDa, ~ 20 kDa and ~ 27 kDa in SDS-PAGE, respectively. The recombinant proteins had a concentration range between 0.8 and 1.0 mg/mL. Moreover, the recombinant pEGFP-E7, pEGFP-Hsp20 and pEGFP-Hsp27 constructs were confirmed by enzyme digestion on agarose gel as clear bands of ~297 bp, ~ 582 bp and ~ 720 bp for E7, Hsp20 and Hsp27 genes, respectively (data not shown).

3.2. Characterization of MSCs

The MSCs were prepared successfully from the bone marrow of mouse femurs. The flow cytometry analysis indicated that the percentage of negative surface marker (CD45) and positive surface marker (CD90.2) was 0.43% and 99.96%, respectively (data not shown). Thus, the stem cells expressed CD90.2 as a MSC marker, and did not express CD45 as a hematopoietic cell marker.

3.3. Characterization of DCs

The murine DCs were provided successfully from bone marrow precursors of C57BL/6 mice and harvested for use after 5-day culture in medium containing GM-CSF and IL-4 cytokines. The DCs were detected by Giemsa stain. Moreover, DCs were stained to analyze surface expression of the DC associated marker (CD11c), MHC II, and co-stimulatory molecules (CD86 and CD40) before pulsing with the recombinant proteins by flow cytometry. The expression levels of CD86, CD11c, CD40, MHCII and CD83 in immature DCs were 57%, 62.2%, 38%, 63% and 16.8%, respectively (data not shown). Then, the recombinant proteins were used to activate immature DCs and induce their maturation *ex vivo*. Most DCs significantly expressed CD83 maturation marker (~67-71%). This marker was stably expressed in DCs pulsed with the recombinant E7, Hsp20 and Hsp27 proteins. The potency of the modified DCs was assessed by immune responses induced post-vaccination.

3.4. E7, Hsp20 and Hsp27 expression under chemical and heat treatment in MSCs

The flow cytometry results showed that the expression of E7, Hsp20 and Hsp27 genes transfected into MSCs by lipofectamine one time were about 6.22 ± 1.21, 7.43 ± 0.69 and 8.98 ± 2.01%, respectively. These ratios were significantly increased after lipofection twice in MSCs as observed in Table 3 ($p < 0.05$). It was observed that heating at 42°C for 2 h induced increasing amounts of E7, Hsp20 and Hsp27 expression after lipofection twice ($p < 0.05$). Indeed, heat shock led to enhance the penetration of plasmids and further expression of three proteins. On the other hand, the delivery of pEGFP-Hsp20 and pEGFP-Hsp27 along with pEGFP-E7 increased the rate of GFP population in the cells indicating high transfection efficiency using lipofection followed by heat shock two times at 48 h after second transfection (Table 3, $p < 0.05$). Furthermore, the expression of Hsp27-GFP, Hsp20-GFP, E7-GFP and GFP proteins was detectable using anti-GFP antibody in western blotting as clear bands of ~ 54 kDa, ~ 47 kDa, ~ 50 kDa and ~ 27 kDa (Supplementary 1).

Table 3
: Flow cytometry analysis of E7, Hsp20 and Hsp27 expression in MSCs.

Groups	DNA	Method	Percentage
1	–	chemical	2.31 ± 0.51%
2	pEGFP-N1	chemical	35.80 ± 1.40%
3	pEGFP-N1	chemical/heat	68.22 ± 1.71%
4	pEGFP-E7	chemical	26.53 ± 1.04%
5	pEGFP-E7	chemical/heat	51.87 ± 2.02%
6	pEGFP-Hsp20	chemical	31.64 ± 3.12%
7	pEGFP-Hsp20	chemical/heat	59.92 ± 1.16%
8	pEGFP-Hsp27	chemical	34.05 ± 0.89%
9	pEGFP-Hsp27	chemical/heat	60.58 ± 2.46%
10	pEGFP-E7 + pEGFP-Hsp20	chemical	39.98 ± 2.01%
11	pEGFP-E7 + pEGFP-Hsp20	Chemical/heat	71.01 ± 1.67%
12	pEGFP-E7 + pEGFP-Hsp27	chemical	40.23 ± 3.11%
13	pEGFP-E7 + pEGFP-Hsp27	Chemical/heat	77.50 ± 1.54%

