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ORIGINAL ARTICLE

A therapeutic multi-epitope protein vaccine targeting HPV16 E6 E7 elicits potent tumor regression and cytotoxic immune responses

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ABSTRACT

Objective: Cervical cancer caused by persistent high-risk human papillomavirus (hrHPV) infection remains a leading cause of cancer-related mortality in women. As prophylactic HPV vaccines cannot eliminate existing infections, developing therapeutic vaccines targeting HPV E6/E7 oncoproteins is critical for reversing precancerous lesions. This study aimed to design a novel multi-epitope vaccine against HPV16, incorporating newly identified immunodominant epitopes and evaluating the therapeutic efficacy.

Methods: The multi-epitope vaccine HSP70-12P was bioinformatically designed to include cytotoxic T lymphocyte (CTL) and helper T lymphocyte (HTL) epitopes from HPV16 E6/E7, which were fused to the C-terminal domain (residues 359–610) of *Mycobacterium tuberculosis* HSP70 as an adjuvant. Two formulations were used, as follows: (1) protein-based Pro-HSP70-12P; and (2) DNA-based DNA-HSP70-12P. Therapeutic efficacy was evaluated in TC-1 tumor-bearing mouse models. Tumor regression, survival rates, and immune correlates (T cell responses and cytokine profiles) were assessed. Immunodominant epitopes were identified using ELISpot.

Results: The Pro-HSP70-12P protein vaccine induced strong immune responses and provided lasting antitumor protection. The vaccine activated cell-mediated immunity and stimulated effector memory T cells in the HPV-16-related tumor mouse model, resulting in strong tumor clearance effects. Pro-HSP70-12P demonstrated superior performance compared to the DNA-HSP70-12P vaccine, achieving complete regression of small tumors (diameter < 2 mm) with a single dose and conferring long-lasting protection in TC-1 rechallenge experiments. Three novel immunodominant epitopes were identified (E6-38-45, E6-124-132, and E7-50-57). The E6 epitopes address a critical gap in E6-targeted vaccine design.

Conclusions: The multi-epitope protein vaccine, Pro-HSP70-12P, represents a potent therapeutic candidate against HPV-driven malignancies, which has the capacity to induce tumor regression and long-term immunity. These findings support further clinical development.

KEYWORDS Human papillomavirus; cervical cancer; therapeutic vaccine; E6/E7 oncoproteins; immuno-oncological therapy

Introduction

Persistent infection with high-risk human papillomavirus (hrHPV), particularly HPV16, drives > 95% of cervical cancers and the majority of HPV-associated malignancies[1,2]. While prophylactic vaccines prevent new infections, therapeutic interventions remain critical for existing cases. The oncoproteins, E6 and E7, constitute ideal targets for therapeutic vaccination because of the essential roles in HPV-induced carcinogenesis[3].

Currently, therapeutic HPV vaccines in clinical stages fall into four main categories: vector vaccines; peptide and protein vaccines; nucleic acid vaccines; and whole cell vaccines[4]. Peptide- and protein-based vaccines are safe, stable, and easy to produce. However, single-peptide vaccines result in limited T-cell induction, possibly due to anergic CD8+ T cells. This anergy may result from the exogenous loading of short epitopes, which are directly presented to CD8+ T cells, bypassing dendritic cell (DC) processing and co-signaling[5].

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Full-length proteins preferentially activate MHC-II pathways and carry oncogenic risks[6]. DNA-based cancer vaccines also represent a promising approach to activate the immune system against cancer[7]. Plasmid DNA immunization is safe and has induced broad immune responses in preclinical and early clinical trials. However, effective DNA vaccine requires advanced techniques to deliver plasmid DNA to target cells. Thus, multi-epitope protein vaccines are considered an attractive strategy. Indeed, multi-epitope protein vaccines target multiple HPV oncoprotein epitopes and promote HPV-specific T cell responses. Preclinical studies have shown that multi-epitope vaccines effectively induce HPV-specific T-cell immunity[8].

The differential expression of tumor-associated and tumor-specific antigens on normal and cancer cells is utilized in the design of peptide-based cancer vaccines[9-11]. Epitope vaccines can precisely trigger cytotoxic T lymphocyte (CTL) or helper T lymphocyte (HTL) responses, leading to strong immune reactions[12]. Identifying suitable epitopes is a key step in peptide vaccine design. Immunoinformatics effectively predicts epitopes for vaccine research. Viral antigens guide epitope-based vaccine design for virus-induced cancers[13-15]. After predicting, multiple CTL and HTL epitopes are combined into an engineered protein immunogen, creating multi-epitope vaccines. These vaccines are then analyzed for allergenicity, antigenicity, and physicochemical properties[16]. Peptides restricted by common HLA class I alleles are included to ensure broad ethnic coverage in vaccine design. Preclinical or *in vitro* studies using cell lines or animal models follow. Dasari et al.[17] reported a vaccine with an EBV gp350 glycoprotein and an engineered EBV multi-epitope protein containing 20 CD8+ T cell epitopes from EBV antigens that provided better protection against EBV-related diseases. In another study, Zhao et al.[18] constructed a DNA vaccine encoding multi-epitope antigen and IL-2, which provoked efficient and long-term immunity against echinococcosis. Rcheulishvili et al.[19] predicted multiple epitopes using bioinformatics and designed a multi-epitope mRNA universal flu vaccine with the IL-6 cytokine. Several epitope-specific therapeutic HPV vaccines have been tested in clinical trials. With new delivery systems and adjuvants, some of them have shown promising clinical results. However, other epitope-specific therapeutic HPV vaccines have shown modest clinical responses[20-24].

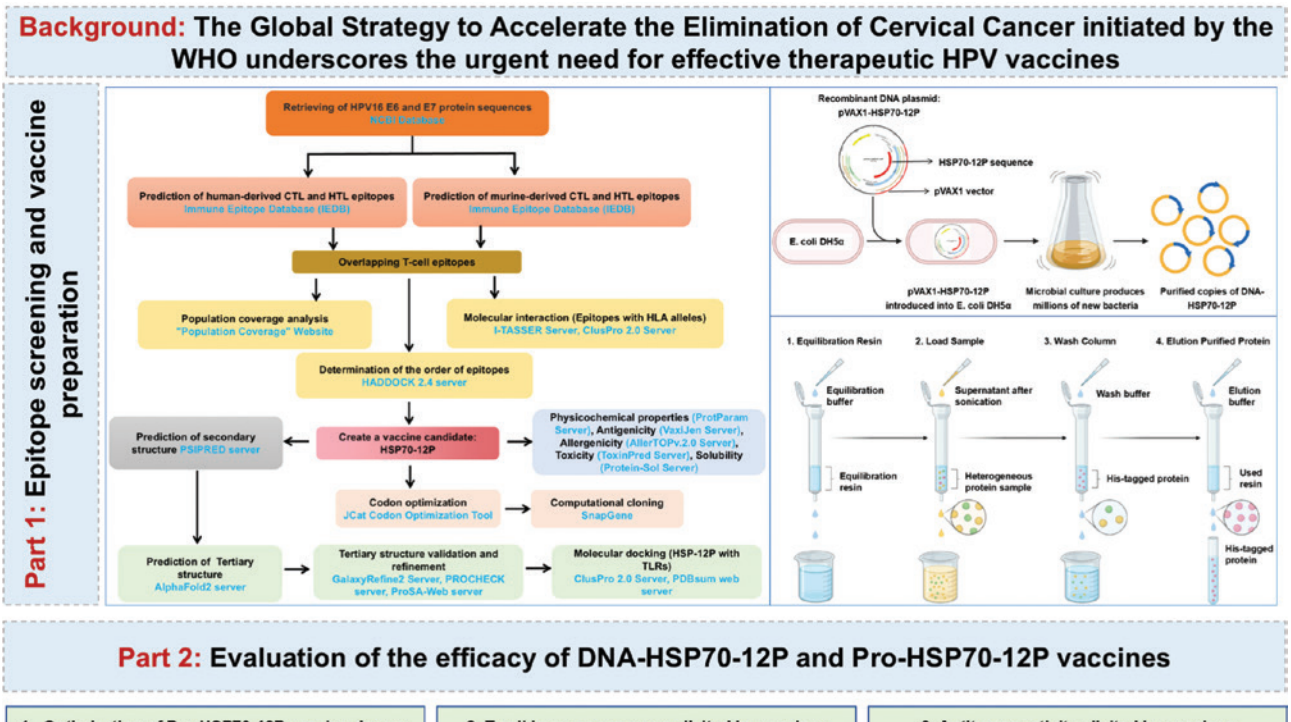
Integrating multiple specific epitopes and effective adjuvants can induce and sustain strong immune responses against cancer, showing great clinical potential[25]. These adjuvants enhance the ability of the vaccine to activate innate and adaptive immunity, especially CD8+ T cell responses. Built-in adjuvants improve immunogenicity by serving as delivery systems and stimulating the necessary innate immune response. Built-in adjuvants provide T helper (Th) cell epitopes or pathogen-associated molecular patterns to activate innate immunity. Non-toxic molecular adjuvants or carriers can help present pathogen epitopes[26-28]. *Mycobacterium tuberculosis* heat shock protein 70 (mHSP70) is widely used as a molecular adjuvant in epitope vaccines. The C-terminal peptide-binding region (HSP70 aa 359–610) binds to CD40 receptors, stimulating Th1-polarizing cytokines, such as interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α), and NO and inducing DC maturation[29-31]. For example, fusion of Hantavirus glycoprotein and nucleocapsid protein with the C-terminus of HSP70 induces more specific immune responses[32]. HSP70 alone induces weak immune responses when not fused with other epitopes. As a built-in adjuvant, HSP70 has entered preclinical and clinical trials for various cancers[33,34].

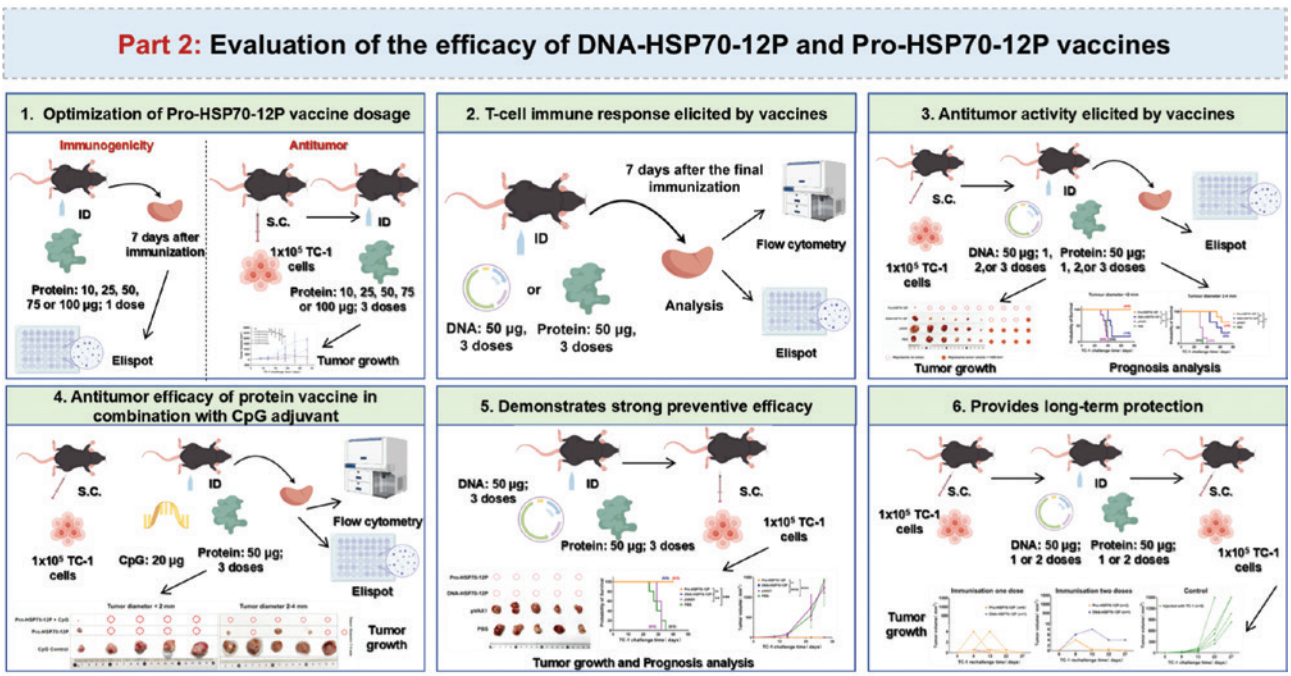
The HPV16 oncogenic proteins, E6 and E7, were targeted in the current study using predicted and selected T-cell epitopes known for strong immunogenicity and broad population coverage[35]. These epitopes were concatenated to construct a vaccine (HSP70-12P), which was combined with internally effective adjuvants. Recombinant protein expression produced the multi-epitope vaccine, Pro-HSP70-12P; a DNA counterpart, DNA-HSP70-12P, was also prepared. By integrating these two vaccine types with internal and exogenous adjuvants, several strategies were designed to stimulate effective and long-lasting immune responses, and the immunogenicity and antitumor effects were evaluated using mouse models (**Study Flowchart**).

