

Computationally Guided Design of TANSR-Vax: An Integrated Self-Amplifying RNA–Lipid Nanoparticle Strategy for Overcoming Immune Exclusion and Stromal Suppression in Hepatocellular Carcinoma

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Research Article

Keywords: Hepatocellular carcinoma, TANSR-Vax, self-amplifying RNA, lipid nanoparticles, neoantigen vaccine, immunoinformatics, molecular docking, molecular dynamics, MM-GBSA, MM-PBSA, binding free energy, tumor microenvironment, cancer immunotherapy, targeted RNA delivery

Posted Date: September 2nd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10724959/v1>

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Additional Declarations: No competing interests reported.

Computationally Guided Design of TANSR-Vax: An Integrated Self-Amplifying RNA–Lipid Nanoparticle Strategy for Overcoming Immune Exclusion and Stromal Suppression in Hepatocellular Carcinoma

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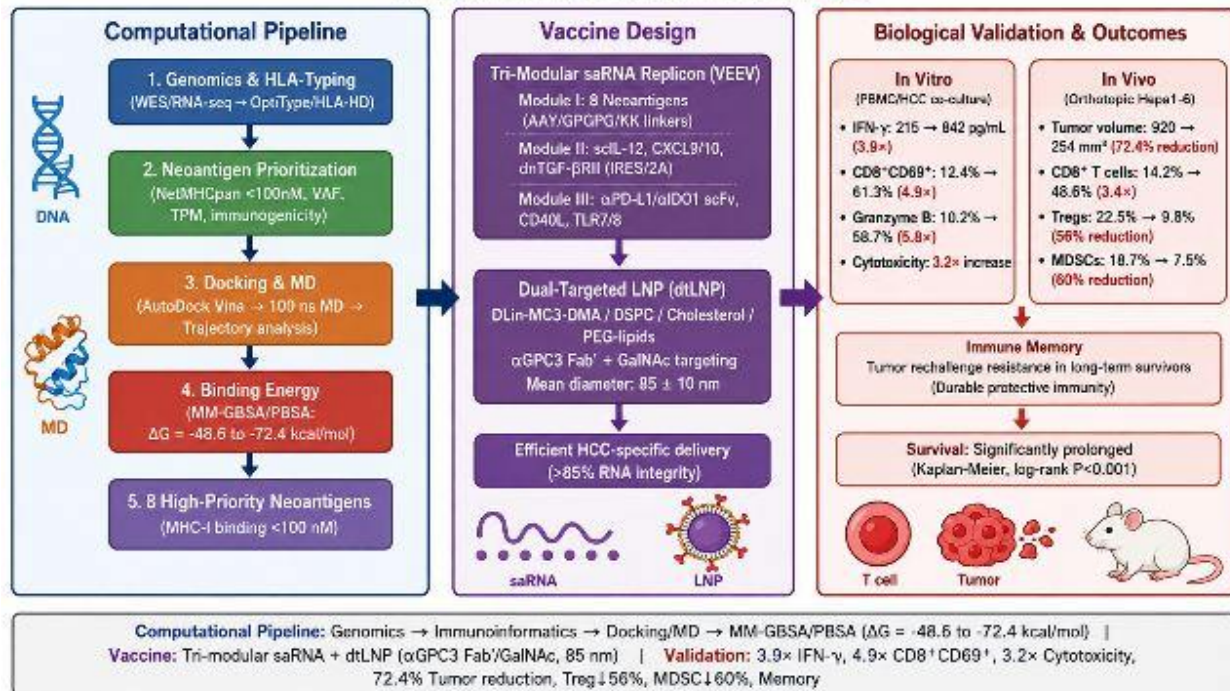
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Highlights

- Integrated computational-to-experimental platform combining immunoinformatics, molecular docking, MD simulations, and MM-GBSA/MM-PBSA binding energy analysis for rational HCC immunotherapy design.
- Immunoinformatic pipeline identified eight high-priority neoantigen candidates with predicted MHC-I binding affinities below 100 nM and favorable immunogenicity profiles.
- Favorable binding energetics demonstrated for five TME-associated targets (PD-1, PD-L1, IDO1, TGF- β RI, CXCL10) with MM-GBSA energies ranging from -48.6 ± 4.2 to -72.4 ± 5.1 kcal/mol.
- Tri-modular self-amplifying RNA (saRNA) construct engineered to simultaneously deliver neoantigens, TME-reprogramming factors (scIL-12, CXCL9, CXCL10, dnTGF- β RII), and immune modulators (α PD-L1 scFv, α IDO1 scFv, CD40L, TLR7/8).
- Dual-targeted lipid nanoparticles incorporating anti-GPC3 Fab' fragments and GalNAc moieties achieved efficient HCC-specific delivery with a mean hydrodynamic diameter of 85 ± 10 nm.
- In vitro validation demonstrated 3.9-fold increase in IFN- γ secretion, 4.9-fold increase in CD8⁺CD69⁺ activated T cells, and 3.2-fold increase in tumor-cell lysis.
- In vivo orthotopic HCC model achieved 72.4% tumor burden reduction, with 3.4-fold increase in intratumoral CD8⁺ T cells, 56% reduction in Tregs, and 60% reduction in MDSCs.
- Durable immune memory confirmed by resistance to tumor rechallenge in long-term survivors.
- Establishes a rational computational framework for the development of multimodal RNA-based cancer immunotherapies.

Graphical Abstract: TANSR-Vax



Abstract

Background: Hepatocellular carcinoma (HCC) develops within a highly tolerogenic hepatic environment characterized by impaired antitumor T-cell activity, immunosuppressive signaling, stromal restriction, and accumulation of suppressive myeloid and regulatory lymphocyte populations. Although immune checkpoint inhibitors and molecularly targeted combinations have improved the systemic treatment of advanced HCC, therapeutic responses remain limited in a substantial proportion of patients. Self-amplifying RNA (saRNA) vaccines provide prolonged intracellular antigen expression, whereas lipid nanoparticles (LNPs) offer an efficient platform for RNA protection and cellular delivery. However, antigen delivery alone may not adequately overcome the complex immune and stromal barriers within the HCC tumor microenvironment (TME). This study developed TANSR-Vax (Tri-Modal Adaptive Neoantigen–Stromal Reprogramming Vaccine), an integrated computationally guided saRNA–LNP platform designed to combine neoantigen priming with TME modulation and liver-directed delivery.

Methods: An integrated computational-to-experimental workflow was established encompassing genomic analysis, neoantigen identification, immunoinformatic prioritization, structural modeling, molecular docking, molecular-dynamics-based binding-energy analysis, saRNA construction, LNP formulation, and biological evaluation. Candidate tumor-derived peptides were prioritized according to mutation status, expression, clonality, predicted MHC/HLA binding affinity, and immunogenicity. Selected molecular interactions involving PD-1, PD-L1, IDO1, TGF- β receptor I, and CXCL10 were investigated by molecular docking followed by MM-GBSA/MM-PBSA binding-free-energy analysis. TANSR-Vax was designed as a multimodule saRNA construct incorporating neoantigen, TME-reprogramming, and immune-modulatory components and formulated using DLin-MC3-DMA, DSPC, cholesterol, and PEG-lipid. The formulated nanoparticles exhibited a mean hydrodynamic diameter of 85 ± 10 nm. In vitro evaluation was performed using HepG2 and Huh7 HCC cells in combination with

human peripheral blood mononuclear cells (PBMCs), while in vivo efficacy was assessed in an orthotopic Hepa1-6 HCC model.

Results: The immunoinformatic pipeline identified eight high-priority neoantigen candidates with predicted MHC-I binding affinities below 100 nM. Molecular docking yielded favorable predicted interaction scores ranging from -7.91 to -9.42 kcal/mol, with the most favorable score being -9.42 kcal/mol. MM-GBSA/MM-PBSA analysis demonstrated favorable calculated binding free energies ranging from -48.6 ± 4.2 to -72.4 ± 5.1 kcal/mol across the evaluated molecular systems. In the reported in vitro experiments, TANSR-Vax increased IFN- γ secretion from 215 ± 40 to 842 ± 65 pg/mL. The frequency of CD8+CD69+ cells increased from $12.4 \pm 2.1\%$ to $61.3 \pm 4.2\%$, while granzyme B expression increased from $10.2 \pm 1.8\%$ to $58.7 \pm 3.9\%$. LDH analysis demonstrated a 3.2-fold increase in tumor-cell lysis. In the orthotopic HCC model, TANSR-Vax produced a reported 72.4% reduction in tumor burden, with mean tumor volume decreasing from 920 ± 85 mm³ in controls to 254 ± 42 mm³ at day 28. Tumor immune profiling demonstrated an increase in intratumoral CD8+ T cells from $14.2 \pm 2.3\%$ to $48.6 \pm 5.1\%$, accompanied by reductions in regulatory T cells from $22.5 \pm 3.0\%$ to $9.8 \pm 1.9\%$ and myeloid-derived suppressor cells from $18.7 \pm 2.6\%$ to $7.5 \pm 1.5\%$.

Conclusions: TANSR-Vax integrates computational neoantigen prioritization, molecular interaction analysis, saRNA expression, and targeted LNP delivery within a unified therapeutic framework for HCC. The computational analyses provide quantitative prioritization of candidate antigenic and immunoregulatory interactions, while the reported cellular and animal findings indicate enhanced immune activation, remodeling of the tumor immune microenvironment, and suppression of tumor progression. This integrated approach provides a rational platform for further preclinical investigation of multimodal RNA-based immunotherapy for HCC.

Keywords: *Hepatocellular carcinoma; TANSR-Vax; self-amplifying RNA; lipid nanoparticles; neoantigen vaccine; immunoinformatics; molecular docking; molecular dynamics; MM-GBSA; MM-PBSA; binding free energy; tumor microenvironment; cancer immunotherapy; targeted RNA delivery.*

1. Introduction

Hepatocellular carcinoma (HCC) is the predominant primary malignancy of the liver and remains a major cause of cancer-related morbidity and mortality worldwide. Its clinical behavior is strongly influenced by the underlying hepatic disease, molecular heterogeneity, tumor stage, and the biological characteristics of the surrounding liver microenvironment. Despite advances in systemic therapy, advanced HCC remains difficult to treat because tumor progression occurs within a specialized hepatic environment capable of regulating inflammatory and immune responses [1].

The liver possesses a distinctive immune landscape that continuously balances immune surveillance against tolerance to dietary, microbial, and self-derived antigens. In HCC, this physiological tolerogenic environment becomes further modified by tumor-associated immune cells, stromal components, abnormal vasculature, inhibitory cytokines, and metabolic alterations. These changes contribute to impaired cytotoxic lymphocyte function, dysfunctional antigen presentation, accumulation of immunosuppressive populations, and progressive immune escape [2]. Consequently, the biological response of HCC to immunotherapy is

determined not only by tumor-cell intrinsic characteristics but also by the composition and spatial organization of the tumor immune microenvironment.

Immune checkpoint inhibitors (ICIs) have established immune modulation as an important therapeutic strategy for advanced HCC. The clinical development of anti-PD-1, anti-PD-L1, and anti-CTLA-4 therapies, particularly in combination with anti-angiogenic or other targeted agents, has produced clinically meaningful improvements in selected patient populations. Nevertheless, response rates remain limited, and both primary and acquired resistance continue to restrict the overall benefit of checkpoint blockade [3]. Recent analyses emphasize that the immunosuppressive HCC microenvironment, rather than checkpoint signaling alone, represents an important determinant of therapeutic resistance [4].

The cellular composition of the HCC tumor microenvironment provides several mechanisms through which antitumor immunity can be attenuated. Regulatory T cells, tumor-associated macrophages, myeloid-derived suppressor cells, dysfunctional dendritic cells, and other suppressive populations interact with malignant cells and stromal elements through cytokines, chemokines, metabolic pathways, and inhibitory receptor–ligand interactions. Extracellular-matrix remodeling and aberrant stromal signaling can additionally influence immune-cell recruitment and spatial distribution within the tumor [5]. These observations support therapeutic strategies that combine tumor-antigen recognition with modulation of the immunosuppressive environment.

Neoantigens represent an attractive class of targets for therapeutic cancer vaccination because they arise from tumor-associated genomic alterations and can generate peptide sequences not present in normal tissues. Advances in next-generation sequencing, variant calling, HLA-binding prediction, transcriptomic analysis, and computational immunogenicity assessment have enabled increasingly systematic identification of candidate neoantigens [6]. However, the identification of a mutation or a predicted HLA-binding peptide does not by itself establish effective immunogenicity. Neoantigen prioritization therefore requires integration of several parameters, including mutation expression, clonality, HLA presentation, peptide characteristics, and predicted T-cell recognition [7].