3.5. Antibody responses

The serum levels of total IgG and the related subclasses (IgG1, IgG2a & IgG2b) were detected against rE7 protein in different groups. Our data indicated that the levels of total IgG, IgG2a and IgG2b in the sera of mice vaccinated by DCs pulsed with E7 + Hsp20 and E7 + Hsp27 as homologous (G4 and G5) and heterologous (G9 and G10) regimens were significantly higher than other groups ($p < 0.05$, Fig. 1**A, E, G**). Moreover, the levels of IgG2a and IgG2b in the sera of mice vaccinated by MSCs transfected with E7 + Hsp27 as homologous (G5) and heterologous (G10) regimens were significantly higher than other groups ($p < 0.05$, Fig. 1**B, F, H**). It was shown that the groups vaccinated by homologous or heterologous DCs had higher levels of total IgG, IgG2a and IgG2b than the groups vaccinated by homologous or heterologous MSCs ($p < 0.05$). Moreover, the groups vaccinated by E7 protein (G11) showed higher levels of IgG1 and total IgG compared to other groups (**Figure 1B & D**). However, Hsp27 along with E7 protein (G15) could significantly direct immune responses toward IgG2a and IgG2b compared to E7 protein. No significant anti-E7 antibody responses could be detected in the sera of control groups and the groups vaccinated by Hsp20 or Hsp27 alone.