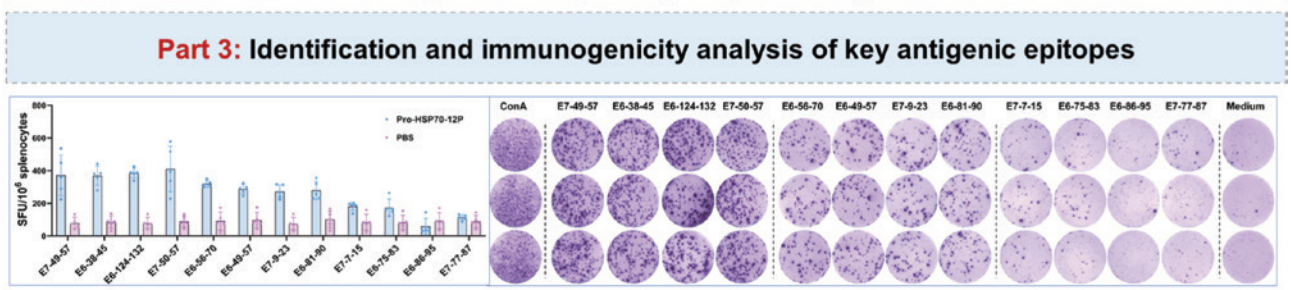
Materials and methods

Bioinformatics prediction

Ten CTL epitopes and two HTL epitopes were identified in our study[35] using a unique bioinformatics prediction method. These epitopes exhibited favorable immunologic properties and high population coverage (**Table S1**). These epitopes were subsequently linked based on their order and the selection of linkers. Human β-defensin 3 was selected as an adjuvant in our prior research. However, considering the differential applicability of adjuvants in DNA and protein vaccines, β-defensin 3 was replaced with an mHSP70 C-terminal sequence in this study[36]. The resulting vaccine sequence was designated HSP70-12P and the bioinformatic properties were







Conclusion: The multi-epitope protein vaccine Pro-HSP70-12P represents a potent therapeutic candidate against HPV16-driven malignancies

Study Flowchart A Therapeutic Multi-Epitope Protein Vaccine Targeting HPV16 E6 E7 Elicits Potent Tumor Regression and Cytotoxic Immune Responses. **Part 1.** A total of 10 CTL epitopes and 2 HTL epitopes were identified and selected; furthermore, DNA (DNA-HSP70-12P) and protein (Pro-HSP70-12P) vaccines incorporating the C-terminal sequence of HSP70 as a built-in adjuvant were successfully constructed. **Part 2.** The results showed that the Pro-HSP70-12P protein vaccine induced strong immune responses and provided long-lasting antitumor protection. The vaccine activated cell-mediated immunity and stimulated effector memory T cells in the HPV-16-related tumor mouse model, leading to potent tumor clearance. Pro-HSP70-12P demonstrated superior efficacy compared to the DNA-HSP70-12P vaccine, achieving complete regression of small tumors (diameter < 2 mm) with a single dose and sustained protection in TC-1 rechallenge experiments. Moreover, the DNA and protein vaccines exhibited strong preventive effects. **Part 3.** The results of the identification and immunogenicity analysis of key antigenic epitopes in the Pro-HSP70-12P multiepitope vaccine revealed three novel immunodominant epitopes (E6-38-45, E6-124-132, and E7-50-57). Notably, the identified E6 epitopes help to address a critical gap in E6-targeted vaccine design. CTL, Cytotoxic T Lymphocyte; HTL, Helper T Lymphocyte; ID, Intradermal; IEDB, Immune Epitope Database; NCBI, National Center for Biotechnology Information; S.C, Subcutaneously.

analyzed from several aspects, including immunologic and physicochemical properties, secondary structure, three-dimensional (3D) structure prediction and refinement, and molecular docking with Toll-like receptor 2 (TLR2), 3 (TLR3), 4 (TLR4), and 8 (TLR8), as shown in **Table S2**.

Production of vaccines

The HSP70-12P sequence was codon-optimized for eukaryotic and prokaryotic expression using JCat (http://www.jcat.de/), then synthesized by Generay Biotechnology (Shanghai, China). The optimized sequence for DNA-HSP70-12P was cloned and inserted into pVAX1 (35-1049; Invitrogen, Carlsbad, CA, USA) *via* EcoRI/XbaI sites, preceded by a Kozak sequence (**GCCACCATG**) and a piece of the IgE leader sequence (**DWTWILFLVAAATRVHS**). Plasmid DNA was purified using an endotoxin-free maxi-prep kit (DP117; TIANGEN, Beijing, China). The sequence for Pro-HSP70-12P was inserted into pCold-TF (VT1301; YouBio, Wuhan, China) [BamHI/XbaI] and expressed in BL21 *E. coli* cells with 0.3 mM IPTG at 15°C overnight. The protein was then purified using an His-tag protein purification kit (P2229S; Beyotime, Shanghai, China) according to the manufacturer's instructions, yielding a protein with the TF tag. The TF tag was subsequently removed by HRV 3C protease (7360; TAKARA, Shiga, Japan) cleavage and purified using BeyoGold His-tag Purification Resin (P2233; Beyotime). The final products were validated by SDS-PAGE and western blotting.

Western blotting

The Pro-HSP70-12P protein concentration was determined with a Pierce BCA protein assay kit™ (Thermo Fisher Scientific, Waltham, MA, USA). The samples (5 μg) were denatured in SDS buffer with β-mercaptoethanol (100°C for 10 min), separated on 12% SDS-PAGE gels, and transferred to PVDF membranes using the Trans-blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk at room temperature for 2 h, followed by an overnight incubation at 4°C with rabbit anti-HPV16 E6 polyclonal primary antibodies (GTX132686; GeneTex, Irvine, CA, USA) at a 1:2,000 dilution. The membranes were then incubated for 1 h at room temperature with a goat anti-rabbit IgG-HRP (H+L) secondary antibody (SA00001-2; Proteintech, Rosemont, IL, USA) at a 1:5,000 dilution. The signal was detected using the Clarity Western ECL Substrate (#1705061; Bio-Rad) and visualized with the ChemiDoc MP Imaging System (Bio-Rad).

Cell line and mice

C57BL/6 mice (female, 6–8 weeks old) were obtained from the Central Animal Service Center [Institute of Medical Biology, Chinese Academy of Medical Sciences (IMBCAMS), Kunming, China] and housed in specific pathogen-free (SPF) animal experimental barrier systems. All animal-related experiments were approved by the Laboratory Animal Ethics Committee of the IMBCAMS (Approval No. DWSP202306014).

The TC-1 cell line was used to establish an HPV16-related murine tumor model. This cell line was derived from C57BL/6 mouse primary lung epithelial cells that were immortalized with HPV16 E6 and E7 oncoproteins and transformed with the c-Ha-ras oncogene[37], kindly provided by Professor Yanbing Ma (Chinese Academy of Medical Sciences & Peking Union Medical College, Peking,

China). TC-1 cells were cultured with RPMI-1640 medium (Corning, NY, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) in an incubator at 37°C with 5% CO_2.

Tumor challenge and immunization

The mice were immunized with 50 μg of DNA-HSP70-12P or Pro-HSP70-12P intradermally using MicronJet600 microneedles (NanoPass Technologies, Yokne'am Illit, Israel)[38,39]. The CpG-adjuvant groups received 20 μg of CpG mixed with the vaccine. The mice were immunized at weekly intervals three times for the immunogenicity experiment (n = 5 mice per group). The mice were injected into the left flanks with TC-1 cells (1×10^5 cells per mouse) subcutaneously in a mixture of 50 μL of PBS and 50 μL of matrix gel for the therapeutic (n = 10 mice per group) and survival experiments (n = 6 or 7 mice per group). Different treatments were administered to each group when the tumor diameter was < 2 mm or 2–4 mm. Tumor growth was monitored and the tumor volume was measured every 3–5 d with calipers. The tumor volume was calculated by the following formula: $(L \times W^2)/2$, where L is the length and W is the width of the tumor. The mice were euthanized by cervical dislocation when exhibited signs of distress or when the average tumor volume exceeded 1,500 mm^3. After euthanasia, the spleens and tumors were collected and placed in pre-chilled RPMI-1640 medium, followed by preparation of single-cell suspensions of the spleen and filtration through a 70-μm filter (CLS431751; Corning).

Flow cytometry

Spleen single-cell suspensions were subjected to flow cytometry staining for cell analysis. The following monoclonal antibodies were used for extracellular staining: CD3 (363-0032-82; Invitrogen); CD4 (100406; BioLegend, SAN Diego, CA, USA); CD8 (100752; BioLegend); NK1.1 (108761; BioLegend); CD62L (104412; BioLegend); CD44 (103007; BioLegend); CD11b (101205; BioLegend); Gr-1 (108412; BioLegend); γδ-T (118116; BioLegend); and CD25 (102043; BioLegend). The following antibodies were used for intracellular staining: anti-IL2 (503808; BioLegend); anti-IL17A (506926; BioLegend); anti-IL4 (554436; BioLegend); anti-granzyme B (372204; BioLegend); anti-interferon-gamma [IFN-γ] (505808; BioLegend); and anti-TNF-α (506328; BioLegend). Nuclear staining was performed with an antibody against FoxP3 (126404; BioLegend).

Spleen cells (5×10^6/well) were stimulated overnight with a peptide pool composed of 12 short peptides (10 μg/mL each) for 4 h, followed by stimulation with Brefeldin A (420601; BioLegend) 96-well-plate. Non-specific signals were blocked using TruStain FcXM (101320; BioLegend) on ice for 15 min. Viability staining was performed with the Zombie NIR Fixable Viability Kit™ (423106; BioLegend) according to the manufacturer's instructions. Surface markers were subsequently stained. Fixation and permeabilization were performed before intracellular cytokine staining, followed by an incubation with cytokine-specific antibodies. Flow cytometry data were acquired on a Beckman Coulter flow cytometer (Brea, CA, USA) and analyzed using FlowJo 10.8.1 software (FlowJo, LLC, Ashland, OR, USA).

The gating strategy for flow cytometry analysis is shown in **Figure S1**.

ELISpot assay

Mouse IFN-γ precoated ELISpot plates (3321-4AST-10; MABTECH, Stockholm, Sweden) were washed, incubated with RPMI-1640 medium, then loaded with spleen cells (2×10^5 cells per well) and 12 short peptides (final concentration per peptide: 5 μg/mL). Spot-forming units of IFN-γ secretion were detected using the corresponding antibodies and substrate solution after a 40-h incubation at 37°C with 5% CO_2. Spot counts were obtained with an automated ELISpot image analyzer (S6 FluoroCore; CTL, Cleveland, Ohio, USA) and the results are presented as spot-forming units per 1×10^6 cells after the negative control values were subtracted.

Statistical analysis

GraphPad Prism v.9.0 software (GraphPad Software, Boston, MA, USA) was used for graphing and statistical analyses. Comparisons of multiple groups were performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Comparisons between two groups were performed using unpaired two-tailed t-tests. The survival benefit of the tumor-bearing mice was analyzed with the log-rank test (Mantel–Cox) and a $P < 0.05$ was considered statistically significant (*$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$). The data are presented as the means ± standard deviation (SD).

Results

Vaccine design and prediction of the immunologic and physiochemical properties

The design of the vaccine construct is shown in **Figure 1A** and the sequence is shown in **Figure 1B**. The solubility score of 0.599 (threshold value = 0.45) suggests the protein is likely to be soluble (**Figure 1C**). Further immunologic analysis revealed that HSP70-12P exhibited antigenicity, lacked allergenicity, and showed no signs of toxicity. In addition, an analysis of the physicochemical properties of the vaccine revealed thermal stability with a GRAVY score of −0.406, indicating its hydrophilic nature (**Table S3**).

In silico cloning and vaccine construction and preparation

The HSP70-12P nucleotide sequences were cloned into the vectors, pVAX1 and pCold-TF, using SnapGene software (**Figure 2A**, **2B**). The results of the double restriction enzyme digestion confirmed the successful cloning of the optimized HSP70-12P nucleotide sequences into the pVAX1 (**Figures 2C, S2A**) and pCold-TF (**Figures 2D, S2A**) expression vectors.

Protein expression analysis subsequently showed that the target protein with the soluble protein tag, TF, was highly expressed in a soluble form with an approximate molecular weight of 100.67 kDa (**Figures 2E, S2B**). The recombinant subunit vaccine, Pro-HSP70-12P, was obtained after HRV-3C protease cleavage and purification. A visible band corresponding to Pro-HSP70-12P, approximately 50.11 kDa in size, was detected (**Figures 2F, S2C**), which was consistent with the predicted size. The vaccine, Pro-HSP70-12P, was further verified using western blotting analysis (**Figures 2G, S2D**).

