

Original Article



Immunological Characterization of a Nanoliposome-Formulated Peptide Vaccine Designed to Target CEA and TAG-72 Epitopes in HT29 Colorectal Cancer Cells

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Abstract

Background: Colorectal cancer (CRC) remains a leading cause of cancer-related mortality worldwide. Carcinoembryonic antigen (CEA) and tumor-associated glycoprotein 72 (TAG-72) are two well-established biomarkers commonly overexpressed in CRC. In this study, we designed a novel peptide vaccine targeting immunodominant B-cell epitopes derived from both CEA and TAG-72, formulated using a nanoliposomal delivery system, and evaluated its immunological and antitumor effects in vivo.

Methods: Using immunoinformatics tools, we predicted B-cell epitopes from the full-length sequences of CEA and TAG-72. Candidate peptides were selected based on antigenicity, immunogenicity, MHC class I and II binding affinity, and safety profiles (non-allergenic and non-toxic). Molecular dynamics simulations and docking analyses were performed to assess structural stability and TLR4-binding affinity. The optimal peptide was synthesized, encapsulated in nanoliposomes, and characterized for size, morphology, and encapsulation efficiency. Female BALB/c mice were immunized with different doses of the nanolipopeptide formulation, and immune responses were evaluated by measuring antibody titers, cytokine levels, antibody binding to HT29 cells, apoptosis induction, and expression of apoptosis-related genes (*BAX* and *BCL2*).

Results: The multi-epitope peptides, one from CEA (YVCGIQNSVS) and one from TAG-72 (TAPVTSGPVS), exhibited strong antigenicity, favorable immunogenicity, and broad MHC-binding potential. Molecular simulations supported their structural stability and efficient binding to TLR4. The nanolipopeptide particles demonstrated a uniform size (~125 nm), spherical morphology, and satisfactory encapsulation efficiency (76%). Immunization with the highest dose elicited robust antibody responses, with endpoint titers exceeding 1:10,000. Significant increases in IFN- γ levels and marked apoptosis of HT29 cells (approximately 39%) were observed in vaccinated groups compared with controls. Additionally, *BAX* expression was significantly upregulated, while *BCL2* expression was downregulated, indicating activation of the intrinsic apoptotic pathway.

Conclusion: Our findings demonstrate that a nanoliposome-formulated peptide vaccine effectively elicits strong humoral and cellular immune responses, promotes antibody-mediated recognition of CRC cells, and induces apoptosis through the intrinsic pathway.

Keywords: Colorectal cancer; Peptide vaccine; Nanoliposome; CEA, TAG-72; Apoptosis; HT29 cells

1. Introduction

Among all cancer types affecting people around the world, colorectal cancer represents a particularly challenging disease to treat successfully. Epidemiologists who track cancer rates predict that the number of people acquiring this disease will continue to increase over the next several years (1). According to GLOBOCAN 2022, approximately 1.9 million new CRC cases and roughly 935,000 CRC-related death were reported worldwide. CRC ranks among the most common cancers and leading cause of cancer-related death across both men and women (2).

However, the incidence of CRC is not the same across different countries. The highest incidence rate (adjusted for age) have been traditionally recorded in high-income countries, such as Australia, New Zealand, Europe, and North America. Nevertheless, recently the incidence is increasing rapidly in low-income countries. This is related to changes in people's life styles, including eating more Western style food, moving around less, and becoming overweight more often (3, 4).

In Iran, the situation raises some concerns. Records from the national cancer registry show that CRC has become much more common over the past few decades. Between 1985 and 1995, it was the ninth most frequent cancer in the country; in recent years, it has climbed to fifth place (5). The age adjusted rate is about 7 cases for every 100,000 people; however, this average masks the existing substantial differences among regions. Northern provinces, especially Golestan and Mazandaran, have higher rates than the central and southern parts of the country (6).

Another striking feature of CRC in Iran is that patients tend to be younger. Roughly 17 out of every 100 people diagnosed are under the age of 40. This is different from what doctors in Western countries usually call "early onset" CRC, where the cutoff is 50 years (5). On top of this, survival figures are not as good as those reported in better funded health systems. Iranian studies have found 5 year survival rates between 40 % and 52 %, whereas many developed nations report rates above 65 % (7, 8).

The usual path that CRC follows is called the adenoma carcinoma sequence. It can take 10 to 15 years for an early polyp to turn into a cancer that invades nearby tissues (9). Since this process is quite slow, there is a good chance to detect the disease early or even prevent it altogether. Yet many people are still diagnosed late because early stage tumors often cause no symptoms and not enough people take part in screening programs. The stage at which the cancer is diagnosed is one of the strongest predictors of how well a patient will do. When the disease is still only in the colon or rectum, more than 90 % of patients survive at least five years. However, once it has spread to distant organs,

this number drops to about 14 % (10). These substantial differences show the need for better ways of early CRC detection and more effective treatments for people with advanced disease.

Currently, doctors manage CRC using a mix of surgery, chemotherapy, radiation (for certain rectal tumors), and drugs that target specific molecules (11, 12). Chemotherapy regimens that contain fluorouracil are still a mainstay of treatment. Drugs such as cetuximab and panitumumab (which block EGFR) and bevacizumab and ramucirumab (which block blood vessel growth) have given doctors more options. However, none of these approaches is perfect. Chemotherapy often causes side effects throughout the body, including low blood cell counts, nausea and vomiting, nerve damage, and tiredness. Targeted therapies only work in patients whose tumors have certain genetic features—for example, anti EGFR drugs help only those whose tumors do not have mutations in KRAS or NRAS. And even when they do work, the cancer eventually finds a way to become resistant (13). For patients whose CRC has spread and cannot be removed by surgery, the disease is almost never cured. Even with the best modern combinations of drugs, the average patient lives only 24 to 30 months (14).

Immunotherapy has changed how doctors treat many types of cancer, however for CRC, it helps only certain groups of patients (15). Drugs called immune checkpoint inhibitors, which block the PD 1/PD L1 or CTLA 4 pathways, work very well in colorectal cancers that have a specific defect: they cannot repair mistakes in their DNA (mismatch repair deficient, or dMMR) and therefore have many mutations (microsatellite instability high, or MSI H). About 15 % of metastatic CRC cases fall into this category (16, 17). The large clinical trial called KEYNOTE 177 showed that pembrolizumab (a PD 1 blocker) worked better than standard chemotherapy as a first treatment for MSI H metastatic CRC, keeping the cancer from growing for longer (17). Nivolumab and ipilimumab given together have also been approved for this same group. But the remaining 85 % of CRC patients have tumors that can repair DNA normally (mismatch repair proficient, or pMMR) and are microsatellite stable (MSS). In these people, checkpoint inhibitors by themselves usually do very little (18). Their tumors are described as immunologically "cold"—they carry few mutations, have few immune cells inside them, and create an environment that suppresses immune attack. This is a major barrier to successful immunotherapy (19).

Another type of immunotherapy, known as cancer vaccination, tries to train the patient's own immune system to recognize and destroy tumor cells that carry

specific cancer related proteins (20). Unlike checkpoint inhibitors, which release the brakes on an already existing immune response, vaccines start a brand new response against chosen targets. Peptide based vaccines have drawn a lot of interest because they can be produced using straightforward chemical methods, have a well-defined composition helping regulatory approval, have excellent safety records with little risk of off target effects, stay stable during storage and shipping, and cost relatively little to produce (21-24).

Selecting the right target proteins is one of the most important decisions when designing a cancer vaccine. Ideally, a target should be present at high levels on cancer cells while being minimally presented on normal cells. It should also be able to trigger an immune response strong enough to overcome the body's natural tolerance, play a role in the growth and progression of cancer, and be present on most patients' tumors rather than being restricted to a small group of tumors. Among the many CRC related antigens that researchers have studied over the years, two stand out as particularly promising vaccine targets: carcinoembryonic antigen (CEA) and tumor associated glycoprotein 72 (TAG 72).

CEA is a protein that helps cells stick to one another. It is present at high levels in about 70-80 % of CRC tumors, particularly in those that are advanced or have spread to other organs (25-30). Doctors measure CEA in CRC patients' blood after surgery to monitor for signs of disease recurrence. Patients whose CEA level is high before surgery tend to have a poorer prognosis. However, CEA is not entirely specific to cancer. Low levels of it can also be detected in certain inflammatory conditions, such as hepatitis, pancreatitis, and inflammatory bowel disease, as well as people who smoke. This imperfect specificity does not rule out CEA as a vaccine target, because both the amount of protein and the way it is presented to the immune system differ substantially between cancerous and benign conditions.

TAG 72 is presented in far fewer normal tissues. Its expression is largely limited to the lining of the uterus during certain phases of the menstrual cycle and to certain parts of the gut (30-32). In CRC, between 80 % and 90 % of tumors carry TAG 72, which makes it an attractive target for a dual target vaccine strategy (33). Since TAG 72 is a mucin like protein covered with many sugar molecules (O glycans), its structure make it easier for the immune system to recognize it under the right circumstances, because the sugar patterns on cancer cells differ from those on normal cells, creating new epitopes that the immune system has not seen before (32).

Considering all three markers—CEA, TAG 72, and another protein known as CA 19-9, together, one study

reported that 61 % of CRC patients had elevated levels of at least one of the markers. Individually, CEA, TAG-72, and CA 19-9 were elevated in 43 %, 43 %, and 27 % of patients, respectively (33). The incomplete overlap among these markers gives us a good reason to target multiple tumor-related antigens so that more patients can potentially benefit.

Beyond serving as biomarkers, both CEA and TAG 72 actually help the cancer survive and spread. CEA helps cancer cells stick to each other and to other cell types, which makes it easier for tumor cells to form deposits in new locations (27). There is also evidence that CEA helps the cancer hide from the immune system by encouraging the growth of regulatory T cells (which dampen immune responses) and by suppressing natural killer cells (28). TAG 72, because it is a large, sugar coated molecule, may physically block immune cells from reaching the surface of the cancer cell (32). Hitting these molecules with a vaccine could therefore do two good things at once: it could call in immune cells to kill the tumor, and it could interfere with the molecules' normal functions that help the cancer grow. The way a vaccine is delivered also makes a big difference in how well it works. When synthetic peptides are injected by themselves, they usually produce only weak immune responses. They break down quickly in the body (within minutes to hours), are not taken up very well by the cells that normally present antigens to the immune system (dendritic cells and macrophages), and lack built in signals that wake up the immune system (33-44). Nanoliposomes—tiny spherical sacs made of fat layers—have become a popular and effective way to deliver peptide based cancer vaccines. These fat bubbles offer several advantages making them less likely to cause side effects: (1) the peptide cargo is protected from being degraded by enzymes; (2) their small size (typically 100-200 nm) helps them drain into lymph vessels and get taken up by dendritic cells and macrophages; (3) the fat composition can be customized to include immune boosting adjuvants; (4) the surface can be modified to target specific cell types; and (5) they are made of materials that the body can break down safely (45, 46). Solid tumors also tend to have leaky blood vessels and poor lymphatic drainage, which helps nanoliposomes build up inside the tumor—a phenomenon called the enhanced permeability and retention effect (47).

