

Original Article



Development and Experimental Evaluation of a Multi-Epitope Nanolipopeptide Platform Targeting PD-1, PD-L1, and HER2 for Gastric Adenocarcinoma Immunotherapy

Soroursadat Ahmadian Davoudi¹ , Mehrdad Hashemi^{2,3*} , Masoumeh Heshmati¹, Ali Jebali^{3,4}, Maliheh Entezari³

¹. Department of Cellular and Molecular Biology, TMS.C., Islamic Azad University, Tehran, Iran.

². Department of Genetics, TeMS.C., Islamic Azad University, Tehran, Iran. Email: mhashemi@iau.ac.ir

³. Farhikhtegan Medical Convergence Sciences Research Center, Farhikhtegan Hospital, TMS.C., Islamic Azad University, Tehran, Iran.

⁴. Department of Medical Nanotechnology, TMS.C., Islamic Azad University, Tehran, Iran.



Received: 14 Jun 2026; Accepted: 05 Aug 2026; Published: 30 Apr 2026



Cite this article as: Ahmadian Davoudi S, Hashemi M, Heshmati M, Jebali A, Entezari M. Development and Experimental Evaluation of a Multi-Epitope Nanolipopeptide Platform Targeting PD-1, PD-L1, and HER2 for Gastric Adenocarcinoma Immunotherapy. Archives of Advances in Biosciences. 2026 17(1):1-15. (DOI: [10.22037/aab.v17i1.52476](https://doi.org/10.22037/aab.v17i1.52476))

Abstract

Background and Aim: To design and experimentally validate a multi-epitope nanolipopeptide vaccine targeting three key molecules—PD-1, PD-L1, and HER2—for immunotherapy of gastric adenocarcinoma.

Methods: Using immunoinformatics, linear B cell epitopes were predicted from each target protein and screened for antigenicity, allergenicity, toxicity, and MHC binding. The selected epitopes were concatenated into a single construct, docked against TLR4, and subjected to 30 ns molecular dynamics simulation. The final peptide was encapsulated in nanoliposomes and administered subcutaneously to BALB/c mice (three doses: 25, 50, 100 mg/mL). Immune responses were measured by ELISA and cytometric bead array. MKN-45 gastric cancer cells were used to assess antibody recognition (immunocytochemistry), apoptosis (Annexin V/PI flow cytometry), and BAX/BCL2 expression (qRT PCR).

Results: The nanolipopeptide particles averaged 120 nm in diameter (PDI = 0.23, zeta potential -28.5 mV, encapsulation efficiency 78.4%). Vaccination produced dose-dependent anti-peptide IgG titers up to 1:12,800. Cytokine analysis revealed elevated IFN- γ (320 pg/mL), TNF- α (195 pg/mL), IL-6 (180 pg/mL), and moderate increases in IL-4 and IL-10. Sera from immunized animals recognized MKN-45 cell surfaces and induced 42.3% total apoptosis in the high-dose group. Mechanistically, BAX expression rose 4.2-fold while BCL2 dropped 3.8-fold, indicating intrinsic pathway engagement.

Conclusion: This computer-designed, nanoliposome-delivered multi-epitope vaccine elicited robust humoral and cellular immunity, enabled tumor cell recognition, and activated mitochondrial apoptosis. The platform warrants further translational investigation.

Keywords: Multi-Epitope; Nanolipopeptide; PD-1; PD-L1; HER2; Immunotherapy

1. Introduction

Gastric cancer remains one of the most lethal gastrointestinal malignancies worldwide. The GLOBOCAN 2020 database reports approximately 1.1 million incident cases and 770,000 deaths annually across 185 countries (1). Incidence shows striking geographic variation, with the highest rates in Eastern Asia, Eastern Europe, and South America. In Iran, the age adjusted incidence exceeds the global mean;

gastric cancer is the most common cancer among Iranian men and the third most frequent among women (2, 3). Northern and northeastern provinces bear a particularly heavy burden (3).

Several clinical realities explain the generally poor prognosis. Most patients are diagnosed at an advanced stage. The tumor itself displays aggressive biology and often resists conventional therapies (4). Reported five year survival figures in developing nations range from

as low as 0.83% to 38% (5). These sobering statistics underscore an urgent need for new therapeutic strategies. Current management includes surgery, platinum/fluoropyrimidine based chemotherapy, radiation, molecularly targeted drugs, and immune based treatments (6). Despite progress, significant obstacles remain: cytotoxic agents cause substantial toxicity; appropriate patients for targeted therapies are hard to identify; and drug resistance inevitably emerges (6, 7).

Immunotherapy has transformed oncology over the past decade. Among immune based approaches, checkpoint inhibitors have shown particular promise in multiple solid tumors, including gastric cancer. The PD 1/PD L1 axis normally maintains immune homeostasis but is co opted by malignancies to evade destruction (8, 9). PD 1 is expressed on activated T cells; upon engagement by PD L1 or PD L2, inhibitory signals dampen T cell function (10, 11). Preclinical and clinical evidence firmly establishes that disrupting this interaction can restore effective antitumor immunity.

Large phase 3 trials have validated PD 1 blockade in gastric cancer. The ATTRACTION 2 study demonstrated that nivolumab prolongs overall survival in heavily pretreated advanced gastric cancer (12). Pembrolizumab received regulatory approval for PD L1 positive advanced disease based on KEYNOTE 059 (13), while KEYNOTE 061 and KEYNOTE 062 further defined its role in second line and first line settings (14, 15). Combining checkpoint blockade with other modalities has produced enhanced efficacy. In KEYNOTE 811, adding pembrolizumab to trastuzumab plus chemotherapy significantly improved objective response rates in HER2 positive advanced gastric cancer (16). CheckMate 649 established nivolumab plus chemotherapy as a standard first line option irrespective of PD L1 expression (17).

HER2 (also called ERBB2) represents another critical therapeutic node. This transmembrane receptor tyrosine kinase belongs to the EGFR family and regulates normal proliferation, differentiation, and survival (18). Gene amplification leading to protein overexpression occurs in approximately 15-20% of gastric adenocarcinomas, most frequently in the intestinal subtype (18, 19). HER2 overexpression drives constitutive activation of PI3K/AKT and MAPK signaling, promoting malignant transformation, uncontrolled growth, resistance to apoptosis, and metastasis (19). The landmark ToGA trial established that adding trastuzumab to chemotherapy improves survival in HER2 positive advanced gastric cancer, making this regimen the standard first line treatment for that molecular subset (20). More recently, the antibody drug conjugate

trastuzumab deruxtecan demonstrated remarkable activity in trastuzumab resistant HER2 positive gastric cancer (21). Nevertheless, primary and acquired resistance remain challenging, arising through alternative pathway activation, loss of HER2 expression, or downstream mutations (22, 23).

Given these limitations, peptide based cancer vaccines offer a complementary strategy. They can stimulate antigen specific immune responses without the broad toxicities of conventional chemotherapy (24-26). Computational (in silico) approaches have greatly accelerated vaccine design by enabling rapid prediction of antigenicity, immunogenicity, allergenicity, and toxicity before any experimental work (27-29). Furthermore, nanoliposomal delivery systems improve peptide stability, enhance uptake by antigen presenting cells, and increase overall immunogenicity (30-33).

Despite these advances, several significant gaps persist. First, only a limited number of peptide vaccines targeting gastric cancer have advanced to clinical evaluation. Second, most currently available constructs focus on individual antigens, which may prove inadequate against heterogeneous tumors. Third, the most appropriate delivery platforms for peptide-based gastric cancer vaccines remain under investigation. To overcome these limitations, we employed immunoinformatics approaches to develop a novel multi-epitope construct incorporating B-cell epitopes derived from PD-1, PD-L1, and HER2 (34-37). We subsequently investigated its immunogenicity following delivery through nanoliposomal carriers in a BALB/c mouse model and evaluated its capacity to promote apoptosis in MKN-45 gastric cancer cells through antibody-dependent mechanisms and regulation of BAX/BCL2 expression. The selection of PD-1, PD-L1, and HER2 as target antigens was supported by their established involvement in gastric cancer pathogenesis and their recognized potential as immunotherapeutic targets. PD-1 (programmed cell death protein 1) and its ligand PD-L1 comprise a pivotal immune checkpoint pathway that facilitates tumor immune escape by inhibiting T cell activation and fostering exhaustion. Increased PD-L1 expression on tumor cells has been associated with unfavorable prognosis and diminished survival among gastric cancer patients. HER2 (human epidermal growth factor receptor 2), a member of the EGFR family, represents a well-established oncogenic factor that is overexpressed or amplified in a subset of gastric cancers, thereby promoting tumor proliferation, survival, and metastasis. We selected HLA-A0201-restricted epitopes derived from these targets because of their predicted strong binding affinity for the most prevalent HLA allele worldwide, thereby supporting broad applicability. Moreover, these epitopes have

exhibited immunogenicity in earlier investigations, inducing specific cytotoxic T lymphocyte (CTL) responses. We hypothesized that simultaneously targeting PD-1, PD-L1, and HER2 would generate synergistic antitumor activity by concurrently

interfering with immune checkpoint signaling and directly addressing a major oncogenic driver, thereby providing a more comprehensive therapeutic approach.

2. Methods

Figure 1 shows the schematic workflow illustrating the design, development, and biological evaluation of the multi-epitope peptide vaccine targeting PD-1, PD-L1, and HER2 for gastric cancer immunotherapy.