Optimization of the Pro-HSP70-12P vaccine dosage for immunogenicity and antitumor efficacy

The immunogenicity of Pro-HSP7012P was first assessed in mice to determine the optimal dosage (**Figure S3A**). The

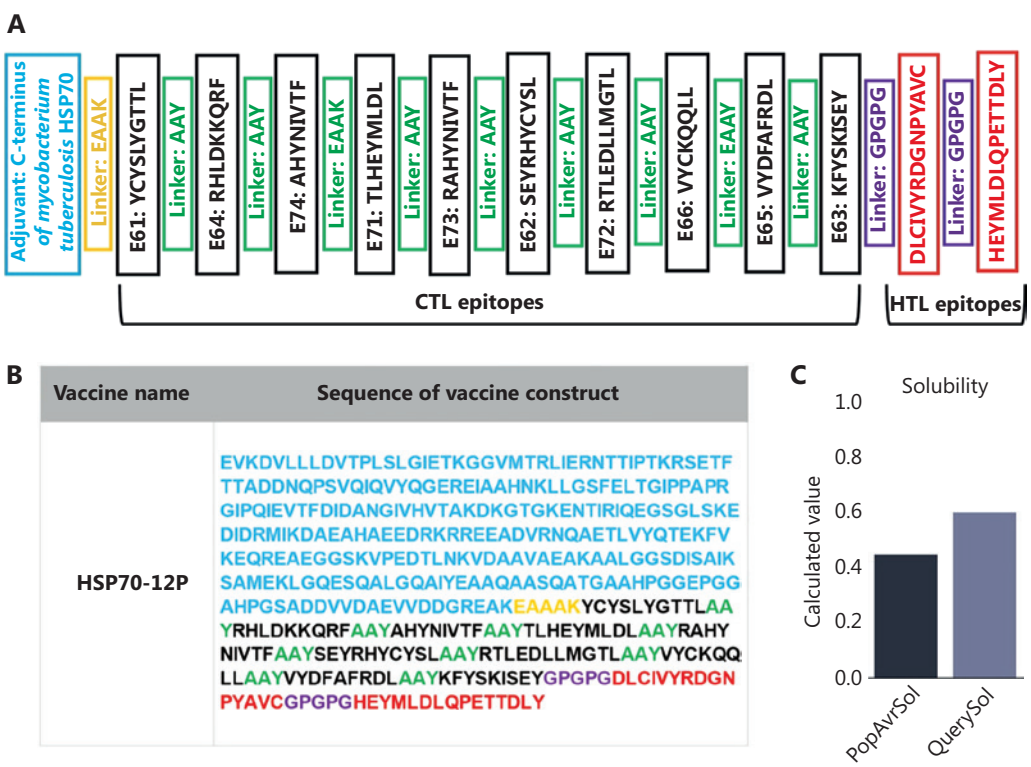


Figure 1 The design, sequence, and structural information of HSP70-12P. (A) Structural arrangement of the final HSP70-12P vaccine construct; (B) amino acid sequence information of the HSP70-12P vaccine construct; and (C) solubility information of the HSP70-12P vaccine construct (Protein-sol software). CTL, Cytotoxic T Lymphocyte; HTL, Helper T Lymphocyte.

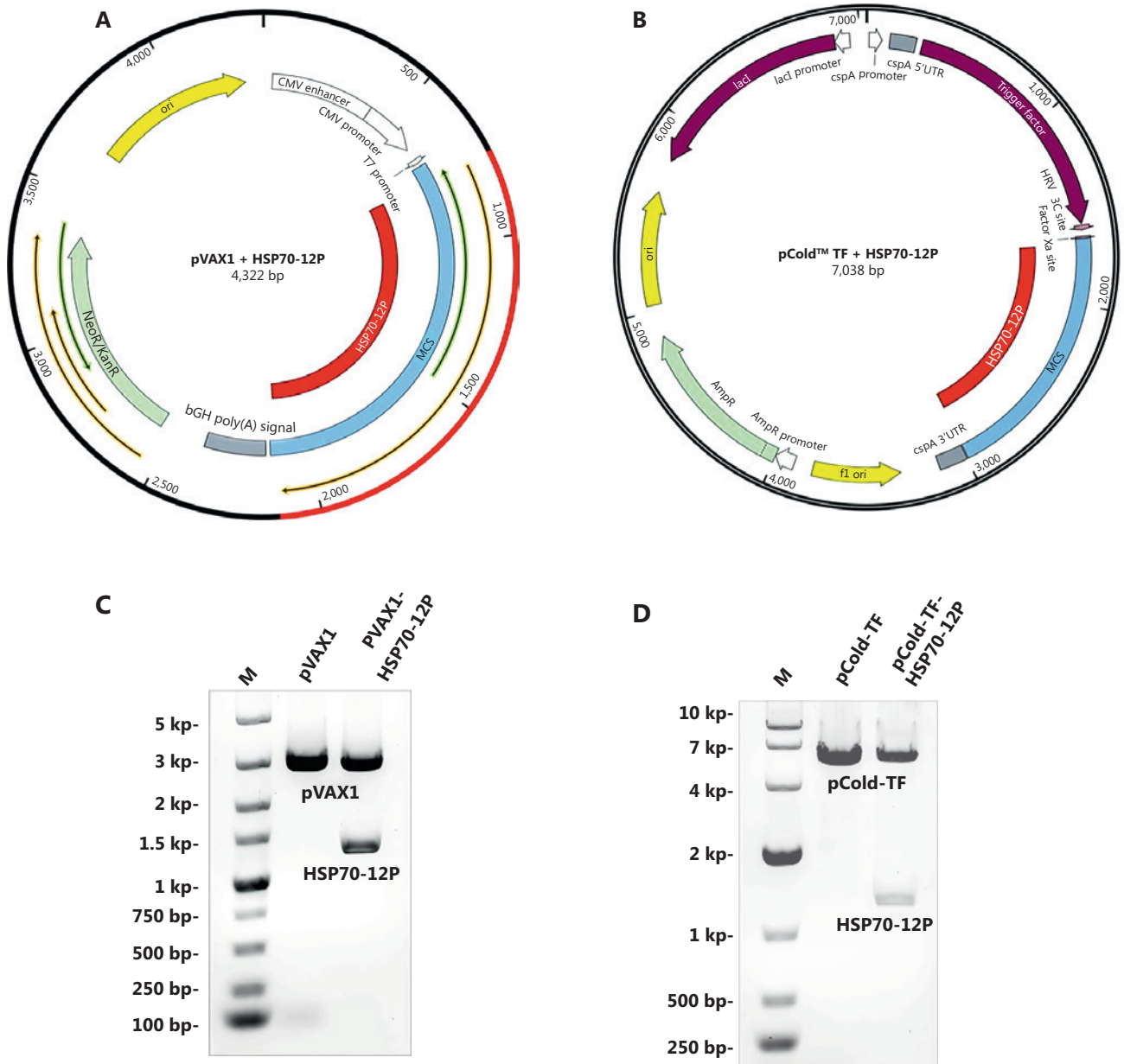


Figure 2 Continued

ELISpot analysis demonstrated that a single immunization with ≥ 25 μg of the vaccine induced robust IFN-γ secretion, whereas 10 μg failed to elicit significant response (**Figure S3B**). All tested doses were well-tolerated with no significant weight changes observed (**Figure S3C**). Antitumor efficacy was subsequently evaluated in a TC-1 tumor model (**Figure S3A**). Although a single 10 μg dose had weak immunogenicity, a 3-dose regimen at this concentration delayed tumor progression and induced complete tumor regression in 2 of 10 mice, indicating that booster immunizations potentiate immune responses (**Figure S3D, S3E**). Higher doses (25–100 μg) exhibited dose-dependent antitumor effects with 50 μg demonstrating optimal efficacy [90% tumor clearance rate (9/10 mice)]. The clearance rates for 25, 75, and 100 μg were 60% (6/10), 40% (4/10), and 70% (7/10), respectively. Based on these findings, 50 μg was selected as the optimal dose for subsequent studies.

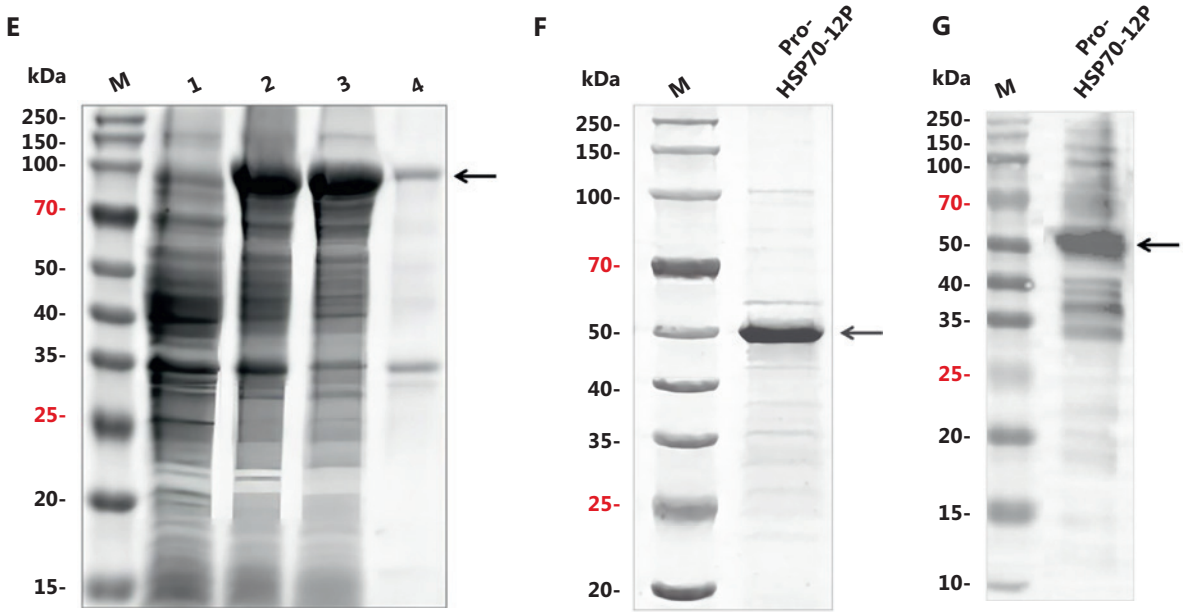


Figure 2 Construction and identification of the Pro-HSP70-12P and DNA-HSP70-12P vaccines. (A, B) *In silico* cloning of HSP70-12P sequence into the pVAX1 vector (full length, 4,322 bp) and the pCold-TF vector (full length, 7,038 bp). The red portion represents the vaccine construct, while the black portion represents the vector backbone (SnapGene software). (C) EcoR1 and Xba1 double restriction enzyme digestion was used to confirm the presence of the HSP70-12P fragment in the pVAX1 vector. The linear pVAX1 vector sequence is 2,966 bp and the HSP70-12P sequence is 1,356 bp, including the Kozak and IgE leader sequences. (D) BamH1 and Xba1 double restriction enzyme digestion was used to confirm the presence of the HSP70-12P fragment in the pCold-TF vector. The linear pCold-TF vector sequence is 5,745 bp, and the HSP70-12P sequence is 1293 bp. (E) Induced expression of Pro-HSP70-12P protein, including the TF tag. 1: Before induction; 2: induction with 0.3 mM IPTG at 15°C overnight; 3: supernatant obtained after sonication and lysis; and 4: pellet obtained after sonication and lysis (SDS–PAGE analysis). (F) Purification of the Pro-HSP70-12P protein [50.11 kDa] (SDS–PAGE analysis). (G) Identification of the Pro-HSP70-12P protein [50.11 kDa] (western blot analysis). M, Marker.

The T cell immune response elicited by the Pro-HSP70-12P vaccine is more potent than the immune response induced by the DNA-HSP70-12P vaccine

The splenic T cell immune features induced by Pro-HSP70-12P and DNA-HSP70-12P were directly compared to evaluate the preliminary immunogenicity of the designed multi-epitope vaccine, demonstrate the relative effectiveness of the multi-epitope vaccine in protein and DNA forms, and determine which form is more suitable for the development of multi-epitope vaccines (**Figure 3**).