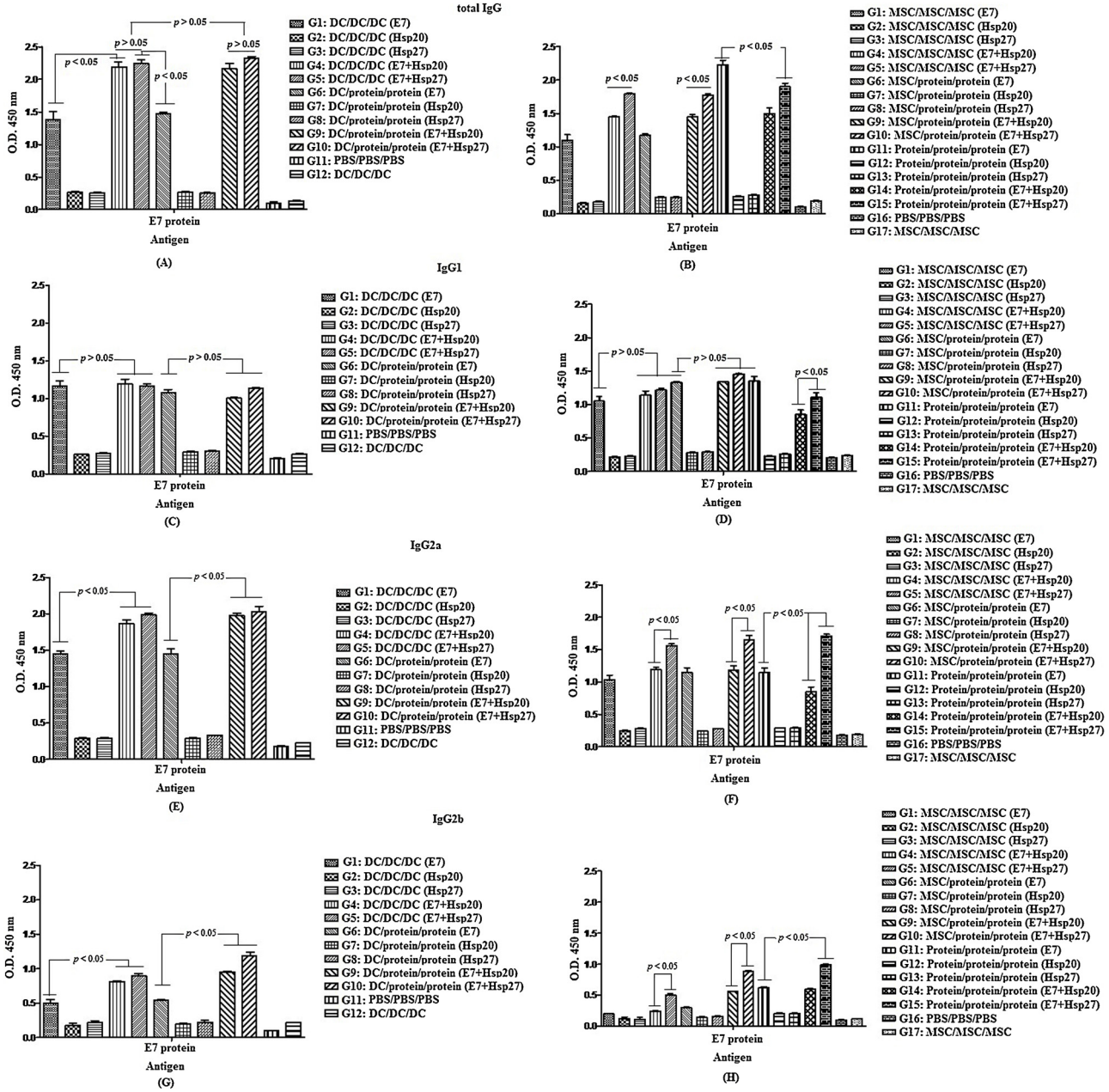

Fig. 1. Antibody responses (total IgG, IgG1, IgG2a and IgG2b) against rE7 protein in different regimens: All analyses were performed in duplicate for each sample. The results were shown as mean absorbance at 450 nm ± SD.

3.6. Secretion of cytokines and Granzyme B

The cytokine results of each group indicated that the levels of E7-specific IFN-γ secretion in the groups vaccinated by DCs pulsed with E7 + Hsp20 and E7 + Hsp27 as homologous (G4 and G5) and heterologous (G9 and G10) regimens were significantly higher than those in other groups ($p < 0.05$, Fig. 2A). This result was obtained in the groups vaccinated by MSCs transfected with E7 + Hsp27 as homologous (G5) and heterologous (G10) regimens compared to other groups ($p < 0.05$, Fig. 2B). Our data indicated that Hsp27 was more effective than Hsp20 as an adjuvant in the groups vaccinated with MSCs and also proteins for stimulation of IFN-γ secretion (G5 *vs* G4; G10 *vs* G9; G15 *vs* G14; $p < 0.05$). Among all the test groups, the groups vaccinated by E7 protein (G11), E7 DC (G1) and E7 MSC (G1) showed a significant IL-5 response (˜ 50, ˜ 70 and ˜ 70 pg/mL, respectively) compared to other groups (˜ 10–20 pg/ml, $p < 0.05$, data not shown). Moreover, the secretion of IL-10 in the splenocytes re-stimulated with rE7 protein was significantly higher in the groups vaccinated by DCs pulsed with E7 + Hsp20 and E7 + Hsp27 as heterologus regimens (G9 and G10) and MSCs transfected with E7 + Hsp27 and E7 + Hsp20 as heterologus regimens (G9 and G10) as well as protein regimens (G14 and G15) than that in other groups ($p < 0.05$, **Figure 2C & D**). Indeed, Hsp20 and Hsp27 could significantly induce IL-10 production compared to other groups ($p < 0.05$). As known, IL-10 can directly act on $CD4^+$ T cells, inhibiting the production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α (Couper et al., 2008). It seems that IL-10 could affect the levels of IL-5, because Hsp27 or Hsp20 showed no significant IL-5 secretion. Generally, our data indicated that the Hsp27 adjuvant could significantly activate Th1 response in all regimens.

