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Production, purification, and *in vivo* evaluation of a novel multiepitope peptide vaccine consisted of immunodominant epitopes of SYCP1 and ACRBP antigens as a prophylactic melanoma vaccine

Ashkan Safavi[a], Amirhosein Kefayat[b], Fattah Sotoodehnejadnematalahi[a], Mansoor Salehi[c], Mohammad Hossein Modarressi[a,*]

[a] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[b] Department of Oncology, Cancer Prevention Research Center, Isfahan University of Medical Sciences, Isfahan 81746-73461, Iran
[c] Division of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

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ABSTRACT

Melanoma cells are significantly resistance to the current treatments. Therefore, the best option for high-risk populations is prevention. Recently, many preventive cancer vaccines have been developed. In our previous study, several bioinformatic tools were employed for selection of the most immunodominant epitopes of acrosin binding protein (ACRBP) and synaptonemal complex protein 1 (SYCP1) antigens to design multiepitope DNA and peptide cancer vaccines. In the current study, the final construct of the multiepitope DNA vaccine was placed into a pcDNA3.1 vector and then, subcloned into a pET-28a (+) expression vector for transfecting BL21 *E. coli* strain. The recombinant multiepitope peptide vaccine, weighing 6.35 kDa, was purified by Fast protein liquid chromatography technique (FPLC) and detected by western blotting. Subsequently, C57BL/6 mice were immunized by a mixture of the peptide vaccine and incomplete Freund's adjuvant (IFA) (four vaccinations with one-week intervals). Two weeks after the last vaccination, the serum levels of the peptide-specific IgG total, IgG2a, and IgG1 were measured by enzyme-linked immunosorbent assays (ELISA). Also, the immunized mice splenocytes efficacy for producing interleukin-4 (IL-4) and interferon-γ (IFN-γ) after stimulation with the peptide vaccine was evaluated. At last, the prophylactic effect of the peptide vaccine immunization was evaluated in B16-F10 murine melanoma model. The peptide vaccine immunization caused a significant increase in the serum levels of IgG1, IgG2a, and IgG2a. Also, the immunized mice splenocytes exhibited significantly higher ability to produce IL-4 (10-fold) and IFN-γ (16-fold) after stimulation with the peptide vaccine, in comparison with the PBS and IFA groups. The peptide immunized mice exhibited 50.2% and 43% decrease in the mean tumors' volume in comparison with PBS and IFA groups. Also, the mean survival time for the peptide immunized mice was 33 ± 1.3 days which was 5 and 6 days more than the PBS and IFA groups, respectively. The obtained results exhibit high efficacy of the designed multiepitope peptide vaccine for the immune system activation and anti-tumor prophylactic effects in the murine melanoma model.

1. Introduction

Melanoma is the most dangerous skin cancer in the world. This skin cancer kills > 10,000 patients every year in the United States. Also, thousands of new cases are diagnosed each year [1]. Unresectable or metastatic melanoma are known as advanced melanoma. These tumors usually exhibit high resistance against radiation therapy and chemotherapy. The median survival time for these patients is about 6–9 months and only one-fourth of them will survive for 1 year. Therefore, the best options for the high-risk populations are prevention and early detection of melanoma [2].

Cancer vaccines have gained lots of attention for cancer prevention and treatment. They can cause recognition and elimination of malignant cells by the immune system [3–7]. Also, cancer vaccines have exhibited high safety and biocompatibility. Many studies have reported the advantages of prophylactic melanoma vaccines [8,9]. In our

Abbreviations: ACRBP, acrosin binding protein; SYCP1, synaptonemal complex protein; CTA, cancer/testis antigen; IFA, incomplete Freund's adjuvant; FPLC, fast protein liquid chromatography

* Corresponding author.
E-mail address: modaresi@tums.ac.ir (M.H. Modarressi).

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previous study, multiepitope DNA and peptide vaccines consisted of the immunodominant epitopes of acrosin binding protein (ACRBP) and synaptonemal complex protein 1 (SYCP1) antigens were designed *in silico* [10]. SYCP1 and ACRBP are two well-known members of the cancer/testis antigens (CTAs) family. These antigens are ideal targets for designing melanoma vaccines due to high expression at cancer cells, high immunogenicity, extremely low expression at the normal tissues, and extensive contribution at cancer progression [11,12]. According to our previous *in silico* study, this multiepitope peptide vaccine has high binding affinity to MHCs and contains several cytotoxic T lymphocytes' (CTL) epitopes based on immunoinformatic analyses. Also, it can stimulate the humoral immune response. Also, the multiepitope DNA vaccine has appropriate codon usage (codon adaptation index for mouse and bacteria were 0.92 and 0.87, respectively) for expression in the prokaryote hosts [10].

The main aim of the present study is to produce and purify an *in silico* designed multiepitope peptide vaccine containing immunodominant epitopes of SYCP1 and ACRBP antigens to investigate its efficacy as a prophylactic melanoma vaccine in C57BL/6 mouse melanoma model.