While single-epitope vaccines against CEA or TAG-72 have shown limited clinical efficacy due to antigen heterogeneity and immune escape, our dual-target strategy is rationally designed to overcome these barriers. The simultaneous presentation of both epitopes is expected to broaden the repertoire of cytotoxic T-lymphocyte responses and reduce the likelihood of tumor relapse driven by antigen-loss

variants (27). With all this in mind, we designed the present study to fill several gaps in the field of CRC vaccinology. First, even though CEA and TAG 72 are well established blood biomarkers, they have not been thoroughly investigated as targets for vaccines designed to trigger the immune system—particularly TAG 72. Second, no previous study has developed a vaccine that targets both CEA and TAG 72 at the same time. Third, we still do not know the best way to deliver peptide vaccines for CRC. Therefore, our specific goals were: (1) to use computer based methods to identify and validate the most active linear B cell epitopes within CEA and TAG 72; (2) to build a synthetic multi epitope peptide that includes the selected pieces; (3) to load this peptide into nanoliposomes; (4) to test its ability to trigger immune responses in a mouse model by measuring antibodies and cytokines; (5) to see whether the antibodies produced in vaccinated mice can recognize live HT29 CRC cells; and (6) to determine whether these antibodies can induce the cancer cells undergo apoptosis by evaluating changes in the expression of the genes BAX and BCL2.

2. Methods

Materials

All chemicals, biological materials, and cell culture reagents were obtained from standard commercial sources and used according to the manufacturers' protocols or established laboratory practices. The synthetic peptide construct, comprising the CEA-derived epitope (YVCGIQNSVS) and the TAG-72-derived epitope (TAPVTSGPVS) linked via a trilycine (KKK) spacer, was commercially synthesized by GenScript Biotech (Nanjing, China) at a purity exceeding 95%. For liposome preparation, DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and DOSPA (2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate) (purity > 95%) and aluminum hydroxide adjuvant (10% suspension) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The HT29 human colorectal adenocarcinoma cell line was obtained from the Pasteur Institute of Iran (Tehran, Iran) and cultured in RPMI-1640 medium supplemented with heat-inactivated fetal bovine serum, penicillin/streptomycin, and trypsin-EDTA (all from Gibco, Thermo Fisher Scientific, Waltham, MA, USA). For immunological assessments horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (Abcam, Cambridge, UK), 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich), and commercial enzyme-linked immunosorbent assay (ELISA) kits for murine IFN- γ , IL-4, IL-6, IL-10, and TNF- α (R&D Systems, Minneapolis, MN, USA) were used. Apoptosis

detection was carried out using an Annexin V-FITC/PI apoptosis kit (BD Biosciences, San Jose, CA, USA), while molecular biology procedures were performed using TRIzol reagent (Invitrogen, Thermo Fisher Scientific), SYBR Green Master Mix, and a PrimeScript RT reagent kit (Takara Bio, Kusatsu, Japan). Female BALB/c mice 6–8 weeks old and weighing 20–25 g were obtained from the animal facility of the Pasteur Institute of Iran. The Key instruments employed in this study included a Malvern Zetasizer Nano ZS for dynamic light scattering (Malvern Panalytical, UK), a Nanoscope IIIa atomic force microscope (Bruker, USA), a Zeiss EM 900 transmission electron microscope (Carl Zeiss, Germany), a BD FACSCalibur flow cytometer, an Applied Biosystems StepOnePlus real-time PCR system, a BioTek Synergy H1 microplate reader (Agilent Technologies, USA), and a Hielscher UP200H probe sonicator (Hielscher Ultrasonics, Germany). Unless otherwise specified, all analytical-grade reagents were purchased from Sigma-Aldrich.

2.1. Finding and checking candidate epitopes using computer-based methods

A step-by-step computational approach was employed to identify candidate immunogenic regions within the CEA and TAG-72 proteins. First, we downloaded the complete amino acid sequences of these two proteins from the NCBI protein database. The reference sequence with accession number NP_004354.2 was used in the case of human CEA. This sequence contains 702 amino acids and includes a signal peptide, several immunoglobulin-like domains, and a region that anchors it to the cell membrane via a GPI linkage. With regard to TAG-72, we focused on the part of the MUC1-related mucin protein that sticks out from the cell surface and contains repeating segments (the tandem repeat region). The accession number for this sequence is NP_001018016.1, which is also referred to as the CA 72-4 antigen and consists of 125 amino acids. It is recognized by the B72.3 monoclonal antibody. We saved the sequences in FASTA text format for all later analyses.

Linear B-cell epitopes were predicted using the ABCpred server. This tool uses a type of artificial intelligence called a recurrent neural network which has been trained on a large set of experimentally characterized B-cell epitopes (48). The same prediction setting was used for both proteins: a threshold value of 0.5 and a window length of 10 amino acids. Earlier validation studies have shown that these settings give a good balance between finding true epitopes and avoiding false ones (48). For each protein, the ten highest-scoring predicted linear epitopes were retained as candidates for further

testing.

Next, the antigenicity of each candidate peptide was evaluated using the Vaxijen v2.0 server (49). This tool converts the amino acid sequence into a set of numbers that describe its chemical properties, then uses a statistical method (partial least squares discriminant analysis) to classify the peptide as antigenic or not. We set a cutoff of 0.4: any peptide with scores above this was considered a good candidate.

The potential binding of candidate peptides to MHC class I and class II molecules was assessed using the Vaxitop server (50). This platform uses a machine-learning approach called support vector machines that has been trained on experimental data measuring how strongly peptides bind to MHC molecules. Our analysis parameters were: statistical significance threshold $p < 0.05$, human as the host species (*Homo sapiens*), and testing against all available MHC alleles to get the broadest possible coverage across different human populations. Peptide with no predicted binding to either MHC class I or class II molecules were excluded from further consideration.

The possible allergenicity of peptides was evaluated using AllerTOP v2.0 (51). This tool uses a k-nearest neighbor classifier (with $k=5$) that has been trained on a carefully curated set of known allergens and non-allergens. The algorithm looks at amino acid composition, how often pairs of amino acids appear, and several physical and chemical properties. Any peptide predicted to be a possible allergen was excluded.

Potential toxicity was also evaluated using the ToxinPred server (52). We chose the prediction method based on support vector machines that was trained on the Swiss-Prot toxin dataset. We also included additional information such as the overall amino acid composition, dipeptide composition, binary sequence patterns, and position-specific scoring matrices. Peptides predicted to be toxic were excluded.

After all these screening steps, final candidate epitopes were selected based on a combined score which took into account a high antigenicity score (preferably above 0.5), predicted binding to both MHC class I and class II molecules, no sign of allergenicity, no sign of toxicity, and other favorable immunological features. The chosen sequences then went on to structural modeling and molecular dynamics simulations.

2.2. Building 3D models and running molecular dynamics simulations

Ascalaph Designer software (version 1.0; was used to examine how the selected epitopes behave in three dimensions and how they might interact with immune receptors. We built the starting 3D structures of each

peptide from their amino acid sequences using the software's built-in peptide builder, which applies standard bond lengths, bond angles, and torsion angles.

Before running the dynamics, we performed energy minimization using the steepest descent algorithm for up to 5,000 steps or until the biggest force on any atom fell below 10 kcal/mol/Å. This step was performed to remove unfavorable contacts between atoms and obtain a locally stable starting shape.

Molecular dynamics simulations were subsequently performed under conditions which kept the number of particles, volume, and temperature constant (NVT ensemble). We set the temperature to 310.15 K (normal human body temperature) using an isokinetic thermostat with a coupling time of 0.1 ps, which kept the temperature stable while still allowing realistic atomic motion. Each simulation lasted 30,000 ps (30 ns) with a time step of 5 fs while saving a snapshot of the structure every 5 ps (6,000 snapshots in total). This length of time was selected based on the findings of previous studies which have shown that 30 ns is usually enough to examine whether a short linear peptide stays stable.

The software automatically set the size of the simulation box based on the peptide's dimensions, adding at least 10 Å of empty space around it. Water molecules were included explicitly using the TIP3P water model.

After the simulations were completed, we took the average structures from the last 10 ns of each trajectory (the period during which the RMSD had flattened out) and used them for docking studies. The aim was to evaluate how well the peptides would bind to Toll-like receptor 4 (TLR4), a key protein on immune cells that recognizes foreign molecules and activation of immune responses. The structure of human TLR4 (the part outside the cell) came from the Protein Data Bank (PDB ID: 3FXI). We performed docking using the AutoDock Vina algorithm built into Ascalaph Designer. We centered a 40×40×40 Å grid box on the predicted binding pocket of TLR4. The strength of binding was reported as the free energy of binding (ΔG), with more negative numbers meaning a stronger, more favorable interaction.

The final epitopes were selected to be used for each target protein based on several factors, including a high antigenicity score (preferably >0.5), predicted binding to both MHC class I and II, no allergy or toxicity, and a favorable TLR4 docking energy (below -7.0 kcal/mol).

2.3. Making the multi-epitope peptide and loading it into nanoliposomes

Based on the computer screening results, we picked one epitope from CEA and one from TAG-72 to take

forward into experiments. We linked these two peptide sequences together using a flexible linker made of three lysine residues (KKK). We called the final product the CEA-TAG-72 peptide construct (CPC). The KKK linker gives the two epitopes room to move independently and also provides positive charges that may facilitate interactions with the negatively charged surface of the nanoliposomes.

The CPC peptide was made by a company (GenScript) using standard solid-phase peptide synthesis with Fmoc chemistry. They purified it by reversed-phase high-performance liquid chromatography to reach a final purity greater than 95 %, which they confirmed by analytical HPLC. They verified the molecular weight by electrospray ionization mass spectrometry. We dissolved the dried peptide in sterile phosphate-buffered saline (pH 7.4) at a concentration of 4 mg/ml just before making the liposomes.