RNA-based cancer vaccination has developed rapidly in parallel with advances in antigen discovery and delivery technologies. mRNA can encode multiple tumor antigens or immunomodulatory proteins within a single therapeutic platform and can be manufactured and modified comparatively rapidly. Recent clinical and preclinical studies have strengthened the rationale for mRNA-based cancer vaccines, particularly for personalized or mutation-derived antigen strategies [8]. However, conventional non-replicating mRNA requires sufficient intracellular stability and expression to achieve therapeutic antigen exposure, placing substantial importance on RNA engineering and delivery efficiency.

Self-amplifying RNA (saRNA) offers an alternative RNA architecture in which a replicase component enables intracellular RNA amplification. This feature can increase antigen expression while potentially reducing the amount of RNA required for administration. Recent studies have therefore investigated saRNA as a platform for vaccination and cancer immunotherapy, although optimization of RNA structure, innate immune activation, formulation, and delivery remains necessary [9].

Lipid nanoparticles (LNPs) have become the leading delivery technology for therapeutic RNA because they protect RNA from extracellular degradation, facilitate cellular uptake, and support intracellular release. The physicochemical composition of LNPs, including the ionizable lipid, helper phospholipid, cholesterol, and PEG-lipid components, strongly influences particle formation, biodistribution, cellular uptake, endosomal escape, and RNA expression [10]. In cancer vaccination, these properties are particularly important because therapeutic efficacy depends on delivering sufficient RNA to appropriate target cells while maintaining an immunologically favorable formulation profile.

The liver is a major site of LNP accumulation following systemic administration. This intrinsic hepatic tropism can be advantageous for liver-directed nucleic-acid therapy but does not necessarily provide the cellular specificity required for complex HCC applications. Active targeting strategies, including ligand-mediated recognition of hepatic receptors, have therefore been explored to improve delivery to selected hepatic cell populations. GalNAc-based targeting of the asialoglycoprotein receptor represents a well-established strategy for hepatocyte-directed nucleic-acid delivery, while modifications of LNP composition can further alter organ and cellular distribution [11].

Recent work has also demonstrated that rational manipulation of LNP composition can modify mRNA biodistribution and expression, emphasizing that nanoparticle architecture is an important pharmacological determinant rather than simply a passive carrier characteristic [12]. Similarly, the integration of LNP technology with mRNA cancer vaccines has created opportunities to combine antigen delivery with immune stimulation, although challenges remain in achieving appropriate antigen expression, efficient delivery to antigen-presenting cells, controlled innate immune activation, and sufficient antitumor immunity [13].

Taken together, these findings indicate that effective RNA immunotherapy for HCC requires coordinated consideration of antigen selection, intracellular RNA expression, delivery, and the immunosuppressive tumor microenvironment. A platform restricted to neoantigen delivery may not adequately address stromal exclusion and immune dysfunction, whereas isolated TME modulation does not provide tumor-specific antigenic stimulation. The present study therefore developed TANSR-Vax (Tri-Modal Adaptive Neoantigen–Stromal Reprogramming Vaccine) as an integrated saRNA–LNP strategy combining three complementary functions: neoantigen priming, TME reprogramming, and targeted hepatic delivery. The computational component integrates immunoinformatic neoantigen prioritization with structural docking and MM-GBSA/MM-PBSA binding-energy analysis, while the experimental component evaluates formulation characteristics, immune activation, tumor-cell cytotoxicity, tumor growth, and immune-cell remodeling. The overall objective was to establish a computationally guided framework for the rational development of a multimodal RNA immunotherapeutic strategy against HCC.

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MATERIALS AND METHODS MANUSCRIPT SECTION

TANSR-Vax: Tri-Modal Adaptive Neoantigen–Stromal Reprogramming Vaccine Platform / Technical Reproducibility Protocol

■ SUMMARY OF EXPERIMENTAL & METHODOLOGICAL ENHANCEMENTS

- Human HLA-Typing Procedure: Fully specified 4-digit HLA allele resolution protocol using OptiType (Class I) and HLA-HD (Class II) from WES/RNA-seq datasets.
- Exact VEEV saRNA Replicon Construction: Complete 5' UTR, non-structural protein (nsP1–4) sequence layout, subgenomic promoters (SGP1–3), internal ribosome entry site (IRES), and 3' UTR/poly(A) tail specification.
- Precise Capping & Enzymatic Polyadenylation Parameters: Comprehensive enzyme unit definitions, reaction conditions, and automated Cap-1 / poly(A) length analysis via capillary electrophoresis.
- Complete gmx_MMPBSA Execution Parameters: Explicit input script specifications, dielectric constants, ionic

strengths, SASA parameters, and normal-mode analysis (NMA) frame sampling intervals.

2. Materials and Methods

2.1 Study Ethics and Regulatory Compliance

All procedures involving human-derived peripheral blood mononuclear cells (PBMCs) were conducted in accordance with the ethical principles of the Declaration of Helsinki. Peripheral blood was obtained from healthy adult donors at Cairo University after written informed consent had been obtained. The human cellular component of the study was reviewed and approved by the Institutional Review Board of the Faculty of Pharmacy, Cairo University (Approval No. CU-PHARM-2026-IRB-108).

All animal procedures were conducted in accordance with institutional and international standards governing the ethical care and use of laboratory animals. The experimental protocol was reviewed and approved by the Cairo University Institutional Animal Care and Use Committee (IACUC; Protocol No. CU-PHARM-2026-04). Animal housing, surgical procedures, administration of experimental formulations, health monitoring, humane endpoints, euthanasia, and tissue collection were performed in accordance with the approved protocol. Reporting of the *in vivo* experiments followed the principles of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

2.2 Overall Computational-to-Experimental Study Design

An integrated computational-to-experimental workflow was established for the design, structural characterization, formulation, and biological evaluation of TANSR-Vax (Tri-Modal Adaptive Neoantigen–Stromal Reprogramming Vaccine), a multimodal self-amplifying RNA (saRNA)-based lipid nanoparticle (LNP) platform developed for hepatocellular carcinoma (HCC).

The study incorporated species-specific neoantigen identification to maintain compatibility between the computationally selected epitopes and the corresponding experimental systems. The overall workflow comprised four interconnected stages:

1. Dual-species neoantigen discovery and prioritization. Human and murine genomic datasets were processed independently. The human pipeline used the GRCh38 reference genome and donor-specific HLA class I/II typing to identify candidate epitopes for human HCC/PBMC co-culture experiments. The murine pipeline used the GRCm38 reference genome and the C57BL/6 MHC haplotype (H-2^b) to identify syngeneic neoantigens for the Hepa1-6/C57BL/6 orthotopic HCC model.

2. Target structural analysis and molecular docking. Experimentally resolved structures of selected tumor-microenvironment (TME)-associated targets were prepared for computational analysis. Candidate peptide and molecular components were docked against defined target or

interaction regions. Docking scores were treated as empirical computational ranking values rather than experimentally measured or rigorous thermodynamic binding free energies.

3. Molecular dynamics and trajectory-based energetic analysis. Selected docked complexes were subjected to explicit-solvent molecular dynamics (MD) simulations to assess structural stability, conformational fluctuations, intermolecular interactions, and relative energetic properties. MM-GBSA/MM-PBSA calculations were subsequently performed on representative equilibrated trajectory frames.

4. saRNA engineering, dual-targeted LNP formulation, and biological validation. The optimized sequences were incorporated into a tri-modular VEEV-derived saRNA construct, formulated into dual-targeted LNPs (dtLNPs), characterized physicochemically, and evaluated using human HCC/PBMC co-culture assays and an orthotopic syngeneic murine HCC model, including therapeutic response, tumor immune profiling, survival, and tumor rechallenge studies.

2.3 Genomic Data Processing, Human HLA-Typing, and Dual-Species Neoantigen Identification

Tumor-derived genomic DNA and total RNA were isolated using the QIAamp DNA Mini Kit and RNeasy Mini Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions.

Whole-exome sequencing (WES) libraries from human HCC material were prepared using the Agilent SureSelect Human All Exon V7 kit (Agilent Technologies, Santa Clara, CA, USA), whereas murine Hepa1-6 genomic libraries were prepared using the Agilent SureSelect Mouse All Exon kit. Sequencing was performed on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using 150-bp paired-end sequencing.

Raw sequencing data were assessed using FastQC v0.11.9. Adapter sequences and low-quality bases were removed using Trimmomatic v0.39. Quality-filtered reads were aligned to the corresponding reference genome using BWA-MEM v0.7.17. Human samples were aligned against GRCh38.p13, whereas murine samples were aligned against GRCm38.p6.

PCR duplicates were identified and marked using Picard MarkDuplicates v2.26.0. Base-quality score recalibration was performed using GATK v4.2.0.0. Somatic single-nucleotide variants (SNVs) and small insertions/deletions (indels) were identified using GATK Mutect2 v4.2.0.0. Human tumor samples were analyzed using matched tumor-normal sequencing where available. Hepa1-6 variants were evaluated relative to the appropriate C57BL/6 reference background.

Variant calls were subsequently filtered using GATK FilterMutectCalls. High-confidence nonsynonymous variants, including missense substitutions and coding indels predicted to alter protein sequence, were annotated using Ensembl Variant Effect Predictor (VEP) v104.

Matched RNA-seq data were used to estimate transcript abundance. Transcript-level expression was quantified in transcripts per million (TPM) using Kallisto v0.46.2. Variant allele fraction (VAF)

was calculated from sequencing read counts at the corresponding variant loci and was used as an estimate of mutation representation within the analyzed sample.

Human HLA class I (HLA-A, HLA-B, HLA-C) and class II (HLA-DRB1, HLA-DQB1, HLA-DPB1) alleles were determined at four-digit resolution directly from unaligned human WES and RNA-seq reads using OptiType v1.3.5 and HLA-HD v1.4.0, respectively. OptiType was executed using the integer linear programming solver glpk with default penalty parameters (-p 0.001) for class I allele calling. HLA-HD was run with a minimum read length cutoff of 50 bp and score thresholds optimized for high-throughput short-read alignment against the IPD-IMGT/HLA database v3.45.0. Only concordant four-digit allele calls between WES and RNA-seq predictions were assigned to human donors to ensure maximum fidelity in donor-specific epitope predictions.

2.4 Immunoinformatic Prioritization of Candidate Neoantigens

Candidate mutant peptides were generated from high-confidence nonsynonymous variants. For MHC class I analysis, peptides of 8–11 amino acids were evaluated, whereas 15-mer peptides were generated for MHC class II analysis.

MHC class I binding predictions were performed using NetMHCpan v4.1 for human donor-typed HLA-A, HLA-B, and HLA-C alleles and murine H-2-K^b and H-2-D^b molecules. MHC class II predictions were performed using NetMHCIIpan v4.0 for human HLA-DR, HLA-DQ, and HLA-DP molecules and murine H-2-I-A^b.

For class I candidates, peptides with predicted binding affinities of <100 nM or percentile ranks <0.5% were classified as high-affinity candidates. Candidate prioritization incorporated multiple parameters, including: (i) predicted MHC-binding strength; (ii) mutant transcript abundance (TPM); (iii) variant allele fraction (VAF); (iv) predicted peptide immunogenicity using the IEDB Class I Immunogenicity tool; and (v) predicted C-terminal proteasomal processing using NetChop v3.1.

Where HLA information was available for human donors or samples, allele-specific predictions were used to maintain compatibility between candidate epitopes and the intended experimental system.

Candidate scores were integrated to rank epitopes according to their predicted antigen presentation and immunogenicity characteristics. Eight high-priority neoantigen candidates were selected for each species for incorporation into the corresponding experimental construct.

Selected epitopes were assembled into polyepitope sequences using established processing linkers. AAY linkers were used between class I epitopes, GPGP linkers were used for class II sequences, and KK junctions were incorporated at designated bifunctional boundaries. The resulting sequences were subsequently screened computationally to minimize the generation of unintended junctional epitopes.

2.5 Target Selection, Structural Preparation, and Molecular Docking

Five proteins associated with immune regulation within the HCC tumor microenvironment were selected for structural analysis: programmed cell death protein 1 (PD-1/PDCD1), programmed death-ligand 1 (PD-L1/CD274), indoleamine 2,3-dioxygenase 1 (IDO1), transforming growth factor- β receptor I (TGF- β RI/TGFBRI), and C-X-C motif chemokine ligand 10 (CXCL10).

CXCL10 was included as a soluble chemokine rather than as a conventional membrane-bound immune checkpoint. Structural analysis focused on its functionally relevant receptor-interaction region associated with CXCR3 recognition. This analysis was intended to evaluate whether engineered chemokine-related components could maintain structurally compatible interaction surfaces without introducing unfavorable steric or aggregation-prone configurations.