The mice were immunized intradermally once per week for a total of three immunizations to evaluate the induced T cell immunogenicity (**Figure 3A**). The splenocytes were stimulated on day 28 with the HPV16 E6 E7-specific antigen peptide pool *in vitro* for IFN-γ ELISpot analysis (**Figure 3B**). The IFN-γ T cell response to specific antigen peptides was minimal in the pVAX1 vector and PBS control groups. Mice immunized with Pro-HSP70-12P or DNA-HSP70-12P vaccine showed significantly higher T cell immunogenicity than the respective control groups; the Pro-HSP70-12P vaccine immunogenicity was higher than the DNA-HSP70-12P vaccine (**Figure 3C**). Splenocytes were stimulated *in vitro* with an HPV16 E6- and E7-specific antigen peptide pool for flow cytometry analysis. The results showed a significant increase in the proportion of CD4+ and CD8+ T cells in the splenocyte population after vaccination. In addition, the number of CD8+ and CD4+ T cells that produced IFN-γ, granzyme B, or TNF-α were significantly increased after vaccination, although the release of TNF-α was not significantly different from the control group. The IFN-γ level in the CD4+ T cells of vaccinated mice was increased, indicating a Th1-biased immune responses (**Figure 3D**). In addition, the proportion of CD4+ T cells that produced IL2 was significantly increased after vaccination, whereas the proportions of CD4+ T cells that produced IL4 and IL17A did

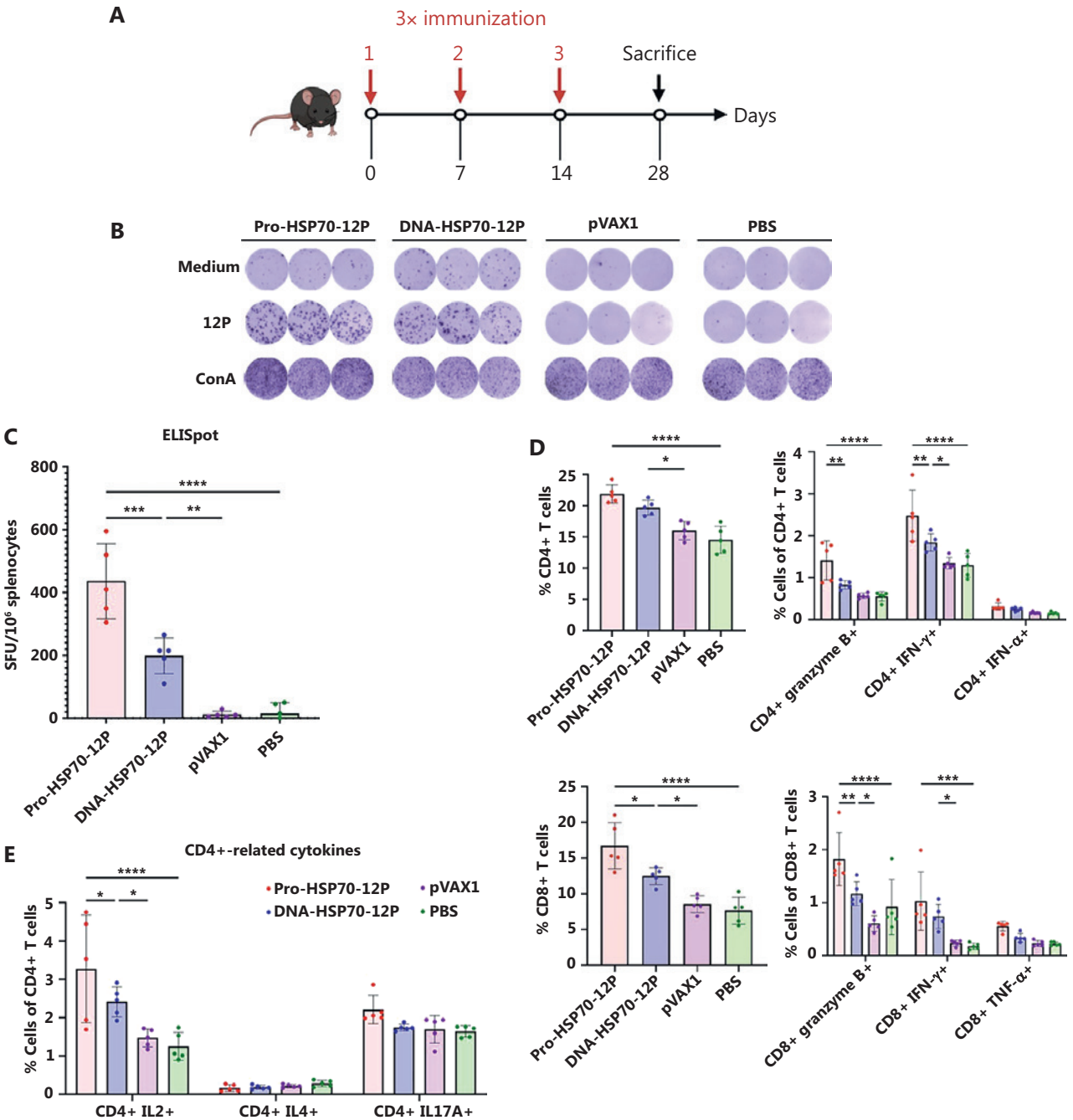


Figure 3 Continued

not change significantly (**Figure 3E**). In general, individuals with lower proportions of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) or a higher CD8+ T cell:Treg ratio tended to have better clinical outcomes[40,41]. Immunization with Pro-HSP70-12P or DNA-HSP70-12P significantly increased the number of CD4+ and CD8+ T cells in the spleen compared to the respective control groups, whereas the proportions of Treg cells and MDSCs decreased, although the reductions were not statistically significant (**Figure 3F**). However, the CD8+ T cell:Treg cell ratios in the Pro-HSP70-12P and DNA-HSP70-12P immunization groups were significantly increased compared to the control groups (**Figure 3G**). These findings suggested that immunization with Pro-HSP70-12P or DNA-HSP70-12P can modulate the immune response toward a more cytotoxic microenvironment. In addition, a significant differentiation of effector memory T (Tem) cells

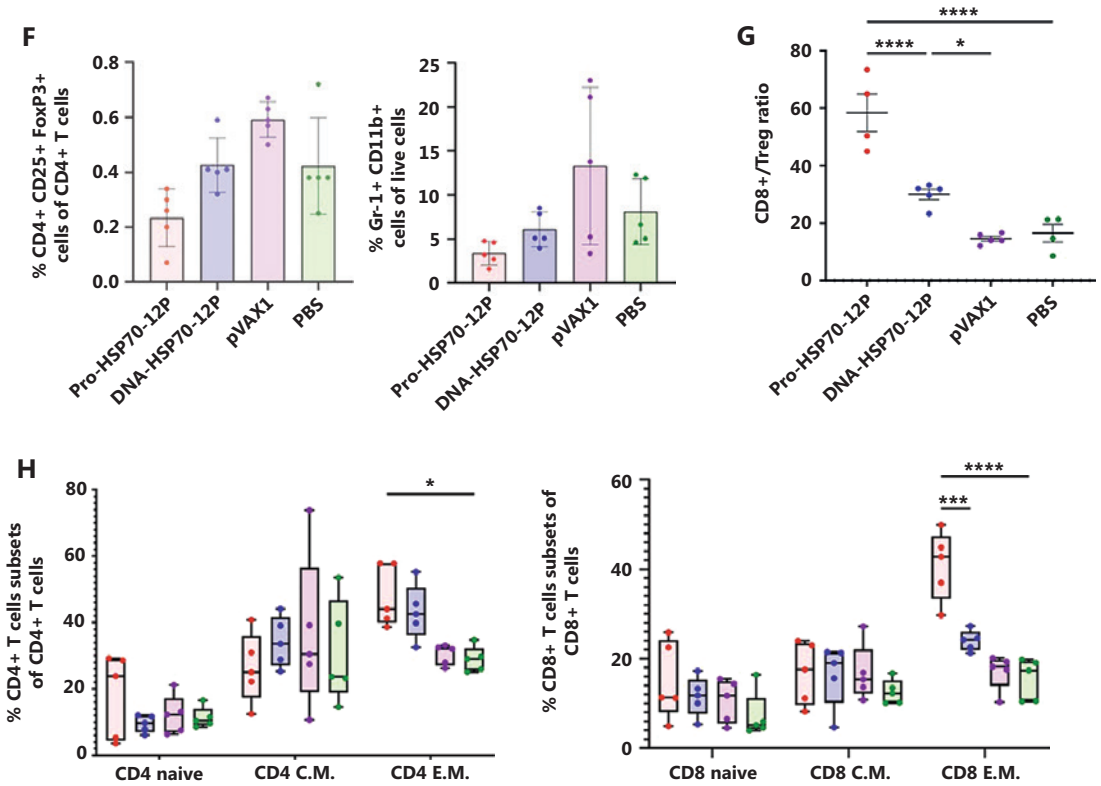


Figure 3 Splenic T-cell immune features induced by the Pro-HSP70-12P and DNA-HSP70-12P vaccines. Each group was immunized once per week for a total of 3 times (Pro-HSP70-2P/DNA-HSP70-12P 50 μg/mouse; injection volume, 100 μL/mouse; 5 mice/group). (A) Experimental workflow; (B, C) images and quantification of IFN-γ levels measured by ELISpot. SFUs denote spot-forming units. (D) Flow cytometry analysis of the levels of IFN-γ, granzyme B, or TNF-α secreted from CD4+ or CD8+ T cells isolated from splenocytes. (E) Levels of cytokines secreted by CD4+ T cells. (F) Proportions of Treg cells and MDSC cells in splenocytes. (G) The CD8+ T cell:Treg cell ratio. (H) Comprehensive analysis of naïve T cells ($CD44^{low}$ $CD62L^{high}$), effector memory T cells ($CD44^{high}$ $CD62L^{low}$), and central memory T cells ($CD44^{high}$ $CD62L^{high}$) in CD4+ T cells and CD8+ T cells (one-way ANOVA, means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). C.M., Central Memory T cell; E.M., Effector Memory T cell; ConA, Concanavalin A; SFU, Spot-Forming Unit; 12P, 12 short peptides.

was observed in CD8+ and CD4+ T cells in the Pro-HSP70-12P and DNA-HSP70-12P groups, indicating the development of potential immune memory. However, no notable differentiation of central memory T (Tcm) cells was observed (**Figure 3H**).

The antitumor activity elicited by the Pro-HSP70-12P vaccine is more potent than the antitumor activity elicited by the DNA-HSP70-12P vaccine

A mouse tumor model was established by implanting TC-1 tumor cells subcutaneously to evaluate the therapeutic efficacy of the vaccines and further compare the relative antitumor effectiveness of the protein and DNA forms of the multi-epitope vaccines. The antitumor effects and T cell responses induced by Pro-HSP70-12P and DNA-HSP70-12P were directly compared in this model (**Figure 4**).

C57BL/6 mice (n = 10/group) were subcutaneously engrafted with 1×10^5 TC-1 cells in the right flank. The mice were immunized 3 times from the 8^{th} day after TC-1 cell implantation, at which point all the mice had visible tumors with a diameter of 1–4 mm (**Figure 4A**). The Pro-HSP70-12P and DNA-HSP70-12P vaccines significantly reduced the TC-1 tumor volume compared to the control groups with Pro-HSP70-12P vaccine achieving complete tumor regression in 90% of the mice on day 33 after TC-1 cell challenge [the study endpoint] (**Figure 4B, 4C**) and demonstrating superior antitumor efficacy over the DNA-HSP70-12P vaccine (**Figure 4B**). In contrast, the mice in the control group had larger tumor volumes or were euthanized because of large tumor volumes within 30 d (**Figure 4C**). In addition, the ELISpot results

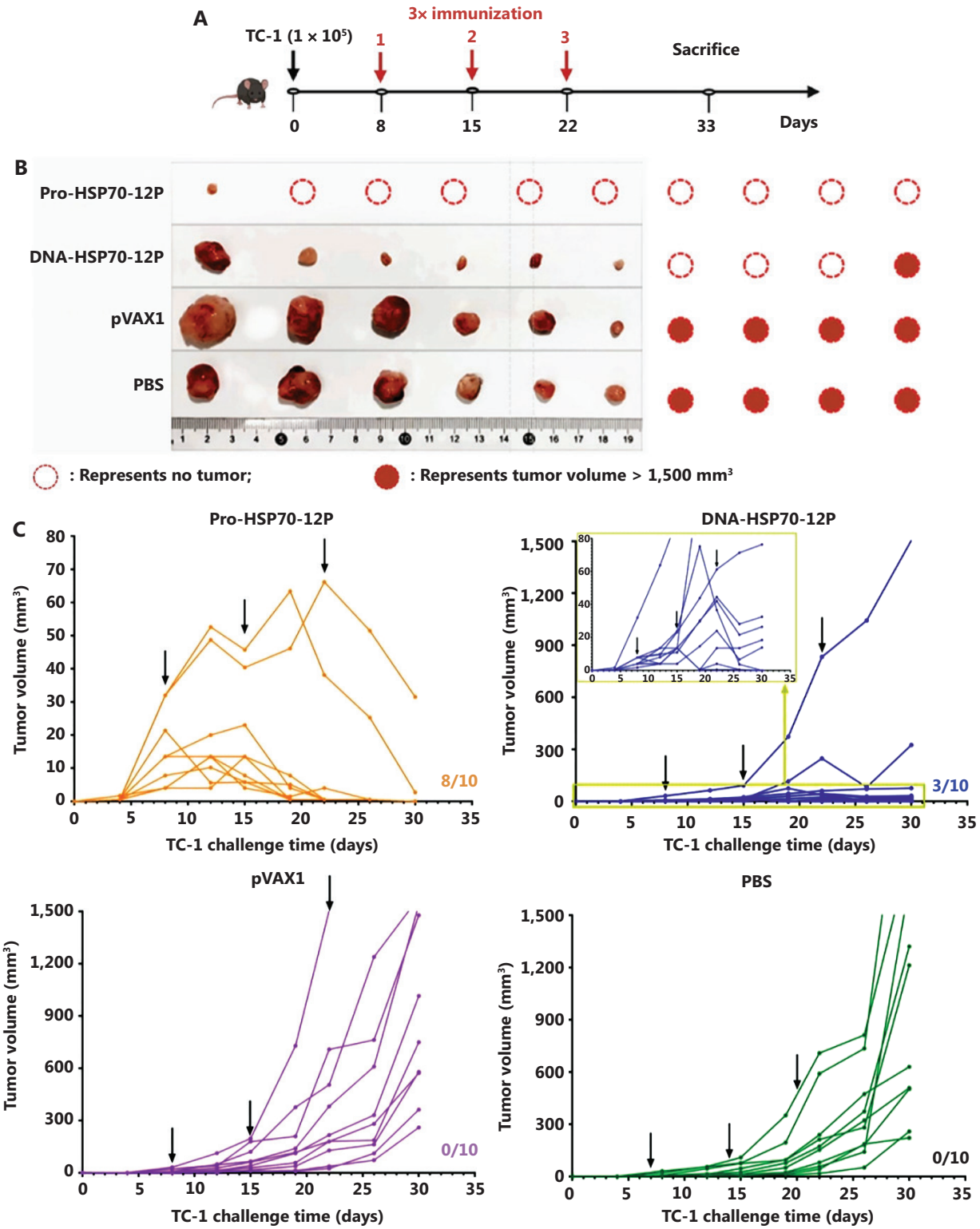


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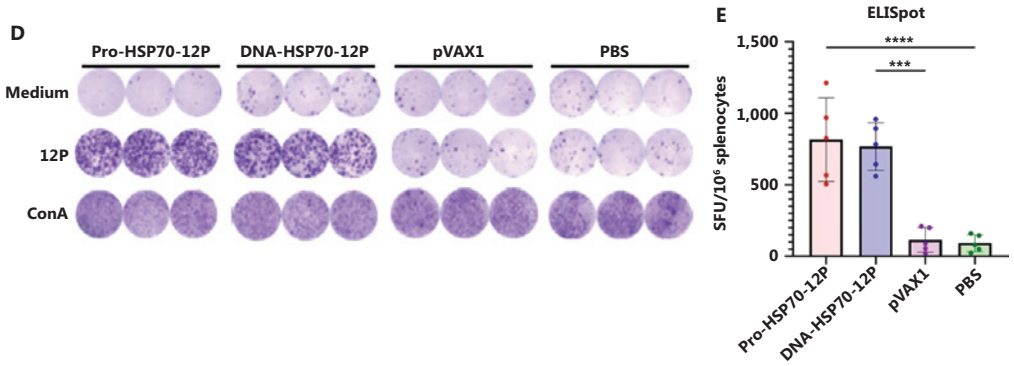