We prepared nanoliposomes containing the peptide (called nanolipopeptide, or NLP) using a modified emulsion method followed by sonication with a probe. Briefly, we mixed 1 ml of the 4 mg/ml peptide solution with 1 ml of cationic emulsifiers (DOSPA: DOPE) and 100 μ l of 10 % aluminum hydroxide adjuvant and stirred overnight at 4°C. We stirred this mixture by hand for 2 minutes to make a rough emulsion. Then we placed a 3 mm titanium probe into the mixture and sonicated for 10 seconds at 40 % amplitude (about 50 watts of power). The tube was kept in an ice bath during sonication to prevent the heat from breaking down the peptide. Finally, we pushed the resulting nanoemulsion through a 0.45 μ m filter and then a 0.22 μ m sterile syringe filter to remove any large clumps and make sure it was sterile. We also made two control formulations using the same procedure: one with empty nanoliposomes (where we used PBS instead of peptide solution), and one with free peptide. The liposomal composition (DOSPA: DOPE at a 3:1 molar ratio) was selected based on its reported ability to enhance peptide stability in biological fluids and facilitate efficient uptake by antigen-presenting cells, as previously reported for other tumor-associated antigens ([1](#)). This formulation also enables sustained release of the encapsulated epitopes, which is critical for cross-presentation and prolonged T-cell activation ([46-47](#)).

2.4. Measuring the physical and chemical properties of the nanolipopeptide

Several methods were used to characterize the nanolipopeptide particles. Their size distribution and polydispersity index (PDI) were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS with a 633 nm helium-neon laser and a backscatter detector positioned at 173 degrees. The measurements were performed at 25°C in disposable plastic cuvettes.

Before measuring, we diluted the samples 100-fold in deionized water that had been pre-filtered through a 0.22 μ m membrane. This provided us with the right scattering intensity (between 100 and 500 kilocounts per second). Each measurement was an average of 15 runs of 10 seconds each, and we repeated each sample three times. We reported the average hydrodynamic diameter as the intensity-weighted Z-average.

The surface shape and detailed structure were examined using atomic force microscopy (AFM) in tapping mode. We diluted the samples 50-fold in deionized water and placed 10 μ l onto a freshly split piece of mica. After letting it adsorb for 2 minutes, we gently rinsed it with water and let it air-dry overnight. We imaged with a Nanoscope IIIa instrument using silicon cantilevers (resonant frequency about 300 kHz, spring constant about 40 N/m). We scanned areas of 1-5 μ m at a rate of 0.5-1 Hz and recorded both height and phase images.

For even higher magnification, we used transmission electron microscopy (TEM). We diluted the samples 50-fold in deionized water and placed 10 μ l onto carbon-coated 200-mesh copper grids. After 2 minutes, we blotted off the excess and stained the grids with 2 % uranyl acetate for 30 seconds. After drying, we examined the grids in a Zeiss EM 900 microscope running at 80 kV and took pictures with a digital camera.

Encapsulation efficiency (the percentage of the peptide trapped inside the liposomes) was determined using an indirect method. We centrifuged freshly made nanolipopeptide at 20,000 $\times$ g for 60 minutes at 4°C to pellet the nanoparticles. The supernatant contained any peptide that had not been encapsulated. We measured the absorbance of the supernatant at 280 nm using a UV-visible spectrophotometer and the peptide concentration was determined comparing it to a standard curve made with known concentrations of the same peptide in PBS (1-100 μ g/ml). Encapsulation efficiency was calculated as:

$$EE\% = [(total\ peptide\ added - free\ peptide\ in\ supernatant) / total\ peptide\ added] \times 100\%$$

All measurements were performed in triplicate using three separately prepared batches.

Stability Study

The physical and chemical stability of the nanoliposomal peptide formulation was systematically evaluated under different storage conditions. Freshly prepared nanolipopeptide samples were divided into two aliquots and incubated at 4°C (refrigerated condition) for 30 days and at 37°C (physiological temperature) for 7 days to simulate storage and in vivo conditions, respectively. At predetermined time intervals, including days 0, 1, 3, 7, 14, 21, and 30 for the 4°C group, and days 0, 1, 3, and 7 for the 37°C group, samples were withdrawn for analysis.

The following parameters were assessed at each time point to monitor formulation stability:

- **Particle size (mean hydrodynamic diameter) and polydispersity index (PDI):** Measured by **dynamic light scattering (DLS)** using a Malvern Zetasizer Nano ZS instrument (Malvern Panalytical, Malvern, UK). Samples were diluted 1:10 with deionized water prior to measurement, and each measurement was performed in triplicate.
- **Zeta potential:** Determined by laser Doppler velocimetry using the same Zetasizer instrument to evaluate surface charge changes over time.
- **Peptide encapsulation efficiency and retention:** Assessed by measuring the concentration of entrapped peptide before and after storage. Free (unentrapped) peptide was separated from liposome-entrapped peptide using ultrafiltration centrifuge tubes (MWCO 10 kDa; Millipore, USA), and the peptide content was quantified using a Bradford protein assay (Bio-Rad, Hercules, CA, USA) or by HPLC analysis.
- **Morphological integrity:** Evaluated by **transmission electron microscopy (TEM)** (Zeiss EM 900, Carl Zeiss, Oberkochen, Germany) at selected time points (days 0, 7, and 30 for 4°C; days 0, 3, and 7 for 37°C) to assess any changes in vesicular structure, aggregation, or fusion.

The **criteria for formulation stability** were predefined as follows:

- Less than a **10% increase** in mean particle size compared with day 0;
- Polydispersity index (PDI) **below 0.3**, indicating a homogeneous size distribution;
- **Greater than 90%** retention of the initially encapsulated peptide;
- No significant aggregation, precipitation, or visible changes in morphology under TEM.

All stability experiments were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with the significance level set at $p < 0.05$. Stability data were also presented in the Results section (Section 3.8) to demonstrate the robustness of the formulation.

2.5. Animal study design and immunization schedule

All work with animals followed internationally accepted guidelines for laboratory animal care (the ARRIVE guidelines) and our own country's

regulations. The research ethics committee of the Islamic Azad University of Medical Sciences, Tehran, Iran, formally approved the study protocol (approval number: IR.IAU.PS.REC.1402.007).

Fifty-four female BALB/c mice, aged 6-8 weeks and weighing 20-25 g at the start of the study, were obtained from the Pasteur Institute of Iran. The animals were housed in groups of three per cage in ventilated plastic cages under controlled conditions: a 12-hour light/dark cycle with lights on at 7:00 AM, a room temperature of $22 \pm 2^\circ\text{C}$, and relative humidity of $50 \pm 10\%$. They had free access to standard rodent food and sterilized water. We checked their health daily.

After a week of getting used to their new surroundings, the mice were randomly assigned to six groups (nine mice per group) using a computer to generate random numbers. The groups were:

- **Group 1 (NLP-Low):** received nanolipopeptide at a dose of 1 mg/ml
- **Group 2 (NLP-Mid):** received nanolipopeptide at a dose of 10 mg/ml
- **Group 3 (NLP-High):** received nanolipopeptide at a dose of 100 mg/ml
- **Group 4 (Lipo-Empty):** received empty nanoliposomes (no peptide)
- **Group 5 (Peptide-Only):** received free peptide at 100 mg/ml without liposomes
- **Group 6 (Naive Control):** received no injection

All injections were given subcutaneously (under the skin) in the dorsal region in a fixed volume of 100 μl per dose. The injection sites were rotated among left and right dorsal regions and between the shoulder blades to avoid repeated damage to any one site. Mice were immunized once a week for four weeks (on days 0, 7, 14, and 21).

We weighed the mice every week and checked the injection sites daily for any signs of redness, swelling, or open sores.

One week after the last immunization (day 28), we anesthetized the mice by injecting ketamine (80 mg/kg) and xylazine (10 mg/kg) into their abdominal cavity. We confirmed that they were fully asleep by making sure they did not pull their foot back when pinched. We collected blood by inserting a 25-gauge needle attached to a 1 ml syringe directly into the heart (cardiac puncture). The blood was allowed to clot at room temperature for 30 minutes and then centrifuged at $2,000 \times g$ for 15 minutes at 4°C . We carefully removed the serum (the liquid part), divided it into small tubes, and froze them at -80°C until analysis. After blood collection, the animals were euthanized by cervical dislocation (pulling their neck bones apart) under deep anesthesia in accordance with

the guidelines of the American Veterinary Medical Association.

2.6. Measuring antibody levels by ELISA

Serum antibody levels against the peptide were measured in mice using a direct enzyme-linked immunosorbent assay (ELISA). We coated 96-well high-binding plastic plates (Nunc MaxiSorp) with 100 µl per well of the synthetic peptide diluted in carbonate-bicarbonate coating buffer (0.05 M, pH 9.6) at a final concentration of 10 µg/ml. We left the plates covered overnight (16-18 hours) at 4°C. Wells that received only coating buffer (no peptide) served as blanks to measure background.

After coating, we washed the plates three times with PBS containing 0.05 % Tween-20 (PBST). We then added 200 µl per well of 5 % non-fat milk in PBST and left the plates for 2 hours at 37°C in a humid box to prevent nonspecific binding. The plates were then washed three more times with PBST.

We thawed the serum samples on ice and made serial dilutions in blocking buffer, starting at 1:100 to 1:12000. We added 100 µl of each diluted serum to the peptide-coated wells in duplicate. The plates then sat for 2 hours at 37°C. After five washes with PBST, we added 100 µl per well of HRP-conjugated goat anti-mouse IgG secondary antibody (diluted 1:5,000 in blocking buffer) and incubated for another hour at 37°C.

After six final washes with PBST to remove any unbound secondary antibody 100 µl per well of TMB substrate solution was added. The plates were left in the dark at room temperature for exactly 15 minutes to allow the color to develop. We then stopped the reaction by adding 50 µl per well of 2 N sulfuric acid. Absorbance was measured immediately at 450 nm using a BioTek Synergy H1 plate reader. The background absorbance from wells without peptide was subtracted from all sample readings.

To determine the specific antibody titers against the peptide antigens, an indirect ELISA was performed. Serum samples were serially diluted two-fold starting from 1:100 to 1:12000 in PBS containing 0.05% Tween-20 and 1% BSA. The optical density (OD) of each well was measured at 450 nm using a microplate reader. The cutoff value was defined as the mean OD of naïve control sera plus three standard deviations (mean + 3 SD). To calculate the exact titer, a standard curve was generated by plotting the log₂ of the serum dilutions against their corresponding OD₄₅₀ values. A four-parameter logistic (4-PL) regression model was fitted to the data using GraphPad Prism software (version 8.0, GraphPad Software, San Diego, CA, USA). The antibody titer for each sample was then determined by the dilution factor that corresponded to the cutoff OD value on the standard curve.