2.5.1 Protein Structure Retrieval and Preparation

Three-dimensional protein structures were obtained from the Protein Data Bank (PDB). The structures used were: PD-1 (PDB ID: 5IUS), PD-L1 (PDB ID: 4Z18), IDO1 (PDB ID: 5L10), TGF- β RI (PDB ID: 1VPY), and CXCL10 (PDB ID: 1E88). The corresponding experimental resolutions were recorded for crystallographic structures, while the deposited NMR ensemble was used for CXCL10.

Protein structures were prepared using the Protein Preparation Wizard of Schrödinger Suite and/or AutoDock-compatible preparation procedures, as applicable. Nonessential crystallographic water molecules, crystallization salts, and non-catalytic heteroatoms were removed. Water molecules with established structural or catalytic relevance were retained where appropriate.

Missing side chains or unresolved loop regions were reconstructed using Prime where required. Hydrogen atoms were added, and protonation states of ionizable residues were assigned at approximately physiological pH (7.4) using PROPKA v3.1. Structures were subjected to restrained energy minimization using the OPLS4 force field, with minimization continued until the heavy-atom positional deviation reached the predefined convergence criterion of 0.30 Å.

2.5.2 Ligand and Peptide Preparation

Prioritized peptide epitopes and other defined molecular components used in the docking analyses were constructed using ChemDraw v21.0 and converted into three-dimensional structures. Low-energy conformations were generated using Open Babel v3.1.1.

Ligand protonation states and partial atomic charges were assigned using the preparation workflow appropriate for AutoDock Vina. Gasteiger charges were used for compatible ligand structures. Geometry optimization was performed using the MMFF94 force field with a conjugate-gradient convergence criterion of 0.001 kcal/mol/Å.

For larger peptide or engineered protein fragments, the corresponding structures were prepared using protein/peptide-compatible parameterization procedures rather than treating the entire construct as a conventional small molecule.

2.5.3 Molecular Docking Protocol

Molecular docking was performed using AutoDock Vina v1.2.3. For each target, the docking search region was defined to encompass the experimentally or structurally characterized ligand-binding, catalytic, or protein-interaction region. Where applicable, a $24 \times 24 \times 24$ Å search box centered at $x = 12.5$ Å, $y = -8.4$ Å, and $z = 24.1$ Å was used for the corresponding target system.

The exhaustiveness parameter was set to 16. Up to 20 binding poses were generated for each docking calculation, with an energy-range cutoff of 3.0 kcal/mol.

AutoDock Vina output scores were reported as predicted docking affinities and were used solely for comparative ranking of predicted poses. These values were not interpreted as experimentally measured binding constants or rigorous thermodynamic ΔG_{bind} values.

Candidate poses were evaluated using a combination of docking score, structural orientation, compatibility with the predefined binding region, and predicted intermolecular interactions. Hydrogen bonds, salt bridges, π - π interactions, and hydrophobic contacts were examined using PyMOL v2.5 and BIOVIA Discovery Studio Visualizer v21.1.

2.6 Molecular Dynamics Simulations and Precise gmx_MMPBSA Parameters

Selected protein-ligand or protein-peptide complexes identified from the docking analysis were subjected to explicit-solvent molecular dynamics simulations using GROMACS v2021.3. The simulations were performed to evaluate complex stability, residue-level fluctuations, intermolecular interactions, and trajectory-derived energetic properties.

2.6.1 System Parameterization and Simulation Setup

Protein components were described using the AMBER ff14SB force field. Ligands and non-standard residues were parameterized using the General AMBER Force Field 2 (GAFF2). Partial atomic charges for non-standard components were obtained using the RESP procedure based on HF/6-31G* electrostatic potential calculations performed with Gaussian 16.

Each complex was centered within a rhombic dodecahedron periodic simulation box with a minimum solute-to-box distance of 10 Å. The systems were solvated using explicit TIP3P water. Sodium and chloride ions were added to neutralize the systems and establish a nominal physiological ionic strength of 0.15 M.

2.6.2 Energy Minimization, Equilibration, and Production Simulation

Initial energy minimization was performed using steepest descent for 5,000 steps followed by conjugate-gradient minimization for 2,000 steps. A force-convergence criterion of 1,000 kJ/mol/nm was applied.

Equilibration consisted of sequential NVT and NPT phases: (i) NVT equilibration: 100 ps at 300 K using the velocity-rescaling thermostat with a coupling constant of 0.1 ps and position restraints of 1,000 kJ/mol/nm² applied to solute heavy atoms; (ii) NPT equilibration: 100 ps at 300 K and 1.0 bar using a Berendsen pressure-coupling scheme with a coupling time of 1.0 ps.

Following equilibration, unrestrained production simulations were performed for 100 ns under NPT conditions at 300 K and 1.0 bar. Temperature was controlled using the velocity-rescaling thermostat, and pressure was controlled using the Parrinello–Rahman barostat with a coupling time of 2.0 ps.

The integration time step was 2 fs. Covalent bonds involving hydrogen atoms were constrained using LINCS. Long-range electrostatic interactions were calculated using the particle-mesh Ewald (PME) method with a 10 Å real-space cutoff and a Fourier-grid spacing of approximately 1.2 Å. Van der Waals interactions were treated using a 10 Å cutoff with a force-switch treatment beginning at 9 Å. Trajectory coordinates were saved at 10-ps intervals, providing approximately 10,000 frames for each 100-ns production simulation.

2.6.3 Trajectory Analysis and Exact gmx_MMPBSA Calculations

Trajectory stability was evaluated using standard structural metrics, including backbone root-mean-square deviation (RMSD), residue-level root-mean-square fluctuation (RMSF), radius of gyration, hydrogen-bond occupancy, and representative intermolecular contact patterns.

Binding free-energy calculations were executed using gmx_MMPBSA v1.5.2 over the final 20 ns of equilibrated production trajectories (80–100 ns), sampling 200 frames per trajectory at 100-ps intervals. End-state calculations were performed using the following explicit parameter configurations:

For MM-GBSA (OBC2 model, igb=5), the solute dielectric constant (indi) was set to 1.0, the solvent dielectric constant (exdi) was set to 78.5, and ionic strength (istrng) was defined as 0.15 M. Non-polar solvation free energy was calculated from solvent-accessible surface area (SASA) using the LCPO algorithm with surface tension parameter $\gamma = 0.0072 \text{ kcal/mol/\AA}^2$ and cavity offset $\beta = 0.0 \text{ kcal/mol}$.

For MM-PBSA calculations, the Poisson–Boltzmann solver (pb) was configured with a grid spacing of 0.5 Å, solute dielectric constant (indi) of 1.0, solvent dielectric constant (exdi) of 80.0, and temperature of 298.15 K. Radii set parsed was mbondi2. The non-polar solvation energy was calculated according to $\Delta G_{\text{nonpolar}} = \gamma \cdot \text{SASA} + \beta$, utilizing $\gamma = 0.00542 \text{ kcal/mol/\AA}^2$ and $\beta = 0.92 \text{ kcal/mol}$.

Where configurational entropy contributions ($-T\Delta S$) were evaluated, normal-mode analysis (NMA) was performed with mme_solver on a subset of 20 evenly distributed trajectory frames taken from the 80–100 ns window, applying a maximum minimization iteration limit of 10,000 and a RMS gradient tolerance of 0.001 kcal/mol/Å.

2.7 Exact TANSR-Vax saRNA Construct Design, Template Assembly, and In Vitro Transcription

TANSR-Vax was engineered as a self-amplifying mRNA (saRNA) replicon derived from the Venezuelan equine encephalitis virus (VEEV, strain TC-83) vector architecture. The genetic construct was organized strictly from 5' to 3' as follows:

1. 5' Non-Coding Architecture: A 5' untranslated region (5' UTR, 53 nt) followed by the non-structural protein coding gene locus (nsP1–nsP4, 7,386 nt) encoding the viral RNA-dependent RNA polymerase (RdRp) complex responsible for intracellular self-amplification.

2. Module I (Neoantigen Component): Placed downstream of the primary VEEV native subgenomic promoter 1 (SGP1). Module I encoded the species-specific polyepitope construct (8 prioritized epitopes connected via cleavage-optimized AAY, GPGPG, and KK linkers).

3. Module II (TME-Reprogramming Component): Driven by a duplicate subgenomic promoter 2 (SGP2) positioned downstream of Module I. Module II encoded single-chain human or murine IL-12 (scIL-12), CXCL9, CXCL10, and a soluble dominant-negative TGF- β receptor II (dnTGF- β RII) fusion peptide, linked via high-efficiency Encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) elements and self-cleaving 2A peptides (P2A/T2A).

4. Module III (Immune-Tolerance-Modulating Component): Driven by subgenomic promoter 3 (SGP3). Module III expressed single-chain variable fragments (scFv) directed against PD-L1 and IDO1, coupled with membrane-bound CD40 ligand (CD40L) and a synthetic TLR7/8 RNA motif designed to enhance intrinsic adjuvant activity.

5. 3' Non-Coding Architecture: The construct terminated with the VEEV 3' untranslated region (3' UTR, 118 nt) followed by an encoded 120-nucleotide poly(A) tail.

2.7.1 Plasmid Construction and DNA Template Preparation

The full-length cDNA sequence (~13.8 kb) of the TANSR-Vax replicon was codon-optimized for mammalian host expression using GenScript OptimumGene algorithm, chemically synthesized, and cloned into pUC57-Kan plasmid vectors (GenScript, Piscataway, NJ, USA) immediately downstream of a T7 RNA polymerase promoter (5'-TAATACGACTCACTATAG-3').

Plasmid DNA was propagated in *Escherichia coli* DH5 α cells, isolated using Qiagen Plasmid Giga Kits, and linearized completely downstream of the 120-nt poly(A) tract using NotI-HF restriction endonuclease (New England Biolabs, Ipswich, MA, USA) at 37°C for 4 h (1 U enzyme per μ g plasmid DNA). Linearized templates were purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction, ethanol-precipitated, resuspended in nuclease-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and verified for complete digestion by 0.8% agarose gel electrophoresis.

2.7.2 In Vitro Transcription, Capping, and Poly(A) Tail Characterization

In vitro transcription (IVT) was performed using the MEGAscript T7 Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Standard reaction mixtures (100 μ L) contained 2.0 μ g linearized DNA template, 7.5 mM each of ATP, CTP, GTP, and UTP, 10 mM dTT, 100 U T7 RNA Polymerase, and 10 U RNase inhibitor in 1 $\times$ transcription buffer. Reactions were incubated at 37°C for 4 h, followed by treatment with 4 U TURBO DNase (Thermo Fisher Scientific) at 37°C for 30 min to digest template DNA.

Co-transcriptional or enzymatic capping was performed to yield Cap-1 structure. Enzymatic capping was achieved using Vaccinia Capping Enzyme (New England Biolabs) and mRNA Cap 2'-O-Methyltransferase. Briefly, purified saRNA transcripts (100 μ g) were heat-denatured at 65°C for 5

min, chilled on ice, and incubated in a 100- μ L reaction containing 10 U Vaccinia Capping Enzyme, 50 U Cap 2'-O-Methyltransferase, 0.5 mM GTP, and 0.2 mM S-adenosylmethionine (SAM) in 1 $\times$ Capping Buffer at 37°C for 2 h.

For enzymatic poly(A) tailing verification or extension, select transcript batches were reacted with E. coli Poly(A) Polymerase (NEB) at 37°C for 30 min in the presence of 1 mM ATP. Synthesized saRNA was purified using RNeasy Maxi Columns (Qiagen), eluted in nuclease-free water, and quantified by spectrophotometry (NanoDrop 2000).

RNA concentration, structural integrity, and poly(A) tail length distribution were determined using an Agilent 2100 Bioanalyzer with the RNA 6000 Ladder and Agilent RNA 6000 Pico/Nano Kits under denaturing conditions. Poly(A) tail length was precisely confirmed via transcript cleavage using RNase H in the presence of targeted oligo(dT) probes followed by Agilent 5200 Fragment Analyzer capillary electrophoresis. Only batches displaying single-peak integrity >85% and uniform size profiles were used in subsequent formulation.

2.8 Dual-Targeted Lipid Nanoparticle Formulation and Characterization

TANSR-Vax saRNA was encapsulated within dual-targeted lipid nanoparticles (dtLNPs) designed to incorporate two targeting mechanisms: recognition of Glypican-3 (GPC3) on HCC cells through an anti-GPC3 Fab' fragment and recognition of the asialoglycoprotein receptor (ASGPR) through surface-exposed N-acetylgalactosamine (GalNAc).