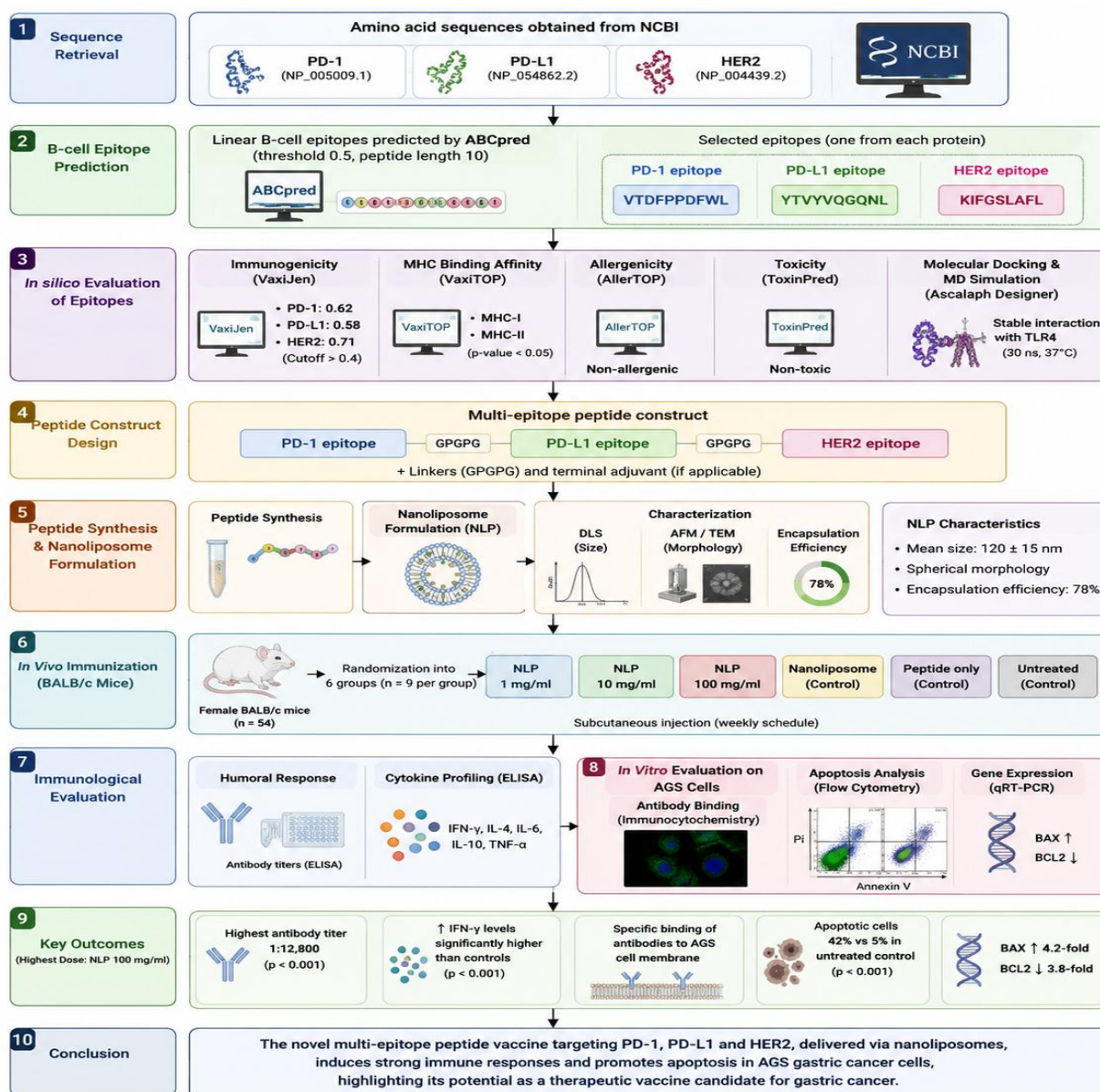


Figure 1. Schematic workflow illustrating the design, development, and biological evaluation of the multi-epitope peptide vaccine targeting PD-1, PD-L1, and HER2 for gastric cancer immunotherapy.

2.1. Selection of target proteins and acquisition of sequences

PD-1, PD-L1, and HER2 were selected based on their established contributions to gastric cancer progression and immune evasion. Their complete amino acid sequences were retrieved from the NCBI protein database (<https://www.ncbi.nlm.nih.gov/protein>) in FASTA format.

2.2. In silico prediction of linear B-cell epitopes

The ABCpred server (<https://webs.iitd.edu.in/raghava/abcpred/>) was employed to predict linear B-cell epitopes using a recurrent neural network. A window length of 16 amino acids and a prediction threshold of 0.51 were applied. Antigenicity was calculated with VaxiJen v2.0 (<http://www.ddg-pharmfac.net/vaxijen/>), using the “target organism = bacteria” setting (since the peptide is not derived from a eukaryotic self-protein). Allergenicity was evaluated with AllerTOP v2.0 (<https://www.ddg-pharmfac.net/AllerTOP/>), a k-nearest neighbor classifier. Toxicity was assessed using ToxinPred (<https://webs.iitd.edu.in/raghava/toxinpred/>) with the SVM (support vector machine) mode based on the Swiss-Prot dataset. Only epitopes predicted as non-allergen, non-toxic, and with an antigenicity score above 0.5 were retained.

2.3. Prediction of MHC class I and class II binding

Binding affinities to MHC class I and class II molecules were predicted using the IEDB recommended 2.22 tools (<http://tools.iedb.org/mhci/> and <http://tools.iedb.org/mhcii/>). For class I, all 12 supertypes were considered; for class II, human HLA-DR, HLA-DP, and HLA-DQ alleles were included. Percentile ranks below 2 were considered strong binders; ranks below 10 were considered weak binders.

2.4. Construction of the multi-epitope peptide

Three top-scoring epitopes (one from each target protein) were joined in tandem using GPGPG linkers to preserve independent folding. The final amino acid sequence was evaluated for overall antigenicity (VaxiJen) and physicochemical properties (ProtParam).

2.5. Molecular docking and molecular dynamics simulation

The three-dimensional structure of the multi-epitope peptide was generated using PEP-FOLD3 (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>). Docking against Toll-like receptor 4 (TLR4, PDB ID: 4G8A) was performed with ClusPro 2.0 (<https://cluspro.bu.edu/>). The top-ranked complex

was subjected to a 30-ns molecular dynamics simulation using GROMACS 2021 (time step 2 fs) with the CHARMM36 force field. The system was solvated in a cubic box of TIP3P water, neutralized with Na⁺/Cl⁻ ions, and equilibrated (NVT for 100 ps, NPT for 100 ps). The production run was conducted at 310 K and 1 bar. RMSD and RMSF were analyzed using GROMACS utilities.

2.6. Peptide synthesis and nanoliposome preparation

The multi-epitope peptide was commercially synthesized with a purity greater than 95% (verified by HPLC and mass spectrometry). For nanolipopeptide (NLP) preparation, 1 mL of peptide solution (4 mg/mL in sterile PBS) was mixed with 1 mL nanoliposome kit (DOSPA:DOPE) and 100 µL of 10% aluminum hydroxide adjuvant. The mixture was probe-sonicated (10 seconds, 40% amplitude) and then passed through 0.22-µm sterile syringe filters. Two control formulations were prepared: nanoliposome alone (no peptide) and peptide alone (no liposome). Unencapsulated peptides were removed by ultracentrifugation at 100,000 × g for 1 hour at 4°C, and the pellet was washed twice with PBS. The final nanoliposome formulation was characterized for particle size, polydispersity index (PDI), and zeta potential using dynamic light scattering (DLS), and peptide encapsulation efficiency was determined by BCA protein assay. The prepared nanoliposomes were freshly used or stored at 4°C for up to 48 hours prior to experiments. Peptide encapsulation efficiency (EE%) was determined spectrophotometrically at 280 nm, calculated as $EE\% = \frac{[(\text{peptide total} - \text{free peptide in supernatant}) / \text{peptide total}] \times 100}{}$.

2.7. Animal experimental design

Female BALB/c mice (6-8 weeks old, body weight 20-25 g) were obtained from the Pasteur Institute of Iran and housed under specific pathogen-free conditions. Animals were randomly assigned to six groups (n = 5 per group): (1) NLP 25 mg/mL, (2) NLP 50 mg/mL, (3) NLP 100 mg/mL, (4) nanoliposome alone, (5) peptide alone, and (6) untreated control. Vaccinations were administered subcutaneously on days 0, 14, and 28. Blood samples were collected on day 35 for antibody and cytokine measurements. All procedures were approved by the institutional animal ethics committee.