2. Materials and methods

2.1. Designing of the multiepitope DNA and peptides vaccines

Based on our previous study [10], the most immunodominant fragments of SYCP1 and ACRBP antigens were recognized by NetMHCpan, ProPred-I, and MHCpred. Also, the NetCTLpan, NetCTL, and CTLPred servers were used to find their CTL epitopes. The RCQHKIAEMVALMEKHKHQYDKI fragment from SYCP1 and YIQYPNYCSFKSQQCL fragment from ACRBP antigens were selected. The fragments were fused by a furin-sensitive linker (RVRR). Furthermore, the sequence was modified to form the final construct of the multiepitope peptide vaccine. The DNA sequence was obtained by reverse translation and codon-optimization of the peptide sequence. Also, the Kozak sequence was added to the 5′ end of the nucleotide sequence. At last, appropriate restriction enzymes' sites (*Nhe*I, *Nco*I, and *Xho*I) were added at the 5′ and 3′ ends. The constructed DNA sequence with 171 bps was purchased in the pcDNA3.1 expression vector (GeneCust Company, Dudelange, Luxemburg).

2.2. Subcloning of the peptide vaccine sequence into the pET-28a expression vector (pcDNA3.1/PEP)

The competent *E. coli* Top10 strain was incubated with the pcDNA3.1/PEP vector (GeneCust, Luxembourg) after vector preparation. Then, the bacteria were transfected by the heat shock method and left for 60 min in LB broth culture media (Sigma, USA) at 37 C°. The transfected bacteria were plated onto LB agar plates containing ampicillin (100 μg/mL) (Sigma, USA) and tetracycline (10 μg/mL) (Sigma, USA). Then, the plates were incubated for 16 h standard culture condition. The presence of the pcDNA3.1/PEP at the white colonies was confirmed by colony PCR. The utilized forward and reverse primers were GCTAGCACCATGGGTAGGTG and GGCACTGCTGGCTCTTGAA, respectively. The plasmids were extracted from the confirmed colonies by miniperp kit (QIAGEN, USA) and then, digested by Nco1 (Invitrogen, USA) and Xho1 (Invitrogen, USA) restriction enzymes. 1 μL of the plasmid DNA (1 μg/μL), 2 μL of Xho1, 2 μL of Nco1, 2 μL of 10 X tango buffer, and 16 μL of nuclease-free water in the total volume of 23 μL were incubated for 7 h at 37 °C. The multiepitope peptide sequence in pcDNA3.1/PEP was extracted from agarose gel and subcloned into the pET-28a (+) expression vector (GeneCust, Luxembourg) which was digested by the same restriction enzymes. The ligation reaction was performed by 125 ng of digested pET-28a (+), 0.5 pmol of purified fragments sequence, 2 μL of rapid 2× ligation buffer (Promega, USA), and 3 units of T4 DNA ligase (Promega, USA) in the total volume of 10 μL. The final solution was incubated overnight at 4 °C. Heat shock method was utilized for the transformation of *E. coli* BL21 (DE3) strain by the recombinant pET-28a (pET-28a/PEP). After 1 h of recovery time in LB broth, the transformed bacteria were cultured onto LB agar containing Kanamycin (50 μg/mL) (Sigma, USA) for 16 h at 37 °C. The colony PCR was utilized to screen the obtained colonies and one of them was confirmed by DNA sequencing. The recombinant plasmid was purified and used for transformation of *E. coli* BL21 (DE3) strain for the peptide production.

2.3. The recombinant multiepitope peptide vaccine production and purification

A single transformed colony was cultured into LB broth, containing Kanamycin (50 μg/mL) (Sigma, USA) and IPTG (Sigma, USA) was used to induce the peptide expression. Then, the bacteria were harvested by centrifugation. For digesting cell wall, dry ice/ethanol bath and neutral (N)-lysis buffer (50 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl and 0.5 mM EDTA) were utilized according to previous studies [13,14]. DNA was removed by DNase (10 μg/mL) and the resulting *E. coli* lysate was placed into a dialysis bag (10 kDa molecular weight cut-off). At the end of the dialysis, centrifugation was employed to remove the cell debris. The purification of the recombinant His-tagged peptide vaccine was done by employing a DEAE-Sepharose Fast Flow column (GE Healthcare, Piscataway, NJ) followed by nickel affinity chromatography according to previously published articles [15–17]. The purification of the peptide vaccine was based on the His-tag protocol. Different steps of purification (*i.e.* ion exchange and affinity chromatography) were monitored by SDS-PAGE, and the peptide vaccine presence was confirmed by immunoblotting. Amount of the total extracted peptide vaccine was determined using the Bradford method (Start Quick Assay Protein Bradford™ kit).

2.4. Immunoblotting

For western blot analyses, hosts protein solutions were obtained through 5 min boiling in a sample buffer containing Tris-HCl (pH = 6.8, 0.5 M), SDS (10% W/V), Glycerol (50% V/V), and Bromo Phenol Blue (0.5% W/V) (Sigma, USA). 40 μL of each sample was loaded in SDS-PAGE (13%) under non-reducing conditions to separate protein bonds. The protein constituents were electrophoretically blotted onto a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked in 5% skim milk/0.1% Tween 20 in PBS (pH 7.4), and then the reactivity of the transferred protein(s) was evaluated with 1 μg/mL monoclonal anti-poly Histidine−Peroxidase antibody. Finally, the detection was performed with tetramethylbenzidine substrate (Sigma, USA).

2.5. Cell culture and preparation

Murine melanoma cell line (B16-F10) was purchased from the Pasteur Institute of Tehran, Iran. The cells were cultured in DMEM medium (Sigma, USA) containing 10% fetal bovine serum (FBS) (Sigma, USA) and 1% antibiotics mixture containing penicillin (Sigma, USA) and streptomycin (Sigma, USA). The cells were incubated at 37 °C in a humidified incubator in 5% CO_2 atmosphere.