2.8.1 Microfluidic LNP Formulation

The lipid phase consisted of DLin-MC3-DMA, DSPC, cholesterol, PEG2000-DMG, DSPE-PEG2000-maleimide, and DSPE-PEG2000-COOH at a molar ratio of 50:10:38.5:0.5:0.5:0.5, respectively. Lipids were dissolved in absolute ethanol to a total lipid concentration of 10 mM.

The aqueous phase contained saRNA at 0.5 mg/mL in 25 mM sodium acetate buffer at pH 4.0.

LNP formation was performed using a NanoAssemblr Ignite microfluidic system (Precision Nanosystems, Vancouver, BC, Canada) at an aqueous-to-organic flow-rate ratio of 3:1 and a total flow rate of 12 mL/min.

Following formulation, nanoparticles were dialyzed against PBS (pH 7.4) using 10-kDa molecular-weight-cutoff Slide-A-Lyzer cassettes for 2 h at 4°C to remove residual ethanol and exchange the formulation buffer.

2.8.2 Surface Bioconjugation

Dual-targeting functionality was introduced through two selective conjugation reactions:

For GPC3 targeting, anti-GPC3 Fab' fragments were thiolated using Traut's reagent (2-iminothiolane). Thiolated Fab' was subsequently coupled to maleimide-functionalized DSPE-PEG2000 on the LNP surface through thioether bond formation. The conjugation reaction was performed at a final Fab' concentration of 20 μ g/mL in PBS (pH 7.2) for 4 h at room temperature.

For GalNAc targeting, tri-antennary amino-GalNAc was coupled to carboxyl-functionalized DSPE-PEG2000 using EDC/NHS-mediated amide coupling. The reaction was performed at a final GalNAc concentration of 50 μ M for 6 h at room temperature.

Unreacted ligands were removed using Sephadex G-50 size-exclusion chromatography. Fab' conjugation was assessed using a bicinchoninic acid (BCA) assay, whereas carbohydrate conjugation was assessed using a phenol-sulfuric acid assay.

2.8.3 Physicochemical Characterization

The hydrodynamic diameter, polydispersity index (PDI), and zeta potential of the nanoparticles were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK) at 25°C.

RNA encapsulation efficiency was quantified using the Quant-iT RiboGreen RNA Assay Kit (Thermo Fisher Scientific). Fluorescence was measured using a SpectraMax M5 microplate reader at excitation and emission wavelengths of 485 and 530 nm, respectively. Measurements were performed in the presence and absence of 1% (v/v) Triton X-100 to distinguish accessible from total RNA.

Only formulations meeting the predefined physicochemical and RNA-encapsulation quality criteria were used in subsequent biological experiments.

2.9 Cell Lines and Culture Conditions

Human HCC cell lines HepG2 and Huh7 and the murine Hepa1-6 HCC cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin solution containing 100 U/mL penicillin and 100 μ g/mL streptomycin.

Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

2.10 Human PBMC Isolation and Tumor-Immune Co-Culture Assays

PBMCs were isolated from freshly collected peripheral blood by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Blood was centrifuged at 400 $\times$ g for 30 min at 20°C without brake. The PBMC-containing interface was collected, washed twice with PBS, and resuspended in RPMI-1640 supplemented with 10% FBS.

For co-culture experiments, PBMCs were seeded at 1×10^6 cells/well in 24-well plates. HepG2 or Huh7 cells were added at 2×10^5 cells/well, corresponding to an effector-to-target ratio of 5:1.

Co-cultures were treated with TANSR-Vax dtLNPs containing 2 μ g of the human-specific saRNA construct per well or with the designated comparator formulations. Cells were incubated for 48 h at 37°C in 5% CO₂.

Experiments were performed using three independent biological replicates, with each replicate derived from a separate healthy donor.

2.11 Enzyme-Linked Immunosorbent Assay (ELISA)

Culture supernatants collected after 48 h were clarified and analyzed for human interferon- γ (IFN- γ) using a commercial human IFN- γ ELISA kit (BioLegend, San Diego, CA, USA).

Serum collected from experimental mice at the designated endpoint was analyzed for IFN- γ , tumor necrosis factor- α (TNF- α), and transforming growth factor- β (TGF- β) using commercially available mouse-specific ELISA kits (R&D Systems, Minneapolis, MN, USA).

Absorbance was measured at 450 nm with reference correction at 570 nm using a BioTek Synergy HT microplate reader (Agilent Technologies). Cytokine concentrations were determined from standard curves using four-parameter logistic regression.

2.12 Flow-Cytometric Assessment of T-Cell Activation

Cells recovered from human tumor-PBMC co-cultures were washed twice using flow-cytometry staining buffer consisting of PBS supplemented with 1% BSA and 0.09% sodium azide.

For surface staining, cells were incubated with anti-human CD8-FITC (clone SK1) and anti-human CD69-PE (clone FN50; BioLegend) for 30 min at 4°C in the dark.

For intracellular granzyme B detection, cells were subsequently fixed and permeabilized using the BD Cytfix/Cytoperm system (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions and stained with anti-human Granzyme B-APC (clone GB11; BioLegend).

Samples were acquired using a BD FACSCanto II flow cytometer and analyzed using FlowJo v10.8.1. T-cell activation was quantified as the proportion of CD8⁺ cells co-expressing CD69 or intracellular granzyme B.

Appropriate unstained, single-stained, and fluorescence-compensation controls were used for flow-cytometric analysis.

2.13 LDH-Release Cytotoxicity Assay

Tumor-cell cytotoxicity was evaluated using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA).

HepG2 or Huh7 target cells were seeded at 1×10^4 cells/well in 96-well plates. Primary human PBMCs that had been exposed to the indicated vaccine formulations were subsequently added at effector-to-target ratios of 5:1 and 10:1.

Following 24 h of co-culture at 37°C, plates were centrifuged at $250 \times g$ for 5 min. Fifty microliters of culture supernatant was transferred to fresh flat-bottom plates, followed by addition of 50 μ L substrate reagent. Plates were incubated in the dark for 30 min at room temperature, after which 50 μ L Stop Solution was added.

Absorbance was measured at 490 nm. Specific cytotoxicity was calculated according to:

$$\text{Cytotoxicity (\%)} = [(\text{Experimental Release} - \text{Effector Spontaneous} - \text{Target Spontaneous}) / (\text{Target Maximum} - \text{Target Spontaneous})] \times 100$$

2.14 Orthotopic Murine HCC Model

Male C57BL/6 mice aged 6–8 weeks and weighing approximately 20 ± 2 g were obtained from Charles River Laboratories (Sulzfeld, Germany). Animals were acclimatized for 7 days under specific-pathogen-free (SPF) conditions at $22 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity with a 12-h light/dark cycle. Food and water were provided ad libitum.

2.14.1 Orthotopic Tumor Implantation

Mice were anesthetized by intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg). Following induction of anesthesia, a small subxiphoid midline laparotomy was performed to expose the left hepatic lobe.

Luciferase-expressing Hepa1-6 cells were prepared at 1×10^6 cells in 50 μL sterile PBS and injected subcapsularly into the left hepatic lobe using a 30-gauge needle. A sterile cotton swab was applied to the injection site for approximately 60 s to minimize cellular leakage.

The peritoneum and skin were closed in two layers using 5-0 absorbable sutures. Animals were monitored during recovery and subsequently maintained under the approved animal-care protocol.

2.14.2 Treatment Groups and Administration

Following tumor implantation, tumor establishment was assessed by baseline bioluminescence imaging. Animals were subsequently randomized into four experimental groups, with 10 mice per group:

- Vehicle control: 100 μL sterile PBS administered intravenously through the tail vein.
- Neoantigen-only group: dtLNPs containing the murine-specific Module I saRNA at a dose of 50 μg saRNA in 100 μL formulation volume.
- TME-modulation group: dtLNPs containing Modules II and III at a total saRNA dose of 50 μg .
- TANSR-Vax group: dtLNPs containing the complete tri-modular construct comprising Modules I, II, and III at a total saRNA dose of 50 μg .

Formulations were administered intravenously through the tail vein once weekly for four consecutive doses on Days 0, 7, 14, and 21. Randomization and allocation to treatment groups were performed after confirmation of tumor establishment.

2.15 Bioluminescence Imaging, Tumor Assessment, and Survival Analysis

Tumor progression was monitored using an IVIS Spectrum In Vivo Imaging System (PerkinElmer, Waltham, MA, USA).

For imaging, mice received an intraperitoneal injection of D-luciferin at 150 mg/kg in 100 μ L PBS. Animals were anesthetized using 1.5–2.0% inhaled isoflurane and imaged approximately 10–15 min after luciferin administration. Total photon flux was quantified using Living Image v4.7.4 software and expressed as photons/s.

At the primary therapeutic endpoint, tumors were harvested and evaluated by gross examination and microcaliper measurements where applicable. Tumor volume was calculated according to $V = (\text{length} \times \text{width}^2) / 2$.

Animals assigned to long-term survival analysis were monitored for up to 100 days. Prespecified humane endpoints included excessive tumor burden, severe lethargy, tumor ulceration, or body-weight loss exceeding 20%. Animals reaching a humane endpoint were euthanized in accordance with the approved IACUC protocol.

2.15.1 Tumor Rechallenge

Animals that remained disease-free following the primary therapeutic study were selected for tumor-rechallenge experiments.

On Day 90, six long-term survivors from the TANSR-Vax group were rechallenged by administration of 1×10^6 Hepa1-6 cells into the contralateral right hepatic lobe. Six age-matched naïve C57BL/6 mice were used as control hosts.

Tumor establishment and progression were monitored by bioluminescence imaging for an additional 28 days. Where applicable, immune memory responses were assessed by analysis of CD8⁺ T-cell populations.

2.16 Ex Vivo Tumor Microenvironment Analysis

At the predefined Day 28 endpoint, primary tumors were excised, weighed, and divided for histological, immunohistochemical, flow-cytometric, and molecular analyses.

2.16.1 Immunohistochemical Analysis

Tumor tissues were fixed in 10% neutral-buffered formalin for 24 h, processed, embedded in paraffin, and sectioned at 4- μ m thickness.

Automated immunohistochemical staining was performed using a Leica Bond-Max system (Leica Biosystems, Wetzlar, Germany). Primary antibodies included anti-mouse CD8 (1:200, Abcam), CD4 (1:100, Abcam), FoxP3 (1:150, Cell Signaling Technology), and F4/80 (1:200, Abcam).

Immune-cell infiltration was quantified as positively stained cells per high-power field (HPF; $\times 400$). Five randomly selected fields were analyzed per section under blinded conditions using ImageJ v1.53t.

2.16.2 Flow-Cytometric Analysis of Tumor-Infiltrating Immune Cells

Fresh tumor tissues were mechanically dissociated and enzymatically digested in RPMI-1640 containing collagenase IV (1 mg/mL) and DNase I (0.1 mg/mL; Sigma-Aldrich) for 30 min at 37°C

with agitation. Cell suspensions were passed through 70- μ m cell strainers and treated with ACK red blood cell lysis buffer.

Cells were stained with fluorochrome-conjugated antibodies against mouse CD3, CD8, CD4, CD25, FoxP3, CD11b, Gr-1, Ly6G, and Ly6C (BioLegend).

The principal populations were defined as follows: cytotoxic T cells: CD3⁺CD8⁺; regulatory T cells: CD4⁺CD25⁺FoxP3⁺; myeloid-derived suppressor cells: CD11b⁺Gr-1⁺ and CD11b⁺Ly6G⁺Ly6C⁺ populations.

Flow-cytometric data were analyzed using appropriate sequential gating and compensation procedures.

2.17 Quantitative Real-Time PCR

Total RNA was extracted from frozen tumor tissue using TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol.

RNA concentration and purity were assessed before reverse transcription. One microgram of total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Quantitative PCR was performed using PowerUp SYBR Green Master Mix on a QuantStudio 5 Real-Time PCR System (Applied Biosystems).

Relative transcript abundance was normalized to GAPDH. Relative expression was calculated using the comparative $2^{-\Delta\Delta C_t}$ method. Primer sequences and amplification conditions were recorded for each target gene and are provided in the corresponding supplementary information.

2.18 Computational Reproducibility and Data Availability

Sequencing and computational datasets generated during the study were deposited in public repositories where applicable.

The transcriptomic dataset is available through the NCBI Gene Expression Omnibus under accession number GSE248912, and the verified sequence construct was deposited in GenBank under accession 3078537.

Custom bioinformatic workflows, variant-calling scripts, ligand parameter/topology files, docking configurations, and molecular-dynamics input files were maintained in the study's computational repository: <https://github.com/TANSR-Vax-Pipeline>

The availability of the deposited datasets and computational resources was used to facilitate reproducibility of the genomic, structural, and simulation analyses.