On the other hand, the results of Granzyme B secretion in each group showed that the groups vaccinated by DCs pulsed with E7 + Hsp27 and E7 + Hsp20 as homologous and heterologous regimens (G4,

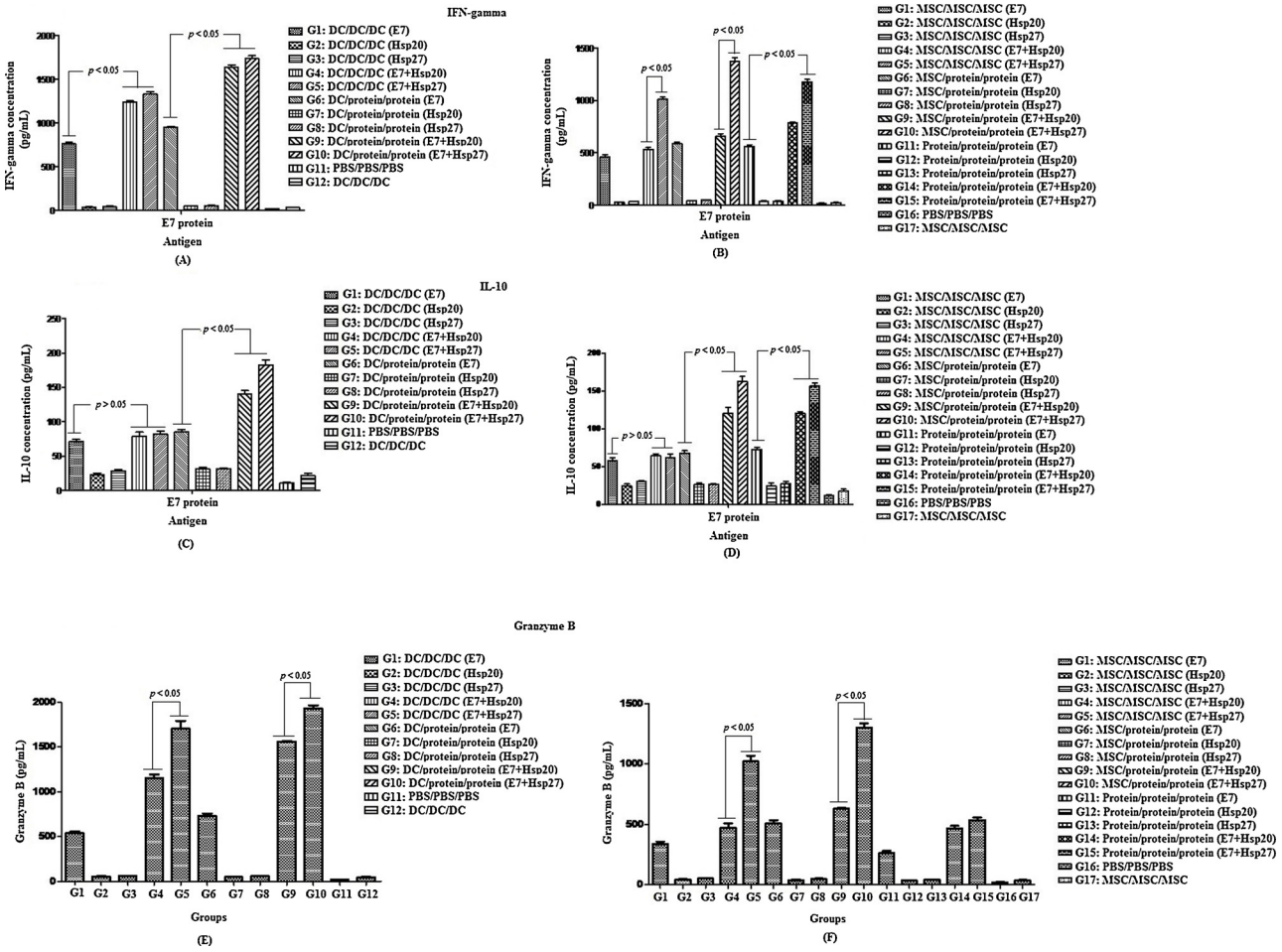

Fig. 2. IFN-γ, IL-10 and Granzyme B secretion in immunized groups with various formulations: The levels of IFN-γ (A, B), IL-10 (C, D) and Granzyme B (E, F) were determined by ELISA as mean absorbance at 450 nm ± SD for each set of samples. All analyses were performed in duplicate for each sample.