2.6. RNA isolation and Real-Time PCR analyses

Total RNA was isolated from B16-F10 cells *in vitro* using TRIzol. Then, the samples were treated with DNase I (Invitrogen, USA) to avoid DNA contamination. The cDNA was generated from 500 ng of total RNA using SuperScript III reverse transcriptase (Invitrogen, USA) with oligo (dT)12–18 as the primer. The utilized primers for Real-Time PCR were as follow: SYCP1, 5-CGAAGATTGCTTTGGAGACTG -3 (forward) and 5-TTCAGGTGATTCCAGTAAAGATG-3 (reverse); ACRBP, 5-TTGGTACCA

GATGGTGCTGTC-3 (forward) and 5-TGGTCATGGAAGTGGGAGG-3(reverse) beta-actin, 5-GAAGATCAAGATCATTGCTCCTC-3 (forward) and 5-AACGCAGCTCAGTAACAGTCC-3 (reverse). Each reaction (95 °C, 2 min; 95 °C, 10 s; 60 °C, 30 s; 72 °C, 30 s, 40 cycles) was performed using QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) in an Mx3000P QPCR System (Stratagene, CA). Cycle threshold (Ct) values were normalized with respect to beta-actin expression.

2.7. Animal care and ethics

This study was approved by the institutional review committee of Islamic Azad University of Medical Sciences and all procedures were reviewed and approved by the Institutional Animal Care and Ethics Committee of Islamic Azad University of Medical Sciences according to their guidelines for care and use of the laboratory animal. 48 Female C57BL/6 mice (age: 6–8 weeks, weight: 22 ± 2 g) were purchased from the Pasteur Institute of Tehran, Iran. The mice were maintained at 24 ± 2 °C temperature, 50 ± 10% relative humidity, and 12 h light/12 h dark cycle condition with complete access to the standard mouse chow and water. The mice were acclimated for at least 10 days before the start of the study. If any signs of pain, massive tumor necrosis, hemorrhage, and diffuse metastasis was observed during each step of the study, the animal was sacrificed. The neck dislocation was used for scarifying the mice.

2.8. Mice immunization

The mice were randomly divided into three groups (n = 16). The 1st group was s.c injected with 100 μL PBS. The 2nd group was s.c injected with 100 μL incomplete Freund's adjuvant (IFA) which was formulated with PBS in 50% V/V. Mice of the 3rd group were immunized by a mixture of the multiepitope peptide vaccine (100 μg in 50 μL of PBS) emulsified with 50 μL IFA 50% V/V. Each mouse at each group underwent four vaccinations with one-week intervals. The vaccination schedule is illustrated in Table 1.

2.9. Antibodies determination by enzyme-linked immunosorbent assay (ELISA)

14 days after the last injection, blood samples were collected for determination of the serum levels of the peptide vaccine-specific antibodies at each group (n = 5) by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with the peptide vaccine (10 μg/well) and incubated at 4 °C overnight. Then, the plates were washed three times with special ELISA solution and blocked with PBS containing 1% BSA for 2 h at room temperature, followed by three times washes. Then, the plates were incubated with PBS-diluted mouse sera for 1 h at 37 °C. After washing, plates were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (diluted 1:4000 in PBS-1% BSA), IgG1 (1:2000), and IgG2a (1:2000) for 1 h at 37 °C and then washed with ELISA solution. Then, the plates were incubated in the dark for 30 min at 37 °C, and the reaction was stopped by adding 2 M H_2SO_4. The optical density (OD) was measured at 490 nm using an ELISA reader. All samples were run four times. Three wells were used for each sample and the culture media (containing 10% FBS) was used as the negative control.

Table 1
Vaccination schedule at different groups.

Group	1st injection	2nd injection	3rd injection	4th injection
1 (n = 16)	PBS	PBS	PBS	PBS
2 (n = 16)	IFA	IFA	IFA	IFA
3 (n = 16)	Peptide-IFA	Peptide-IFA	Peptide-IFA	Peptide-IFA

IFA: Incomplete Freund's adjuvant, PBS: Phosphate buffer solution, n: Number of mice per group, Peptide: The multiepitope peptide vaccine.

2.10. Cytokines assay

The immunized mice spleen was aseptically harvested for assessment of the cytokines production (n = 3), 14 days after the last vaccination. The splenocytes were cultured in 96-well cell culture plates and the cell-free supernatants were collected and restimulated with the peptide vaccine (10 mg/mL) and incubated for 60 h at standard cell culture condition. Subsequently, the supernatants were collected to investigate the cytokines' level. Interleukin-4 (IL-4), interferon gamma (IFN-γ), and granzyme B (GrB) were measured by ELISA kits (R&D Systems, USA), following the manufacturer's instruction. Three wells were used for each sample and the culture media (containing 10% FBS) was used as the negative control.