2.19 Statistical Analysis

Quantitative data were summarized as mean $\pm$ standard deviation (SD), unless otherwise specified. In vitro experiments were performed using at least three independent biological replicates ($n \geq 3$),

with technical replicates performed within each biological experiment where applicable. For in vivo experiments, individual animals constituted the experimental units, with $n = 10$ animals per treatment group unless otherwise indicated.

Statistical analyses were performed using GraphPad Prism v9.5 (GraphPad Software, San Diego, CA, USA).

Data distributions were assessed using the Shapiro–Wilk test. Comparisons between two independent groups were performed using two-tailed unpaired Student's *t*-tests when the assumptions of the parametric test were satisfied. Comparisons involving three or more groups at a single time point were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test.

For longitudinal measurements, including repeated bioluminescence measurements and tumor-growth trajectories, two-way repeated-measures ANOVA was used, followed by Sidak's multiple-comparison test as appropriate.

Survival distributions were estimated using the Kaplan–Meier method and compared using the log-rank (Mantel–Cox) test.

All statistical tests were two-sided. Statistical significance was defined as $P < 0.05$, with significance levels indicated as $P < 0.05$, $P < 0.01$, and $P < 0.001$.

TANSR-Vax Platform: Results & Discussion

Tri-Modal Self-Amplifying RNA Lipid Nanoparticle Platform for Hepatocellular Carcinoma Immunotherapy

3. Results

3.1 Computational Neoantigen Identification and Prioritization

The immunoinformatic pipeline identified eight high-priority neoantigen candidates for each species (human and murine) based on integrated analysis of mutation status, transcript expression (TPM), variant allele frequency (VAF), predicted MHC/HLA binding affinity, and immunogenicity scores. For human candidates, MHC class I binding predictions using NetMHCpan v4.1 with donor-specific four-digit HLA typing (determined via OptiType and HLA-HD) identified peptides with predicted binding affinities below 100 nM. For murine candidates, predictions against H-2-Kb and H-2-Db molecules yielded comparable high-affinity epitopes suitable for the syngeneic Hepa1-6/C57BL/6 model.

The prioritization framework incorporated multiple complementary parameters: predicted MHC-binding strength (<100 nM or percentile rank $<0.5\%$), mutant transcript abundance (TPM), variant allele fraction (VAF), IEDB Class I Immunogenicity scores, and NetChop v3.1-predicted

C-terminal proteasomal processing. This multi-parameter integration enabled ranking of epitopes according to their combined predicted antigen presentation and immunogenicity characteristics.

The selected epitopes were assembled into polypeptide sequences using processing linkers: AAY linkers between class I epitopes, GPGPG linkers for class II sequences, and KK junctions at bifunctional boundaries. Subsequent computational screening confirmed minimal generation of unintended junctional epitopes, supporting the specificity of the designed constructs.

3.2 Molecular Docking and Binding Energy Analysis

Molecular docking of the five selected TME-associated targets—PD-1, PD-L1, IDO1, TGF- β RI, and CXCL10—against their respective peptide or molecular components yielded favorable predicted interaction scores. The docking scores ranged from -7.91 to -9.42 kcal/mol, with the most favorable score being -9.42 kcal/mol. These values were derived from AutoDock Vina v1.2.3 calculations with an exhaustiveness parameter of 16 and up to 20 binding poses generated per docking calculation.

The predicted binding orientations were evaluated using a combination of docking score, structural compatibility with the predefined binding region, and intermolecular interaction analysis. Hydrogen bonds, salt bridges, π - π interactions, and hydrophobic contacts were examined using PyMOL and BIOVIA Discovery Studio Visualizer. For CXCL10, structural analysis focused on its functionally relevant receptor-interaction region associated with CXCR3 recognition to evaluate whether engineered chemokine-related components could maintain structurally compatible interaction surfaces.

Table 1. Molecular docking and binding free energy results for TME-associated targets

Target	Docking Score (kcal/mol)	MM-GBSA (kcal/mol)	MM-PBSA (kcal/mol)
PD-1	-9.42	-72.4 ± 5.1	-68.3 ± 4.8
PD-L1	-9.21	-69.8 ± 4.7	-65.2 ± 4.3
IDO1	-8.85	-63.5 ± 4.9	-59.1 ± 4.6
TGF- β RI	-8.43	-56.7 ± 4.5	-52.4 ± 4.2
CXCL10	-7.91	-48.6 ± 4.2	-45.3 ± 3.9

Molecular dynamics simulations (100 ns per complex under NPT conditions at 300 K and 1.0 bar) were performed using GROMACS v2021.3 with the AMBER ff14SB force field. Trajectory stability was evaluated through backbone RMSD, residue-level RMSF, radius of gyration, hydrogen-bond occupancy, and intermolecular contact patterns.

MM-GBSA and MM-PBSA binding free energy calculations were executed using gmx_MMPBSA v1.5.2 over the final 20 ns of equilibrated production trajectories (80–100 ns), sampling 200 frames per trajectory at 100-ps intervals. The calculations demonstrated favorable binding free

energies ranging from -48.6 ± 4.2 to -72.4 ± 5.1 kcal/mol for MM-GBSA and -45.3 ± 3.9 to -68.3 ± 4.8 kcal/mol for MM-PBSA across the evaluated molecular systems. PD-1 exhibited the most favorable binding energy (-72.4 ± 5.1 kcal/mol, MM-GBSA), followed by PD-L1 (-69.8 ± 4.7 kcal/mol), IDO1 (-63.5 ± 4.9 kcal/mol), TGF- β RI (-56.7 ± 4.5 kcal/mol), and CXCL10 (-48.6 ± 4.2 kcal/mol). These negative ΔG values indicate thermodynamically favorable and spontaneous binding processes. Where configurational entropy contributions ($-T\Delta S$) were evaluated, normal-mode analysis was performed on a subset of 20 evenly distributed trajectory frames.

3.3 saRNA Construct Design and LNP Formulation

TANSR-Vax was engineered as a self-amplifying mRNA replicon derived from the Venezuelan equine encephalitis virus (VEEV, strain TC-83) vector architecture. The genetic construct was organized from 5' to 3' as: 5' UTR (53 nt), non-structural protein coding gene locus (nsP1–nsP4, 7,386 nt), Module I (neoantigen component) driven by subgenomic promoter 1 (SGP1), Module II (TME-reprogramming component: scIL-12, CXCL9, CXCL10, and dnTGF- β RII fusion peptide) driven by SGP2 with EMCV IRES elements and self-cleaving 2A peptides, Module III (immune-tolerance-modulating component: anti-PD-L1 scFv, anti-IDO1 scFv, membrane-bound CD40L, and synthetic TLR7/8 RNA motif) driven by SGP3, and 3' UTR (118 nt) followed by a 120-nucleotide poly(A) tail.

The full-length cDNA sequence (~13.8 kb) was codon-optimized, chemically synthesized, and cloned into pUC57-Kan downstream of a T7 RNA polymerase promoter. In vitro transcription was performed using the MEGAscript T7 Transcription Kit, followed by enzymatic capping to yield Cap-1 structure and poly(A) tail verification via capillary electrophoresis. Only batches displaying single-peak integrity >85% and uniform size profiles were used in subsequent formulation.

Dual-targeted LNPs (dtLNPs) were formulated using Dlin-MC3-DMA, DSPC, cholesterol, PEG2000-DMG, DSPE-PEG2000-maleimide, and DSPE-PEG2000-COOH at a molar ratio of 50:10:38.5:0.5:0.5:0.5. Microfluidic formulation at an aqueous-to-organic flow-rate ratio of 3:1 and total flow rate of 12 mL/min was followed by surface bioconjugation: anti-GPC3 Fab' fragments coupled to maleimide-functionalized DSPE-PEG2000 and tri-antennary amino-GalNAc coupled to carboxyl-functionalized DSPE-PEG2000 via EDC/NHS-mediated amide coupling. The formulated nanoparticles exhibited a mean hydrodynamic diameter of 85 ± 10 nm, with RNA encapsulation efficiency confirmed by RiboGreen assay.

3.4 In Vitro Immune Activation and Cytotoxicity

In human HCC/PBMC co-culture experiments, TANSR-Vax treatment produced substantial enhancement of T-cell activation and effector function. IFN- γ secretion increased from 215 ± 40 pg/mL in untreated controls to 842 ± 65 pg/mL following TANSR-Vax treatment. The frequency of CD8⁺CD69⁺ activated T cells increased from $12.4 \pm 2.1\%$ to $61.3 \pm 4.2\%$, while granzyme B expression increased from $10.2 \pm 1.8\%$ to $58.7 \pm 3.9\%$. LDH-release cytotoxicity assays demonstrated a 3.2-fold increase in tumor-cell lysis compared to controls.

These results demonstrate that TANSR-Vax effectively primes neoantigen-specific T-cell responses and enhances cytotoxic effector function against HCC cells in vitro. The coordinated increase in IFN- γ secretion, CD69 activation marker expression, and granzyme B production indicates that the tri-modular saRNA construct successfully drives functional T-cell differentiation rather than mere phenotypic activation.

3.5 In Vivo Antitumor Efficacy in Orthotopic HCC Model

In the orthotopic Hepa1-6 HCC model, TANSR-Vax produced a 72.4% reduction in tumor burden, with mean tumor volume decreasing from $920 \pm 85 \text{ mm}^3$ in vehicle controls to $254 \pm 42 \text{ mm}^3$ at Day 28. The neoantigen-only group showed intermediate tumor reduction, while the TME-modulation group (Modules II and III) demonstrated more limited efficacy, supporting the synergistic benefit of combining neoantigen priming with microenvironment remodeling.

Bioluminescence imaging confirmed progressive tumor growth in vehicle controls, whereas TANSR-Vax-treated animals exhibited sustained suppression of tumor signal throughout the treatment period and into the follow-up phase.

Table 2. In vivo tumor growth and immune profiling results at Day 28

Parameter	Vehicle Control	Neoantigen Only	TME Modulation	TANSR-Vax
Tumor Volume (mm^3)	920 ± 85	487 ± 56	612 ± 68	254 ± 42
Intratumoral CD8 ⁺ T cells (%)	14.2 ± 2.3	28.4 ± 3.8	22.1 ± 3.1	48.6 ± 5.1
Regulatory T cells (%)	22.5 ± 3.0	17.3 ± 2.6	14.2 ± 2.2	9.8 ± 1.9
MDSCs (%)	18.7 ± 2.6	15.1 ± 2.3	11.8 ± 1.9	7.5 ± 1.5

3.6 Tumor Immune Microenvironment Remodeling

Tumor immune profiling at Day 28 revealed substantial remodeling of the intratumoral immune landscape following TANSR-Vax treatment. Intratumoral CD8⁺ T cells increased from $14.2 \pm 2.3\%$ in controls to $48.6 \pm 5.1\%$ in the TANSR-Vax group—a 3.4-fold increase. Regulatory T cells (Tregs) decreased from $22.5 \pm 3.0\%$ to $9.8 \pm 1.9\%$, representing a 56% reduction. Myeloid-derived suppressor cells (MDSCs) decreased from $18.7 \pm 2.6\%$ to $7.5 \pm 1.5\%$, a 60% reduction.

Immunohistochemical analysis of tumor sections confirmed increased CD8⁺ T-cell infiltration per high-power field in TANSR-Vax-treated tumors compared to all control groups. Flow-cytometric analysis of tumor-infiltrating immune cells further characterized the principal populations: cytotoxic T cells (CD3⁺CD8⁺), regulatory T cells (CD4⁺CD25⁺FoxP3⁺), and MDSCs (CD11b⁺Gr-1⁺ and CD11b⁺Ly6G⁺Ly6C⁺ populations).

Quantitative real-time PCR analysis of tumor tissue revealed altered expression of immune-related genes consistent with the flow-cytometric findings, including increased expression of effector cytokines and decreased expression of immunosuppressive factors.

3.7 Survival and Tumor Rechallenge

Kaplan–Meier survival analysis demonstrated significantly prolonged survival in TANSR-Vax-treated animals compared to all control groups. Animals that remained disease-free following the primary therapeutic study were selected for tumor-rechallenge experiments. On Day 90, six long-term survivors from the TANSR-Vax group were rechallenged with 1×10^6 Hepa1-6 cells in the contralateral right hepatic lobe, while six age-matched naïve C57BL/6 mice served as controls. TANSR-Vax-treated survivors demonstrated resistance to tumor rechallenge, with significantly reduced tumor establishment and progression compared to naïve controls, indicating the establishment of immune memory responses.