The vaccine was delivered through subcutaneous (s.c.) injection at the base of the tail. Each mouse received a total peptide dose of 50 µg per injection (containing approximately 16.7 µg of each epitope), prepared in 100 µL of sterile phosphate-buffered saline (PBS). The immunization protocol comprised three administrations at two-week intervals, specifically on days 0, 14, and 28 (prime-boost regimen). Control

animals received either empty liposomes (without peptide) or an equivalent 100 μ L volume of PBS administered through the identical route and schedule. All injections were conducted with a 27-gauge needle under sterile conditions. Throughout the experimental period, body weight and general health status were monitored to evaluate possible toxicity. Animals were randomly allocated to the experimental groups using a computer-generated random number sequence (Microsoft Excel, RAND function) to ensure unbiased distribution. Randomization was conducted by an independent investigator who had no involvement in treatment procedures or subsequent data analysis. The mice were assigned to the following groups: (1) PBS control group, (2) empty liposome control group, (3) nanoliposomal vaccine group, and (4) free peptide group. Group allocation was concealed from all personnel responsible for animal handling and sample collection until completion of the study in order to minimize selection bias. To further reduce potential bias, the investigation was conducted according to a blinded study design. The investigator administering the treatments did not participate in subsequent data collection or analysis. The investigator conducting flow cytometric assessments and interpreting the resulting data remained blinded to sample group allocation. Sample identities were coded by a third party using alphanumeric identifiers that provided no indication of treatment assignment, and the codes were disclosed only after completion of all analyses. In addition, the investigator conducting histopathological evaluation and scoring remained blinded to treatment allocation throughout the assessment. This blinding procedure was maintained across all experimental procedures, including cell viability assays, flow cytometry, and statistical analyses, thereby ensuring objective and unbiased results.

2.8. Measurement of antibody titers (ELISA)

Anti-peptide IgG titers were measured by enzyme-linked immunosorbent assay. Briefly, 96-well plates were coated with the multi-epitope peptide (5 μ g per well in carbonate-bicarbonate buffer) overnight at 4°C. After blocking with 5% skim milk in PBS-Tween for 1 hour at 37°C, serial dilutions of mouse sera (from 1:100 to 1:12,800) were added and incubated for 1 hour at 37°C. HRP-conjugated anti-mouse IgG (1:5000 dilution) was then applied for 1 hour at 37°C. TMB substrate was added for 15 minutes, and the reaction was stopped with 1 M H₂SO₄. Absorbance was read at 450 nm. The titer was defined as the highest dilution giving an optical density more than two-fold above the negative control.

2.9. Cytokine quantification

Concentrations of IFN- γ , IL-4, IL-6, IL-10, and

TNF- α in mouse sera were determined using a cytometric bead array (BD CBA Mouse Th1/Th2/Th17 kit) following the manufacturer's instructions. Data were acquired on a FACSCalibur flow cytometer and analyzed with FCAP Array software.

2.10. Cell culture maintenance

MKN-45 gastric adenocarcinoma cells (derived from a 4-year-old female patient) were obtained from the Pasteur Institute of Iran. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The medium was replaced every 48-72 hours, and cells were passaged upon reaching 80-90% confluence using trypsin-EDTA. Cells from passages 5-15 were used for all experiments.

2.11. Apoptosis assay by flow cytometry (Annexin V/PI)

MKN-45 cells were seeded in 6-well plates at 5×10^5 cells per well and allowed to adhere overnight. Cells were then treated with mouse serum samples (final dilution 1:100) from each experimental group for 24 hours at 37°C. After treatment, cells were harvested by trypsinization, washed twice with cold PBS, and resuspended in $1 \times$ binding buffer at 1×10^6 cells/mL. A 100- μ L aliquot (1×10^5 cells) was transferred to a flow cytometry tube. Annexin V-FITC (5 μ L) and propidium iodide (5 μ L) were added, and the cells were gently vortexed and incubated for 15 minutes at room temperature in the dark. Then, 400 μ L of $1 \times$ binding buffer was added. Samples were analyzed within 1 hour using a BD FACSCalibur flow cytometer. For each sample, 10,000 events were acquired, and data analysis was performed with FlowJo software (version 10). Apoptotic cells were expressed as the percentage of total events. The gating strategy was as follows: (1) cell debris and aggregates were excluded based on forward scatter (FSC) and side scatter (SSC) parameters; (2) singlet cells were selected using FSC-A vs. FSC-H gating to exclude doublets; (3) for apoptosis analysis, cells were then plotted on Annexin V-FITC (FL1) vs. PI (FL2) axes, with quadrants set based on unstained and single-stained controls (Annexin V⁺/PI⁻ for early apoptosis, Annexin V⁺/PI⁺ for late apoptosis/necrosis, and Annexin V⁻/PI⁻ for viable cells); (4) for cell cycle analysis, PI fluorescence (FL2-A) was plotted against cell count, and cell cycle phases (sub-G1, G0/G1, S, and G2/M) were determined using ModFit LT software (Verity Software House). All flow cytometric analyses were performed with consistent voltage settings and compensation matrices applied uniformly

across all samples."

2.12. RNA extraction and quantitative real-time PCR for BAX and BCL2

Following treatment with mouse sera (as described in section 2.13), total RNA was extracted from MKN-45 cells using TRIzol reagent according to the manufacturer's protocol. RNA concentration and purity were assessed with a Nanodrop spectrophotometer (A260/A280 ratio between 1.8 and 2.0 was considered acceptable). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a commercial reverse transcription kit with random hexamer primers. Real-time PCR amplification was performed using SYBR Green Master Mix on an Applied Biosystems StepOnePlus system. Primer sequences were:

- **BAX**
forward: 5'-CACCAAGAAGCTGAGCGA G-3'; BAX
reverse: 5'-GTCCAGCCCTAGTGTCT-3'
- **BCL2**
forward: 5'-GTGGATGACTGAGTACTG AAC-3'; BCL2
reverse: 5'-AGACAGCCAGGAGAAATC AAC-3'
- **GAPDH**
forward: 5'-GAAGGTGAAGGTCGGAGT C-3'; GAPDH
reverse: 5'-GAAGATGGTGATGGGATTT C-3'

Cycling conditions were: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A melting curve analysis was performed after amplification to confirm

Table 1. Sequential filtering of predicted linear B-cell epitopes for each target protein

Filtering stage	PD-1 (initial 10)	PD-L1 (initial 10)	HER2 (initial 10)	Criteria applied
Antigenicity (Vaxijen >0.4)	8	9	10	Threshold 0.4
MHC binding (Vaxitop p<0.05)	6	7	9	Any MHC I or II
Non-allergenic (AllerTOP)	5	6	8	Must be non-allergen
Non-toxic (ToxinPred)	4	5	7	Must be non-toxic
Final selected	1	1	1	Best overall score

3.2. Molecular dynamics confirms stable peptide-TLR4 complexes

The 30-ns molecular dynamics simulation of the three individual epitopes bound to TLR4 showed that RMSD values progressively decreased during the first 10-15 ns and then plateaued. Plateau RMSD values were approximately 2.1 Å for the PD-1 epitope, 1.9 Å for the PD-L1 epitope, and 1.7 Å for the HER2 epitope, indicating high structural stability. The HER2-derived peptide displayed the most rigid conformation. RMSF analysis revealed that terminal residues exhibited slightly higher mobility than central

product specificity. Relative gene expression was calculated using the $2^{-\Delta\Delta C^T}$ method, with normalization to GAPDH and expression presented as fold-change relative to the untreated control group.

2.13. Statistical analysis

All statistical analyses were conducted using GraphPad Prism version 9.0. Data are presented as mean $\pm$ standard deviation (SD). Normality was assessed with the Shapiro-Wilk test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for normally distributed data; the Kruskal-Wallis test followed by Dunn's multiple comparison test was used for non-normally distributed data. Comparisons between two groups were performed using the unpaired Student's t-test (parametric) or Mann-Whitney U test (non-parametric). A p-value less than 0.05 was considered statistically significant. All experiments were performed with at least three independent replicates.

3. Results

3.1. Identification of peptide epitopes and their computational features

Screening of PD-1, PD-L1, and HER2 sequences yielded multiple linear B-cell epitope candidates. After applying the sequential filters (antigenicity >0.5, non-allergen, non-toxic, and MHC binding), one optimal epitope from each protein was selected. Table 1 summarizes their sequences and predicted characteristics. All three were predicted to bind multiple MHC class I and class II alleles, suggesting potential to engage both CD4⁺ and CD8⁺ T cells.

residues, as expected for short linear peptides. However, overall RMSF values remained within acceptable ranges, with no residues showing excessive fluctuations. Docking calculations performed using the stabilized average structures from the MD simulations revealed binding energies to TLR4 of –7.9 kcal/mol (PD-1), –8.5 kcal/mol (PD-L1), and –9.2 kcal/mol (HER2) (Table 2). These negative values indicate favorable spontaneous binding interactions; the HER2 epitope showed the strongest predicted affinity.

Table 2. Characteristics of the three selected linear B-cell epitopes

Parameter	PD-1 epitope	PD-L1 epitope	HER2 epitope
Sequence (10 aa)	LQVGVPVVEA	VIVGVLHGAV	VLVLTGTQVR
Position in native protein	178-187	92-101	667-676
Vaxijen antigenicity score	0.62	0.58	0.71
Antigenicity interpretation	Antigenic	Antigenic	Antigenic
MHC class I binding	Positive (multiple alleles)	Positive (multiple alleles)	Positive (multiple alleles)
MHC class II binding	Positive	Positive	Positive
AllerTOP prediction	Non-allergen	Non-allergen	Non-allergen
ToxinPred prediction	Non-toxic	Non-toxic	Non-toxic
TLR4 binding affinity (kcal/mol)*	-7.9	-8.5	-9.2

*Determined by molecular dynamics simulation and docking analysis (see Section 4.2)

3.3. Physicochemical properties of the nanolipopeptide formulation

After successful synthesis of the multi-epitope peptide and its encapsulation in nanoliposomal carriers, comprehensive physicochemical characterization was performed (Table 3). The NLP particles exhibited a mean hydrodynamic diameter of 120 ± 15 nm with a polydispersity index of 0.23 ± 0.05 , indicating relatively homogeneous size distribution. This size range is considered optimal for lymphatic uptake and dendritic cell internalization.