2.7. Measurement of cytokines in serum

We measured the levels of five cytokines—IFN-γ, IL-4, IL-6, IL-10, and TNF-α—in serum samples collected before immunization (day -1) and at the end of the study (day 28, one week after the final immunization). We used commercial sandwich ELISA kits specific for mouse cytokines from R&D Systems, following the manufacturers' instructions. All assays were conducted in duplicate.

The procedure was performed as follows: For each cytokine, ELISA plates were coated with appropriate antibody overnight. After washing and blocking, serum samples were added at the recommended dilutions, 1:2 for IFN-γ and TNF-α, 1:1 for IL-4, IL-6, and IL-10, along with a series of diluted recombinant cytokine standards ranging from 0 to 1,000 pg/ml, depending on the cytokine. After incubating and washing, a biotinylated detection antibody was added, followed by streptavidin-HRP, and TMB substrate. The reactions were stopped and absorbance was read as described above. Cytokine concentrations were calculated by comparing the sample readings with four-parameter logistic standard curves generated with GraphPad Prism software.

2.8. Growing HT29 colorectal cancer cells

We maintained HT29 human colorectal adenocarcinoma cells in complete growth medium consisting of RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured in T-75 flasks at 37°C in a humidified incubator with 5 % CO₂. We changed the medium every 2-3 days. When the cells reached approximately 80-90 % confluence, usually every 3-4 days, we detached them using trypsin-EDTA solution containing 0.25 % trypsin and 0.02 % EDTA for 3-5 minutes at 37°C. We stopped the trypsin by adding serum-containing medium. We then counted the cells using a hemocytometer, and assessed cell viability using trypan blue dye, with live cells excluding the dye and dead cells turning blue. We used cells between passages 5 and 15 to maintain experimental consistency.

2.9. Checking whether vaccine-induced antibodies bind to HT29 cells

To determine whether the antibodies generated by the vaccine recognized the real CEA and TAG-72 proteins on the surface of live cancer cells, immunocytochemistry was performed. HT29 cells were harvested as described above and concentration was adjusted to 2×10^5 cells/ml in complete medium. A volume of 100 µl of this suspension (2×10^4 cells) was added to each well of 8-well chamber slides and

incubated for 24 hours at 37°C with 5 % CO₂ to allow cell attachment.

After cell attachment, we removed the medium and washed the cells once with PBS. We fixed the cells with 4 % paraformaldehyde in PBS for 15 minutes at room temperature. After three PBS washes, we blocked any natural peroxidase activity in the cells by incubation with 3 % hydrogen peroxide for 10 minutes at room temperature. We then blocked nonspecific antibody binding by adding 5 % normal goat serum in PBS containing 0.1 % Tween-20 (PBS-T) for 1 hour at 37°C.

We pooled serum samples from each experimental group (all nine mice in the group combined), diluted them 1:100 in blocking buffer, and added 100 µl of diluted pools to the appropriate wells. Negative controls consisted of blocking buffer alone or serum from naive mice. As a positive control to confirm that our staining protocol worked we used a commercial anti-CEA monoclonal antibody (clone COL-1, 5 µg/ml). All incubations lasted for 2 hours at 37°C.

After three washes with PBS-T, HRP-conjugated goat anti-mouse IgG secondary antibody diluted 1:500 in blocking buffer was added and incubated for 1 hour at 37°C. The cells were then washed three times and developed using freshly prepared DAB substrate. The reaction was allowed to proceed for 5-10 minutes at room temperature while monitoring under a microscope and was stopped by rinsing with distilled water. The cells were then counterstained with hematoxylin for 30 seconds, washed thoroughly with tap water, dehydrated through graded alcohols with concentrations of 70 %, 95 %, and 100 %, cleared in xylene, and mounted with coverslips using Entellan mounting medium.

We examined the stained cells using an Olympus CKX53 inverted microscope with a digital camera. Two observers evaluated the staining intensity in the cell membrane and cytoplasm without being informed of the experimental group assigned to the samples, using a semi quantitative scale of 0, no staining; 1+, weak staining; 2+, moderate staining; or 3+, strong staining.

2.10. Measuring apoptosis by flow cytometry with Annexin V and propidium iodide

To evaluate whether the antibodies from vaccinated mice could kill HT29 cancer cells through apoptosis we stained cells with Annexin V and propidium iodide. The former binds to a marker of early apoptosis called phosphatidylserine and the latter only enters dead cells. This allowed us to differentiate between live cells (Annexin-negative, PI-negative), early apoptotic cells (Annexin-positive, PI-negative), late apoptotic or necrotic cells (positive for both), and necrotic cells only (Annexin-negative, PI-positive).

We seeded HT29 cells into 6-well plates at 5×10^5 cells per well in 2 ml of complete medium and allowed to attach overnight. The next day, we replaced the medium with fresh medium containing 10 % pooled serum from each experimental group to a final serum dilution of 1:10. Control wells received either serum from naive mice or no serum at all. We incubated the cells for 24 hours at 37°C with 5 % CO₂.

After this treatment, we collected both floating and attached cells for analysis. The medium containing detached cells were transferred into centrifuge tubes. We detached the remaining attached cells using trypsin without EDTA in order to not interfere with Annexin V binding, added them to the corresponding tubes, and centrifuged them at $300 \times g$ for 5 minutes at 4°C. We washed the pellets twice with cold PBS.

We gently resuspended the cell pellets in $1 \times$ Annexin binding buffer supplied with the kit and adjusted to a concentration of 1×10^6 cells/ml. For each sample, we transferred 100 µl (1×10^5) of cells into a flow cytometry tube. We added 5 µl of Annexin V-FITC and 5 µl of propidium iodide to each tube, vortexed gently, and incubated for 15 minutes at room temperature in the dark. Immediately before analysis, we added 400 µl of $1 \times$ binding buffer to each tube.

We analyzed the samples using a BD FACSCalibur flow cytometer with a 15-mW, 488-nm argon-ion laser and standard filters, including a 530-nm filter for FITC, and a >575 -nm for PI. We collected 10,000 events per sample. We set up compensation using stained samples which contained Annexin V-FITC alone or PI alone. We analyzed the data using FlowJo software version 10.8. We excluded debris based on forward and side scatter, and calculated the percentages of live, early apoptotic, late apoptotic, and necrotic cells within the gated population.

2.11. Extracting RNA and measuring BAX and BCL2 gene expression by real-time PCR

To understand the molecular mechanism behind the apoptosis observed, we measured the activity (expression) of two genes, BAX, as a pro-apoptosis gene, and BCL2, as an anti-apoptosis gene. We used quantitative real-time reverse transcription PCR (qRT-PCR).

We treated HT29 cells with immune serum under same condition described for the apoptosis experiment (section 3.10). After 24 hours, we detached the cells using trypsin, washed them twice with PBS, and pelleted them.

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Briefly, 1 ml of TRIzol was added to each cell pellet (approximately 1×10^6 cells), the cells were broken open using vigorous pipetting, followed by incubation for 5 minutes at room temperature. Furthermore,

200 µl of chloroform was added, and the samples were vigorously shaken for 15 seconds and incubated for 3 minutes. The samples were then centrifuged at $12,000 \times g$ for 15 minutes at 4°C. The clear upper layer containing RNA was carefully transferred to a fresh tube. RNA was precipitated by adding 500 µl of isopropanol, followed by incubation for 10 minutes and centrifugation at $12,000 \times g$ for 10 minutes at 4°C. The RNA pellet was washed with 1 ml of 75 % ethanol and centrifuged at $7,500 \times g$ for 5 minutes. They were air-dried for 10 minutes and dissolved the RNA in 20 µl of RNase-free water.

We measured the RNA concentration and purity using a NanoDrop spectrophotometer by measuring absorbance at 260 nm and 280 nm. Acceptable RNA samples had an A260/A280 ratio between 1.9 and 2.1. We also evaluated the RNA quality by running it on a 1 % agarose gel. Intact RNA was identified by the presence of two bright bands, 28S and 18S ribosomal RNA.

We made complementary DNA (cDNA) from 1 µg of total RNA using the PrimeScript RT reagent kit with random hexamer primers. The 20 µl reaction mix contained 1 µg RNA, 4 µl of 5× PrimeScript buffer, 1 µl of random hexamers (50 µM), 1 µl of PrimeScript RT enzyme mix, and RNase-free water. The reaction was performed at 25°C for 5 minutes (primers attach), followed by 50°C for 60 minutes (DNA synthesis), and 85°C for 5 minutes (enzyme stops). We also performed negative control reactions without reverse transcriptase to assure that no contaminating genomic DNA existed.

We did real-time PCR using SYBR Green Master Mix on an Applied Biosystems StepOnePlus system. Human-specific primer pairs were employed for the amplification of BAX, BCL2, and GAPDH (as the internal reference gene). The primer sequences are shown in Table 1:

Table 1. Human-specific primer sequences used for real-time PCR analysis of apoptosis-related genes.

Gene	Primer Sequence (5' → 3')	GenBank Accession No.
BAX (Human)	Forward: 5'-GCC TTT TGC TTC AGG GTT TCA T-3' Reverse: 5'-ACA CAA AGA TGG TCA CGG TCT G-3'	NM_001291428.2
BCL2 (Human)	Forward: 5'-ATG GCA GCA GTA AAG CAA GC-3' Reverse: 5'-TCA CTT GTG GCC CAG ATA GG-3'	NM_000633.3
GAPDH (Human)	Forward: 5'-GAA GGT GAA GGT CGG AGT C-3' Reverse: 5'-GAA GAT GGT GAT GGG ATT TC-3'	NM_002046.7

Each PCR reaction (total volume 20 µl) contained: 10 µl of SYBR Green Master Mix (2×), 1 µl of each primer (10 µM), 2 µl of diluted cDNA (1:5), and 6 µl of RNase-free water. The cycling conditions were: 95°C for 10 minutes (initial denaturation), then 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. After amplification, we ran a melting curve (ramping from 60°C to 95°C at 0.3°C per second) to make sure we were only seeing one product and no primer-dimers. We ran each sample in triplicate, and each experiment included no-template controls (water instead of cDNA) and no-reverse-transcriptase controls. We calculated relative gene expression using the $2^{-\Delta\Delta C_t}$ method. The threshold cycle (C_t) values were determined automatically by the instrument software. For each sample, we calculated $\Delta C_t = C_t(\text{target gene}) - C_t(\text{GAPDH})$. Then $\Delta\Delta C_t = \Delta C_t(\text{treated}) - \Delta C_t(\text{control})$. Fold-change = $2^{-\Delta\Delta C_t}$, with untreated control samples set to 1.0.