Figure 4 Pro-HSP70-12P and DNA-HSP70-12P vaccine efficacy in the TC-1 tumor-bearing mouse model. (A) Experimental workflow. (B) The tumors of the mice were isolated and photographed on day 33 after TC-1 cell challenge (the red dashed circles indicate no tumors). (C) Tumor growth curve of each mouse in each group subjected to different treatments. The black arrows indicate the time points of vaccine immunization. The bottom right score represents the percentage of mice with a tumor volume of 0. (D, E) Images and quantification of IFN-γ levels measured by ELISpot. SFUs denote spot-forming units. Some of the mice were euthanized by cervical dislocation before the end of the experiment because the tumor volume exceeded the ethical limit (1 mouse from the DNA-HSP70-12P group, 4 mice from the pVAX1 group, and 4 mice from the PBS group). Five mice were selected from the remaining mice for ELISpot analysis to standardize the sample size in each group (one-way ANOVA, means ± SD, ***P < 0.001, ****P < 0.0001). ConA, Concanavalin A; SFU, Spot-Forming Unit; 12P, 12 short peptides.

showed that the splenocytes from the mice immunized with the Pro-HSP70-12P and DNA-HSP70-12P vaccines secreted large amounts of IFN-γ upon stimulation with the HPV16 E6 E7-specific antigen peptide pool, whereas the splenocytes from the control group secreted minimal amounts of IFN-γ upon stimulation with the specific antigen peptide pool (**Figure 4D, 4E**).

Pro-HSP70-12P exerts antitumor effects in the absence of CpG

The efficacy of Pro-HSP70-12P was confirmed by comparing the T cell immune response and antitumor effects of the protein and DNA forms of the multi-epitope vaccine. A strategy that combined endogenous and exogenous adjuvants, specifically CpG, was explored to increase the antitumor effects and potency of the epitope-based vaccine. Based on our previous study[35], which indicated that shorter vaccination intervals reduced the duration of immune response, a 3-dose regimen with 1- and 2-week intervals to balance the response strength and duration was selected and evaluations in 2 mouse models with different tumor sizes were performed.

All the mice were immunized 3 times from day 7 after TC-1 cell implantation (**Figure 5A**, **5B**). The Pro-HSP70-12P vaccine significantly reduced the TC-1 tumor volume and achieved a greater rate of complete tumor eradication compared to the CpG control, regardless of the co-administration of CpG (**Figure 5C–5F**). Notably, one mouse with a tumor diameter of approximately 5 mm (tumor volume = 64 mm^3) eventually experienced tumor disappearance after receiving 3 doses of Pro-HSP70-12P (**Figure 5F**). Furthermore, the ELISpot results revealed substantial IFN-γ secretion from splenocytes isolated from mice vaccinated with Pro-HSP70-12P or Pro-HSP70-12P + CpG (**Figure 6A**, **6B**). The flow cytometry analysis performed after stimulation with the HPV16 E6 E7-specific antigen peptide pool showed that the proportions of CD8+ and CD4+ T cells in the spleen were significantly increased in the Pro-HSP70-12P+CpG and Pro-HSP70-12P groups compared to the CpG control group. Additionally, the proportion of CD8+ T cells that secreted IFN-γ, granzyme B, and TNF-α, as well as the proportion of CD4+ T cells that secreted IFN-γ, were also significantly increased. However, no significant differences in these cell or cytokine proportions were detected between the Pro-HSP70-12P+CpG and Pro-HSP70-12P groups (**Figure 6C**), which was consistent with the observation that tumor sizes between the two groups were not significantly different. Interestingly, mice vaccinated with Pro-HSP70-12P presented a notable increase in the proportion of NK cells, a phenomenon not observed in mice vaccinated with Pro-HSP70-12P+CpG. The proportion of γδ-T cells was increased in the Pro-HSP70-12P+CpG and Pro-HSP70-12P groups compared to the CpG

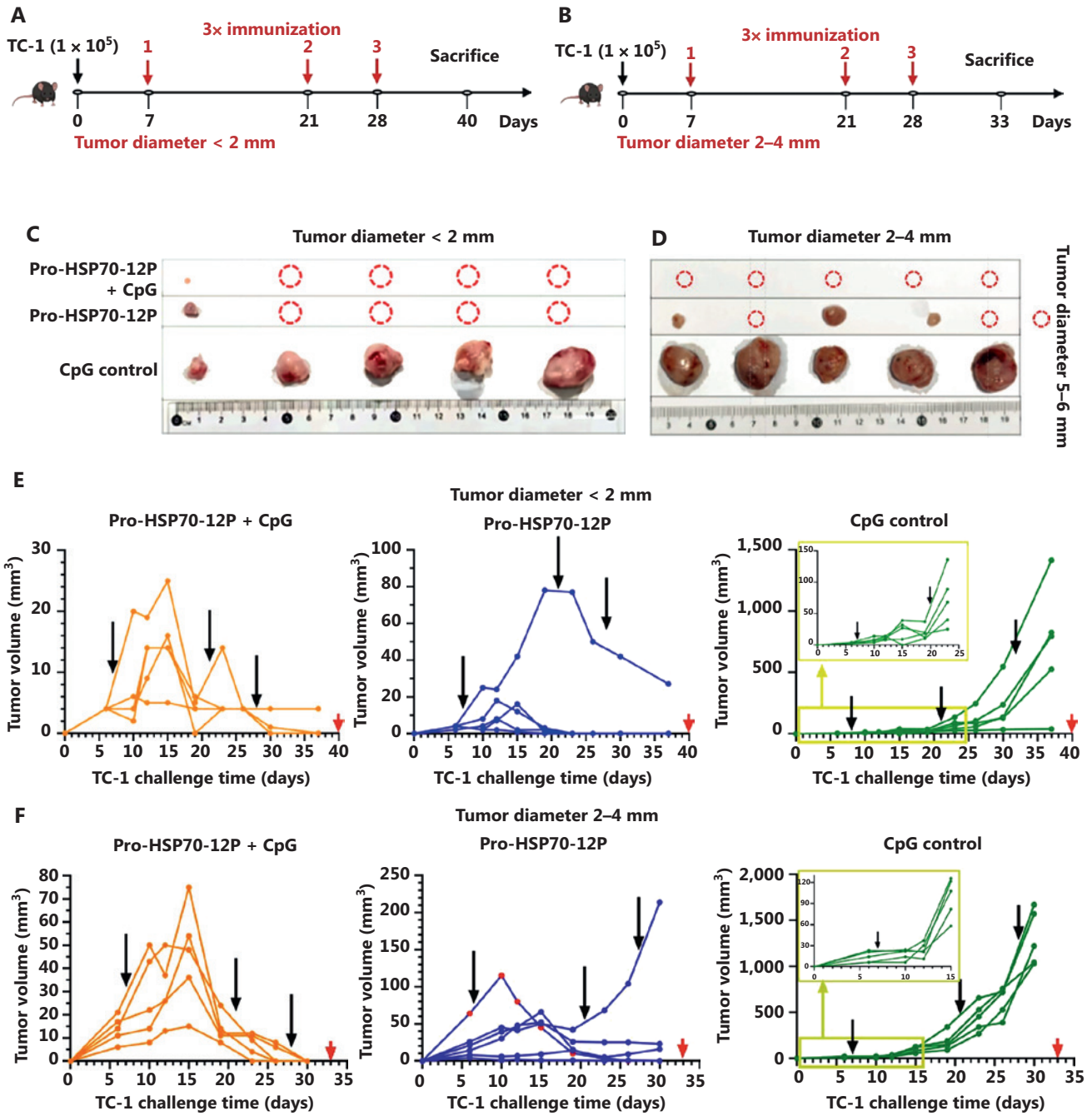


Figure 5 The therapeutic antitumor effects were characterized for two different tumor sizes in TC-1 tumor-bearing mice. Female C57BL/6 mice (6–8 weeks old; 10 mice/group) were subcutaneously injected with 1 × 10^5 TC-1 cells on day 0. On day 7, when all mice had developed tumors, the mice with tumors ranging from 2 to 4 mm in diameter were randomly divided into 3 groups (5 mice/group). Similarly, the mice with tumors < 2 mm in diameter were randomly divided into 3 groups (5 mice/group). This grouping was performed to ensure that the tumor sizes in each group were as similar as possible. The mice were then injected three times with Pro-HSP70-12P+CpG, Pro-HSP70-2P or the CpG control (50 μg/mouse Pro-HSP70-2P, 20 μg/mouse CpG, and 100 μL/mouse injection volume) intradermally. (A, B) Experimental workflow. (C, D) The tumors of the mice were isolated and photographed (the red dashed circles indicate no tumors). (E, F) Tumor growth curve of each mouse in each group subjected to different treatments (the blue dashed line with red dots represents the tumor growth curve of mice with an initial tumor diameter of 5–6 mm). The black arrows indicate the time points of vaccine immunization and the red arrows indicate the end points of the experiment.

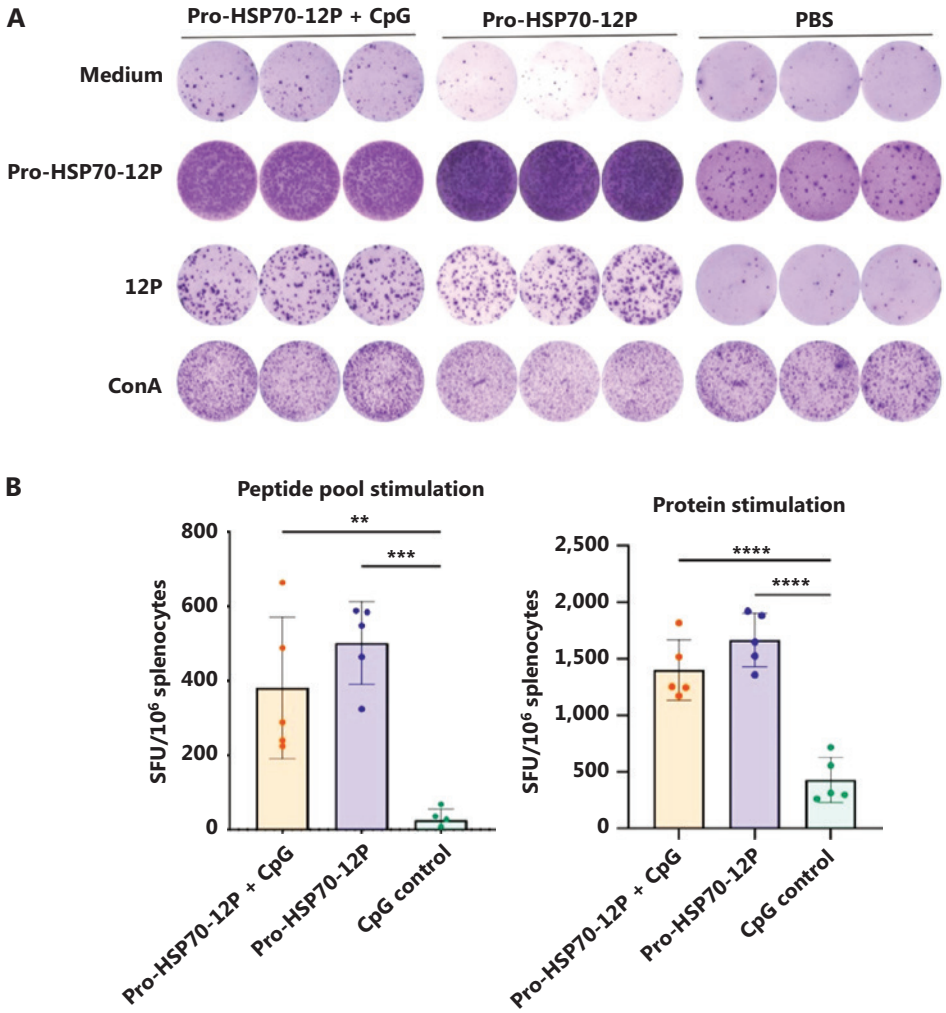


Figure 6 Continued

control group, although the differences were not significant (**Figure 6C**). Based on these findings, Pro-HSP70-12P without CpG was selected for further study.