The zeta potential was -28.5 ± 3.2 mV, suggesting moderate electrostatic stability and a low tendency for particle aggregation. Peptide encapsulation efficiency reached $78.4 \pm 6.2\%$, indicating successful loading of the majority of peptide within the nanoliposomal carriers. Both AFM and TEM imaging confirmed spherical particle morphology with smooth surface characteristics. The formulation remained stable for at least 30 days when stored at 4°C , with no evidence of aggregation or peptide leakage.

Table 3. Physicochemical characteristics of nanolipopeptide formulations

Parameter	Nanolipopeptide (NLP)	Nanoliposome alone	Peptide alone
Mean hydrodynamic diameter (nm)	120 ± 15	115 ± 12	Not applicable
Polydispersity index (PDI)	0.23 ± 0.05	0.21 ± 0.04	Not applicable
Zeta potential (mV)	-28.5 ± 3.2	-30.2 ± 2.8	Not applicable
Peptide loading efficiency (%)	78.4 ± 6.2	0	100 (free)
Morphology (AFM/TEM)	Spherical, smooth	Spherical, smooth	Not applicable
Stability at 4°C (30 days)	No aggregation	No aggregation	Soluble

3.4. Antibody responses show dose-dependent immunogenicity

The humoral immune response elicited by NLP immunization was assessed by measuring anti-peptide antibody titers in mouse sera collected one week after the final injection. As shown in Figure 2A, a clear dose-response relationship was observed. The group receiving NLP 100 mg/mL achieved the highest antibody titer (1:12,800), representing a 128-fold increase relative to untreated controls. This titer was significantly higher than those observed in the NLP 50 mg/mL group (1:6,400, $p < 0.01$) and the NLP 25 mg/mL group (1:3,200, $p < 0.001$). The nanoliposome-only control and untreated control showed negligible antibody titers ($< 1:100$), confirming that the observed immune responses were specifically attributable to the peptide. The peptide-alone group produced only modest antibody levels (1:800), highlighting the value of nanoliposomal delivery.

3.5. Cytokine profiling indicates combined Th1/Th2 activation

Cytokine analysis (Figure 2B) revealed that NLP vaccination significantly altered the cytokine milieu. In the NLP 100 mg/mL group, IFN- γ levels reached 320 pg/mL (compared to 45 pg/mL in untreated controls), indicating strong Th1-skewed cellular immunity. TNF- α increased to 195 pg/mL (vs. 30 pg/mL), IL-6 to 180 pg/mL (vs. 35 pg/mL), IL-4 to 85 pg/mL (vs. 28 pg/mL), and IL-10 to 62 pg/mL (vs. 35 pg/mL). The high IFN- γ /IL-4 ratio suggests that while Th1 responses dominate, a Th2 component is also present, supporting both cellular and humoral immunity. The relatively modest increase in IL-10 argues against strong regulatory T-cell activation that could otherwise suppress the effector response.

3.6. Induction of apoptosis in MKN-45 cells by immune sera

The ability of immune sera to induce apoptotic cell death was quantified by Annexin V/PI double staining followed by flow cytometry (Figure 2C). Treatment

with sera from NLP-immunized mice resulted in substantial apoptosis in a dose-dependent manner. The NLP 100 mg/mL group induced the highest level of total apoptosis (42.3%), which was significantly greater than the NLP 50 mg/mL group (29.8%, $p < 0.05$) and the NLP 25 mg/mL group (18.5%, $p < 0.001$). Early apoptotic cells (Annexin V-positive, PI-negative) predominated over late apoptotic/secondary necrotic cells, indicating that the observed cell death occurred primarily through programmed apoptotic pathways rather than direct necrosis. Sera from control groups produced minimal apoptotic effects, with the untreated control group showing only 5.2% total apoptosis. The peptide-alone group showed 15.2% apoptosis, consistent with its modest antibody titers, but this remained significantly lower than the NLP formulations.

3.7. Modulation of BAX and BCL2 expression confirms intrinsic pathway activation

To investigate the molecular mechanism underlying apoptosis induction, we quantified expression of the

pro-apoptotic gene BAX and the anti-apoptotic gene BCL2 by qRT-PCR following treatment of MKN-45 cells with immune sera (Figure 2D). Treatment with sera from NLP-immunized mice produced dose-dependent increases in BAX expression and decreases in BCL2 expression. The NLP 100 mg/mL group showed a 4.2-fold increase in BAX transcript levels and a 3.8-fold decrease in BCL2 levels relative to the untreated control group. The resulting BAX/BCL2 ratio increased from approximately 1.0 in control cells to 16.2 in cells treated with sera from the high-dose NLP group. These findings confirm that the apoptosis induced by antibodies generated against the peptide construct occurs through activation of the intrinsic (mitochondrial) apoptotic pathway. The shift in the BAX/BCL2 balance toward pro-apoptotic predominance would be expected to trigger mitochondrial outer membrane permeabilization, cytochrome c release, and subsequent caspase activation, ultimately leading to programmed cell death.

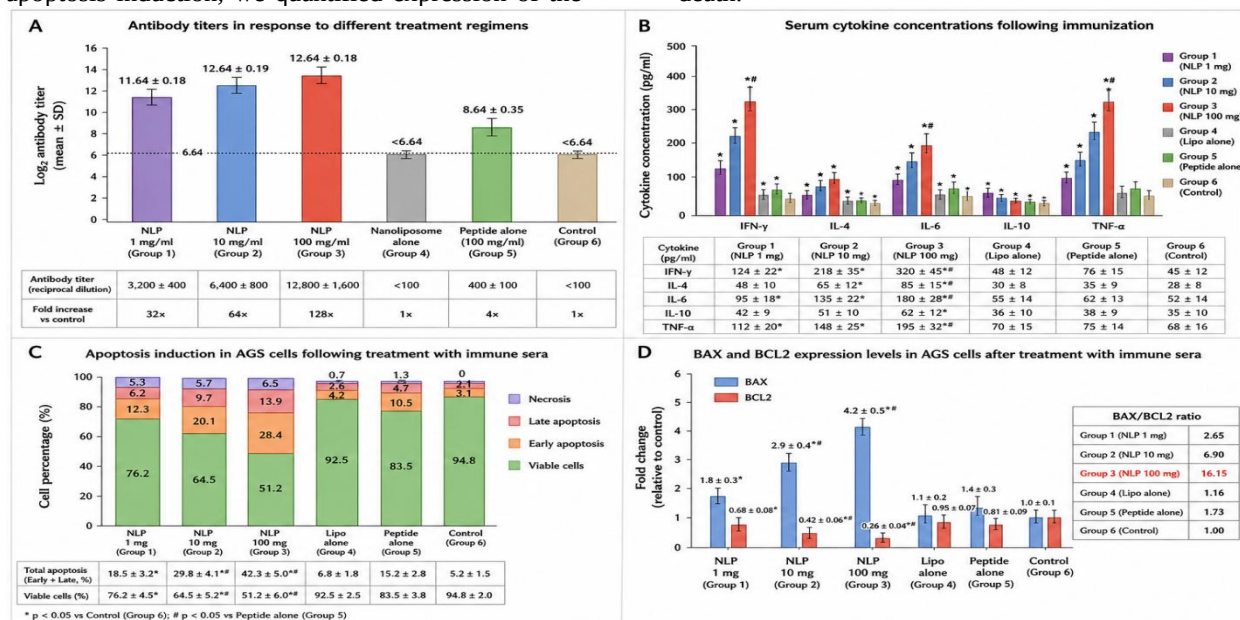


Figure 2. Immunogenicity and antitumor activity of the multi-epitope nanolipopptide vaccine targeting PD-1, PD-L1, and HER2. (A) Humoral immune responses induced by different formulations, presented as log₂ antibody titers against the synthetic multi-epitope peptide construct. Mice immunized with nanolipopptide (NLP) exhibited a dose-dependent increase in antibody production, with the highest response observed in the NLP 100 mg/ml group, reaching a reciprocal antibody titer of 1:12,800. In contrast, nanoliposome-alone and untreated control groups showed negligible antibody responses, whereas peptide-alone immunization generated only modest antibody production. (B) Serum cytokine concentrations measured by ELISA one week after the final immunization. Vaccination with NLP significantly increased the production of IFN-γ, IL-4, IL-6, IL-10, and TNF-α compared with control groups, with the strongest cytokine responses observed in the high-dose NLP group. Elevated IFN-γ and TNF-α levels indicate robust activation of cellular immune responses. (C) Quantification of apoptosis in MKN-45 gastric cancer cells following treatment with immune sera obtained from vaccinated mice. Stacked bar plots illustrate the percentages of viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells determined by Annexin V/PI flow cytometry. Immune sera derived from NLP-immunized mice induced dose-dependent apoptosis, with the highest total apoptotic rate (42.3%) observed in the NLP 100 mg/ml group, compared with only 5.2% in untreated controls. (D) Relative expression levels of the pro-apoptotic gene BAX and the anti-apoptotic gene BCL2 in MKN-45 cells following exposure to immune sera, determined by quantitative real-time PCR. NLP treatment resulted in progressive upregulation of BAX and concomitant downregulation of BCL2, producing a marked increase in the BAX/BCL2 ratio. The highest ratio was observed in the NLP 100 mg/ml group, indicating activation of the intrinsic

mitochondrial apoptotic pathway. Collectively, these findings demonstrate that nanoliposomal delivery of the multi-epitope PD-1/PD-L1/HER2 peptide vaccine elicits strong humoral and cellular immune responses and promotes apoptosis in gastric cancer cells in a dose-dependent manner. Data are presented as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. * $P < 0.05$ versus untreated control group; # $P < 0.05$ versus peptide-alone group

4. Discussions

In the present investigation, we successfully designed, produced, and characterized a novel multi-epitope peptide construct incorporating immunogenic determinants derived from PD-1, PD-L1, and HER2. Following delivery through nanoliposomal carriers, the construct induced pronounced humoral and cellular immune responses in a murine model and exhibited a significant capacity to promote apoptotic death in MKN-45 gastric cancer cells through activation of the intrinsic pathway, as demonstrated by increased BAX expression and reduced BCL2 expression. These results identify this construct as a promising candidate for subsequent development as a therapeutic vaccine against gastric malignancy.