2.11. Antigen-specific proliferation assay

The cultivated splenocytes were cultured in triplicate in a 96-well plate. Each well was seeded by 1.5×10^5 cells. Subsequently, the cells were stimulated with the peptide vaccine (2 μg/well) as recall antigen. Unstimulated wells were used as negative controls. Also, phytohemagglutinin-A (PHA) (5 μg/mL) was utilized as a positive control. After 72 h incubation at standard cell culture condition, the proliferation was measured by incorporation of 5-bromo-2 deoxyuridine (BrdU) into the peptide vaccine stimulated splenocytes using a cell proliferation ELISA kit (Abcam, USA). The stimulation index (SI) for cell activity was calculated using the following formula (1):

$$SI = (\text{Stimulated wells'OD} - \text{blanks'OD})/(\text{unstimulated wells'OD} - \text{blanks'OD}) \quad (1)$$

2.12. Tumor challenge

The mice were injected subcutaneously with 1×10^6 B16-F10 cells suspended in 50 μL of DMEM-F12 (Sigma, USA) into their left flank. The vaccination site was shaved and sterilized before injection. To determine tumors' growth progression, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) of the tumors were measured every 3 days for 18 days. Then, the tumor's volume was calculated by the tumor volume Eq. (2). For survival analysis, the tumor-bearing mice were observed for 40 days after tumor implantation. The animals' death was recorded every day. The standardized humane endpoint was used to euthanize animals which weren't able to eat or drink for over 3 days.

$$\text{Tumor volume} = (\text{Tumor length}) \times (\text{Tumor width})^2 \times 0.52 \quad (2)$$

2.13. Statistical analysis

Statistical analyses were performed using JMP 11.0 (SAS Institute, Japan). All the data were analyzed by One-way ANOVA through Tukey's *post hoc* tests. Also, survival times were analyzed by Kaplan-Meyer using log-rank test. Statistical significance was set at $P < 0.05$ (*: $P < 0.05$, ns: not significant).

3. Results

3.1. The multiepitope DNA vaccine construct

The multiepitope DNA vaccine construct encodes the immunodominant fragments of SYCP1 and ACRBP antigens, while connected by a furin-sensitive linker. Also, it contains Nhe1, *Nco*I, *Xho*I restriction sites, and a Kozak sequence (Fig. 1).

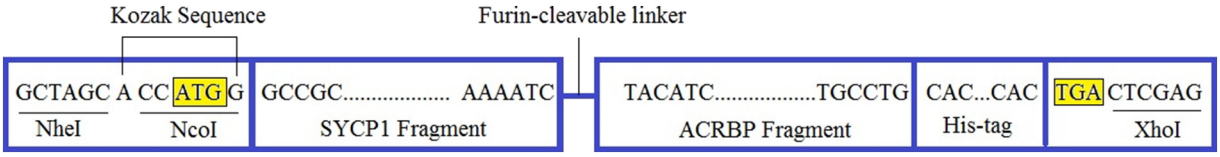

Fig. 1. Schematic illustration of the DNA construct of the multiepitope peptide vaccine.

3.2. Production and purification of the peptide vaccine

The pcDNA3.1 was transfected to *E. coli* Top10 strain (Fig. S1). Then, the peptide vaccine-related DNA sequence was subcloned into the pET-28a and transfected to *E. coli* BL21 (Fig. S2). The recombinant peptide vaccine was produced by the transfected *E. coli* BL21 (Fig. S3). Subsequently, the peptide vaccine was purified with > 90% purity (Fig. S4). The concentration of the purified peptide vaccine was assessed by Bradford method and converted to molarity. The final yield of the recombinant peptide vaccine was 2.5 mg/mL in a volume of 2 mL which was calculated according to the Bradford method. Also, the peptide was detected by western blotting (Fig. S5).

3.3. SYCP1 and ACRBP mRNAs expression at the melanoma cancer cells in vitro

Total mRNA from the cultured B16-F10 cells was extracted and used as the template for Real-Time PCR analyses. Strong expression of SYCP1 and ACRBP mRNAs were detected in the B16-F10 cells *in vitro*. The Ct for the Beta-actin, SYCP1, and ACRBP were 24, 22, and 30, respectively. The amplification plot is illustrated in Fig. 2.

3.4. Humoral immune response in the immunized mice

The humoral immune response was evaluated by measuring the serum levels of the peptide vaccine-specific IgG total and subclasses antibodies. As Fig. 3 illustrates, the peptide vaccine immunized mice sera contained significantly ($P < 0.001$) higher IgG1, IgG2a, and IgG total levels in comparison with the PBS and IFA groups. The serum levels of IgG1 and IgG2a were assayed to investigate the Th1 and/or Th2 response provocation. The peptide vaccine immunized mice exhibited IgG1 predominance which is related to Th2 shift response (Fig. 3).

3.5. Cytokines production and cytotoxic activity evaluations

The cytokines' level was measured in the splenocytes' supernatant after stimulation with the peptide vaccine (Fig. 4). The splenocytes of the peptide vaccine immunized mice exhibited significantly ($P < 0.0001$) more IL-4 and IFN-γ production in comparison with the PBS and IFA groups (Fig. 4A, B). IL-4 is a cytokine which induces

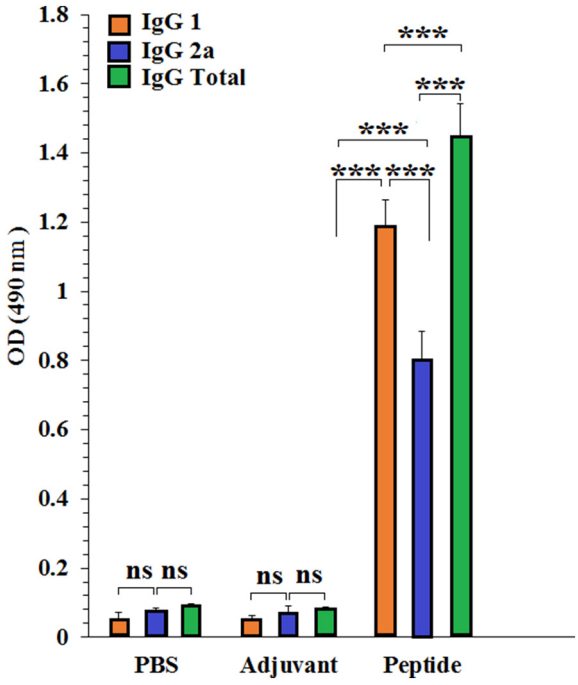

Fig. 3. Comparison of the serum levels of the peptide-specific (A) IgG1, (B) IgG2a, and (C) IgG total at different groups, 14 days after the last vaccination (n = 5). (*: $P < 0.05$, ***: $P < 0.001$, ns: not significant).