G5, G9, G10) produced significantly higher concentrations of Granzyme B than other groups ($p < 0.05$, Fig. 2E). However, Hsp27 was more effective than Hsp20 as an adjuvant in Granzyme B secretion ($p < 0.05$). After these groups, the groups vaccinated by MSCs transfected with E7+ Hsp27 as homologous and heterologous regimens (G5 and G10) significantly secreted higher concentrations of Granzyme B than other groups ($p < 0.05$, Fig. 2F).

3.7. Hsp27 and Hsp20 could enhance mice protection against E7-expressing tumor cells

For evaluation of vaccine efficiency, tumor growth was measured in all groups. As shown in Fig. 3, tumor growth was significantly reduced for the groups vaccinated by modified DCs and MSCs with E7, E7 + Hsp20 and E7 + Hsp27 as well as by proteins (E7, E7 + Hsp20 and E7 + Hsp27) compared to other groups ($p < 0.05$). All mice developed tumor growth on approximately 7–28 days in control groups. As indicated in Fig. 3, vaccination by modified DCs with E7 + Hsp27 or E7 + Hsp20 as homologous (G4 and G5) and heterologous (G9 and G10) regimens as well as modified MSCs with E7 + Hsp27 as homologous and heterologous regimens (G5 and G10) could protect all mice from tumor growth.

3.8. Treatment by DCs and MSCs pulsed with E7 + Hsp27 effectively eradicated the tumor growth in mice

Mice with pre-established E7-expressing tumors were treated by modified DCs with E7 + Hsp27 or E7 + Hsp20 as homologous and heterologous regimens as well as modified MSCs with E7 + Hsp27 as homologous and heterologous regimens with protection of 100%, one week after inoculation with TC-1 tumor cells. Among these groups, the groups vaccinated by modified DCs and MSCs with E7 + Hsp27 as homologous and heterologous regimens displayed complete regression and remained tumor-free > 60 days following treatment. However, the groups vaccinated by modified DCs with E7 + Hsp20 could eradicate the tumor growth nearly 66.67% (data not shown). Thus, Hsp20 could be less effective compared to Hsp27 for tumor therapy.

4. Discussion

DC-based HPV vaccines involved loading the DCs with HPV antigens *ex vivo* and delivering those DCs to the infected host (Yang et al., 2016). Moreover, the ability of modified mesenchymal stem cells (MSCs) to express and secrete antigens, and induce immune responses has been attracted a special interest. However, it is required to use efficient transfection approaches in these cells (Haleem-Smith et al., 2005). Our goal in this study was the evaluation of immune responses and anti-tumor effects generated by DCs and MSCs modified with HPV16 E7, sHsp 27 and sHsp20 in tumor mouse model. In this study, DCs and MSCs derived from mouse bone marrow were used to develop therapeutic vaccines against HPV-related cancers for the first time. Antigen-pulsed DCs as adjuvants could actively prime T-dependent immunity in rodents. Indeed, the protective anti-microbial and anti-tumor immunity was elicited using antigen-pulsed DCs without other adjuvants. These DCs used in these experiments undergo maturation *ex vivo* prior to injection (Strome et al., 2002). In the recent study, we generated DCs