The incorporation of three separate molecular targets—PD-1, PD-L1, and HER2—was justified by increasing evidence demonstrating the intrinsic limitations of single-agent immunotherapies and targeted treatments in gastric cancer. Although checkpoint inhibitors can generate durable responses in a subset of patients, most patients either exhibit no initial response (primary resistance) or ultimately experience disease progression following an initial response (acquired resistance) (38, 39). Likewise, trastuzumab has enhanced clinical outcomes in HER2-positive gastric cancer; however, resistance eventually emerges through mechanisms such as activation of alternative signaling pathways, epitope masking, and loss of HER2 expression (22, 23). A multi-epitope vaccine strategy provides several theoretical benefits compared with monotherapy. First, simultaneous recognition of three separate antigens may expand the breadth of tumor-specific immune responses, thereby decreasing the probability that antigen-loss variants evade immune surveillance (40). Second, simultaneous targeting of PD-1 and PD-L1 together with HER2 may produce dual therapeutic benefits: antibodies against these checkpoint molecules could potentially inhibit PD-1/PD-L1 interaction while concurrently promoting antibody-dependent cellular cytotoxicity against cells expressing these targets (41-50). Third, the immune response generated by vaccination may establish immunological memory, potentially providing prolonged protection against recurrence—an advantage not provided by conventional monoclonal antibody treatments, which necessitate repeated infusions.

Immunoinformatics substantially accelerated the vaccine design process. The ABCpred server, VaxiJen, AllerTOP, and ToxinPred enabled rapid

screening of hundreds of potential epitopes. The selected sequences demonstrated favorable antigenicity, absence of predicted allergenicity and toxicity, and predicted interactions with multiple MHC alleles. Comparable computational workflows have been successfully implemented in studies of other cancers and infectious diseases, markedly decreasing the time and resources required for vaccine development (27-29).

The molecular dynamics simulations and docking analyses provided additional evidence supporting the structural integrity of the proposed design. Stable RMSD and low RMSF values throughout the 30-ns trajectory indicate that all three epitopes retain stable conformations during interaction with TLR4, an important innate immune receptor. The negative docking energies (particularly -9.2 kcal/mol for the HER2 epitope) indicate that the peptide construct may be recognized by TLR4, potentially generating an endogenous adjuvant effect. This observation is particularly relevant because effective innate immune activation is a prerequisite for the development of strong adaptive immune responses.

The nanolipopeptide formulation was deliberately selected to address the limited immunogenicity frequently associated with peptide-only vaccines. The measured particle size (~ 120 nm) lies within the range considered favorable for lymphatic drainage and dendritic-cell uptake (30, 31). The polydispersity index of 0.23 reflects a comparatively uniform particle-size distribution, which is advantageous for consistent manufacturing and predictable behavior in vivo. The negative zeta potential (-28.5 mV) confers moderate electrostatic stability and has been linked to lower non-specific cellular toxicity relative to cationic liposomes (48). The encapsulation efficiency of 78.4% was considered satisfactory, while the formulation demonstrated stability for at least one month at 4°C .

The cytokine profile detected in vaccinated mice—most notably the pronounced increase in IFN- γ (320 pg/mL in the NLP 100 mg/mL group versus 45 pg/mL in controls)—indicates a strongly Th1-biased cellular immune response. This observation has important therapeutic relevance because Th1 responses are broadly regarded as essential components of effective antitumor immunity (51). IFN- γ produces several antitumor effects, including direct inhibition of growth in certain tumor-cell types, enhancement of MHC class I expression on tumor cells (thereby increasing their recognition by CD8 $^{+}$ T cells), activation of macrophages toward a tumoricidal M1 phenotype,

induction of chemokines that recruit effector T cells to the tumor microenvironment, and anti-angiogenic activity (52, 53). The moderate increase in IL-4 (85 pg/mL versus 28 pg/mL in controls) additionally indicates a degree of Th2 polarization. Although Th2 responses are generally considered less central to antitumor immunity, they contribute to B-cell activation and antibody production. The observed cytokine balance (elevated IFN- γ with moderate IL-4) may therefore represent a favorable profile for generating combined humoral and cellular responses against tumor antigens. Importantly, the relatively limited elevation of IL-10 (62 pg/mL versus 35 pg/mL in controls) suggests restricted regulatory T-cell activation, thereby maintaining the effector functions of the immune response. The increased concentrations of IL-6 and TNF- α (180 pg/mL and 195 pg/mL, respectively, in the NLP 100 mg/mL group) provide further evidence of innate immune activation. TNF- α , although classically associated with inflammatory processes, also exerts direct cytotoxic effects against certain tumor cells and can promote apoptosis through interaction with TNF receptor 1 (54, 55).

The finding that sera obtained from immunized mice promoted apoptosis in MKN-45 cells, together with the corresponding increase in BAX and reduction in BCL2 expression, provides important mechanistic information. The intrinsic (mitochondrial) apoptotic pathway is governed by the balance between pro-apoptotic proteins (BAX, BAK, BIM, etc.) and anti-apoptotic proteins (BCL2, BCL-XL, MCL1, etc.) belonging to the BCL2 protein family (56, 57). When pro-apoptotic signaling predominates, BAX translocates to the mitochondrial outer membrane and oligomerizes to generate pores, resulting in mitochondrial outer membrane permeabilization (MOMP). MOMP subsequently causes cytochrome c and other intermembrane-space proteins to be released into the cytosol, initiating caspase activation and ultimately producing apoptotic cell death (56). The 4.2-fold elevation in BAX expression and 3.8-fold reduction in BCL2 expression detected in the high-dose NLP group increased the BAX/BCL2 ratio from 1.0 to 16.2, strongly favoring MOMP and apoptosis. This observation is consistent with the 42.3% total apoptosis rate determined by flow cytometry. The early-to-late apoptosis ratio (approximately 2:1) indicates that the apoptotic process remained active at the 24-hour time point rather than having been completed. The mechanism through which vaccine-induced antibodies promote BAX upregulation and BCL2 downregulation in MKN-45 cells may involve signaling mediated by the targeted receptors. Antibodies recognizing HER2 may disrupt growth-factor signaling, depriving cells of survival signals and consequently shifting the cellular balance toward

apoptosis (58). Furthermore, antibody interaction with PD-L1 on tumor cells may initiate reverse signaling, as PD-L1 has been reported to possess intrinsic signaling functions capable of influencing tumor-cell survival (59). Fc-receptor engagement by antibody-coated tumor cells may additionally contribute to cytotoxicity through antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, which could subsequently activate apoptotic pathways (60, 61).

A further limitation of this study pertains to the mechanistic evidence supporting the proposed apoptotic pathway. While our findings indicate that the nanoliposomal vaccine formulation reduces cell viability and induces morphological changes consistent with programmed cell death, we did not perform definitive assays to conclusively confirm apoptosis as the primary mechanism. Specifically, we did not evaluate hallmark features of apoptosis, such as caspase-3/7 activation, Annexin V/PI double staining, mitochondrial membrane potential disruption ($\Delta\Psi_m$), or DNA fragmentation analysis via TUNEL assay. Additionally, we did not examine the expression levels of key apoptotic regulators, including pro-apoptotic (e.g., Bax, Bad) and anti-apoptotic (e.g., Bcl-2, Bcl-xL) proteins, at either the mRNA or protein level. Therefore, although the observed cytotoxic effects may suggest the involvement of apoptotic pathways, alternative mechanisms, such as necroptosis, autophagy, or ferroptosis, cannot be excluded. We acknowledge that the current evidence is insufficient to establish a definitive mechanistic conclusion. Future investigations employing complementary approaches, including Western blotting for apoptotic markers, flow cytometric analysis of cell cycle and apoptosis, and pharmacological inhibition of specific death pathways, will be necessary to fully elucidate the precise molecular mechanism(s) responsible for the observed antitumor activity. Nevertheless, the present findings provide a solid foundation for subsequent mechanistic studies.