differentiation of naive helper T cells to Th2 cells [18]. Also, IFN-γ is a key role player in cellular immunity and considerably stimulates $CD8^+$ T cell activation [19]. In addition, granzyme B (GrB) production by the cultured splenocytes was measured after stimulation with the peptide vaccine to evaluated the cytotoxic activity (Fig. 4C). GrB level at the peptide immunized mice splenocytes' culture media was significantly ($P = 0.0001$) higher than the PBS and IFA groups.

3.6. Evaluation of lymphocytes proliferation in response to the peptide vaccine stimulation

Lymphocyte proliferation was evaluated as the stimulation index

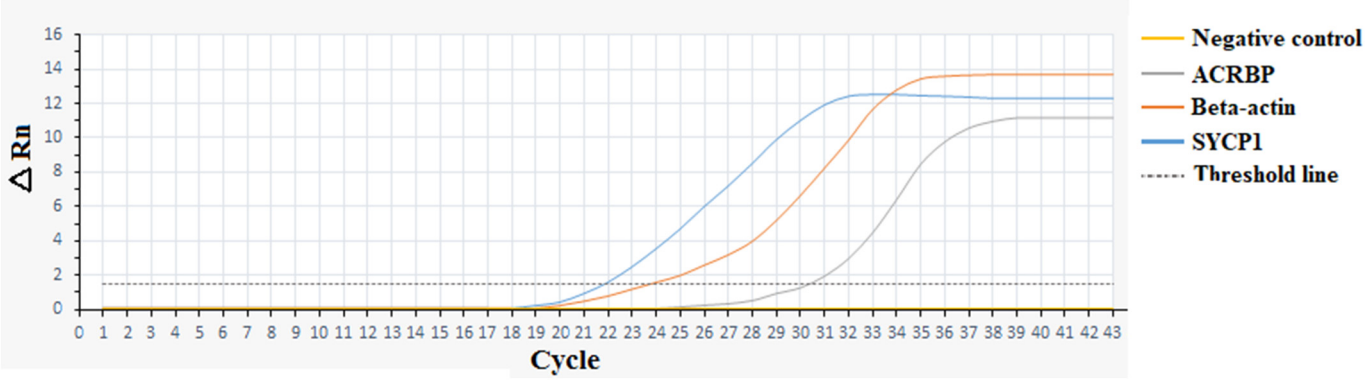

Fig. 2. Amplification plot of the SYCP1, ACRBP, and beta-actin at B16-F10 melanoma cells *in vitro*.

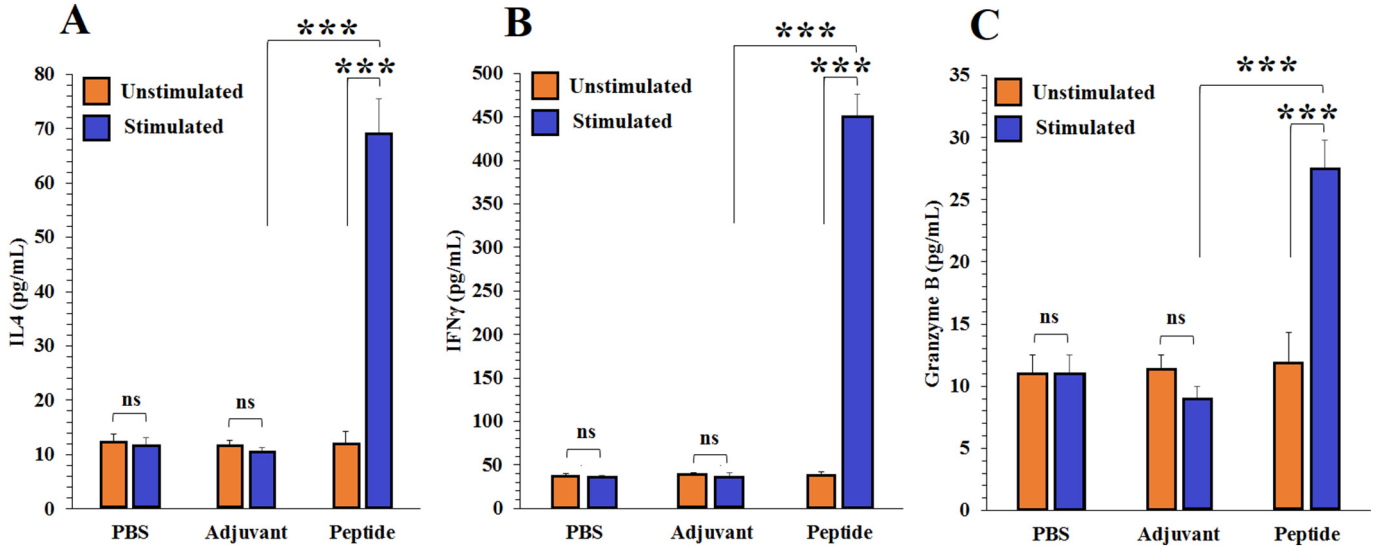

Fig. 4. Cytokine production by the immunized mice splenocytes with and without stimulation with the peptide vaccine *in vitro*. (A) IL-4, (B) IFN-γ, and (C) granzyme B concentrations in the culture media. (*: $P < 0.05$, ***: $P < 0.001$, ns: not significant).