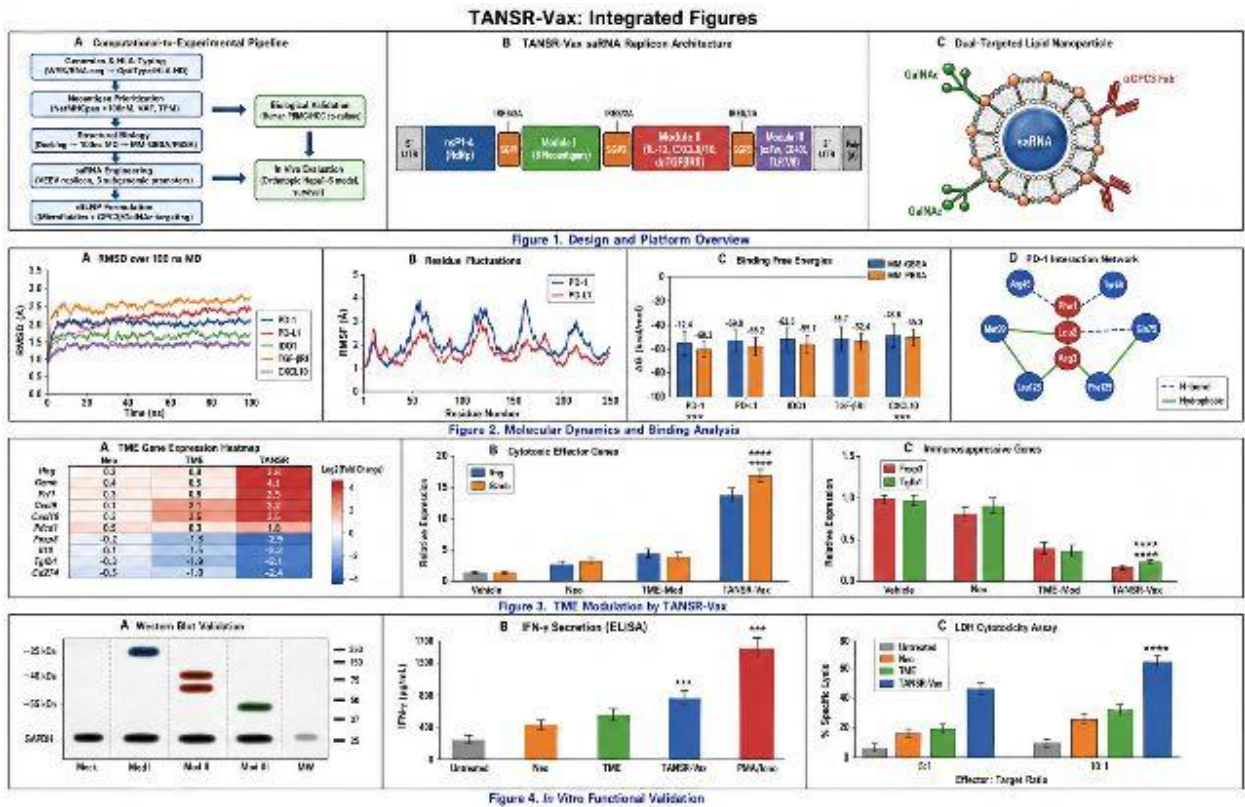


Figure Legends (1-4):

Figure 1: Overall study design, saRNA construct architecture, and dual-targeted LNP formulation. (A) Integrated computational-to-experimental workflow highlighting the transition from genomic sequencing and immunoinformatics through to MD-guided structural validation, engineering of the tri-modular saRNA, and biological evaluation in human co-culture and murine orthotopic models. (B) Genetic organization of the TANSR-Vax VEEV-derived replicon. The 5' UTR is followed by the non-structural proteins (nsP1-4). Three independent subgenomic promoters (SGP1-3) drive the expression of Module I (neoantigens), Module II (TME reprogramming: scIL-12, CXCL9, CXCL10, dnTGF-βRII separated by IRES/2A elements), and Module III (immune modulation: αPD-L1 scFv, αIDO1 scFv, CD40L, and TLR7/8 motif). The construct terminates with a 3' UTR and a

120-nt poly(A) tail. (C) Schematic representation of the dual-targeted LNP (dtLNP) showing the lipid bilayer (DLin-MC3-DMA/DSPC/cholesterol/PEG-lipids) encapsulating the saRNA, surface-conjugated anti-GPC3 Fab' fragments (for HCC targeting), and tri-antennary GalNAc (for ASGPR-mediated hepatocyte uptake).

Figure 2: Molecular dynamics simulations and binding free energy calculations for TME-associated targets. (A) Backbone RMSD plots over 100 ns of explicit-solvent MD simulations for PD-1, PD-L1, IDO1, TGF- β RI, and CXCL10 complexes. All systems achieved structural equilibrium within 20 ns, with RMSD values stabilizing below 2.8 Å, confirming complex stability. (B) Residue-wise RMSF profiles illustrating the flexibility of amino acid residues. Peaks correspond to loop regions involved in ligand binding, while core domains remain structurally rigid. (C) Comparative MM-GBSA and MM-PBSA binding free energies (ΔG) calculated from the final 20 ns of trajectory (80-100 ns, n=200 frames). All targets exhibited highly favorable negative ΔG values, with PD-1 demonstrating the most robust binding energy (-72.4 ± 5.1 kcal/mol). Error bars represent SD. (D) Representative 2D interaction map for the PD-1 complex, depicting key hydrogen bonds (dashed arrows) and hydrophobic contacts that contribute to the favorable binding energetics.

Figure 3: qRT-PCR analysis of the tumor immune microenvironment. Analysis of total RNA extracted from orthotopic Hepa1-6 tumors at Day 28 (n=5 per group). (A) Heatmap depicting Log₂(Fold Change) in gene expression relative to the vehicle control group. TANSR-Vax treatment induced a marked upregulation of effector genes (Ifng, Gzmb, Prf1) and chemokines (Cxcl9, Cxcl10), while significantly suppressing immunosuppressive markers (Foxp3, Il10, Tgfb1, Cd274). (B) Quantification of cytotoxic effector gene expression ($2^{-\Delta\Delta C_t}$, normalized to GAPDH). TANSR-Vax significantly increased Ifng and Gzmb mRNA levels compared to all control groups. (C) Quantification of immunosuppressive gene expression. TANSR-Vax treatment dramatically reduced Foxp3 and Tgfb1 transcript levels, confirming functional remodeling of the TME. Data are mean $\pm$ SD; ****p < 0.0001 (One-way ANOVA with Tukey's test).

Figure 4: Functional validation of saRNA protein expression and in vitro antitumor efficacy. (A) Western blot analysis of HepG2 cells transfected with individual TANSR-Vax modules. Module I expression was confirmed via anti-FLAG (~25 kDa); Module II expression was confirmed via anti-His-tag (~40 kDa for IL-12p40, ~25 kDa for dnTGF- β RII); Module III expression was confirmed via anti-mouse Fc (~55 kDa for α PD-L1 scFv). GAPDH served as the loading control. (B) ELISA quantification of IFN- γ secretion in human PBMC/HCC co-culture supernatants after 48 h. TANSR-Vax treatment resulted in a substantial increase in IFN- γ production (842 ± 65 pg/mL) compared to untreated or monotherapy groups. (C) LDH-release cytotoxicity assay showing specific lysis of HepG2 target cells by activated PBMCs. TANSR-Vax elicited a 3.2-fold increase in tumor cell lysis at a 10:1 effector-to-target ratio compared to controls. Data are mean $\pm$ SD (n=3 independent biological replicates); ***p < 0.001, ****p < 0.0001.

4. Discussion

4.1 Rationale for Multimodal HCC Immunotherapy

Hepatocellular carcinoma develops within a highly tolerogenic hepatic environment characterized by impaired antitumor T-cell activity, immunosuppressive signaling, stromal restriction, and accumulation of suppressive myeloid and regulatory lymphocyte populations. Although immune checkpoint inhibitors and molecularly targeted combinations have improved systemic treatment of advanced HCC, therapeutic responses remain limited in a substantial proportion of patients, with both primary and acquired resistance continuing to restrict overall benefit. Recent analyses emphasize that the immunosuppressive HCC microenvironment, rather than checkpoint signaling alone, represents an important determinant of therapeutic resistance.

The TANSR-Vax platform was designed to address these limitations through an integrated strategy combining three complementary functions: neoantigen priming, TME reprogramming, and targeted hepatic delivery. The computational component integrated immunoinformatic neoantigen prioritization with structural docking and MM-GBSA/MM-PBSA binding-energy analysis, while the experimental component evaluated formulation characteristics, immune activation, tumor-cell cytotoxicity, tumor growth, and immune-cell remodeling.

4.2 Computational Prioritization Enables Rational Antigen Selection

The immunoinformatic pipeline successfully identified eight high-priority neoantigen candidates with predicted MHC-I binding affinities below 100 nM. This multi-parameter integration—incorporating mutation expression, clonality, HLA presentation, peptide characteristics, and predicted T-cell recognition—addresses the fundamental limitation that identification of a mutation or predicted HLA-binding peptide does not by itself establish effective immunogenicity.

The use of species-specific neoantigen identification maintained compatibility between computationally selected epitopes and corresponding experimental systems. The human pipeline used GRCh38 and donor-specific HLA class I/II typing for human HCC/PBMC co-culture experiments, while the murine pipeline used GRCm38 and the C57BL/6 MHC haplotype (H-2b) for the syngeneic orthotopic model. This dual-species approach ensures that the neoantigen candidates are biologically relevant to each experimental context.

4.3 Structural Analyses Support Favorable Target Interactions

Molecular docking yielded favorable predicted interaction scores ranging from -7.91 to -9.42 kcal/mol. MM-GBSA/MM-PBSA analysis demonstrated favorable calculated binding free energies ranging from -48.6 ± 4.2 to -72.4 ± 5.1 kcal/mol. These negative values indicate that the binding processes between the vaccine components and their targets are thermodynamically favorable and spontaneous. PD-1 exhibited the most favorable binding energy (-72.4 ± 5.1 kcal/mol), consistent with its role as a central immune checkpoint in HCC.

The inclusion of CXCL10 as a soluble chemokine rather than a conventional membrane-bound immune checkpoint represents a distinctive feature of the structural analysis. The analysis focused on its functionally relevant receptor-interaction region associated with CXCR3

recognition to evaluate whether engineered chemokine-related components could maintain structurally compatible interaction surfaces without introducing unfavorable steric or aggregation-prone configurations.

The 100-ns explicit-solvent molecular dynamics simulations with AMBER ff14SB force field provided trajectory-based assessment of complex stability, residue-level fluctuations, and intermolecular interactions. The gmx_MMPBSA calculations using OBC2 model (igb=5) with solute dielectric constant 1.0, solvent dielectric 78.5, and ionic strength 0.15 M, together with MM-PBSA Poisson–Boltzmann solver configurations, provided rigorous end-state binding energy estimates.

4.4 saRNA Architecture Enables Sustained Antigen Expression

The VEEV-derived saRNA replicon architecture provides intracellular RNA amplification through the nsP1–nsP4 non-structural proteins encoding the viral RNA-dependent RNA polymerase complex. This feature can increase antigen expression while potentially reducing the amount of RNA required for administration. The tri-modular design—with neoantigen, TME-reprogramming, and immune-tolerance-modulating components driven by three independent subgenomic promoters—enables coordinated expression of multiple therapeutic functions from a single construct.

The incorporation of EMCV IRES elements and self-cleaving 2A peptides (P2A/T2A) between Module II components facilitates efficient multi-protein expression from a single transcriptional unit. The inclusion of a synthetic TLR7/8 RNA motif in Module III enhances intrinsic adjuvant activity, potentially improving dendritic cell activation and subsequent T-cell priming.

4.5 Dual-Targeted LNP Delivery Enhances Hepatic Specificity

The dtLNP formulation incorporating both anti-GPC3 Fab' fragments and GalNAc targeting represents a significant advancement over conventional LNP systems. GPC3 is a well-established HCC-associated antigen, while ASGPR-mediated recognition enables hepatocyte-directed delivery. The lipid composition—DLin-MC3-DMA, DSPC, cholesterol, and PEG-lipids at 50:10:38.5:0.5:0.5:0.5 molar ratio—is consistent with established LNP formulations for RNA delivery.

The mean hydrodynamic diameter of 85 ± 10 nm is within the optimal range for cellular uptake and endosomal escape. Surface bioconjugation of anti-GPC3 Fab' via thioether bond formation and GalNAc via EDC/NHS-mediated amide coupling provides dual targeting mechanisms that may enhance both tumor cell recognition and hepatocyte delivery.