Compared to current standard of care monoclonal antibody therapies (trastuzumab for HER2 positive gastric cancer; nivolumab/pembrolizumab for PD L1 positive disease), our vaccine approach offers several potential advantages (12, 17, 20). First, vaccines induce active immunity with immunological memory, potentially providing long lasting protection and reducing the need for repeated infusions. Second, manufacturing costs for peptide vaccines are substantially lower than for monoclonal antibodies, which could improve affordability and access. Third, a single vaccine product targeting three antigens could replace the need for multiple separate biologic agents. Fourth, the vaccine approach may be associated with

fewer infusion related reactions and a lower risk of developing anti drug antibodies. However, several challenges must be acknowledged. Peptide vaccines typically induce weaker immune responses than live attenuated vaccines or potent adjuvants, necessitating optimized delivery systems such as the nanoliposomes used here (46). Additionally, the requirement for adjuvant co administration and the need for booster doses may complicate clinical implementation. The relatively slow kinetics of vaccine induced immunity (weeks to develop) compared to passive antibody administration (immediate) may be disadvantageous in rapidly progressive disease.

Several limitations of this study should be acknowledged to appropriately interpret the findings and guide future investigations. First, while the BALB/c mouse model provides valuable preliminary data, it does not fully recapitulate the human immune system or the complex tumor microenvironment of gastric cancer. Future studies should evaluate the construct in humanized mouse models or in non human primates prior to clinical translation. Second, only one gastric cancer cell line (MKN-45) was evaluated. MKN-45 cells are derived from a gastric adenocarcinoma and express HER2, PD L1, and PD 1 to varying degrees, but the expression profile may not represent all gastric cancer subtypes. Future studies should include additional cell lines (e.g., NCI N87, MKN 45, KATO III) and, ideally, patient derived organoids or xenografts to assess heterogeneity in response. Third, while our study demonstrated apoptosis induction in vitro through antibody dependent mechanisms, we did not directly evaluate T cell responses. ELISpot assays, intracellular cytokine staining, and cytotoxicity assays should be performed in future studies to characterize the cellular arm of the immune response. The MHC binding predictions suggest that the epitopes may also activate T cells, but this requires experimental confirmation. Fourth, the duration of the immune response was not assessed. Longitudinal studies tracking antibody titers and T cell responses for extended periods (e.g., 3 6 months post immunization) are needed to evaluate immunological memory. Fifth, therapeutic efficacy studies in tumor bearing mice are needed. While our study demonstrates immunogenicity and in vitro apoptosis, the ultimate test of vaccine efficacy is the ability to inhibit tumor growth, induce regression, and prolong survival in animal models of established gastric cancer. Such studies should include both prophylactic (vaccination before tumor challenge) and therapeutic (vaccination after tumor engraftment) protocols. Sixth, safety studies beyond the acute observation period are needed. While no adverse effects were observed during the four week study period, long term safety studies evaluating potential autoimmune sequelae

(given that PD 1, PD L1, and HER2 are also expressed on some normal tissues) should be performed.

While targeting the PD-1/PD-L1 immune checkpoint axis represents a promising immunotherapeutic strategy, it is imperative to acknowledge the potential autoimmune risks associated with this approach. PD-1 and PD-L1 play a critical physiological role in maintaining peripheral immune tolerance and preventing excessive immune activation against self-antigens. Consequently, their inhibition, whether through monoclonal antibodies or peptide-based vaccines, has been associated with a spectrum of immune-related adverse events (irAEs) in clinical practice. These include immune-mediated colitis, pneumonitis, dermatitis, hepatitis, and endocrinopathies such as thyroiditis and hypophysitis. In the context of our vaccine strategy, which utilizes HLA-A0201-restricted epitopes derived from PD-1 and PD-L1, there is a theoretical concern regarding molecular mimicry and cross-reactivity with structurally similar peptides expressed on normal tissues. Although our in vitro cytotoxicity data did not indicate overt off-target effects on non-malignant cell lines, the possibility of cross-reactive T cell responses against self-antigens cannot be entirely excluded. This is particularly relevant given the widespread expression of PD-L1 on various normal tissues, including the intestinal epithelium, lung epithelium, and pancreatic islet cells. Therefore, we emphasize that the safety profile of our nanoliposomal vaccine requires rigorous evaluation in future studies. Specifically, we recommend comprehensive autoimmune toxicity assessments in appropriate immunocompetent animal models, including histopathological examination of target organs (e.g., gastrointestinal tract, lungs, skin, and endocrine glands), monitoring of autoantibody production, and evaluation of T cell reactivity against tissue-specific antigens. Such investigations will be essential to establish the therapeutic window of this vaccine and to mitigate potential risks before clinical translation.

Despite the promising in vitro findings of this study, several limitations warrant consideration. Notably, the therapeutic efficacy of the developed nanoliposomal vaccine was not evaluated in an in vivo tumor-bearing animal model. While our cell-based assays provide valuable insights into the cytotoxic and apoptotic potential of the formulation, they cannot fully recapitulate the complex tumor microenvironment, immune system interactions, and pharmacokinetic behavior that would occur in a living organism. Therefore, the translational relevance of these findings remains to be established. Future studies should employ appropriate immunocompetent murine tumor models, to assess antitumor efficacy, tumor growth inhibition, and survival benefits. Additionally, such in

vivo investigations would allow for comprehensive evaluation of the vaccine's safety profile, biodistribution, and potential off-target effects. Nevertheless, the current study serves as a critical foundation, providing the necessary preclinical rationale and optimization data to guide subsequent animal studies and, ultimately, clinical translation. Another limitation of the present study is the absence of direct evaluation of cellular immune responses. Although our data demonstrate that the nanoliposomal vaccine formulation induces cytotoxic effects and apoptosis, we did not assess the activation of antigen-specific T lymphocytes, such as CD8⁺ cytotoxic T cells, which are critical mediators of antitumor immunity. Parameters including T cell proliferation, cytokine secretion profiles (e.g., IFN- γ , TNF- α , and IL-2), and degranulation markers (e.g., CD107a) were not measured in this investigation. Consequently, while the observed antitumor activity may be partially attributed to direct peptide-mediated effects, the potential contribution of adaptive cellular immunity remains unexplored. Future studies should incorporate comprehensive immune monitoring approaches, including ELISpot assays, intracellular cytokine staining, and multiparametric flow cytometry, using appropriate in vivo models to elucidate the immunogenic mechanisms underlying the vaccine's therapeutic activity. Such investigations would provide a more complete understanding of the vaccine's mechanism of action and facilitate its optimization for clinical applications.

5. Conclusion

This study successfully designed a multi epitope peptide construct targeting PD 1, PD L1, and HER2 using an integrated immunoinformatics approach. Nanoliposomal delivery of this construct produced a formulation with favorable physicochemical properties (size ~120 nm, PDI 0.23, zeta potential -28.5 mV, encapsulation efficiency 78.4%). Immunization of BALB/c mice with the nanolipoepptide vaccine elicited dose dependent antibody production (titer up to 1:12,800) and a Th1 skewed cytokine response (IFN γ 320 pg/mL). Importantly, antibodies generated by vaccination recognized native antigens on MKN-45 gastric cancer cells, induced significant apoptosis (42.3% total apoptosis in the high dose group), and shifted BAX/BCL2 expression toward a pro apoptotic state (4.2 fold BAX increase, 3.8 fold BCL2 decrease). These results establish the multi epitope nanolipoepptide platform as a promising immunotherapeutic candidate for gastric adenocarcinoma. Future work should focus on

evaluating therapeutic efficacy in tumor bearing animal models, characterizing T cell responses, assessing long term safety and immunological memory, and ultimately translating this construct into clinical studies.

Acknowledgements

The authors gratefully acknowledge the staff of the Farhikhtegan Medical Convergence Sciences Research Center for their technical support and provision of laboratory facilities. We also thank the Pasteur Institute of Iran for providing the MKN-45 cell line and BALB/c mice.

Ethical Considerations and Compliance with Ethical Guidelines

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.1403.431). All experiments were performed in accordance with relevant guidelines and regulations including the ARRIVE guidelines

Conflict of interest

This research was funded by the Islamic Azad University of Medical Sciences, Tehran, Iran.

Author contributions

- Sorouralsadat Ahmadian Davoudi: Conceptualization, investigation, data curation, formal analysis, writing – original draft
- Mehrdad Hashemi: Conceptualization, supervision, methodology, funding acquisition, writing – review & editing
- AliBeman Jebali: Methodology (nanoliposome preparation and characterization), validation, resources
- Masoumeh Heshmati: Methodology (animal experiments, ELISA), project administration
- Maliheh Entezari: Methodology (molecular biology, real-time PCR), visualization, software

All authors reviewed and approved the final version of the manuscript.