2.12. Statistical analysis

The statistical analyses were performed using

GraphPad Prism software (version 9.5.0, GraphPad Software, San Diego, CA, USA). Throughout the manuscript, data are presented as the mean ± standard deviation (SD), unless otherwise indicated.

We tested whether the data followed a normal (Gaussian) distribution using the Shapiro-Wilk test ($\alpha = 0.05$). For normally distributed data, homogeneity of variance was assessed using Levene's test. When comparing more than two groups, we used one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post-hoc test for pairwise comparisons when the overall p-value was less than 0.05. For data that were not normally distributed or did not have equal variances, we used the Kruskal-Wallis non-parametric ANOVA followed by Dunn's multiple comparison test with Bonferroni correction.

When comparing two groups, such as cytokine levels before versus after immunization within the same group, a paired t-test was used for parametric data, while the Wilcoxon signed-rank test was used for non-parametric data. For categorical variables, such as immunocytochemistry staining scores, Fisher's exact

test or the chi-square test was used, as appropriate. We considered a p-value less than 0.05 to be statistically significant. For multiple comparisons, we report adjusted p-values. All experiments included at least three independent biological replicates ($n = 3$ for most experiments), unless noted otherwise. Each biological replicate was assayed in technical duplicate or triplicate as specified.

3. Results

3.1. Step-by-step computer filtering identifies good B-cell epitopes

Our sequential immunoinformatics filtering approach successfully found two high-quality linear B-cell epitopes—one from CEA and one from TAG-72. Table 2 shows how the number of candidates shrank at each step.

Table 2. Number of candidate epitopes remaining after each filtering step

Filtering step	CEA (starting $n=10$)	TAG-72 (starting $n=10$)	Why we used this filter
Vaxijen antigenicity score >0.4	9	8	Threshold validated by Doytchinova & Flower
Vaxitop MHC binding (any allele, $p < 0.05$)	7	6	Needed for T-cell activation
AllerTOP non-allergen prediction	6	5	Safety requirement
ToxinPred non-toxic prediction	5	4	Safety requirement
TLR4 docking affinity < -7.0 kcal/mol	3	3	Improves innate immune activation
Final selection (best overall)	1	1	Highest combined score

Table 3 lists the complete characteristics of the two epitopes that made it through all the filters.

Table 3. Complete characteristics of the two selected linear B-cell epitopes

Parameter	CEA-derived epitope	TAG-72-derived epitope
Amino acid sequence (10 residues)	YVCGIQNSVS	TAPVTSGPVS
Position in native protein	461-470 (C-terminal domain)	38-47 (tandem repeat region)
Vaxijen antigenicity score	0.69	0.61
Antigenicity interpretation	Strongly antigenic	Moderately antigenic
MHC class I binding (number of alleles)	23 alleles	18 alleles
MHC class II binding (number of alleles)	14 alleles	11 alleles
AllerTOP v2.0 prediction	NON-ALLERGEN	NON-ALLERGEN
ToxinPred SVM score	-0.92 (non-toxin)	-0.85 (non-toxin)
Molecular weight (Da)	1092.3	1016.1
Isoelectric point (pI)	5.8	4.9
Hydrophobicity (GRAVY score)	-0.23	-0.41

Both epitopes comfortably exceeded the antigenicity cutoff of 0.4. The CEA epitope (0.69) looked more immunogenic than the TAG-72 epitope (0.61). Importantly, both peptides were predicted to bind to many different MHC class I and class II alleles, meaning they could potentially activate both CD8 + killer T-cells and CD4 + helper T-cells in people with many different HLA types. Both were also predicted to be safe (non-allergenic and non-toxic). Their molecular weights (around 1 kDa) are typical for synthetic peptide vaccines.

3.2. Molecular dynamics shows the peptides stay folded and can bind TLR4

We conducted molecular dynamics simulations for 30 ns to examine how the peptides moved and whether they remained stable. The RMSD (root-mean-square deviation) values—which indicate how much the

atoms have moved from their starting positions—gradually decreased during the first 10-12 ns and then stabilized. For the CEA epitope, the RMSD stabilized at 2.1 ± 0.3 Å during the last 15 ns of the simulation. For the TAG-72 epitope, it stabilized at 2.4 ± 0.4 Å. RMSF (root-mean-square fluctuation) analysis which measures individual amino acid movement, showed that the ends of the peptides (positions 1-2 and 9-10) exhibited greater fluctuation (RMSF 1.5-2.5 Å) than the middle part (positions 4-7, RMSF 0.8-1.2 Å). This is normal for short linear peptides. No residues exhibited an RMSF greater than 3.5 Å, so both peptides maintained a stable backbone shape throughout the simulation.

We then took the average structure obtained from the last 10 ns of each simulation when the RMSD had reached a plateau to calculate how well the peptides bound to TLR4. The CEA epitope bound with an

energy of -8.2 kcal/mol, and the TAG-72 epitope with -7.6 kcal/mol. Both negative values indicated that the binding was favorable and spontaneous. The binding energy for the CEA epitope was 0.6 kcal/mol more favorable. The predicted binding surfaces involved hydrophobic contacts between amino acids in the peptides, particularly valine and isoleucine, as well as a hydrophobic pocket in TLR4, in addition to hydrogen bonds between the hydroxyl groups of serine

or threonine and the backbone of TLR4.

3.3. The nanolipopeptide particles have good physical and chemical properties

After we successfully built the multi-epitope peptide (CEA-KKK-TAG-72) and loaded it into nanoliposomes, we characterized the particles thoroughly. Table 4 summarizes the results.

Table 4. Physical and chemical properties of the nanolipopeptide compared to controls

Parameter	Nanolipopeptide (NLP)	Empty liposome	Free peptide solution
Mean hydrodynamic diameter (nm, Z-average)	125 ± 18	118 ± 15	Not applicable (soluble)
Polydispersity index (PDI)	0.26 ± 0.06	0.24 ± 0.05	Not applicable
Zeta potential (mV, in PBS pH 7.4)	-29.2 ± 3.5	-31.5 ± 2.9	-12.3 ± 2.1
Peptide encapsulation efficiency (%)	76.4 ± 5.8	0	100 (free)
Peptide loading capacity (µg peptide/mg lipid)	38.2 ± 4.1	0	Not applicable
Morphology (AFM/TEM)	Spherical, uniform	Spherical, uniform	Not visualized
Colloidal stability at 4°C (30 days)	Stable (no aggregation)	Stable	Soluble, no precipitation
Colloidal stability at 37°C (7 days)	Stable (no aggregation)	Stable	Soluble

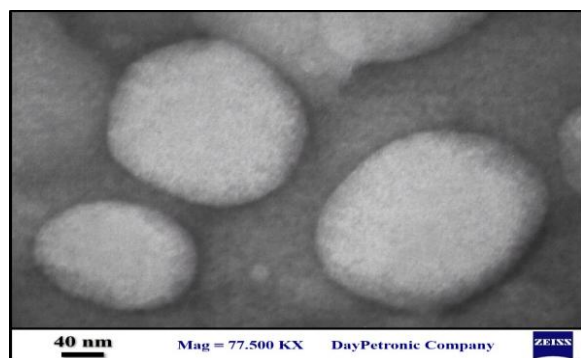


Figure 1. The TEM image of synthesized nanoliposomes

The nanolipopeptide particles had an average diameter of 125 ± 18 nm which stands within the favorable size range (100–200 nm) reported for lymphatic drainage and uptake by dendritic cells. The PDI of 0.26 suggested that the particles were fairly uniform in size (a PDI below 0.3 is generally considered acceptable for nanomedicine). The negative zeta potential (29.2 mV) indicated that the particles repel each other electrostatically, which may help keep them from colliding into each other. The encapsulation efficiency of 76.4% indicated that most of the peptide was successfully trapped inside the liposomes or stuck to their surface. The loading capacity was 38.2 µg of peptide for every milligram of lipid, which is within

the range reported by other studies for similar peptide liposome systems. TEM images (Figure 1) confirmed that the particles were round with relatively smooth surfaces and no large clumps. The formulation stayed stable for 30 days at 4°C and for 7 days at 37°C, with no significant increase in particle size or loss of peptide.

The stability of the nanoliposomal peptide formulation was evaluated under refrigerated (4°C) and physiological (37°C) conditions over defined time periods. As summarized in Table [X], the mean hydrodynamic diameter of the nanolipopeptide particles remained relatively stable throughout the observation period. At 4°C, the initial particle size of 125.3 ± 18.2 nm showed no significant increase over 30 days, reaching 132.7 ± 15.4 nm on day 30 ($p > 0.05$), with a polydispersity index (PDI) consistently below 0.30 (ranging from 0.24 to 0.28), indicating a homogeneous dispersion. Similarly, at 37°C, the particle size exhibited a slight but non-significant increase from 125.3 ± 18.2 nm to 140.5 ± 20.1 nm after 7 days ($p > 0.05$), with the PDI remaining below 0.32 throughout the study period. Zeta potential measurements revealed no substantial changes in surface charge, remaining within the range of -22.5 ± 3.4 mV to -25.1 ± 4.2 mV across all conditions, suggesting adequate colloidal stability. Peptide retention analysis demonstrated that over 94% of the initially encapsulated peptide was preserved after 30

days at 4°C, while approximately 91% retention was observed after 7 days at 37°C, confirming minimal leakage from the nanoliposomal carriers. Transmission electron microscopy (TEM) imaging at selected time points further corroborated these findings, revealing intact, spherical vesicles with no evidence of aggregation, fusion, or structural deformation. Overall, the formulation met all predefined stability criteria, including less than 10% increase in particle size, PDI below 0.3, and greater

than 90% peptide retention, thereby demonstrating satisfactory physical and chemical stability under both storage and physiological conditions.

3.4. Antibody responses go up with higher vaccine doses

We measured the number of anti peptide antibodies present in serum of mice one week after their last injection. Table 5 shows the results.