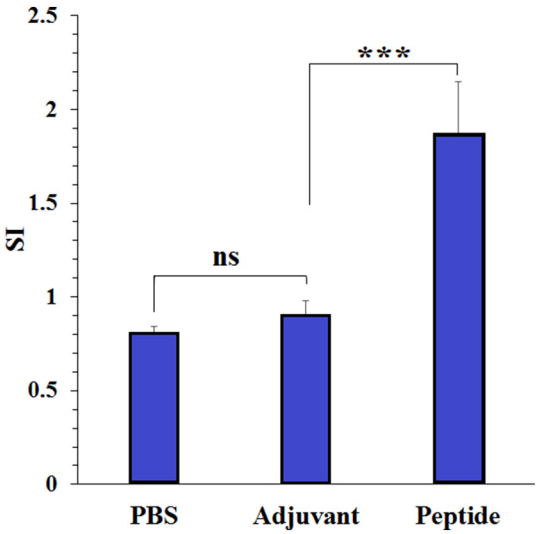

Fig. 5. Splenocytes proliferation after stimulation with the peptide vaccine at different groups. (*: $P < 0.05$, ns: not significant).

(SI). As Fig. 5 illustrates, the peptide vaccine immunized mice splenocytes exhibited significantly higher proliferation after stimulation with the peptide vaccine in comparison with the PBS and IFA groups ($P < 0.001$).

3.7. Prophylactic vaccination with the peptide vaccine

Two weeks after the last vaccination, B16-F10 cancer cells were s.c implanted in the immunized mice to evaluate the efficacy of the peptide vaccine prophylactic vaccination for inhibition of the tumors' growth. As Fig. 6A illustrates, the mean tumors' volume at the PBS and IFA groups were almost the same on the last day of the challenge ($P > 0.05$). However, the peptide vaccine immunized mice exhibited significantly lower mean tumor's volume in comparison with the PBS and IFA groups ($P < 0.05$). Also, the tumor-bearing mice survival time was evaluated in different groups (Fig. 6B). The peptide vaccine immunized mice exhibited the longest mean survival time (33 ± 1.3 days) among all groups (PBS: 28 ± 0.7 days, IFA: 27.8 ± 0.8 days) ($P < 0.05$). Also, none of the immunized mice exhibited any sign of cachexia, anorexia, or loss of mobility among all groups.

4. Discussion

Cancer vaccines have gained lots of attention and different types of cancer vaccines have entered clinical testing [20]. The main aim of cancer vaccination is activating the immune response against cancer cells by presenting the tumor-specific antigens to the immune system. However, selecting of appropriate antigens with high immunogenicity is a determinative step. The selected antigens must exhibit low expression at the normal tissues and high expression at the malignant cells. CTAs are one of the most suitable types of antigens for this purpose [21,22]. CTAs expression is limited to the testis and cancer cells. The blood-testis barrier prepares an immune privilege condition for the testicular cells and sperms to prohibit activation of the immune response against them [23]. On the other hand, CTAs can significantly activate the immune system against cancer cells after being recognized by the immune cells [24,25]. SYCP1 and ACRBP are two well-known CTAs which have high expression in a vast variety of tumors. Also, they can activate both the humoral and cellular immune responses in the autologous host [26–29]. These facts can demonstrate the high immunogenicity of these antigens. Therefore, our multiepitope peptide vaccine which is consisted of the immunodominant epitopes of these antigens can exhibit high efficacy as a prophylactic cancer vaccine. In our previous study [10], this multiepitope peptide vaccine was designed *in silico* by employing several bioinformatic tools. The most immunodominant fragments of these antigens were selected and linked together. The peptide sequence was reverse translated and codon-optimized to design the DNA vaccine (Fig. 1). In the current study, the recombinant peptide vaccine, weighing 6.35 kDa, was produced by prokaryote host and detected through immunoblotting. The pET-28a was used for the production of the peptide vaccine. This vector is an excellent system for production of large amounts of the desired peptide due to containing T7 promoter. Also, it has appropriate restriction enzymes sites [30]. Also, BL21 is the most common strain for protein expression. It doesn't express Lon protease which can degrade foreign proteins. This stain doesn't contain OmpT gene, which codes the outer membrane protease. Lack of these protease protects the extracellular proteins from degradation. Besides, plasmid loss is prohibited due to the *hsd*SB mutation in this strain [31].