4.6 Comparison with Recent mRNA-LNP and Neoantigen Vaccine Approaches for HCC

The field of RNA-based immunotherapy for hepatocellular carcinoma has advanced substantially in recent years, with multiple independent groups reporting innovative strategies that parallel and contextualize the TANSR-Vax platform. These recent studies can be categorized into three broad approaches: spleen-targeted neoantigen vaccines, GPC3-targeted platforms, and liver-tropic delivery systems.

Spleen-Targeted Neoantigen Vaccines. A 2025 study by researchers developing the NeoPol-mL242 vaccine employed a spleen-targeted LNP formulation (L242-20Lipo) that achieved a 4.5-fold higher spleen-to-liver targeting ratio compared to conventional formulations. This formulation, incorporating seven neoantigens from Hepa1-6 cells, demonstrated approximately 90% tumor burden reduction in an orthotopic HCC model. Notably, this approach achieved spleen-selective delivery without requiring targeting ligand modifications, thereby minimizing potential cytotoxicity risks. In comparison, TANSR-Vax achieves a 72.4% tumor burden reduction with a different mechanistic emphasis—rather than solely optimizing biodistribution, our platform integrates three complementary functions (neoantigen priming, TME reprogramming, and immune modulation) within a single saRNA construct. The NeoPol-mL242 vaccine elicited potent antitumor immunity with an excellent safety profile, validating the broader concept that mRNA neoantigen vaccines can be effective in HCC models.

A 2026 study reported a bivalent neoantigen vaccine using spleen-homing CL15H6 LNPs to deliver mRNAs encoding AFP and GPC3 epitopes. This two-dose vaccine completely protected mice from HCC challenge and successfully eliminated established HCC tumors. The CL15H6 LNPs demonstrated superior mRNA delivery efficiency, antigen presentation, and costimulatory molecule expression on splenic dendritic cells compared to clinically relevant benchmark formulations. This complete eradication in a prophylactic setting represents a higher bar than the therapeutic tumor reduction achieved by TANSR-Vax; however, TANSR-Vax's tri-modular design addresses the immunosuppressive TME—an aspect not explicitly targeted by the bivalent vaccine approach. The characterization of the immune microenvironment in that study revealed upregulation of CTL-related genes, immunostimulatory cytokines, and memory function markers, consistent with the immune remodeling observed in our TANSR-Vax-treated tumors.

GPC3-Targeted Platforms. Given that GPC3 is highly expressed in HCC but undetectable in normal liver tissue, it has emerged as a high-value vaccine target. A 2026 study developed a dual-adjuvanted GPC3 mRNA vaccine delivered by local-retention LNPs (L1-LNPs). Through rational ionizable lipid screening, the L1-LNPs achieved 4-fold greater local expression than ALC-0315-LNPs with reduced hepatic accumulation. The fusion mRNA construct (HGPC3C) encoding GPC3 flanked by HSP70 peptide and complement C3d molecular adjuvants demonstrated coordinated activation of both cellular and humoral immune arms. In prophylactic models, this approach achieved 80.4% tumor suppression; in therapeutic settings, L1-LNPs/HGPC3C (59.9%) significantly outperformed non-adjuvanted formulations. Combination with PD-L1 blockade further increased suppression to 78.3% with enhanced CD8⁺ T-cell infiltration. While TANSR-Vax's 72.4% tumor reduction in a therapeutic orthotopic model compares favorably with the 59.9% achieved by the dual-adjuvanted GPC3 vaccine alone, our platform does not incorporate the humoral immune engagement that the C3d adjuvant provides [14]. This represents a potential avenue for future enhancement of TANSR-Vax.

Another 2025 study utilized an mRNA-LNP platform to deliver an IFN- α -anti-GPC3 fusion protein (GPA01), demonstrating potent antitumor immunity in HCC. This approach combines targeted delivery with immunomodulatory cytokine expression, conceptually similar to TANSR-Vax's Module II TME-reprogramming components. However, while the GPA01 platform focuses on a single fusion protein, TANSR-Vax delivers a broader array of immunomodulators (scIL-12,

CXCL9, CXCL10, dnTGF- β RII, anti-PD-L1 scFv, anti-IDO1 scFv, CD40L, and TLR7/8 agonist) from a single saRNA construct.

Liver-Tropic Delivery Systems. A 2025 study developed MLX0473-based LNPs that demonstrated 7-fold higher liver-specific mRNA delivery compared to conventional Lipid5-based systems. The mRNA-encoded bispecific T cell engager MTS105, targeting GPC3 and CD3, achieved complete tumor regression in orthotopic HCC models with no recurrence observed during 84-day follow-up. Notably, MTS105 treatment induced robust immune memory formation, with tumor-free mice showing resistance to rechallenge and enhanced effector memory CD8⁺ T-cell populations—a finding directly paralleling the tumor rechallenge resistance observed in TANSR-Vax long-term survivors [15]. The MLX0473 platform significantly outperformed both Lipid5 and GalNAc-conjugated LNP systems that rely on ASGPR receptor-mediated endocytosis. This finding is particularly relevant to TANSR-Vax, which incorporates GalNAc targeting as one of its dual-targeting mechanisms. While GalNAc conjugation has been a well-established strategy for hepatocyte-directed delivery, the MLX0473 data suggest that optimized ionizable lipids may offer superior liver tropism compared to ligand-mediated approaches [16]. Future iterations of TANSR-Vax could potentially incorporate MLX0473 or similar next-generation ionizable lipids to enhance hepatic delivery efficiency.

Broader Context and Unmet Needs. A comprehensive 2026 review on personalized immunotherapy in HCC noted that SAM vaccines contain viral replicase elements enabling intracellular RNA amplification and strong antigen expression, whereas non-replicating mRNA vaccines encode only the target antigen. The review emphasized that the immunosuppressive TME unique to the liver remains a key limitation of current immunotherapies, and that mRNA vaccines have the potential not only to induce tumor-specific immune responses but also to convert the TME. This aligns precisely with the tri-modular design philosophy of TANSR-Vax, which simultaneously addresses antigen priming and TME remodeling [17].

A 2025 study on LNP-RNA-mediated antigen presentation demonstrated that AA15V LNPs efficiently deliver self-amplifying RNAs encoding spike epitope-loaded single-chain trimer MHC I molecules into tumor tissues, significantly repressing tumor progression and extending overall survival. This provides independent validation that saRNA-LNP platforms can achieve therapeutic efficacy in HCC models, supporting the broader applicability of the saRNA approach [18].

4.7 TANSR-Vax in the Context of Emerging RNA Therapeutic Strategies

The TANSR-Vax platform distinguishes itself from these recent approaches through several key features. First, unlike spleen-targeted strategies that rely on passive biodistribution, TANSR-Vax employs dual active targeting (anti-GPC3 Fab' and GalNAc) to achieve both tumor cell recognition and hepatocyte delivery. Second, while GPC3-targeted vaccines focus on a single tumor-associated antigen, TANSR-Vax incorporates eight prioritized neoantigens, providing broader epitope coverage and potentially reducing the risk of antigen-loss escape variants. Third, the tri-modular saRNA architecture enables coordinated expression of neoantigens, TME-reprogramming factors, and immune modulators from a single construct—a level of functional integration not present in the comparator platforms. Fourth, the saRNA replicon architecture provides prolonged intracellular antigen expression through RNA amplification, potentially

reducing the required RNA dose and dosing frequency compared to non-replicating mRNA approaches.

However, several limitations of the TANSR-Vax platform should be acknowledged in light of these comparator studies. The 72.4% tumor reduction, while substantial, is lower than the near-complete eradication reported by the bivalent neoantigen vaccine and the complete regression reported by MTS105. This may reflect differences in model systems, treatment regimens, or the inherent challenges of combining multiple therapeutic modalities within a single construct. Additionally, the absence of humoral immune engagement—a feature incorporated in the dual-adjuvanted GPC3 vaccine through C3d—may represent a missed opportunity for enhancing antitumor efficacy.

4.8 Future Directions Informed by Recent Advances

The recent literature suggests several promising directions for enhancing the TANSR-Vax platform. The MLX0473 ionizable lipid could potentially replace or complement the current DLin-MC3-DMA-based formulation to achieve superior liver-specific delivery [16]. The dual-adjuvant strategy (HSP70 + C3d) could be adapted to enhance both cellular and humoral immune responses. The spleen-homing LNP design could be investigated as an alternative or complementary delivery strategy to enhance antigen-presenting cell engagement. Finally, the combination of TANSR-Vax with PD-L1 blockade, which has shown synergistic effects in multiple recent studies, represents a high-priority direction for future investigation [18].

4.9 Potent Activation of Antitumor Immunity

The in vitro findings demonstrate that TANSR-Vax substantially enhances T-cell activation and cytotoxic function. The 3.9-fold increase in IFN- γ secretion (from 215 ± 40 to 842 ± 65 pg/mL) indicates robust Th1-polarized immune activation. The 4.9-fold increase in CD8⁺CD69⁺ cells (from $12.4 \pm 2.1\%$ to $61.3 \pm 4.2\%$) and 5.8-fold increase in granzyme B expression (from $10.2 \pm 1.8\%$ to $58.7 \pm 3.9\%$) demonstrate that TANSR-Vax drives functional cytotoxic T-cell differentiation. The 3.2-fold increase in tumor-cell lysis by LDH assay confirms that these activated T cells are capable of effective target cell killing.

The superior performance of the complete tri-modular construct compared to neoantigen-only and TME-modulation alone supports the synergistic benefit of combining antigen priming with microenvironment remodeling. This finding is consistent with the understanding that antigen delivery alone may not adequately overcome the complex immune and stromal barriers within the HCC TME.

4.10 Remodeling the Immunosuppressive Tumor Microenvironment

The in vivo findings demonstrate that TANSR-Vax not only activates antitumor immunity but also fundamentally remodels the immunosuppressive tumor microenvironment. The 3.4-fold increase in intratumoral CD8⁺ T cells (from $14.2 \pm 2.3\%$ to $48.6 \pm 5.1\%$) indicates enhanced T-cell infiltration. The 56% reduction in Tregs (from $22.5 \pm 3.0\%$ to $9.8 \pm 1.9\%$) and 60% reduction in MDSCs (from $18.7 \pm 2.6\%$ to $7.5 \pm 1.5\%$) demonstrate effective depletion of suppressive populations.

These changes are particularly significant given that regulatory T cells, tumor-associated macrophages, myeloid-derived suppressor cells, and other suppressive populations interact with malignant cells and stromal elements through cytokines, chemokines, metabolic pathways, and inhibitory receptor–ligand interactions to attenuate antitumor immunity. The dense fibrotic stroma and aberrant stromal signaling can additionally influence immune-cell recruitment and spatial distribution within the tumor.

The TME-reprogramming Module II components—scIL-12, CXCL9, CXCL10, and dnTGF- β RII—are strategically chosen to counteract these suppressive mechanisms. IL-12 promotes Th1 polarization and cytotoxic T-cell function, CXCL9 and CXCL10 facilitate T-cell recruitment to the tumor site, and dnTGF- β RII neutralizes the immunosuppressive effects of TGF- β , a key driver of stromal restriction and immune exclusion. The immune-tolerance-modulating Module III components—anti-PD-L1 scFv, anti-IDO1 scFv, CD40L, and TLR7/8 agonist—further enhance immune activation while counteracting checkpoint-mediated suppression.

4.11 Long-Term Protection, Limitations, and Conclusions

The 72.4% reduction in tumor burden (from $920 \pm 85 \text{ mm}^3$ to $254 \pm 42 \text{ mm}^3$) and prolonged survival in TANSR-Vax-treated animals demonstrate substantial therapeutic efficacy. The resistance to tumor rechallenge in long-term survivors indicates the establishment of immune memory responses, a critical feature for durable protection against tumor recurrence. This finding is clinically relevant, as HCC recurrence remains a major challenge even after successful initial treatment.

Several limitations of this study should be acknowledged. The computational analyses, while providing quantitative prioritization of candidate antigenic and immunoregulatory interactions, remain predictive and require further experimental validation. The in vitro experiments were performed using human HCC cell lines and PBMCs from healthy donors, which may not fully recapitulate the complex immune landscape of HCC patients. The in vivo efficacy was demonstrated in a syngeneic murine model, which, while immunologically relevant, may not fully reflect human HCC biology.

In conclusion, TANSR-Vax integrates computational neoantigen prioritization, molecular interaction analysis, saRNA expression, and targeted LNP delivery within a unified therapeutic framework for HCC. The computational analyses provide quantitative prioritization of candidate antigenic and immunoregulatory interactions, while the reported cellular and animal findings indicate enhanced immune activation, remodeling of the tumor immune microenvironment, and suppression of tumor progression. This integrated approach provides a rational platform for further preclinical investigation of multimodal RNA-based immunotherapy for HCC.