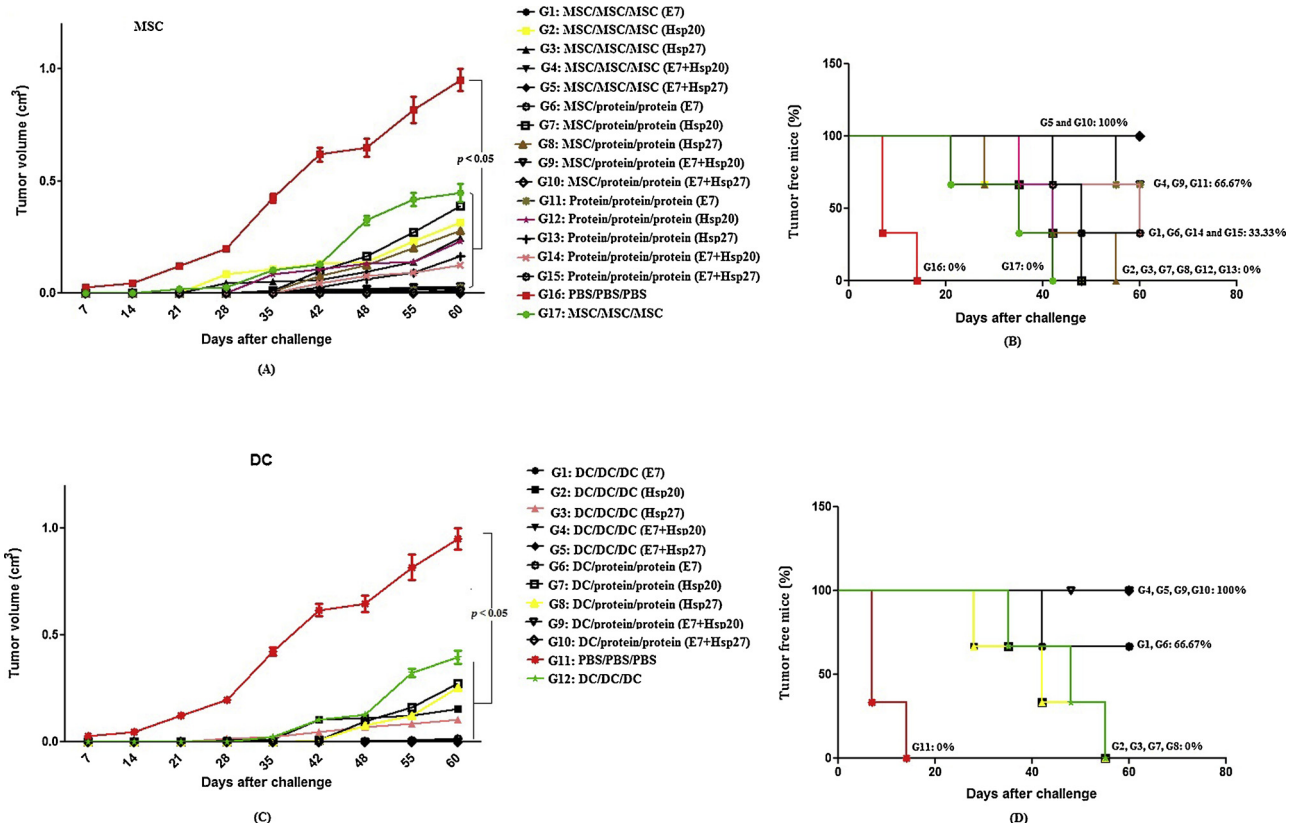

Fig. 3. Preventive studies against TC-1 tumor cells in mice immunized with different vaccine formulations: The mice were challenged with 1×10^5 TC-1 in the right flank 2 weeks after second booster. Tumor volumes were measured twice a week (A, C). Moreover, the percentage of tumor-free mice was determined over time in various groups (B, D).