Expression of the target antigens at the cancer cells is necessary for an effective immune response. Therefore, RT-PCR was used to evaluate

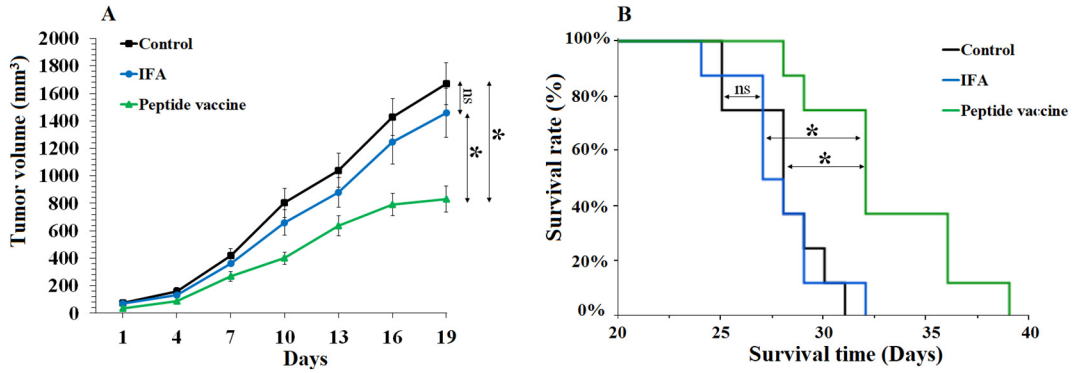

Fig. 6. The effect of prophylactic immunization of the mice on the B16-F10 melanoma tumors' growth progression and survival time of melanoma tumor-bearing mice. (A) B16-F10 melanoma tumors' growth progression at PBS (Control), IFA, and the peptide immunized groups (n = 8). (B) Kaplan-Meier survival curves of the groups (n = 8). (*: $P < 0.05$, ns: not significant).

the SYCP1 and ACRBP genes expression at the B16-F10 cells in comparison with the housekeeping genes. High expression of SYCP1 and ACRBP mRNAs were detected in B16-F10 cell line (Fig. 2). C57BL/6 mice were immunized with the peptide vaccine and the IFA was used as the adjuvant. The PBS and IFA injected mice were used as the control groups. Antibodies levels at the immunized mice sera and cytokines (IL-4 and IFN-γ) production by their splenocytes were measured to evaluate the immune system activation efficacy. As Fig. 4 illustrates, the cytokines' patterns revealed that following the peptide vaccine stimulation, both IL-4 ($P < 0.001$) and IFN-γ ($P < 0.001$) production were increased in comparison with the control groups. IL-4 is a cytokine that induces the differentiation of naive helper T cells to Th2 cells [18]. Also, IFN-γ is a key role player in anti-tumor cellular immunity and strongly activates $CD8^+$ T cells [19]. This cytokine is produced predominantly by Th1 and cytotoxic T lymphocytes (CTL) as the secondary immune response [32]. Th1 cells activate cellular immunity, while Th2 cells mainly trigger humoral responses. However, Th1 and Th2 are not strictly equal with the cell-mediated and humoral immunity, respectively [33]. Moreover, lymphocyte proliferation assay and GrB level evaluations which are widely used to assess the cellular immunity exhibited significantly more lymphocytes proliferation and GrB secretion at the peptide immunized mice splenocytes in comparison with the PBS and IFA groups [34]. Taking together, as predicted in our previous *in silico* study, this multiepitope peptide vaccine can activate both humoral and cellular immune responses (Fig. 7).

Significant inhibition in the melanoma tumors' growth was observed in the peptide vaccine immunized mice in comparison with the control groups (Fig. 6A). Also, the peptide vaccine prophylactic immunization caused a significant increase in the tumor-bearing mice survival time (Fig. 6B). According to these observations, the peptide vaccine immunization can activate the immune system against the murine melanoma. In this study, IFA was used as the immunoadjuvant. It is a water-in-oil emulsion adjuvant which is used in veterinary vaccines and many clinical trials have used it for human vaccines. IFA has exhibited high ability to activate the humoral responses in comparison with other adjuvants like Alum. IFA toxicity has been controlled using high-grade oils and purified surfactants [35].

Malignant melanoma is a lethal form of skin cancer. Individuals with a high risk of developing melanoma including those with prior melanoma, dysplastic melanocytic nevi, large congenital nevi, large number of common acquired nevi, fair skin, familial melanoma, and congenital disorders of DNA repair such as xeroderma pigmentosum will benefit from effective preventive interventions for this disease [36,37]. Current therapeutic approaches are approximately unable to treat advanced melanoma and the best options for the high-risk persons is prevention. Some cancer vaccines are being developed for cancer prevention and inhibition of tumor progression. Prophylactic cancer vaccine will be used for non-tumor bearing individuals with a high risk of developing cancer. Therefore, they should exhibit different properties including simple preparation, practicable administration, applicability in diverse populations, and high safety and efficacy. Also, these prophylactic cancer vaccines should be inexpensive [8]. In this study, we produced and purified an *in silico* designed multiepitope peptide vaccine containing immunodominant epitopes of SYCP1 and ACRBP antigens to investigate its efficacy as a prophylactic melanoma vaccine in a syngeneic melanoma model *in vivo*. While prevention of melanoma is a favorable approach, the investigation of this peptide vaccine in a therapeutic setting would be interesting, too. However, the therapeutic efficacy of a vaccine is difficult to be evaluated in the B16-F10 melanoma model due to its rapid tumor growth. Other murine models with slow-growing tumors may serve as better therapeutic models as they provide sufficient time for the tumor-bearing mice to generate cellular-mediated immune responses against tumors. According to our observations, this peptide vaccine has several obvious advantages as a prophylactic melanoma vaccine due to inexpensive and easy generation. Also, it is completely safe and can be easily administered with subcutaneous injection. Moreover, it can simultaneously stimulate humoral and cellular immune responses. According to our *in silico* analyses [10], this peptide vaccine doesn't require precise knowledge of epitope sequences and their matched HLA types, making the vaccine applicable to broad populations with diverse HLA types (high population coverage). Therefore, the peptide vaccine can be utilized as an effective prophylactic vaccine for high-risk melanoma patients in different countries. Many studies believe that the application of immunotherapy regimens for the treatment of advanced cancer patients is suboptimal [38]. However, cancer vaccines can exhibit high efficacy for preventing tumors' growth in healthy patients with a high risk of developing a specific type of cancer including people with occupational exposure to carcinogens [39] and hereditary mutations in tumor suppressor or oncogenes [40,41]. Also, cancer vaccines can be beneficial for treating cancer recurrence or even, metastasis in patients with minimal residual cancerous lesions. A major problem in developing prophylactic cancer vaccines is the direct translation of these vaccines from preclinical studies to large primary prevention trials in humans. Therefore, many studies have reported that a more feasible way would go from successful primary cancer prevention in murine cancer models to tertiary prevention (*i.e.*, adjuvant therapy) in humans, and only then to primary or secondary prevention [38,42].