A single injection of the Pro-HSP70-12P vaccine achieves complete elimination of tumors with diameters < 2 mm, whereas multiple injections significantly increase the survival rate of mice bearing tumors with diameters ranging from 2–4 mm

Additional studies were conducted to assess the antitumor responses induced by different doses of the Pro-HSP70-12P and DNA-HSP70-12P vaccines in mice bearing TC-1 tumors. The results showed that a single dose of Pro-HSP70-12P vaccine achieved potent tumor regression in mice with tumor diameters < 2 mm (**Figure 7A**). The booster immunization significantly improved the survival rate and prolonged the survival time in mice with tumor diameters between 2 and 4 mm (**Figure 7B, 7C**). A single immunization only prolonged the survival time of DNA-HSP70-12P vaccinated mice (**Figure 7A**) but insufficient to achieve a high probability of tumor regression, whereas multiple immunizations significantly improved the survival rate and prolonged the survival time (**Figure 7B, 7C**). The data indicate that single and multiple immunizations with Pro-HSP70-12P significantly outperformed DNA-HSP70-12P in inhibiting tumor growth, improving survival rates, and extending survival time in mice.

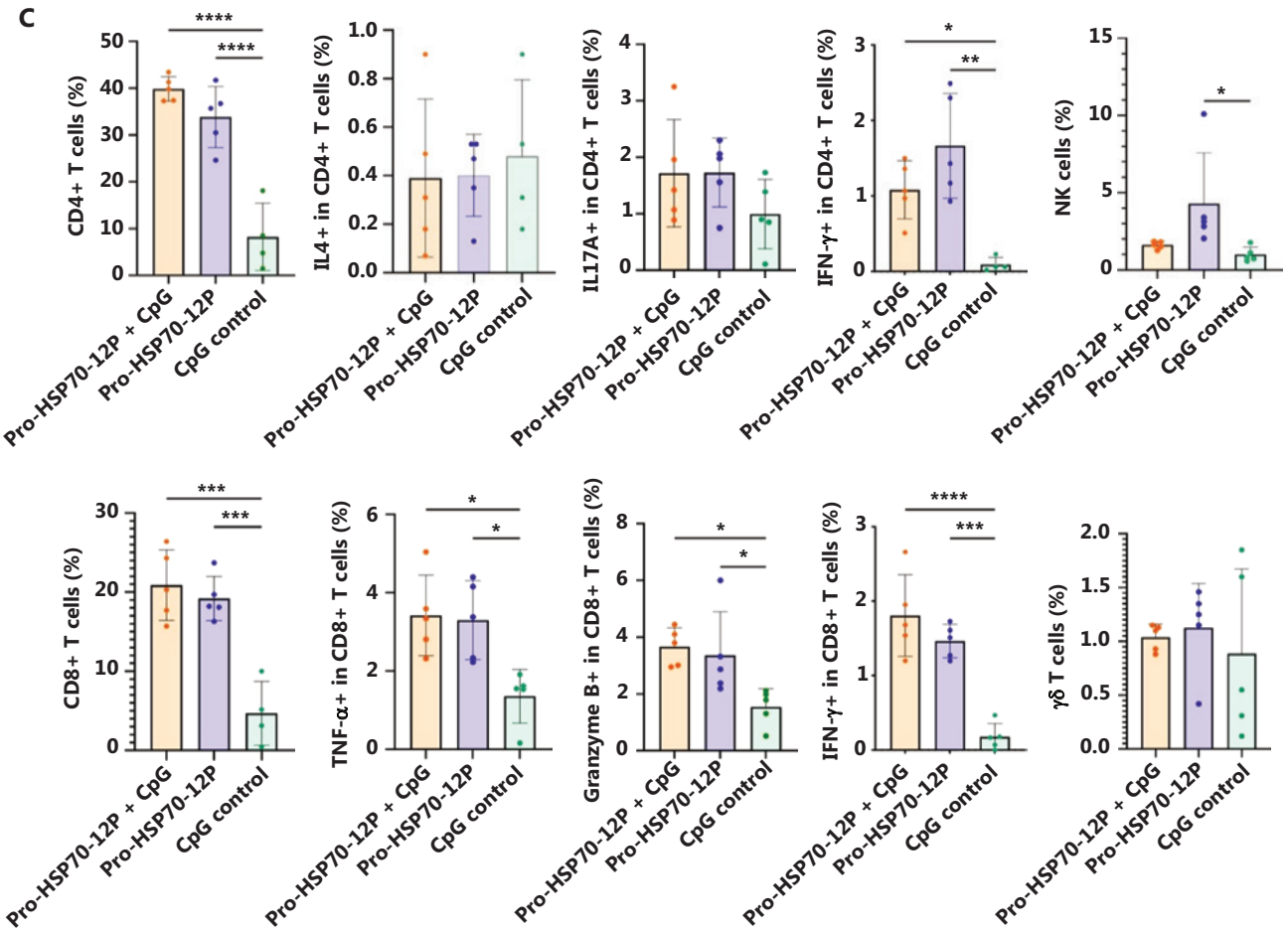


Figure 6 The immune response in TC-1 tumor-bearing mice mediated by HPV antigen-specific CD8+ T cells induced by Pro-HSP70-12P was characterized for a small tumor size. (A) Image of IFN-γ levels measured by ELISpot; each row represents one mouse (Con A: the positive stimulator, concanavalin A). (B) Quantification of IFN-γ levels measured by ELISpot. SFUs denote spot-forming units. (C) Flow cytometry data showing CD8+ T cell subsets that secrete IFN-γ, granzyme B, and TNF-α, as well as CD4+ T cell subsets that secrete IFN-γ, IL4, and IL17A. Additionally, the proportions of CD4+ T, CD8+ T, γδ T, and NK cells were analyzed (5 mice/group, one-way ANOVA, means ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). ConA, Concanavalin A; SFU, Spot-Forming Unit; 12P, 12 short peptides.

Pro-HSP70-12P can induce long-term antitumor immune protection and achieve potent tumor prevention efficacy

In addition, TC-1 rechallenge experiments were conducted in mice that achieved complete tumor regression after one or two doses of the vaccine to determine the durability of protective antitumor immunity. On day 74 after the first TC-1 cells challenge, a second injection of 1×10^5 TC-1 cells was administered to determine whether mice immunized with reduced doses of the vaccine possessed long-term antitumor protection (5 unimmunized mice were selected and injected with the same dose of TC-1 cells as the control group to evaluate whether the TC-1 rechallenge was successful; **Figure S4A**). The results showed that within 27 d after the second TC-1 cells challenge (101 days after the first TC-1 cells challenge), all mice in the Pro-HSP70-12P and DNA-HSP70-12P groups experienced tumor disappearance after initial small-volume growth, whereas the mice in the control group carried larger tumors or were euthanized due to ethical limit within 27 d (**Figure S4B**).

The mice were immunized 3 times with 50 μg of Pro-HSP70-12P or DNA-HSP70-12P with 1-week intervals between each immunization to determine the prophylactic effect of the vaccine. The mice were challenged with TC-1 cells 14 d after the final immunization (**Figure 8A**). The results showed that the number

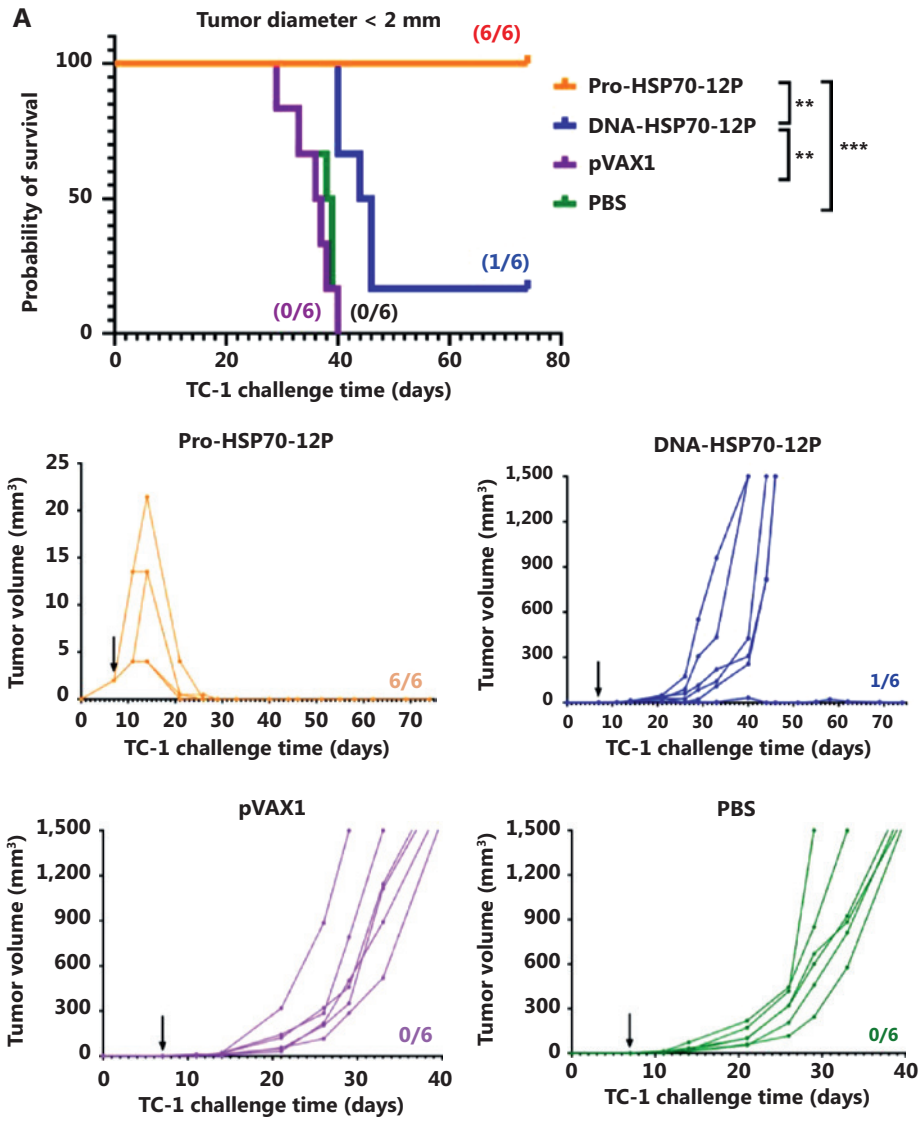


Figure 7 Continued

of mice in the control group approached the ethical limit within 28 d after TC-1 cell challenge, whereas mice in the Pro-HSP70-12P and DNA-HSP70-12P groups exhibited no tumor growth, indicating that the mice achieved potent tumor protection (**Figure 8B, 8C**). These results not only demonstrate the long-term antitumor protective capability of the Pro-HSP70-12P vaccine but also highlight its tremendous potential in preventing tumor growth.

Identification and immunogenicity analysis of key antigenic epitopes in the Pro-HSP70-12P multi-epitope vaccine

C57BL/6 mice were immunized 3 times (50 μg each on days 0, 7, and 14) and IFN-γ ELISpot assays were performed on splenocytes isolated 7 d after the final immunization using individual peptides for stimulation to determine which epitopes in the Pro-HSP70-12P vaccine have crucial roles in inducing immune responses. The results demonstrated that E7-49-57 (a known H2-Db-restricted immunodominant epitope), E6-38-45 (novel), E6-124-132 (novel), and E7-50-57 (novel, a truncated variant of E7-49-57) elicited the strongest IFN-γ secretion and were identified as the dominant antigenic peptides. In addition, two novel HTL epitopes (E6-56-70 and E7-9-23) along with previously reported epitopes (E6-49-57 and E6-81-90) also induced significant IFN-γ production, albeit at slightly lower levels than the dominant epitopes. In contrast, E7-7-15 (reported as HLA-A2-restricted epitope in humans) and E6-75-83 (a reported candidate epitope)