pulsed with E7, Hsp27 and Hsp20 proteins as individual or combined forms. All these recombinant proteins could upregulate CD83 maturation marker. The reports showed that the interaction of Hsps with DCs has led to develop different Hsp-containing vaccines designed to deliver antigens directly to DCs (McNulty et al., 2013). A study indicated that tumor-derived Hsp70 chaperones a tyrosinase peptide for delivering into human immature DCs by receptor-dependent uptake required for MHC class I-restricted cross-presentation and T-cell induction (Noessner et al., 2002). Indeed, the recombinant (r) Hsp70 could bind to immature DCs derived from monocyte precursors and induce their maturation using an increase in CD40, CD86 and CD83 expression. Thus, the inherent ability of rhsp70 was important to stimulate the maturation of immature DC which can be useful as an effective adjuvant in DC-based immunotherapy (Kuppner et al., 2001). As known, small Hsps also possess the roles in differentiation, development, proteasomal degradation, autophagy and immunity (Bakthisaran et al., 2015). The same sequences with α-crystallin in Hsp20 (aa 71–91) and Hsp27 (aa 93–113) showed strong chaperone and anti-apoptotic activities. For instance, these peptide sequences inhibited apoptosis in HeLa cells by blocking cytochrome *c* release from the mitochondria and caspase-3 activation (Nahomi et al., 2015). Yusuf et al demonstrated that treatment of DC cultures with the recombinant Hsp27 could upregulate IL-1β, TNF-α, IL-6, IL-12p70 and IL-12p40 but not IL-23p19 (Yusuf et al., 2009). Furthermore, DCs primed with tumor antigens could effectively reduce the tumor growth in both murine and human models (Strome et al., 2002; Weng et al., 2011). Herein, we showed that E7 as an oncogenic protein and small Hsps as adjuvants or immunomodulators could enhance maturation of DCs, induce immune responses and represent antitumor effects. A phase I study with the full length HPV-16 and -18 E7-pulsed dendritic cells and an immunological tracer molecule, keyhole limpet hemocyanin (KLH), showed an increase in E7-specific $CD4^+$ and $CD8^+$ T cells in patients with stage Ib or IIa cervical cancer (Santin et al., 2008). As similarly, DCs loaded with HPV-16 or -18 E7 co-administered with IL-2 showed E7-specific $CD8^+$ responses in all patients (Santin et al., 2006). Furthermore, pre-immune DCs (PIDCs) pulsed with HPV-16 E6 or E7 induced specific immune responses in 63% (E6) or 58% (E7) of patients. The reports indicated that the use of PIDCs compared to mature DCs has the potency to reduce vaccine cost (Rahma et al., 2014; Yang et al., 2016; Chabeda et al., 2018). Our studies also showed that DCs pulsed with E7 + Hsp27 and E7 + Hsp20 as homologous (DC prime/ DC boost) and heterologous (DC prime/ protein boost) regimens could significantly induce the secretion of IgG2a, IgG2b, IFN-γ, Granzyme B and also the protective effects against TC-1 tumors. However, the anti-tumor effects of E7 + Hsp27 DC/ E7 + Hsp27 DC and E7 + Hsp27 DC/ E7 + Hsp27 protein were confirmed in therapeutic studies (100% tumor-free mice), but the regimens of E7 + Hsp20 DC/ E7 + Hsp20 DC and E7 + Hsp20 DC/ E7 + Hsp20 protein showed only ˜66.67% survival rate in mice. These data showed that Hsp27 along with E7 is more effective than Hsp20 in eliciting immune responses and inducing antitumor effects.

In this study, we used the mesenchymal stem cells (MSCs) transfected with E7, Hsp27 and Hsp20 as individual or combined, as well. Some reports showed that most standard transfection methods had poor transfection efficiency for MSCs (less than 1%). In a study, Haleem-Smith et al extended an electroporation method termed as amaxa Nucleofection™ for delivery of green fluorescent protein (GFP) gene into low passage MSCs derived from adult human bone marrow. The results showed up to 90% transfection efficiency of the viable population of MSCs. At 2 and 3 week post-transfection, about 25% and 10% of the cell population retained GFP expression (Haleem-Smith et al., 2005). Other study used ultrasound-targeted microbubble destruction (UTMD) as a novel technique for hVEGF165 gene transfer in MSCs. The data showed that the mechanic index (MI) 0.6 for 90 s led to the highest level of protein expression (Pu et al., 2011). Also, Liu et al reported that