AI Using Declaration

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

6. References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics

- 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209-249. (DOI: [10.3322/caac.21660](https://doi.org/10.3322/caac.21660))
2. Kalan Farmanfarma K, Mahdavi N, Hassanipour S, Salehiniya H. Epidemiologic study of gastric cancer in Iran: a systematic review. *Clin Exp Gastroenterol*. 2020;13:511-542. (DOI: [10.2147/CEG.S256627](https://doi.org/10.2147/CEG.S256627))
3. Moradian F, Fararouei M, Karami M, Ghasemi Y, Hosseini SV, Rezaianzadeh A, et al. Trend of geographical distribution of stomach cancer in Iran from 2004 to 2014. *BMC Gastroenterol*. 2022;22(1):4. (DOI: [10.1186/s12876-021-02066-z](https://doi.org/10.1186/s12876-021-02066-z))
4. Smyth EC, Nilsson M, Grabsch HI, van Grieken NC, Lordick F. Gastric cancer. *Lancet*. 2020;396(10251):635-648. (DOI: [10.1016/S0140-6736\(20\)31288-5](https://doi.org/10.1016/S0140-6736(20)31288-5))
5. Farahmandi F, Salarabedi MM, Parhizgar P, Ahmadi M, Zare M, Rezaei F, et al. Survival of gastric cancer patients in Iran: a systematic review and meta-analysis. *Gastroenterol Hepatol Bed Bench*. 2023;16(3):245-258. (DOI: [10.22037/ghfbb.v16i2.2674](https://doi.org/10.22037/ghfbb.v16i2.2674))
6. Li K, Zhang A, Li X, Zhang H, Zhao L. Advances in clinical immunotherapy for gastric cancer. *Biochim Biophys Acta Rev Cancer*. 2021;1876(2):188615. (DOI: [10.1016/j.bbcan.2021.188615](https://doi.org/10.1016/j.bbcan.2021.188615))
7. Joshi SS, Badgwell BD. Current treatment and recent progress in gastric cancer. *CA Cancer J Clin*. 2021;71(3):264-279. (DOI: [10.3322/caac.21657](https://doi.org/10.3322/caac.21657))
8. Sharma P, Siddiqui BA, Anandhan S, Yadav SS, Subudhi SK, Gao J, et al. The next decade of immune checkpoint therapy. *Cancer Discov*. 2021;11(4):838-857. (DOI: [10.1158/2159-8290.CD-20-168](https://doi.org/10.1158/2159-8290.CD-20-168))
9. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science*. 2018;359(6382):1350-1355. (DOI: [10.1126/science.aar4060](https://doi.org/10.1126/science.aar4060))
10. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. 2012;12(4):252-264. (DOI: [10.1038/nrc3239](https://doi.org/10.1038/nrc3239))
11. Topalian SL, Taube JM, Pardoll DM. Neoadjuvant checkpoint blockade for cancer immunotherapy. *Science*. 2020;367(6477):eaaz0182. (DOI: [10.1126/science.aaz0182](https://doi.org/10.1126/science.aaz0182))
12. Kang YK, Boku N, Satoh T, Ryu MH, Chao Y, Kato K, et al. Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet*. 2017;390(10111):2461-2471. (DOI: [10.1016/S0140-6736\(17\)31827-5](https://doi.org/10.1016/S0140-6736(17)31827-5))
13. Fuchs CS, Doi T, Jang RW, Muro K, Satoh T, Machado M, et al. Safety and efficacy of pembrolizumab monotherapy in patients with previously treated advanced gastric and gastroesophageal junction cancer: phase 2 clinical KEYNOTE-059 trial. *JAMA Oncol*. 2018;4(5):e180013. (DOI: [10.1001/jamaoncol.2018.001](https://doi.org/10.1001/jamaoncol.2018.001))
14. Shitara K, Özgüroğlu M, Bang YJ, Di Bartolomeo M, Mandalà M, Ryu MH, et al. Pembrolizumab versus paclitaxel for previously treated, advanced gastric or gastro-oesophageal junction cancer (KEYNOTE-061): a randomised, open-label, controlled, phase 3 trial. *Lancet*. 2018;392(10142):123-133. (DOI: [10.1016/S0140-6736\(18\)31257-1](https://doi.org/10.1016/S0140-6736(18)31257-1))
15. Shitara K, Van Cutsem E, Bang YJ, Fuchs C, Wyrwicz L, Lee KW, et al. Efficacy and safety of pembrolizumab or pembrolizumab plus chemotherapy vs chemotherapy alone for patients with first-line, advanced gastric cancer: the KEYNOTE-062 phase 3 randomized clinical trial. *JAMA Oncol*. 2020;6(10):1571-1580. (DOI: [10.1001/jamaoncol.2020.3370](https://doi.org/10.1001/jamaoncol.2020.3370))
16. Janjigian YY, Kawazoe A, Yañez P, Li N, Lonardi S, Kolesnik O, et al. The KEYNOTE-811 trial of dual PD-1 and HER2 blockade in HER2-positive gastric cancer. *Nature*. 2021;600(7890):727-730. (DOI: [10.1038/s41586-021-04161-3](https://doi.org/10.1038/s41586-021-04161-3))
17. Janjigian YY, Shitara K, Moehler M, Garrido M, Salman P, Shen L, et al. First-line nivolumab plus chemotherapy versus chemotherapy alone for advanced gastric, gastro-oesophageal junction, and oesophageal adenocarcinoma (CheckMate 649): a randomised, open-label, phase 3 trial. *Lancet*. 2021;398(10294):27-40. (DOI: [10.1016/S0140-6736\(21\)00797-2](https://doi.org/10.1016/S0140-6736(21)00797-2))
18. Rubin I, Yarden Y. The basic biology of HER2. *Ann Oncol*. 2001;12 Suppl 1:S3-S8. (DOI: [10.1093/annonc/12.suppl_1.s3](https://doi.org/10.1093/annonc/12.suppl_1.s3))
19. Pazo Cid RA, Antón A. Advanced HER2-positive gastric cancer: current and future targeted therapies. *Crit Rev Oncol Hematol*. 2013;85(3):350-362. (DOI: [10.1016/j.critrevonc.2012.08.008](https://doi.org/10.1016/j.critrevonc.2012.08.008))
20. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet*. 2010;376(9742):687-697. (DOI: [10.1016/S0140-6736\(10\)61121-X](https://doi.org/10.1016/S0140-6736(10)61121-X))
21. Shitara K, Bang YJ, Iwasa S, Sugimoto N, Ryu MH, Sakamoto N, et al. Trastuzumab deruxtecan in previously treated HER2-positive gastric cancer. *N Engl J Med*. 2020;382(25):2419-2430. (DOI: [10.1056/NEJMoa2004413](https://doi.org/10.1056/NEJMoa2004413))
22. Zhu Y, Zhu X, Wei X, Tang C, Zhang W. HER2-targeted therapies in gastric cancer. *Biochim Biophys Acta Rev Cancer*. 2021;1876(1):188549. (DOI: [10.1016/j.bbcan.2021.188549](https://doi.org/10.1016/j.bbcan.2021.188549))
23. Janjigian YY, Sanchez-Vega F, Jonsson P, Chatila WK, Hechtman JF, Ku GY, et al. Genetic predictors of response to systemic therapy in esophagogastric cancer. *Cancer Discov*. 2018;8(1):49-58. (DOI: [10.1158/2159-8290.CD-17-0787](https://doi.org/10.1158/2159-8290.CD-17-0787))
24. Liu J, Fu M, Wang M, Wan D, Wei Y, Wei X. Cancer vaccines as promising immuno-therapeutics: platforms and current progress. *J Hematol Oncol*. 2022;15(1):28. (DOI: [10.1186/s13045-022-01247-x](https://doi.org/10.1186/s13045-022-01247-x))
25. Saxena M, van der Burg SH, Melief CJM, Bhardwaj N. Therapeutic cancer vaccines. *Nat Rev Cancer*. 2021;21(6):360-378. (DOI: [10.1038/s41568-021-00346-0](https://doi.org/10.1038/s41568-021-00346-0))
26. Mittendorf EA, Lu B, Melisko M, Price Hiller J, Bondarenko I, Brunt AM, et al. Efficacy and safety analysis of nelipecimut-S vaccine to prevent breast cancer recurrence: a randomized, multicenter, phase III clinical trial. *Clin Cancer Res*. 2019;25(14):4248-4254. (DOI: [10.1158/1078-0432.CCR-18-2867](https://doi.org/10.1158/1078-0432.CCR-18-2867))
27. Brown TA, Mittendorf EA, Hale DF, Myers JW, Peace KM, Jackson DO, et al. Prospective, randomized, single-blinded, multi-center phase II trial of two HER2 peptide