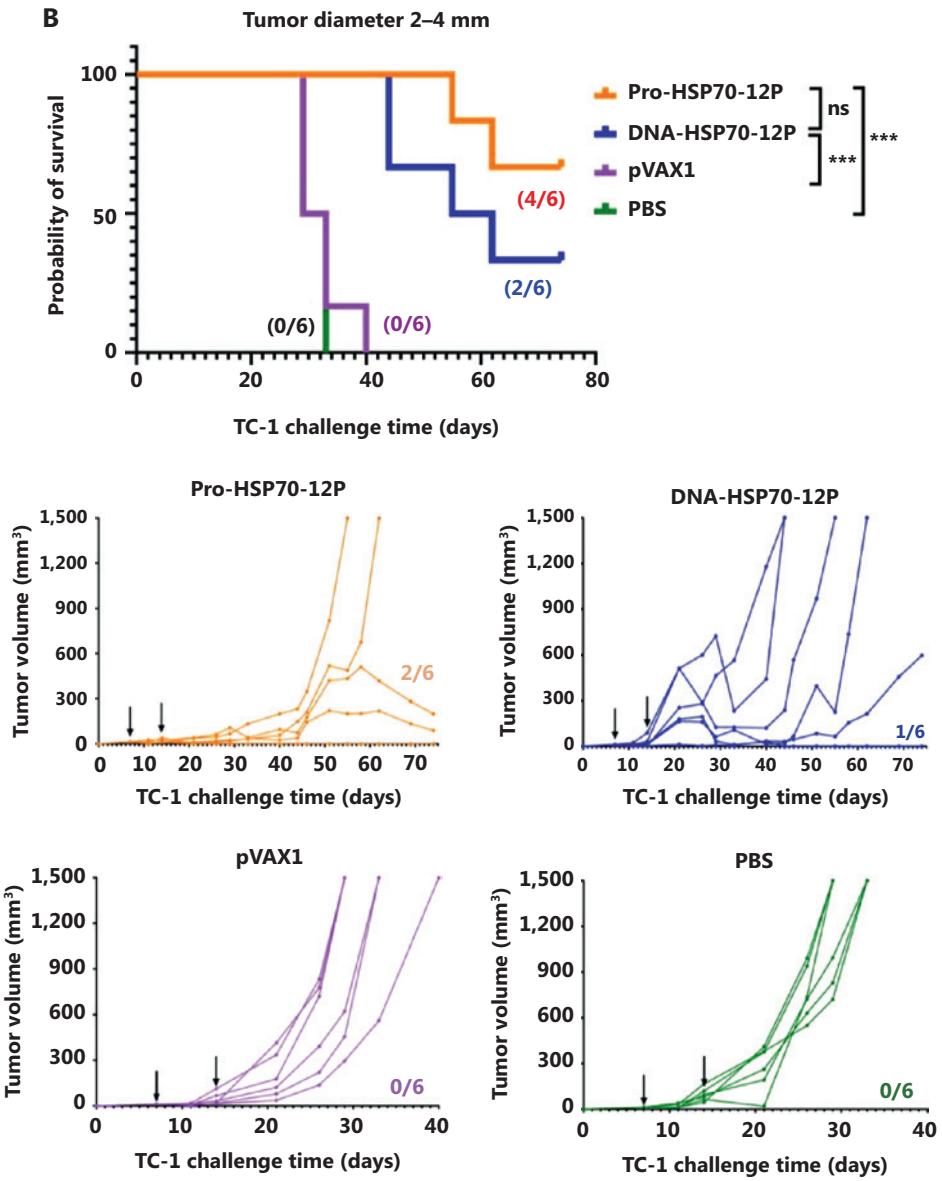


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triggered only weak responses, whereas the newly identified E6-86-95 epitope (novel) and E7-77-87 epitope (a reported candidate epitope) did not induce detectable IFN-γ secretion (**Figure S5**).

Discussion

Multi-epitope vaccine design has been revolutionized by reverse vaccinology approaches leveraging genomic/proteomic data and bioinformatics tools[42]. By integrating multiple MHC-restricted T-cell epitopes, these vaccines achieve three key advantages: (1) eliciting broad-spectrum immune responses with minimal epitopes[43]; (2) avoiding immunodominant but nonprotective epitopes[44,45]; and (3) overcoming the weak immunogenicity of whole-protein vaccines that predominantly activate MHC-II pathways[6]. Several clinical trials are testing the safety and efficacy of polyepitope DNA vaccines for various cancers, including breast, cervical, and ovarian cancer[46]. This strategy is particularly promising for HPV16, where we previously identified 12 high-coverage

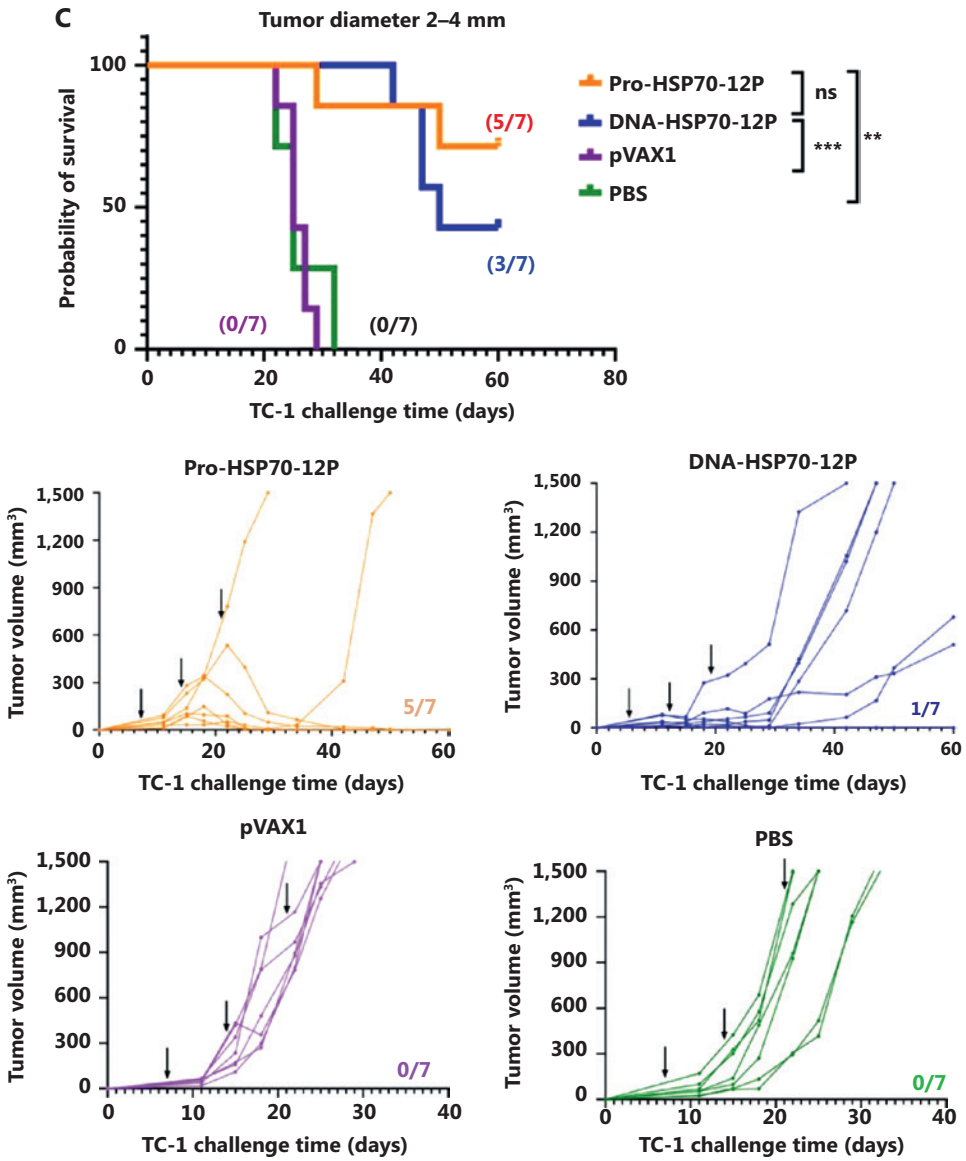


Figure 7 Different doses of the Pro-HSP70-12P and DNA-HSP70-12P vaccines induced antitumor responses in mice harboring TC-1 cells. C57BL/6 mice (19 mice/group) were subcutaneously injected with 1×10^5 TC-1 cells on day 0. All mice had developed tumors by day 7 and mice with tumor diameters ranging from 2 to 4 mm were randomly divided into 4 groups (13 mice/group) with 6 mice in each group receiving 2 doses of vaccine immunization and 7 receiving 3 doses. Similarly, mice with tumor diameters < 2 mm were randomly divided into 4 groups (6 mice/group) and received a single dose of the vaccine. The purpose of this experiment was to ensure that the tumor sizes in each group of mice were as similar as possible. (A) Survival curves of the mice after a single immunization (the bottom right score represents the percentage of surviving mice) and tumor growth curves of single mice (the bottom right score represents the percentage of mice with a tumor volume of 0). (B) Survival curves of mice after two immunizations and tumor growth curves of single mice. (C) Survival curves of mice after three immunizations and tumor growth curves of single mice. Log-rank (Mantel–Cox) test; ns: not significant; $^{**}P < 0.01$, $^{***}P < 0.001$.

epitopes using computational predictions[35]. The HSP70-12P vaccine exemplifies this approach by combining (1) CTL epitopes (for CD8+ T cell cytotoxicity), (2) HTL epitopes (to enhance CD4+ T helper cell)[47,48], and (3) a built-in HSP70 adjuvant (to activate TLR-mediated innate immunity). The HSP70-12P vaccine construct was predicted to have suitable physicochemical properties and safety and was predicted to be a soluble protein. Structural modeling confirmed stability

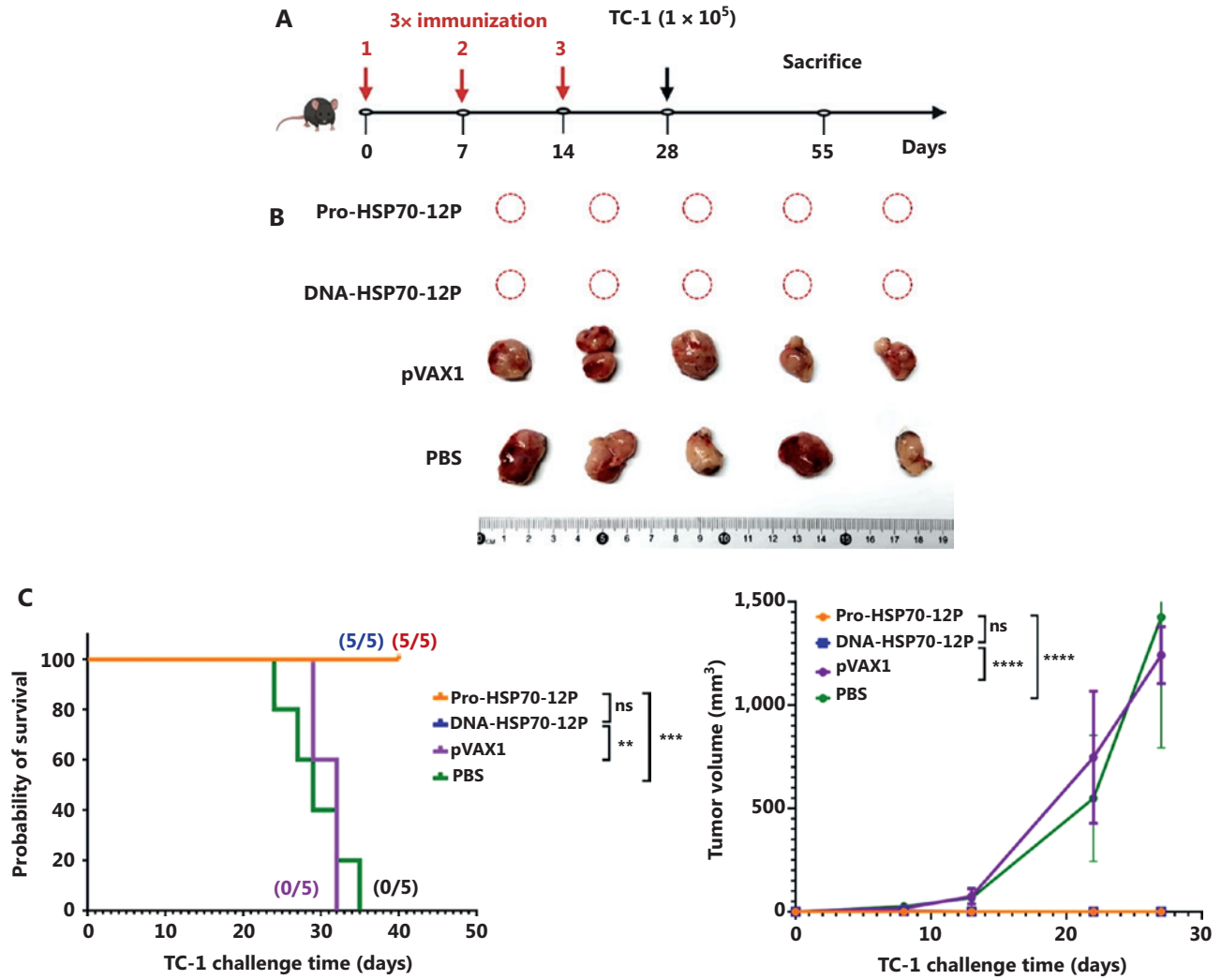


Figure 8 The mice were immunized 3 times with 50 μg of Pro-HSP70-12P or DNA-HSP70-12P with 1-week intervals between each immunization to determine the prophylactic effect of the vaccine. The mice were challenged with TC-1 cells 14 d after the final immunization. (A) Experimental workflow for the prophylactic experiment. (B) Isolation and imaging of mouse tumors in the prophylactic experiment. (C) Survival curves and tumor growth curves of mice after TC-1 challenge. Log-rank (Mantel–Cox) test; ns: not significant; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

(98.4% residues in favored regions; **Figure S6 and Table S4**). Molecular docking studies suggested that effective binding to TLR receptors (TLR2, TLR3, TLR4, and TLR8) is crucial for innate immunity (**Figure S7 and Tables S5, S6**).