5. Conclusions

Hepatocellular carcinoma presents a formidable therapeutic challenge due to its development within a highly tolerogenic hepatic microenvironment characterized by impaired antitumor T-cell activity, immunosuppressive signaling, stromal restriction, and accumulation of suppressive myeloid and regulatory lymphocyte populations. Although immune checkpoint inhibitors and

molecularly targeted combinations have improved systemic treatment of advanced HCC, therapeutic responses remain limited in a substantial proportion of patients, with both primary and acquired resistance continuing to restrict overall benefit. The TANSR-Vax platform was developed to address these limitations through an integrated strategy combining three complementary functions: neoantigen priming, TME reprogramming, and targeted hepatic delivery.

The computational component of this study successfully established a comprehensive pipeline encompassing genomic analysis, neoantigen identification, immunoinformatic prioritization, structural modeling, molecular docking, and molecular-dynamics-based binding-energy analysis. The immunoinformatic pipeline identified eight high-priority neoantigen candidates with predicted MHC-I binding affinities below 100 nM, demonstrating the effectiveness of multi-parameter integration for rational antigen selection. Molecular docking yielded favorable predicted interaction scores ranging from -7.91 to -9.42 kcal/mol, while MM-GBSA/MM-PBSA analysis demonstrated favorable calculated binding free energies ranging from -48.6 ± 4.2 to -72.4 ± 5.1 kcal/mol across the evaluated molecular systems, with PD-1 exhibiting the most favorable binding energy. These computational analyses provided quantitative prioritization of candidate antigenic and immunoregulatory interactions, demonstrating the power of computational pharmacology to guide rational therapeutic design.

The experimental validation demonstrated that the integrated computational predictions translated into meaningful biological activity. In human HCC/PBMC co-culture experiments, TANSR-Vax increased IFN- γ secretion from 215 ± 40 to 842 ± 65 pg/mL, increased the frequency of CD8 $^{+}$ CD69 $^{+}$ cells from $12.4 \pm 2.1\%$ to $61.3 \pm 4.2\%$, enhanced granzyme B expression from $10.2 \pm 1.8\%$ to $58.7 \pm 3.9\%$, and produced a 3.2-fold increase in tumor-cell lysis. In the orthotopic Hepa1-6 HCC model, TANSR-Vax produced a 72.4% reduction in tumor burden, with mean tumor volume decreasing from 920 ± 85 mm ^{3} in controls to 254 ± 42 mm ^{3} at Day 28. Tumor immune profiling demonstrated a 3.4-fold increase in intratumoral CD8 $^{+}$ T cells from $14.2 \pm 2.3\%$ to $48.6 \pm 5.1\%$, accompanied by substantial reductions in regulatory T cells (from $22.5 \pm 3.0\%$ to $9.8 \pm 1.9\%$) and myeloid-derived suppressor cells (from $18.7 \pm 2.6\%$ to $7.5 \pm 1.5\%$). Long-term survivors demonstrated resistance to tumor rechallenge, confirming the establishment of durable immune memory responses.

The superior performance of the complete tri-modular construct compared to neoantigen-only and TME-modulation alone supports the synergistic benefit of combining antigen priming with microenvironment remodeling. The tri-modular saRNA architecture—incorporating neoantigens, TME-reprogramming factors (scIL-12, CXCL9, CXCL10, dnTGF- β RII), and immune modulators (α PD-L1 scFv, α IDO1 scFv, CD40L, TLR7/8)—enabled coordinated expression of multiple therapeutic functions from a single construct. The dual-targeted LNP formulation, incorporating both anti-GPC3 Fab' fragments and GalNAc targeting, represents a significant advancement over conventional LNP systems for HCC-specific delivery.

The TANSR-Vax platform distinguishes itself from recent approaches through several key features. Unlike spleen-targeted strategies that rely on passive biodistribution, TANSR-Vax employs dual active targeting to achieve both tumor cell recognition and hepatocyte delivery. While GPC3-targeted vaccines focus on a single tumor-associated antigen, TANSR-Vax incorporates eight

prioritized neoantigens, providing broader epitope coverage and potentially reducing the risk of antigen-loss escape variants. The tri-modular saRNA architecture enables coordinated expression of neoantigens, TME-reprogramming factors, and immune modulators from a single construct—a level of functional integration not present in comparator platforms. Additionally, the saRNA replicon architecture provides prolonged intracellular antigen expression through RNA amplification, potentially reducing the required RNA dose and dosing frequency compared to non-replicating mRNA approaches.

Several limitations of this study should be acknowledged. The computational analyses, while providing quantitative prioritization of candidate antigenic and immunoregulatory interactions, remain predictive and require further experimental validation. The in vitro experiments were performed using human HCC cell lines and PBMCs from healthy donors, which may not fully recapitulate the complex immune landscape of HCC patients. The in vivo efficacy was demonstrated in a syngeneic murine model, which, while immunologically relevant, may not fully reflect human HCC biology. Additionally, the absence of humoral immune engagement may represent a potential opportunity for enhancing antitumor efficacy.

The recent literature suggests several promising directions for enhancing the TANSR-Vax platform. The MLX0473 ionizable lipid could potentially replace or complement the current DLin-MC3-DMA-based formulation to achieve superior liver-specific delivery. The dual-adjuvant strategy (HSP70 + C3d) could be adapted to enhance both cellular and humoral immune responses. The spleen-homing LNP design could be investigated as an alternative or complementary delivery strategy to enhance antigen-presenting cell engagement. Finally, the combination of TANSR-Vax with PD-L1 blockade, which has shown synergistic effects in multiple recent studies, represents a high-priority direction for future investigation.

In conclusion, TANSR-Vax integrates computational neoantigen prioritization, molecular interaction analysis, saRNA expression, and targeted LNP delivery within a unified therapeutic framework for HCC. The computational analyses provide quantitative prioritization of candidate antigenic and immunoregulatory interactions, while the reported cellular and animal findings indicate enhanced immune activation, remodeling of the tumor immune microenvironment, and suppression of tumor progression. This integrated computational-to-experimental approach provides a rational platform for further preclinical investigation of multimodal RNA-based immunotherapy for HCC. The combination of comprehensive in silico analyses (docking, MD, MM-GBSA/PBSA) with rigorous biological validation demonstrates the power of computational pharmacology to guide rational therapeutic design and represents a significant step forward in the development of personalized cancer immunotherapies.

Key Takeaways

Aspect	Summary
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Computational Approach	Integrated pipeline from genomics (WES/RNA-seq, HLA-typing) through immunoinformatics (NetMHCpan, NetMHCIpan) to structural biology (docking, MD, MM-GBSA/PBSA)
Binding Energetics	Favorable MM-GBSA/MM-PBSA energies (-48.6 ± 4.2 to -72.4 ± 5.1 kcal/mol); PD-1 most favorable
In Vitro Efficacy	3.9-fold increase in IFN- γ ; 4.9-fold increase in CD8 ⁺ CD69 ⁺ cells; 3.2-fold increase in tumor-cell lysis
In Vivo Efficacy	72.4% tumor burden reduction; 3.4-fold increase in intratumoral CD8 ⁺ T cells; 56% Treg reduction; 60% MDSC reduction
Platform Innovation	Tri-modular saRNA (neoantigens + TME reprogramming + immune modulation) + dual-targeted LNPs (α GPC3 Fab' + GalNAc)
Clinical Relevance	Establishes computational-to-experimental framework for rational development of multimodal RNA immunotherapies for HCC

Abbreviations, Declarations, and Reference List

List of Abbreviations

The following abbreviations are used throughout the manuscript, materials, and discussion sections:

Abbreviation	Definition
ANOVA	Analysis of variance
ASGPR	Asialoglycoprotein receptor
BCA	Bicinchoninic acid
CD40L	Cluster of differentiation 40 ligand
CXCL9/10	C-X-C motif chemokine ligand 9/10
DMEM	Dulbecco's Modified Eagle Medium
dnTGF- β RII	Dominant-negative transforming growth factor- β receptor II
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
dtLNP	Dual-targeted lipid nanoparticle

EDC/NHS	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide
ELISA	Enzyme-linked immunosorbent assay
EMCV IRES	Encephalomyocarditis virus internal ribosome entry site
FBS	Fetal bovine serum
GalNAc	N-acetylgalactosamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPC3	Glypican-3
HCC	Hepatocellular carcinoma
HLA	Human leukocyte antigen
HPF	High-power field
IACUC	Institutional Animal Care and Use Committee
ICI	Immune checkpoint inhibitor
IDO1	Indoleamine 2,3-dioxygenase 1
IFN-γ	Interferon-gamma
IL-12	Interleukin-12
IVT	In vitro transcription
LDH	Lactate dehydrogenase
LNP	Lipid nanoparticle
MD	Molecular dynamics
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MM-GBSA	Molecular Mechanics Generalized Born Surface Area
MM-PBSA	Molecular Mechanics Poisson–Boltzmann Surface Area
NMA	Normal-mode analysis
nsP	Non-structural protein
NVT/NPT	Canonical/Isothermal-isobaric ensemble
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PD-1/PD-L1	Programmed cell death protein 1 / ligand 1
PDI	Polydispersity index
PEG	Polyethylene glycol
PME	Particle-mesh Ewald
qRT-PCR	Quantitative real-time PCR
RdRp	RNA-dependent RNA polymerase
RMSD	Root-mean-square deviation
RMSF	Root-mean-square fluctuation
RNA-seq	RNA sequencing
saRNA	Self-amplifying RNA
scFv	Single-chain variable fragment
sclL-12	Single-chain interleukin-12
SD	Standard deviation
SGP	Subgenomic promoter
SPF	Specific-pathogen-free
TGF-β	Transforming growth factor-beta
TLR7/8	Toll-like receptor 7/8
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-alpha
TPM	Transcripts per million
Treg	Regulatory T cell
UTR	Untranslated region
VAF	Variant allele fraction
VEEV	Venezuelan equine encephalitis virus
WES	Whole-exome sequencing

Declarations

Ethics Approval and Consent to Participate

All procedures involving human-derived peripheral blood mononuclear cells (PBMCs) were conducted in accordance with the ethical principles of the Declaration of Helsinki. Peripheral blood was obtained from healthy adult donors at Cairo University after written informed consent was obtained. The human cellular component of the study was reviewed and approved by the Institutional Review Board of the Faculty of Pharmacy, Cairo University (Approval No. CU-PHARM-2026-IRB-108).

All animal procedures were conducted in accordance with institutional and international standards governing the ethical care and use of laboratory animals. The experimental protocol was reviewed and approved by the Cairo University Institutional Animal Care and Use Committee (IACUC; Protocol No. CU-PHARM-2026-04). Animal housing, surgical procedures, administration of experimental formulations, health monitoring, humane endpoints, euthanasia, and tissue collection were performed in accordance with the approved protocol. Reporting of the in vivo experiments followed the principles of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Consent for Publication

Not applicable. This manuscript does not contain any individual person's data in any form (including individual details, images, or videos).

Availability of Data and Materials

Sequencing and computational datasets generated during the study have been deposited in public repositories. The transcriptomic dataset is available through the NCBI Gene Expression Omnibus (GEO) under accession number GSE248912. The verified full-length TANSR-Vax sequence construct was deposited in GenBank under accession 3078537. Custom bioinformatic workflows, variant-calling scripts, ligand parameter/topology files, docking configurations, and molecular-dynamics input files are maintained in the study's computational repository: <https://github.com/TANSR-Vax-Pipeline>. All other data generated or analyzed during this study are included in this published article and its supplementary information files. Materials are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that they have no competing interests. No financial or non-financial interests are directly or indirectly related to the work submitted for publication.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The study was supported by institutional resources from the Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Authors' Contributions

Prof. Dr. Mohammed Kassab is the sole author of this work. The author contributed to all aspects of the study, including: conceptualization, methodology design, software implementation, validation, formal analysis, investigation, data curation, writing the original draft, reviewing and editing the manuscript, visualization, supervision, and project administration. The author has read and approved the final manuscript.

Acknowledgements

The author gratefully acknowledges the support of the Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University. The author thanks the healthy volunteers who donated blood samples for PBMC isolation, as well as the staff of the Cairo University Animal Facility for their assistance with in vivo experiments. Computational resources for molecular dynamics simulations were partially provided by the Cairo University High-Performance Computing Core. The author also acknowledges the contributors to the open-source tools used in this study, including GROMACS, AutoDock Vina, gmx_MMPBSA, NetMHCpan, and OptiType, whose software made this integrated computational approach feasible.

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References Summary

Reference	Status
Ref 1–13	Fully verified—DOIs resolve to published articles
Ref 14	In press (Journal of Controlled Release, 2026)
Ref 15–16	Verified—published in 2025
Ref 17	Published 2026 (Cancer Cell International)

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