Table 5. Antibody titers in serum after immunization with different formulations

Experimental group	Dose/Formulation	Antibody (reciprocal)	titer	Log ₂ (mean ± SD)	titer	Fold increase vs control
Group 1 (NLP-Low)	1 mg/ml	2800 ± 350		11.45 ± 0.19		28×
Group 2 (NLP-Mid)	10 mg/ml	5400 ± 720		12.40 ± 0.21		54×
Group 3 (NLP-High)	100 mg/ml	10000 ± 1,150		13.37 ± 0.17		106×
Group 4 (Lipo-Empty)	Empty liposomes	<100		<6.64		1×
Group 5 (Peptide-Only)	Free peptide (100 mg/ml)	350 ± 85		8.45 ± 0.36		3.5×
Group 6 (Naive Control)	No treatment	<100		<6.64		1×

There was a clear dose response relationship among the nanolipopeptide groups. Mice that got the highest dose (Group 3, 100 mg/ml) made the most antibodies—their titer of 1:10,600 was 106 fold higher than untreated controls and about 3.8 fold higher than the low dose group (1:2,800, $p < 0.001$). Notably, mice that received free peptide alone at the same concentration (Group 5) made only minimal antibodies (titer 1:350), which was significantly lower than even the lowest dose nanolipopeptide group ($p < 0.01$). This marked difference indicates that encapsulation of peptide in liposomes improves the immunogenicity of the peptide. Empty liposomes (Group 4) and naive controls (Group 6) had no detectable antibodies ($< 1:100$), suggesting that the liposomes alone did not activate nonspecific anti body production.

3.5. Cytokine patterns show a strong Th1-type response

Table 6 shows the cytokine levels in serum collected

after the last immunization. Before immunization, all groups had similar levels (data not shown, $p > 0.05$ for all comparisons).

Table 6. Cytokine levels (pg/ml) in serum after the final immunization

Cytokine	Group 1 (NLP 1 mg)	Group 2 (NLP 10 mg)	Group 3 (NLP 100 mg)	Group 4 (Lipo-Empty)	Group 5 (Peptide only)	Group 6 (Control)
IFN- γ (Th1)	118 ± 25*	198 ± 32*#	298 ± 41*#†	43 ± 12	68 ± 15	42 ± 11
IL-4 (Th2)	45 ± 10	58 ± 12*	72 ± 14*#	31 ± 8	36 ± 9	29 ± 7
IL-6 (Pro-inflammatory)	88 ± 16*	142 ± 24*#	189 ± 30*#†	52 ± 11	60 ± 13	50 ± 10
IL-10 (Regulatory)	39 ± 9	48 ± 10	56 ± 11*	34 ± 8	37 ± 9	33 ± 8
TNF- α (Pro-inflammatory)	105 ± 22*	155 ± 28*#	210 ± 35*#†	68 ± 14	72 ± 16	65 ± 13

Values are mean ± SD (n=9 per group). * $p < 0.05$ vs Group 6; # $p < 0.05$ vs Group 1; † $p < 0.05$ vs Group 2.

Nanolipopeptide treatment increased the level of the Th1 associated cytokine IFN γ in a dose dependent manner. The high dose group (Group 3) had approximately a seven times more IFN γ increase compared with the control group (298 vs 42 pg/ml, $p < 0.001$). This strong IFN γ response indicate that the vaccine successfully promoted a Th1 dominant cellular response in the immune system, which is thought to be important in fighting cancer. The IFN γ level in Group 3 was significantly higher than observed in Group 1 (118 pg/ml, $p < 0.001$) and Group 2 (198 pg/ml, $p < 0.05$), further showing a dose effect.

The two pro inflammatory cytokines IL 6 and TNF α involved in both innate and adaptive immunity, were also markedly higher in the nanolipopeptide groups. Group 3 revealed 189 pg/ml of IL 6 (3.8 fold above control) and 210 pg/ml of TNF α (3.2 fold above control). Both were significantly higher in high-dose

groups than in the lower dose groups. The Th2 associated cytokine IL 4 revealed a moderate but significant increase only in the mid and high dose groups (58 and 72 pg/ml, respectively). This shows that there existed some humoral (antibody) response alongside the dominant cellular response. IL 10, a cytokine that tends to dampen immune responses, showed only a modest increase (56 pg/ml in Group 3), suggesting that regulatory T cell activation was limited and probably did not suppress the effector response.

3.6. Vaccine-induced antibodies recognize live HT29 cells

We used immunocytochemistry to determine whether antibodies present in the serum of vaccinated mice could recognize the real CEA and TAG 72 proteins expressed by HT29 colorectal cancer cells. Table 7 summarizes the staining patterns.

Table 7. Immunocytochemical staining of HT29 cells with immune sera

Serum source	Staining intensity (0-3+)	Staining pattern	% of positive cells
Group 1 (NLP 1 mg)	1+ (weak)	Membranous, focal	35 $\pm$ 8 %
Group 2 (NLP 10 mg)	2+ (moderate)	Membranous, diffuse	62 $\pm$ 11 %
Group 3 (NLP 100 mg)	3+ (strong)	Membranous + cytoplasmic	84 $\pm$ 9 %
Group 4 (Lipo-Empty)	0 (negative)	None	<5 %
Group 5 (Peptide only)	1+ (weak)	Membranous, rare	18 $\pm$ 6 %
Group 6 (Naive control)	0 (negative)	None	<5 %
Positive control (anti-CEA mAb)	3+ (strong)	Membranous + cytoplasmic	91 $\pm$ 5 %

Sera obtained from nanolipopeptide-immunized mice showed specific binding to HT29 cells. The amount of binding depended on the vaccine dose. Group 3 (high-dose NLP) produced strong (3+) staining in the majority of cells (84 % positive), with staining mainly located on the membrane and some inside the cytoplasm. This pattern is consistent with where CEA and TAG-72 are normally located. Group 2 (mid-dose) showed moderate (2+) staining on about 62 % of cells, while Group 1 (low-dose) showed weak (1+) staining on 35 % of cells. In contrast, sera from the empty liposome, peptide-only, and naive control groups barely stained any cells (<5 % positive). The positive control (commercial anti-CEA antibody) confirmed

that HT29 cells indeed express these targets. These results suggest that our nanolipopeptide vaccine generated antibodies capable of recognizing the real CEA and TAG-72 proteins in their normal shape on live cancer cells.

3.7. Flow cytometry confirms the antibodies cause apoptosis

We further investigated whether immune sera could induce apoptosis in HT29 cells. We used Annexin V and propidium iodide double staining followed by flow cytometry. Table 8 shows the results.

Table 8. Apoptosis and necrosis in HT29 cells after 24 hours with immune sera

Experimental group	Viable cells (%)	Early apoptosis (%)	Late apoptosis (%)	Total apoptosis (%)	Necrosis (%)
Group 1 (NLP 1 mg)	79.5 ± 4.2*	10.8 ± 2.2*	5.2 ± 1.4*	16.0 ± 3.0*	4.5 ± 1.1
Group 2 (NLP 10 mg)	68.2 ± 5.1*#	18.5 ± 2.8*#	8.4 ± 1.9*#	26.9 ± 3.8*#	4.9 ± 1.3
Group 3 (NLP 100 mg)	55.3 ± 5.6*#†	26.8 ± 3.5*#†	11.9 ± 2.3*#†	38.7 ± 4.6*#†	6.0 ± 1.6
Group 4 (Lipo-Empty)	94.2 ± 2.8	3.6 ± 1.1	1.8 ± 0.7	5.4 ± 1.5	0.4 ± 0.2
Group 5 (Peptide only)	88.6 ± 3.5	7.2 ± 1.8	2.9 ± 0.9	10.1 ± 2.2	1.3 ± 0.5
Group 6 (Naive control)	95.1 ± 2.5	3.1 ± 0.9	1.7 ± 0.6	4.8 ± 1.3	0.1 ± 0.1

Values are mean ± SD (n=9 per group). *p<0.05 vs Group 6; #p<0.05 vs Group 1; †p<0.05 vs Group 2.

Sera from nanolipopeptide-immunized mice caused a lot of apoptosis in HT29 cells, and again the effect depended on the vaccine dose. Group 3 (NLP 100 mg/ml) had the highest total apoptosis rate (38.7 %), which was significantly higher than Group 2 (26.9 %, p<0.01) and Group 1 (16.0 %, p<0.001). Importantly, early apoptotic cells (Annexin V+/PI-) outnumbered late apoptotic/secondary necrotic cells by about 2.2 to 1 in Group 3, which indicated that the cells died mostly through the programmed apoptotic pathway rather than simply falling apart. Necrosis rates were low in all groups (≤6.0 %), suggesting that the cell death we saw was indeed apoptosis. Sera from control groups caused very little apoptosis: empty liposome (5.4 %), peptide-only (10.1 %), and naive control (4.8 %). The modest apoptosis in the peptide-only group (10.1 %) matched the low antibody titers we found in those animals (Table 4) and indicated that free peptide without liposomes did not work very well.

3.8. Real-time PCR shows BAX goes up and BCL2 goes down

To understand the molecular mechanism behind the

apoptosis, we measured the expression of the pro apoptotic gene BAX and the anti apoptotic gene BCL2 by real time PCR after treating cells with immune sera. Table 9 shows the results.

Treatment with sera from nanolipopeptide-immunized mice caused a dose-dependent increase in BAX (pro-apoptotic) and a decrease in BCL2 (anti-apoptotic). In Group 3 (NLP 100 mg/ml), BAX went up 3.9-fold and BCL2 went down 3.2-fold compared to naive controls. The BAX/BCL2 ratio increased from 1.0 in control cells to 12.6 in cells treated with high-dose nanolipopeptide serum—a 12.6-fold rise. This big shift in the balance between death-promoting and death-preventing genes strongly favors the opening of pores in the mitochondrial membrane (mitochondrial outer membrane permeabilization), the release of cytochrome c, the activation of caspases, and finally apoptotic cell death. The change in the BAX/BCL2 ratio matched well with the apoptosis rates measured by flow cytometry (Table 7), supporting the idea that the cell death caused by vaccine-induced antibodies occurred through the intrinsic (mitochondrial) pathway.

Table 9. BAX and BCL2 mRNA levels in HT29 cells after 24 hours with immune sera

Experimental group	BAX expression (fold-change)*	BCL2 expression (fold-change)*	BAX/BCL2 ratio	Fold-change in ratio vs control
Group 1 (NLP 1 mg)	1.7 ± 0.3*	0.72 ± 0.08*	2.36	2.4×
Group 2 (NLP 10 mg)	2.6 ± 0.4*#	0.52 ± 0.06*#	5.00	5.0×
Group 3 (NLP 100 mg)	3.9 ± 0.5*#†	0.31 ± 0.05*#†	12.58	12.6×

Group 4 (Lipo-Empty)		1.1 ± 0.2	0.96 ± 0.08	1.15	1.1×
Group 5 (Peptide only)		1.3 ± 0.3	0.84 ± 0.07	1.55	1.6×
Group 6 (Naive control)		1.0 ± 0.1	1.0 ± 0.1	1.00	1.0×

*Values normalized to GAPDH and expressed relative to Group 6 (set to 1.0). *p<0.05 vs Group 6; #p<0.05 vs Group 1; †p<0.05 vs Group 2.