5. Conclusions

The multiepitope peptide vaccines are gaining lots of attention as prophylactic cancer vaccines. In this study, we tried to produce and purify an *in silico* designed multiepitope peptide vaccine which was designed by bioinformatic tools in our previous study. The multiepitope peptide vaccine was utilized as a prophylactic cancer vaccine in C57BL/

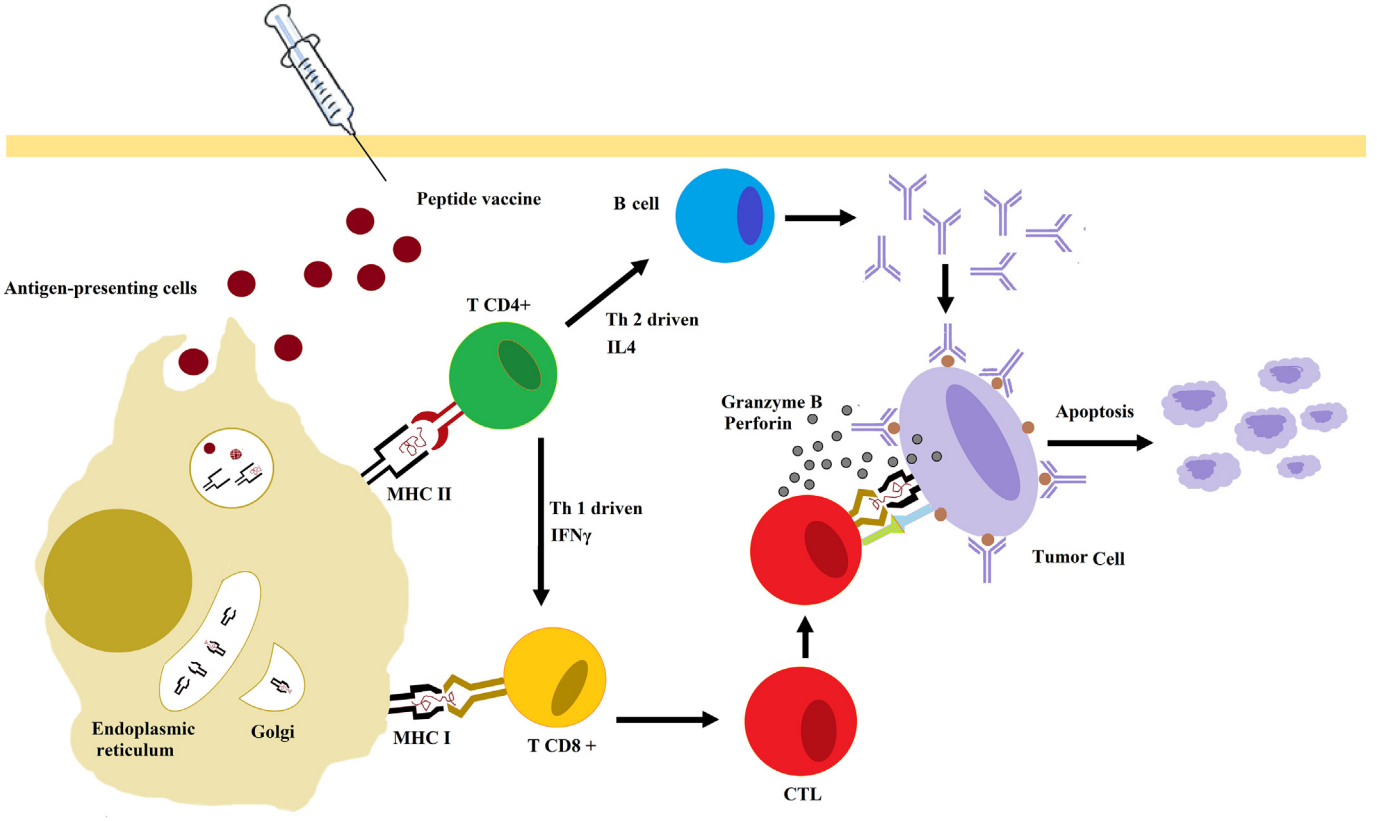

Fig. 7. Schematic illustration of the mechanism of action for multiepitope peptide vaccine to activate the cellular and humoral immune responses against tumor cells.

6 mice melanoma model and caused significant immune system activation and inhibition in the tumor's growth progression. Therefore, utilizing from this prophylactic multiepitope peptide vaccine consisted of immunodominant epitopes of SYCP1 and ACRBP antigens can exhibit significant efficacy for the prevention of melanoma in high-risk populations. However, more experiments and clinical trials are needed to demonstrate this peptide vaccine efficacy in different aspects.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105872.

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