Both the Pro-HSP70-12P subunit vaccine and DNA-HSP70-12P were constructed to assess the effectiveness of the HSP70-12P vaccine. Although cancer vaccines targeting the HPV E6 and E7 genes are currently being developed for therapeutic purposes, this study highlights the advantages of therapeutic formulations. The results indicated that Pro-HSP70-12P and DNA-HSP70-12P have preventive effects on HPV16. During the TC-1 cell challenge conducted 14 d after the final immunization, the mice remained tumor-free for nearly 30 d, whereas the tumors in the control group had already reached the ethical limits. These findings suggested that our vaccine may help prevent the occurrence of HPV16-related cancers. Studies conducted by Kayyal et al.[49] and Qi et al.[8] demonstrated that although the novel multi-epitope DNA or protein vaccines significantly inhibited HPV-related tumor growth in preventive or therapeutic experiments, the multi-epitope DNA or protein vaccines failed to completely prevent or eliminate tumor growth; Kayyal et al. used the C3 tumor model[50]

and Qi et al. used the TC-1 or E5-TC-1 tumor model. In addition, mice treated with Pro-HSP70-12P and DNA-HSP70-12P exhibited significant differences upon stimulation with HPV 16 E6 E7-specific antigen peptide pools compared to the control group, as evidenced by the ELISpot results. The flow cytometry analysis revealed a notable increase in the number of CD4+ and CD8+ T cells secreting IFN-γ, TNF-α, and granzyme B induced by both vaccines, along with Th1-type cytokine (IFN-γ) secretion. Tumors can induce Tregs to suppress the local immune response and promote further tumor growth[51]. In various types of human cancers, an increased number of Tregs, particularly a decreased CD8+ T cell:Treg ratio among tumor-infiltrating lymphocytes, is associated with poor prognosis[40,41]. Although significant reductions in the proportions of Tregs and MDSCs were not detected in the Pro-HSP70-12P and DNA-HSP70-12P groups, the significantly lower Treg:CD8+ T cell ratio indicated a more cytotoxic microenvironment, which aligns with observations in human patients exhibiting better clinical outcomes[40]. Tcm cells usually reside in lymphoid organs and lack immediate cytotoxic function, whereas Tem cells are primarily found in non-lymphoid tissues, exhibit cytotoxic activity, and express high levels of receptors for migration to inflamed tissues[52], enabling the rapid deployment of IFN-γ to exert immune effects[53]. Tem cells have superior immediate effector functions compared to Tcm cells and are key drivers of cytotoxic responses against cancer cells[54,55]. As a method to characterize the cellular immune response further, CD44 and CD62L markers in T cell populations were analyzed to distinguish among naïve, Tcm, and Tem cells. The proportion of Tem cells among CD4+ and CD8+ T cells in the spleens of the mice treated with Pro-HSP70-12P were significantly different compared to the PBS control group, indicating the critical role of this cell population in cell-mediated control during HPV tumorigenesis.

Furthermore, the Pro-HSP70-12P vaccine exhibited remarkable antitumor effects, outperforming the DNA-HSP70-12P vaccine. Among the mice with tumor diameters of 1–4 mm and treated with 3 doses of the Pro-HSP70-12P vaccine, 90% exhibited complete tumor regression on day 33 after TC-1 cell challenge (the study endpoint), whereas the remaining mice presented suppressed tumor growth. In contrast, for mice with tumor diameters < 2 mm, a single dose of the Pro-HSP70-12P vaccine resulted in 100% tumor regression. The booster immunization significantly improved survival rates and extended the survival time for mice with tumor diameters of 2–4 mm. A single immunization of DNA-HSP70-12P vaccine only prolonged the survival time, which was insufficient for a high probability of tumor elimination, whereas multiple immunizations significantly improved survival rates and extended the survival time. Notably, mice with complete tumor regression after one or two immunizations were subjected to TC-1 rechallenge experiments, revealing long-term antitumor protection and tumor growth resistance upon a secondary TC-1 cell challenge. The Pro-HSP70-12P-vaccinated group presented greater infiltration of immune cells compared to the control group, such as CD8+ and effector memory T cells among CD8+ and CD4+ T cells, which are well known for roles in tumor elimination. These results support the finding that the Pro-HSP70-12P vaccine significantly inhibits tumor growth following TC-1 rechallenge.

Furthermore, despite the remarkable antitumor efficacy displayed by Pro-HSP70-12P, strategies to further enhance the potency of multi-epitope vaccines were explored based on Pro-HSP70-12P. Specifically, a combination of endogenous and exogenous mature adjuvants was investigated, particularly CpG, aimed at augmenting the antitumor effects of epitope-based vaccines. CpG is a TLR9 ligand that induces strong Th1 immune responses in mice and was previously demonstrated to be an effective adjuvant for the Rv2034-based subunit vaccine[56]. Thus, the performance of Pro-HSP70-12P was evaluated with or without CpG adjuvant in terms of antitumor efficacy and cellular immune responses in tumor models. The results indicated that while the Pro-HSP70-12P and Pro-HSP70-12P+CpG vaccines significantly outperformed the CpG control in reducing the tumor volume and achieving complete tumor eradication, no significant differences were detected between the Pro-HSP70-12P and the Pro-HSP70-12P+CpG groups. In addition, ELISpot and flow cytometry analyses revealed no significant differences in the cellular immune responses between the Pro-HSP70-12P and Pro-HSP70-12P+CpG groups, although both groups presented significant differences compared to the CpG control group. The results indicated that the addition of the mature exogenous adjuvant CpG did not significantly enhance the antitumor efficacy or immune response of the multi-epitope Pro-HSP70-12P vaccine, which was unexpected. Two reasons may explain this finding. First, the inclusion of an internal adjuvant and the introduction of HTL epitopes may have already stimulated the production of sufficient Th1-polarized cytokines, making the efficacy of the vaccine adequate to suppress tumor growth. Therefore, the addition of exogenous CpG did not further improve efficacy. Second, intradermal immunization with a microneedle to administer CpG together with the multi-epitope vaccine in mice might not fully exploit the

potential of CpG. Notably, CpG was approved by the FDA in 2009 for use in combination with the HBV vaccine Heplisav-B, which is a protein-based hepatitis B vaccine administered *via* intramuscular injection[57]. In other studies and applications, CpG has been combined with protein-based vaccines and administered *via* subcutaneous injection, producing effective results[56,58]. However, these findings remain speculative and require further investigation in future studies.

In the broader context of vaccine development, the HSP70-based built-in adjuvant in our design differs mechanistically from traditional or clinically used adjuvants, such as aluminum salts (alum) and Adjuvant System 04 (AS04). Alum, the most widely used adjuvant in licensed vaccines, is thought to act primarily by activating the NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome, leading to IL-1β and IL-18 production essential for humoral immunity, but alum has limited capacity to induce potent CTL responses[59,60]. AS04, a clinically used adjuvant combining alum with the TLR4 agonist, monophosphoryl lipid A (MPL) (used in vaccines, like Cervarix), enhances humoral and Th1 responses *via* NF-κB activation and antigen-presenting cell (APC) maturation, yet still exhibits relatively modest CTL-inducing potency compared to pathogen-associated molecular patterns (PAMPs), like CpG[61]. Conversely, HSP70 acts as a molecular chaperone facilitating antigen cross-presentation and as a damage-associated molecular pattern (DAMP), engaging receptors, like TLR2/4, enhancing APC activation, and promoting robust CTL responses[62,63]. This mechanistic distinction may underlie why our Pro-HSP70-12P vaccine elicited strong cellular immune responses and complete tumor eradication *in vivo*, even without additional exogenous adjuvants.

When explore the immunologic mechanisms underlying Pro-HSP70-12P, several individual epitopes within the construct were shown to elicit significant IFN-γ responses independently, indicating the important roles in the immune activation process. E7-49-57, a classic H-2Db-restricted immunodominant epitope[50], has been confirmed for its immunogenicity. Previous studies have suggested that E7-49-57 can induce responses in human peripheral blood mononuclear cells (PBMCs)[64,65] and may serve as a B-cell epitope for therapeutic vaccines[66]. Three immunodominant epitopes were identified (E6-38-45, E6-124-132, and E7-50-57). Notably, the two E6 epitopes provide new candidates for targeting E6 in vaccine design because dominant E6 epitopes are rarely reported. E7-50-57, although a shorter version of E7-49-57, maintained similar immunogenicity, suggesting that critical T-cell receptor (TCR) contact residues are situated in the C-terminal region. In addition, the newly discovered HTL epitopes (E6-56-70 and E7-9-23) induced robust IFN-γ production *in vivo*, implying that the HTL epitopes may function through the following mechanisms: classic MHC-II presentation to activate CD4+ Th1 cells; cross-presentation *via* MHC-I to stimulate CD8+ T cells; and the inclusion of embedded CTL epitopes (e.g., E7-7-15 and E7-11-19 within E7-9-23). These findings demonstrated the application potential of HTL epitopes. In addition, several literature-supported peptides (e.g., E6-49-57[67], E6-81-90[68], E7-7-15[69], and E6-75-83[70]), which were previously shown to bind human HLA-I, also exhibited strong responses in our assays, supporting candidacy for inclusion in vaccines. Although some peptides, including the newly identified E6-86-95 and E7-77-87 peptides (which overlap with multiple reported immunogenic epitopes such as E7-79-87[69] and have been detected in mass spectrometry studies of CaSki cells, such as E7-77-87 and E7-78-86[71]), failed to produce significant immune responses in the current experiments, the potential immunogenicity in humans warrants further validation, given the interspecies differences in MHC molecule presentation.

Importantly, the vaccine design procedure herein aims to explore three key challenges in epitope vaccines strategically: weak individual immunogenicity; narrow antigenic targeting; and limited HLA coverage. Antigenic peptides with high binding scores for both human HLA and murine MHC molecules were selected to develop candidate vaccine components with "human–mouse co-presentation" potential. This strategy not only enhances the scientific validity of preclinical evaluation but also provides theoretical support for clinical translation. Currently, several HLA-A*0201-restricted E7 peptides (e.g., E7-11-20, E7-12-20, and E7-86-93) have been tested in clinical trials[72]. However, despite the ability to induce specific T-cell responses, the actual clinical benefits are limited. Therefore, improving the immunogenicity and population coverage of epitope vaccines has become a critical challenge to address. Our work provides both theoretical foundation and practical epitope resources for developing next-generation personalized vaccines based on multiple MHC-restricted epitopes. Future studies should focus on validating the immunogenicity of these epitopes in clinical trials and evaluating potential synergistic effects among different epitope combinations.

Beyond the current single-platform vaccination approach, future studies could explore heterologous prime–boost regimens, such as priming with a DNA vaccine and boosting with the Pro-HSP70-12P protein vaccine, to further enhance the magnitude and quality of antigen-specific immune responses. Such strategies could synergistically combine the strong cellular

immunity typically induced by DNA vaccines with the robust T-cell responses elicited by protein vaccines, thereby increasing the translational potential of this approach. In parallel, optimization of vaccine delivery routes may further improve efficacy. While DNA vaccines have shown clinical promise (NCT01634503 and NCT00788164)[4], the potency often depends on specialized delivery systems, such as electroporation, to ensure nuclear uptake. Intradermal administration *via* microneedles, such as the MicronJet600[73] has been shown to outperform conventional intramuscular injection by reducing the required dose and improving immunogenicity[38,74], largely due to the abundance of skin-resident antigen-presenting cells, including Langerhans and dermal dendritic cells[75]. Incorporating such delivery strategies alongside heterologous prime–boost regimens could amplify both the magnitude and breadth of vaccine-induced immune responses. Furthermore, extending the evaluation of the Pro-HSP70-12P vaccine to more clinically relevant models, such as humanized mouse systems, patient-derived xenograft (PDX) models, or the K14E6 transgenic mouse model, would provide deeper insights into its efficacy and immune mechanisms in settings that more closely recapitulate human HPV-associated disease.

This study has some limitations. First, detailed immunophenotyping within the tumor microenvironment or tumor-draining lymph nodes was not performed. Although robust systemic immune responses were demonstrated in splenocytes, the strong therapeutic efficacy of the vaccine led to rapid and complete tumor regression in most treated mice, limiting tumor tissue availability for analysis at later stages. Second, the contributions of distinct T-cell populations to vaccine-induced antitumor protection remain to be elucidated. While protective responses in the TC-1 tumor model are known to be mediated by CD8+ T cells[76-78], CD4+ T cells are also critical for the effective priming of CD8+ T cells and the establishment of long-lasting immune memory[79,80]. Future studies will investigate the contributions of specific T cell populations to vaccine-induced therapeutic antitumor protection, using either CD4+/CD8+ T cell depletion experiments or knockout mouse models, alongside earlier time-point sampling to characterize immune cell dynamics during the effector phase of tumor regression.

Conclusions

The multi-epitope protein vaccine, Pro-HSP70-12P, represents a potent therapeutic candidate against HPV16-driven malignancies and has the capacity to induce tumor regression and long-term immunity. These findings support further clinical development and highlight the importance of optimizing built-in adjuvant-epitope combinations, focusing on T-cell-centric vaccine design, and developing minimal-dose regimens for clinical translation.

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Conflict of Interest Statement

No potential conflicts of interest are disclosed.

Author contributions

Conceived and designed the analysis: Li Shi, Yufeng Yao, Ming Sun, Lanfang Zhu.
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Data availability statement

The data generated in this study are available upon request from the corresponding author.

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