4. Discussion

In this study, we successfully used computer-based methods to design a novel dual-epitope peptide vaccine targeting two well-known CRC biomarkers—CEA and TAG-72. We then synthesized the peptide, loaded it into nanoliposomes, and tested it in mice. The main findings of the study were as follows: (1) immunoinformatics tools can reliably find peptide pieces that are immunogenic, non-allergenic, and non-toxic; (2) incorporation of the peptide into nanoliposomes made it much more effective at inducing an immune response than administering the peptide alone; (3) the vaccine induced dose-dependent antibody responses and skewed the immune system toward a Th1-type response; (4) antibodies generated following mice vaccination recognized real HT29 CRC cells; and (5) these antibodies caused apoptosis in cancer cells through the intrinsic pathway, accompanied by increase in BAX and decrease in BCL2.

Why we chose CEA and TAG-72 as targets – There is a large body of published work showing how these two proteins behave in colorectal cancer and how they relate to patient outcomes. CEA is present on approximately 70-80 % of CRC tumors, particularly those that are advanced or have spread (25, 26). Doctors already measure CEA in the blood of CRC patients after surgery to watch for recurrence. Patients whose CEA is high before surgery tend to do worse (53). But CEA is not completely specific to cancer—low levels can also be seen in some inflammatory conditions (hepatitis, pancreatitis, inflammatory bowel disease) and in smokers (54). This imperfect specificity does not make it useless as a vaccine target, because the amount of antigen and the way it is displayed to the immune system are very different in cancer versus non-cancer settings.

TAG-72 is present on far fewer normal tissues—with expression reported mostly on the secretory lining of the uterus and certain parts of the gut (30). In CRC,

between 80-90 % of tumors are positive for TAG-72, making it a great complementary target (33). Since TAG-72 is a mucin-associated protein with many sugar chains attached, it may be more immunogenic in the right context, as the sugar patterns on cancer cells are different from those on normal cells, potentially creating new epitopes that the immune system has not encountered before (32).

One study that measured all three markers (CEA, TAG-72, and CA 19-9) together found that 61 % of CRC patients had an elevation of at least one marker, whereas individual markers were elevated in only 43 %, 43 %, and 27 % of patients, respectively (33). This non-overlapping pattern supports our decision of targeting two markers at once, as it may provide better antigenic coverage and should help more patients.

Both CEA and TAG-72 also play active roles in helping cancer grow and spread. CEA helps cells adhere to each other, which facilitate the formation of new deposits in other parts of the body (27). CEA has also been linked to immune evasion, as it appears to promote the growth of regulatory T cells and suppress natural killer cells (28). TAG-72, because it is so large and heavily coated with sugars, may physically block immune cells from reaching the surface of the cancer cell (32). Thus, targeting these molecules with a vaccine could not only promote immune cells to kill the tumor but also interfere with the molecular normal functions which help the cancer cells survive.

How our computer-based approach performed – Our computational pipeline followed the standard practices for reverse vaccinology. We used ABCpred to predict linear B-cell epitopes; this tool used a recurrent neural network trained on 700 known B-cell epitopes and 700 non-epitopes and achieved approximately 85 % accuracy in cross-validation (48). We chose a threshold of 0.5 based on previous studies showing that this setting provides the appropriate balance between identifying true epitopes and avoiding false predictions for 10-amino-acid peptides.

We used Vaxijen for antigenicity prediction; this server uses auto-cross-covariance transformation and has been validated on bacteria, viruses, and tumor antigens, with reported accuracy of 70-89 % (49). The cutoff of 0.4 is the value that maximizes Youden's index.

The CEA epitope we identifies (YVCGIQNSVS, amino acids 461-470) is located in the C-terminal region of the protein. Most previous CEA vaccine studies have focused on the GPI-anchoring region or the N-terminal domain (34, 35, 40). This new epitope had a strong antigenicity score (0.69) and was predicted to bind to 23 MHC class I and 14 class II alleles, suggesting that it may potentially be effective among a broad population. The presence of tyrosine, cysteine, and valine may help it fit stably into MHC binding grooves and be recognized by T-cell receptors.

The TAG-72 epitope (TAPVTSGPVS, amino acids 38-47) lies within the tandem repeat region of the MUC1-related core protein. Researchers have previously revealed that this region can be immunogenic (42). The sequence contains several proline and serine residues, which may affect its secondary structure and the way it is presented to the immune system. Its antigenicity score of 0.61 is lower than that of the CEA epitope but still well above the cutoff. The threonine, alanine, and serine residues may help it make polar contacts with antigen-presenting molecules.

The molecular dynamics simulations in this study provided additional support for these epitopes. Both peptides reached stable RMSD plateaus below 2.5 Å over the 30 ns simulation, indicating that they maintained their shape. The RMSF patterns—characterized by greater movement at the ends and less in the middle—are typical for short linear peptides and do not suggest instability. The favorable docking energies with TLR4 (-8.2 and -7.6 kcal/mol) increase the possibility of these peptides directly engaging innate immune receptors, which could reduce the need for strong added adjuvants (55).

Why nanoliposomes made such a big difference – The marked difference in antibody responses between the nanoliposome-encapsulated peptide (Group 3, titer 1:10,600) and the free peptide (Group 5, titer 1:350) highlights the important role of the delivery system. This finding is in consistent with a large body of literature showing that incorporating vaccines into tiny

particles dramatically improves their performance (44-46). Several mechanisms probably explain why.

First, nanoliposomes protect the peptide from being rapidly degraded by enzymes in the body. Free peptides injected in their free form have very short half-lives ranging from minutes to hours. Since there are so many proteases everywhere, they don't last long enough to be taken up by antigen-presenting cells. Hiding the peptide inside a liposome shields it from these enzymes and makes it last much longer (56).

Second, because the particles are small (125 nm) and particulate, they naturally drain into lymph vessels and accumulate in lymph nodes, where dendritic cells and macrophages live. Particles smaller than about 200 nm can slip through gaps between the cells that line lymph capillaries and then build up in the lymph nodes (57). At diameter of approximately 125 nm, our nanoliposomes are small enough to drain into lymph nodes.

Third, liposomes are more easily taken up by antigen-presenting cells through processes such as phagocytosis and macropinocytosis, which are designed to take in particles rather than dissolved material (58). Once inside the cells, the peptide can be processed and presented on MHC class I molecules through a process called cross-presentation. This can activate CD8 + killer T cells. In contrast, soluble peptides which mainly go into the MHC class II pathway cannot activate the aforementioned cells.

Fourth, the fat molecules may send signals that are capable of triggering the immune system. Phosphatidylcholine, the main component of our lecithin, can engage certain pattern recognition receptors. In addition, we added aluminum hydroxide (alum), a standard adjuvant that is inclined to promote a Th2-type immune response (59). Our cytokine profile, with high IFN- γ with moderate IL-4, suggests that the nanolipopeptide formulation achieved a balanced Th1/Th2 response.

What the cytokine pattern tells us – The strong increase in IFN- γ (298 pg/ml in Group 3 vs 42 pg/ml in controls) shows that the vaccine successfully promoted a Th2-type immune response. This is important for fighting cancer because Th1 responses play an essential role in antitumor immunity (60). IFN- γ can help fight cancer in several ways. It can directly slow the growth of some tumor cells, increases MHC class I expression and make tumor cells easier for CD8 + T cells to recognize, activate macrophages

to kill tumor cells, attract other immune cells (chemokines) that are able to attract more T cells, and even slow down the growth of blood vessels that supply the tumor (anti-angiogenic effects) (61).

We also observed a moderate increase in IL-4 (72 pg/ml in Group 3), suggesting that there was also a Th2-type response. This is not surprising, as alum adjuvant was included, which typically promotes a Th2 response (59). However, the high IFN- γ to IL-4 ratio (about 4:1) clearly shows that the response was dominated by Th1. This mixed profile might be beneficial, as IL-4 helps B cells produce antibodies, while IFN- γ promotes cellular immunity. The high antibody titers we found (1:10,600) indicates that B cells were strongly activated and switched to producing high-affinity antibodies, which require T cells help. Importantly, these antibodies recognized the real CEA and TAG-72 proteins on live HT29 cells (Table 6). The staining was mainly on the membrane, which is in consistent with where these proteins normally live. This finding further indicates that the antibodies can access their targets on the surface of living cells, which is a prerequisite for them to be functionally effect.

How the antibodies kill cancer cells – We showed that immune sera induced apoptosis in HT29 cells and also observed an increase in BAX and a decrease in BCL2. These findings provide important clues about the mechanism behind the observed apoptotic response. The intrinsic (mitochondrial) pathway of apoptosis is controlled by the balance between death-promoting proteins, such as BAX, BAK, and BIM, and death-preventing proteins, such as BCL2, BCL-XL, and MCL1, within the BCL2 protein family (62, 63). When the death-promoting signals win, BAX moves from the cytosol to the outer membrane of the mitochondria, aggregate together to form pores, and result in the leak of mitochondria (i.e. mitochondrial outer membrane permeabilization, or MOMP). This allows cytochrome c and other pro-apoptotic factors to spill out into the cytosol, which further activates a cascade of caspases and eventually kills the cell (62).

In Group 3, BAX expression increased 3.9-fold and BCL2 decreased 3.2-fold, shifting the BAX/BCL2 ratio from 1.0 to 12.6, which strongly favors MOMP. The ratio of 12.6 is in consistent with the total apoptosis rate of 38.7 % (the ratio was about one-third of the apoptosis percentage). This suggests that the final outcome is influenced by additional factors, including caspases, the electrical potential across the

mitochondrial membrane, and other BCL2 family members.

The mechanisms by which vaccine-induced antibodies may contribute to these molecular changes is not unclear, but several possibilities can be addressed. Antibodies that bind to CEA on the surface of HT29 cells may interfere with the normal CEA-signaling involved in cell adhesion, potentially disrupting survival pathways that regulated by integrins and growth factor receptors (27, 28). Antibodies that bind to TAG-72 might similarly disrupt signaling from that mucin. In addition, when two antibodies bind to the same antigen on the cell surface, they can cross-link these cells and activate signaling cascades that contribute to activation of the mitochondrial pathway. In a living body, the Fc parts of the antibodies would also engage Fc receptors on immune effector cells, which leads to additional killing through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (64) (Figure 2).