polyethyleneimine-coated magnetic ferro-ferric oxide nanoparticles showed a higher transfection rate than lipofectamine in rat mesenchymal stem cells (Liu et al., 2016). However, lipids (*e.g.,* lipofectamine) were widely used to transfer genes into various cell types. Lipofection showed high transient transfection efficiency in many cell lines, but however, it could not act very well in some non-cancerous and cancerous cell lines for gene delivery. To overcome this problem, treatment of the cells was performed by a mild heat shock at 42 °C for 10–30 min immediately following lipid transfection. For instance, a 10 min treatment at 42 °C led to a 90%, 96%, 55%, 100% and 52% increase in gene transfection into the SW480, A549, MCF-7, B-16 and 4T1 cells, respectively (Pipes et al., 2005). In addition, increasing the temperature of the cells to 42 °C for a few hours after DNA transfection resulted in an up to 10-fold increase in the frequency of the cells that transiently expressed β-galactosidase gene, but it had only a slightly enhancing effect on the expression of stable transformants. The enhanced expression of β-galactosidase was correlated with increasing the enzyme activity (Takai and Ohmori, 1992). On the other hand, a study indicated that the heat treated DNA had higher gene transfection efficiency than untreated DNA (Hou et al., 2008). However, conventional transient gene expression (TGE) was limited to a short production period of usually about 96 h, thus limiting productivity (Cerver et al., 2014). A method based on repeated transfections at 48 h interval for 168 h was reported. DNA-repeated transfections resulted in high levels of gene delivery with no effect on cell viability. *In vivo* data also confirmed that this method could enhance transfection versus single administration (Montani et al., 2015). Also, a novel gene expression approach termed extended gene expression (EGE) was used to prolong the production period by the combination of medium exchange and repeated transfection of cell cultures with plasmid DNA encoding intracellular GFP, secreted GFP, and Gag-GFP virus-like particles (VLPs) using polyethylenimine (PEI) as transfection reagent. The data showed that the production period was prolonged between 192 and 240 h with a 4-12-fold increase in production levels using EGE strategy (Cerver et al., 2014). In this study, we used a mild heat shock at 42 °C for 2 h immediately following lipofectamine transfection (lipofection). Moreover, transfection was repeated twice with 48 h interval. Our data showed that the efficiency of transfection was increased significantly compared to the use of lipofection, alone. It is possible that heat could increase the number of cells that uptake the plasmid by affecting fluidity of the cell membrane as reported by others (Pipes et al., 2005). In this study, we showed that mouse MSCs transfected with E7 + Hsp27 as homologous (MSC prime/ MSC boost) and heterologous (MSC prime/ protein boost) regimens were able to stimulate $CD4^+$ T cells, produce Th1 instead of Th2 cytokines and activate Granzyme B. The B cells stimulated with these modified MSCs produced more E7-specific IgG2a and IgG2b compared to other groups. These responses were significantly higher in DCs pulsed with E7 + Hsp27 than those in MSCs transfected with E7 + Hsp27. Indeed, E7 + Hsp27 protein-pulsed DCs or E7 + Hsp27-DNA transfected MSCs were also able to direct the immune response from Th2-dominance towards Th1 resulting in decreased IgG1 production. However, E7 + Hsp27 protein-pulsed DCs and E7 + Hsp27-DNA transfected MSCs could generate the same anti-tumor effects (100% tumor-free mice) in both protective and therapeutic experiments.

As known, DC vaccination strategies showed low clinical success for therapeutic cancer vaccines and had high production costs due to necessary individual tailoring (Bhargava et al., 2012; Palucka and Banchereau, 2012). The studies indicated that MSC strategy may improve the problems of DNA and DC-based vaccinations. The ability to prevent graft-versus-host disease (GvHD) also suggested that MSCs expressing foreign antigen might have an advantage over DCs for stimulation of specific immune responses against specific antigen not the donor MSC. Indeed, MSC-based strategy may reduce production time and costs associated with necessary HLA matching as observed by DCs or other cell types (Tomchuck et al., 2012). Wei et al. reported that the E7-expressing MSCs combined with a modified E7 fusion protein showed a decrease in tumor growth and an E7-specific antibody response in a mouse tumor model (Wei et al., 2011). On the other hand, some researchers indicated that the heterologous prime-boost can take various forms and that the order of prime-boost administration may be important, although this may be antigen-dependent and may be influenced by the host species and the types of immune responses to be achieved (Kardani et al., 2016; Lu, 2009).

In conclusion, MSCs transfected with E7 + Hsp27 DNA and DCs pulsed with E7 + Hsp27 protein in homologous and heterologous prime/ boost vaccinations could significantly enhance the E7-specific T-cell responses and suppress tumor growth in mice. These results indicated that DC- and MSC-based vaccinations with specific modalities will be a useful approach for immunotherapy and protection against HPV-associated cancers. However, it is required to obtain histological documents conferring effective protection in mice in near Future and subsequently determine safety and long-term memory for modified MSCs in human.

Author contributions

AB designed the study, supervised the experiments and wrote the paper. SS and EA performed *in vivo* experiments. EA, NH, AH and FA performed *in vitro* experiments and analyzed the data. All authors approved the final version of the manuscript.

Conflict of interest

The authors report no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.02.016.

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