- vaccines, GP2 and AE37, in breast cancer patients to prevent recurrence. *Breast Cancer Res Treat.* 2020;181(2):391-401. (DOI: [10.1007/s10549-020-05638-x](https://doi.org/10.1007/s10549-020-05638-x))
- 28.Clifton GT, Hale D, Vreeland TJ, Hickerson AT, Litton JK, Alatrash G, et al. Results of a randomized phase IIb trial of nelipepimut-S + trastuzumab versus trastuzumab to prevent recurrences in patients with high-risk HER2 low-expressing breast cancer. *Clin Cancer Res.* 2020;26(11):2515-2523. (DOI: [10.1158/1078-0432.CCR-19-2741](https://doi.org/10.1158/1078-0432.CCR-19-2741))
- 29.Wiedermann U, Garner-Spitzer E, Chao Y, Maglakelidze M, Bulat I, Debois M, et al. Clinical and immunologic responses to a B-cell epitope vaccine in patients with HER2/neu-overexpressing advanced gastric cancer—results from phase Ib trial IMU.ACS.001. *Clin Cancer Res.* 2021;27(13):3649-3660. (DOI: [10.1158/1078-0432.CCR-20-3742](https://doi.org/10.1158/1078-0432.CCR-20-3742))
- 30.Sato H, Okonogi N, Nakano T. Rationale of combination of anti-PD-1/PD-L1 antibody therapy and radiotherapy for cancer treatment. *Int J Clin Oncol.* 2020;25(5):801-809. (DOI: [10.1007/s10147-020-01666-1](https://doi.org/10.1007/s10147-020-01666-1))
- 31.Tanaka Y, Wada H, Goto R, Osawa C, Yamamura M, Kubo M, et al. TAS0314, a novel multi-epitope long peptide vaccine, showed synergistic antitumor immunity with PD-1/PD-L1 blockade in HLA-A*2402 mice. *Sci Rep.* 2020;10(1):17284. (DOI: [10.1038/s41598-020-74187-6](https://doi.org/10.1038/s41598-020-74187-6))
- 32.Zhou Y. HER2/neu-based vaccination with li-Key hybrid, GM-CSF immunoadjuvant and trastuzumab as a potent triple-negative breast cancer treatment. *J Cancer Res Clin Oncol.* 2023;149(9):6711-6718. (DOI: [10.1007/s00432-023-04574-9](https://doi.org/10.1007/s00432-023-04574-9))
- 33.Saha S, Raghava GP. Prediction of continuous B-cell epitopes in an antigen using recurrent neural network. *Proteins.* 2006;65(1):40-48. (DOI: [10.1002/prot.21078](https://doi.org/10.1002/prot.21078))
- 34.Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinformatics.* 2007;8:4. (DOI: [10.1186/1471-2105-8-4](https://doi.org/10.1186/1471-2105-8-4))
- 35.He Y, Xiang Z, Mobley HL. Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development. *J Biomed Biotechnol.* 2010;2010:297505. (DOI: [10.1155/2010/297505](https://doi.org/10.1155/2010/297505))
- 36.Dimitrov I, Bangov I, Flower DR, Doytchinova I. AllerTOP v.2—a server for in silico prediction of allergens. *J Mol Model.* 2014;20(6):2278. (DOI: [10.1007/s00894-014-2278-5](https://doi.org/10.1007/s00894-014-2278-5))
- 37.Gupta S, Kapoor P, Chaudhary K, Gautam A, Kumar R, Raghava GP. In silico approach for predicting toxicity of peptides and proteins. *PLoS One.* 2013;8(9):e73957. <https://doi.org/10.1371/journal.pone.0073957>
- 38.O'Donnell JS, Teng MWL, Smyth MJ. Cancer immunoediting and resistance to T cell-based immunotherapy. *Nat Rev Clin Oncol.* 2019;16(3):151-167. (DOI: [10.1038/s41571-018-0142-8](https://doi.org/10.1038/s41571-018-0142-8))
- 39.Jenkins RW, Barbie DA, Flaherty KT. Mechanisms of resistance to immune checkpoint inhibitors. *Br J Cancer.* 2018;118(1):9-16. (DOI: [10.1038/bjc.2017.434](https://doi.org/10.1038/bjc.2017.434))
- 40.Hu Z, Ott PA, Wu CJ. Towards personalized, tumour-specific, therapeutic vaccines for cancer. *Nat Rev Immunol.* 2018;18(3):168-182. (DOI: [10.1038/nri.2017.131](https://doi.org/10.1038/nri.2017.131))
- 41.Li J, Al Faruque H, Li S, Sima M, Sborov D, Hu-Lieskovan S, et al. PD-L1 targeted antibody-polymer-epirubicin conjugate prolongs survival in a preclinical murine model of advanced ovarian cancer. *J Control Release.* 2025;382:113682. (DOI: [10.1016/j.jconrel.2025.113682](https://doi.org/10.1016/j.jconrel.2025.113682))
- 42.Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. *Nature.* 2011;480(7378):480-489. (DOI: [10.1038/nature10673](https://doi.org/10.1038/nature10673))
- 43.de Paula Peres L, da Luz FA, dos Anjos Pultz B, Brigido PC, de Araujo RA, Goulart LR, et al. Peptide vaccines in breast cancer: the immunological basis for clinical response. *Biotechnol Adv.* 2015;33(8):1868-1877. (DOI: [10.1016/j.biotechadv.2015.10.013](https://doi.org/10.1016/j.biotechadv.2015.10.013))
- 44.Zeng W, Pan J, Fang Z, Jia J, Zhang R, He M, et al. A novel PD-L1-containing MSLN targeting vaccine for lung cancer immunotherapy. *Front Immunol.* 2022;13:925217. <https://doi.org/10.3389/fimmu.2022.925217>
- 45.Kaumaya PT. B-cell epitope peptide cancer vaccines: a new paradigm for combination immunotherapies with novel checkpoint peptide vaccine. *Future Oncol.* 2020;16(23):1767-1791. (DOI: [10.2217/fon-2020-0224](https://doi.org/10.2217/fon-2020-0224))
- 46.Sahdev P, Ochyl LJ, Moon JJ. Biomaterials for nanoparticle vaccine delivery systems. *Pharm Res.* 2014;31(10):2563-2582. (DOI: [10.1007/s11095-014-1419-y](https://doi.org/10.1007/s11095-014-1419-y))
- 47.Liu J, Zhang R, Xu ZP. Nanoparticle-based nanomedicines to promote cancer immunotherapy: recent advances and future directions. *Small.* 2019;15(32):1900262. (DOI: [10.1002/smll.201900262](https://doi.org/10.1002/smll.201900262))
- 48.Hou X, Zaks T, Langer R, Dong Y. Lipid nanoparticles for mRNA delivery. *Nat Rev Mater.* 2021;6(12):1078-1094. (DOI: [10.1038/s41578-021-00358-0](https://doi.org/10.1038/s41578-021-00358-0))
- 49.Tenchov R, Bird R, Curtze AE, Zhou Q. Lipid nanoparticles—from liposomes to mRNA vaccine delivery, a landscape of research diversity and advancement. *ACS Nano.* 2021;15(11):16982-17015. (DOI: [10.1021/acsnano.1c04996](https://doi.org/10.1021/acsnano.1c04996))
- 50.Jebali A, Noroozi Karimabad M, Ahmadi Z, Khorramdel H, Kaeidi A, Mirzaei M, et al. Attenuation of inflammatory response in the EAE model by PEGylated nanoliposome of pistachio oils. *J Neuroimmunol.* 2020;347:577352. (DOI: [10.1016/j.jneuroim.2020.577352](https://doi.org/10.1016/j.jneuroim.2020.577352))
- 51.O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4⁺ T cells. *Science.* 2010;327(5969):1098-1102. (DOI: [10.1126/science.1178334](https://doi.org/10.1126/science.1178334))
- 52.Gocher AM, Workman CJ, Vignali DAA. Interferon- γ : teammate or opponent in the tumour microenvironment? *Nat Rev Immunol.* 2022;22(3):158-172. (DOI: [10.1038/s41577-021-00566-3](https://doi.org/10.1038/s41577-021-00566-3))
- 53.Castro F, Cardoso AP, Gonçalves RM, Serre K, Oliveira MJ. Interferon- γ at the crossroads of tumor immune surveillance or evasion. *Front Immunol.* 2018;9:847. <https://doi.org/10.3389/fimmu.2018.00847>
- 54.van Loo G, Bertrand MJM. Death by TNF: a road to inflammation. *Nat Rev Immunol.* 2023;23(5):289-303. (DOI: [10.1038/s41577-022-00792-3](https://doi.org/10.1038/s41577-022-00792-3))
- 55.Choy EH, De Benedetti F, Takeuchi T, Hashizume M, Rose-John S, Kishimoto T. Translating IL-6 biology into effective treatments. *Nat Rev Rheumatol.* 2020;16(6):335-345. (DOI: [10.1038/s41584-020-0419-z](https://doi.org/10.1038/s41584-020-0419-z))
- 56.Singh R, Letai A, Sarosiek K. Regulation of apoptosis in health and disease: the balancing act of BCL-2 family

- proteins. *Nat Rev Mol Cell Biol.* 2019;20(3):175-193. (DOI: [10.1038/s41580-018-0089-8](https://doi.org/10.1038/s41580-018-0089-8))
- 57.Ola MS, Nawaz M, Ahsan H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. *Mol Cell Biochem.* 2011;351(1-2):41-58. (DOI: [10.1007/s11010-010-0709-x](https://doi.org/10.1007/s11010-010-0709-x))
- 58.Meric-Bernstam F, Johnson AM, Ileana Dumbrava EE, Raghav K, Balaji K, Bhatt M, et al. Advances in HER2-targeted therapy: novel agents and opportunities beyond breast and gastric cancer. *Clin Cancer Res.* 2019;25(7):2033-2041. (DOI: [10.1158/1078-0432.CCR-18-2275](https://doi.org/10.1158/1078-0432.CCR-18-2275))
- 59.Lecis D, Sangaletti S, Colombo MP, Chiodoni C. Immune checkpoint ligand reverse signaling: looking back to go forward in cancer therapy. *Cancers (Basel).* 2019;11(5):624. (DOI: [10.3390/cancers11050624](https://doi.org/10.3390/cancers11050624))
- 60.Bournazos S, Gupta A, Ravetch JV. The role of IgG Fc receptors in antibody-dependent enhancement. *Nat Rev Immunol.* 2020;20(10):633-643. (DOI: [10.1038/s41577-020-00410-0](https://doi.org/10.1038/s41577-020-00410-0))
- 61.Gulley JL, Madan RA, Pachynski R, Mulders P, Sheikh NA, Trager J, et al. Role of antigen spread and distinctive characteristics of immunotherapy in cancer treatment. *J Natl Cancer Inst.* 2017;109(4):djw261. (DOI: [10.1093/jnci/djw261](https://doi.org/10.1093/jnci/djw261))