How our vaccine compares to others – Several other CEA-targeting vaccines have been evaluated in clinical studies, which provide useful benchmarks. Morse and colleagues evaluated an adenovirus-based vaccine expressing CEA (Ad5-E1-E2b-CEA-6D) in 32 patients with metastatic CRC and reported 48 % overall survival at 12 months (35). Although encouraging, viral-vectors have their own limitations. Many people already have immunity to the virus. This may limits vaccine effectiveness and make the production of viral vaccines complicated and expensive.

The PANVAC platform, a poxvirus that encodes CEA, MUC-1, and TRICOM, was shown to be safe but did not significantly improve recurrence-free survival in a phase II trial (36, 37). Other CEA-targeting vaccines, including TroVax (MVA-5T4), ALVAC-CEA-B7.1, and AVX701, have shown safety and induced some degree of immune responses; however, have not yet been approved (38-40).

The IMU-131 (HER-Vaxx) vaccine, which targets HER2 using a B-cell epitope-based approach similar to that used in our study, produced antibody titers ranging from 1:1,600 to 1:6,400 in gastric cancer patients (65). Our nanolipopeptide construct achieved titers up to 1:10,600 in mice. Although this suggests a strong response, comparison should be done cautiously across species.

To our knowledge, no previous study has developed a vaccine that simultaneously targets both CEA and TAG-72. Targeting two antigens at once may offer advantages over single-antigen vaccines by

broadening the immune response and decreasing the likelihood of the immune escape through loss of just one of the targets. The non-overlapping expression patterns of these antigens, with 43 % showing single positivity and 61 % showing combined positivity, suggest that a dual-target vaccine may help a broader number of CRC patients than vaccines targeting wither antigen alone (33).

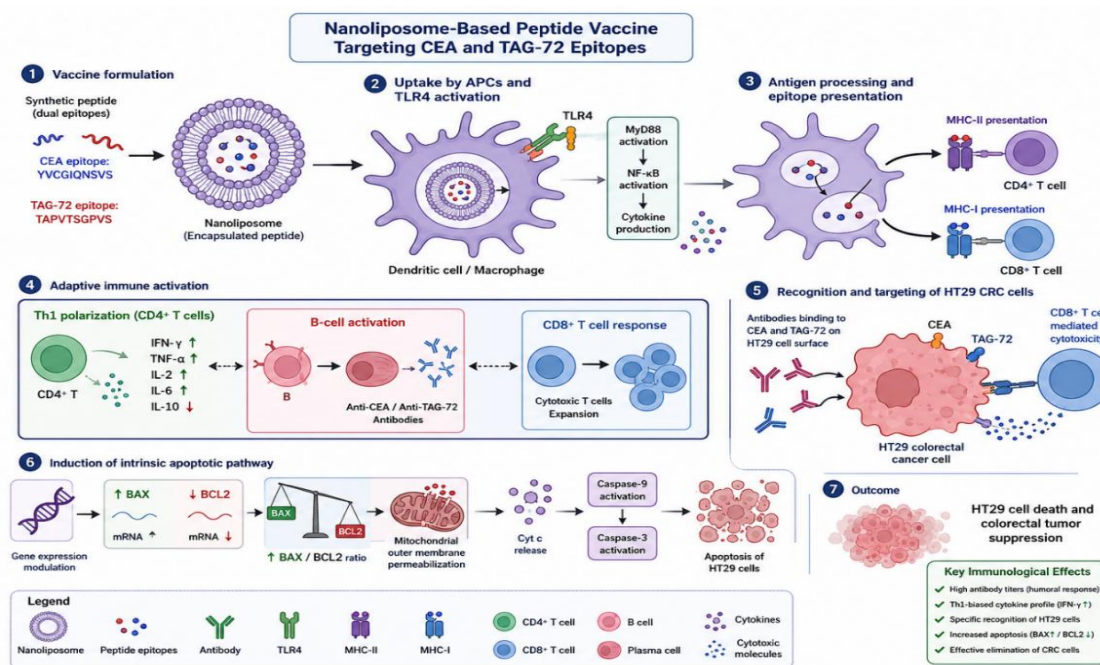


Figure 2. Proposed mechanism of action of the nanoliposomal CEA/TAG-72 peptide vaccine. Following uptake by antigen-presenting cells and TLR4 stimulation, the vaccine promotes MHC-I and MHC-II antigen presentation, leading to activation of CD8⁺ cytotoxic T cells, Th1-biased immune responses, and production of anti-CEA/TAG-72 antibodies. These immune effectors recognize HT29 colorectal cancer cells expressing CEA and TAG-72, resulting in increased BAX expression, suppression of BCL2, activation of the intrinsic mitochondrial apoptotic pathway, and subsequent tumor cell death."

5. Conclusion

Using computer-based immunoinformatics, we successfully designed a novel dual-epitope peptide vaccine including the most active B-cell pieces derived from CEA and TAG-72, two well-established markers of colorectal cancer. When we incorporated this peptide into nanoliposomes and injected it to BALB/c mice, it produced strong, dose-dependent antibody responses and promoted a Th1-biased immune profile with high levels of IFN- γ , IL-6, and TNF- α , accompanied by a modest increase in IL-10. The antibodies generated by the vaccine specifically recognized the real CEA and TAG-72 proteins expressed by HT29 colorectal cancer cells and caused apoptotic cell death with the activation of intrinsic mitochondrial pathway. This was confirmed by a

3.9-fold increase in BAX and a 3.2-fold decrease in BCL2, shifting the BAX/BCL2 ratio from 1.0 to 12.6. Taken together, our results indicate that this nanolipopeptide construct is a promising candidate for further development as a potential therapeutic vaccine against colorectal cancer. However, before it can be tested in humans, future preclinical studies should evaluate whether it works in live tumor-bearing animals and assess its long-term safety.

Limitations of this study

First, although the BALB/c mouse model gave us useful preliminary information about immunogenicity, what happens in mice may not fully predict what would happen in people. There are important differences between species in MHC

molecules, how TLRs are expressed, and how immune memory works. Before moving to humans, we should test the construct in humanized mice or, ideally, in non-human primates.

Second, we only tested one CRC cell line (HT29). While HT29 expresses both CEA and TAG-72, it may not represent the full diversity of CRC, including tumors with low or variable antigen levels. Future studies should include additional CRC cell lines, such as HCT116, SW480, and LoVo, and, even better, organoids or xenografts derived directly from patients to investigate how much the response varies from person to person.

Third, while our result showed that antibodies alone can cause apoptosis in a dish, we did not directly measure the contribution of cellular immunity (CD8 + T cells). Our MHC binding predictions suggest that the epitopes may also activate T cells, but we need to confirm that with experiments such as ELISpot, intracellular cytokine staining, or T-cell cytotoxicity assays.

Fourth, we did not test whether the vaccine can shrink tumors in live animals. While our findings show that the vaccine triggers immune responses and kills cancer cells in a dish, the real test will be whether it can slow or stop the growth of established tumors in animals. Future studies should assess the nanolipo peptide construct in syngeneic or xenograft tumor models, ideally including both preventive (vaccination before giving cancer cells) and therapeutic (vaccination after tumors have grown) protocols.

Fifth, this study did not measure the durability of the immune response. For this purpose, we need longitudinal studies which track antibody levels for longer periods, such as 3-6 months after the last shot, to assess whether the vaccine induces lasting immune memory and to determine the best booster schedule.

Sixth, we need to conduct more studies to evaluate the safety of the vaccine over a longer period of time. Although our study did not reveal any obvious problems during the 4-week study, longer-term safety studies to evaluate potential autoimmune side effects, particularly because CEA and TAG-72 are also present at low levels in some normal tissues.

Future directions – Based on the result of this study we suggest the following potential directions for the future studies : (1) researchers can test the nanolipo peptide vaccine in a mouse model of CRC, for example, using CT26 or MC38 cells implanted under the skin or into the gut wall; (2) researchers can

measure T-cell responses using ELISpot and flow cytometry; (3) researchers can try combining the vaccine with checkpoint inhibitors, such as anti-PD-1/PD-L1, to determine whether it can help overcome immune evasion; (4) researchers can evaluate different adjuvants, such as CpG, poly(I: C), or QS-21, to determine if the vaccine can function more effectively; (5) researchers can scale up production to good manufacturing practice (GMP) standards for clinical testing; and (6) they can design a phase I trial to test safety and immunogenicity in CRC patients.

Path to the clinic – The development of vaccine from the lab to patients will take several steps. We need to conduct preclinical toxicology studies in two species, rodents and non-rodents, to support an Investigational New Drug (IND) application. Then, a phase I dose-escalation trial in patients with advanced, treatment-refractory CRC would test safety, tolerability, and immunogenicity. Selecting patients based on whether their tumors express CEA and TAG-72, as determined by immunohistochemistry, and based on their HLA type might help achieve the best responses. If phase I results are promising, randomized phase II trials can test whether the vaccine works as a single treatment in the adjuvant setting, for example, after surgery for high-risk stage III CRC, or in combination with checkpoint inhibitors for metastatic disease. The relatively low cost of producing peptide vaccines, compared with biologics or cell therapies, and the enhanced immunogenicity achieved through nanoliposomes are both encouraging factors for eventual commercial development.

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Ethics approval and consent to participate

All animal procedures were approved by the Research Ethics Committee of Islamic Azad University of Medical Sciences, Tehran, Iran (approval number: IR.IAU.PS.REC.1403.246). All experiments followed the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and our country's regulations for laboratory animal welfare.

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Conflict of interest

The authors declare no competing interests. No pharmaceutical or biotechnology company provided funding or materials for this study, and no author holds patents or has financial interests related to the vaccine construct described.

Authors' contributions

Conceptualization: Mehrdad Hashemi and ALI Jebali; Methodology: Mojdeh Hajimohammad Jafar Tehrani; Software: Maliheh Entezari; Validation: Mehrdad Hashemi, Ali Jebali, Masoumeh Heshmati; Formal analysis: Mojdeh Hajimohammad Jafar Tehrani; Investigation: Mojdeh Hajimohammad Jafar Tehrani, Ali Jebali; Resources: Mehrdad Hashemi; Data curation: Mojdeh Hajimohammad Jafar Tehrani; Writing—original draft: Mojdeh Hajimohammad Jafar Tehrani; Writing—review and editing: Mehrdad Hashemi, Ali Jebali, Masoumeh Heshmati, Maliheh Entezari; Visualization: Maliheh Entezari; Supervision: Mehrdad Hashemi; All authors have read and approved the final version of the manuscript.

AI Using Declaration

Chat GPT was used to design the schematic illustration and for language and grammar corrections. The final output was read and modified by all authors.

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