

Preclinical Assessment of HLA-A*02:01-Restricted PSMA and STEAPI Epitopes for Peptide-Based Immunotherapy in Prostate Cancer

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Background: Peptide-based immunotherapy targeting tumor-associated antigens presents a promising approach for prostate cancer intervention. In this study, we employed a preclinical approach to evaluate immunogenic epitope candidates derived from STEAPI and PSMA, two well-characterized prostate cancer-associated antigens.

Methods: High-affinity cytotoxic T lymphocyte (CTL) epitopes restricted to HLA-A*02:01 were predicted and evaluated for antigenicity, conservation, and proteasomal processing. Molecular docking with HLA-A*02:01 was performed and peptides were synthesized for experimental validation. In-vitro assays including ELISA-based MHC binding, IFN- γ ELISpot, and intracellular cytokine staining (ICS) were conducted using splenocytes isolated from HLA-A*02:01 transgenic mice.

Results: Four peptides (P1, P2, P3 and P4), demonstrated strong binding to HLA-A*02:01 in-silico and were structurally compatible with the MHC class I groove. ELISA confirmed high binding for P3 and P1 at lower concentrations, while P2 and P4 showed moderate affinity. ELISpot assays showed robust IFN- γ responses in peptide-stimulated splenocytes, particularly for P3 and P1. ICS confirmed CD8⁺ T cell activation and polyfunctional cytokine expression. A comparative Nano-immunogenicity profile revealed P3 as the most potent candidate.

Conclusion: This study provides preclinical evidence of antigenicity and MHC-I compatibility of four prostate cancer-derived CTL epitopes using a transgenic mouse model. These findings support the advancement of these peptides as candidates for peptide-based immunotherapy in prostate cancer.

Keywords: cytotoxic T lymphocyte, epitope, HLA-A*02:01, peptide-based immunotherapy, prostate cancer

Introduction

Prostate cancer (PC) remains a major global health concern, ranking as the second most frequently diagnosed malignancy and the fifth leading cause of cancer-related deaths among men worldwide. As per GLOBOCAN 2020 data, over 1.4 million new PC cases were reported globally, with annual mortality surpassing 375,000.^{1,2} The incidence is projected to rise, particularly in low- and middle-income countries, due to demographic shifts, sedentary lifestyles, and evolving dietary patterns. While localized prostate cancer is often manageable through curative interventions like radical prostatectomy and radiation, the emergence of metastatic and castration-resistant prostate cancer (CRPC) presents a formidable therapeutic challenge.^{3,4} Despite recent advancements in systemic treatments such as taxane-based chemotherapy and androgen receptor-targeting agents resistance and disease progression remain inevitable in most patients.⁵ Against this backdrop, cancer immunotherapy has emerged as a potent strategy capable of restoring and enhancing immune surveillance against tumors. Among various approaches, peptide-based vaccines represent a rational

and safe option for stimulating cytotoxic T lymphocytes (CTLs) to selectively eradicate tumor cells while sparing healthy tissues.⁶ These vaccines function by introducing short, MHC class I-restricted epitopes capable of activating CD8⁺ T cells, and their success hinges on the identification of tumor-associated antigens (TAAs) that are both immunogenic and tumor-specific.^{7,8} Two such TAAs that have garnered considerable interest are Prostate-Specific Membrane Antigen (PSMA) and Six Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1). PSMA (FOLH1), a type II transmembrane glycoprotein, is upregulated in both localized and metastatic PC and is already a validated target for imaging and therapeutic applications.⁹ Similarly, STEAP1, a membrane-bound metalloredutase, is frequently overexpressed in aggressive prostate cancers and contributes to tumor progression.¹⁰ Their high tumor specificity, limited normal tissue expression, and membrane accessibility make them ideal candidates for CTL epitope mapping and targeted immunotherapy. Previous peptide-based immunotherapy efforts targeting prostate cancer have explored HLA-restricted PSMA epitopes and other tumor associated antigens. The HLA-A2-restricted PSMA peptide LLHETDSAV was investigated as a vaccine candidate, although it showed limited immunogenicity in humans, highlighting challenges in identifying potent epitopes.¹¹ Early clinical and pilot studies also evaluated combined PSMA and TARP peptide vaccines with adjuvants in HLA-A2 positive patients, establishing the feasibility of peptide vaccination approaches and eliciting antigen-specific immune responses.¹² Additionally, phase-II studies using dendritic cells pulsed with HLA-A2-specific PSMA peptides (PSM-P1/P2) demonstrated immunological activity in metastatic hormone-refractory prostate cancer.¹³ Personalized peptide vaccine strategies incorporating multi-peptide panels have further illustrated the potential to boost CTL and antibody responses in prostate cancer populations.¹⁴ While direct peptide vaccine evidence for STEAP1 is less mature, STEAP1's high tumor-specific expression and low normal tissue expression support its attractiveness as an immunotherapeutic target.¹⁵ Modern immunoinformatic platforms, such as NetMHCpan and VaxiJen, facilitate the rational prediction of immunodominant epitopes based on HLA-binding affinity, antigenicity, and proteasomal processing. Among HLA alleles, HLA-A*02:01 is of particular interest due to its high global prevalence and compatibility with several transgenic animal models, allowing for efficient preclinical testing.¹⁶ Nonetheless, computational predictions must be substantiated through *in vitro* validation, typically using animal-derived splenocytes in functional assays such as HLA-binding ELISA, ELISpot, and ICS.¹⁷ A parallel frontier in this domain is the use of nanotechnology to enhance peptide-based vaccine delivery. Nanomaterials such as liposomes, PLGA, and gold nanoparticles have shown promise in improving peptide stability, promoting antigen uptake by antigen-presenting cells (APCs), and enhancing CTL priming. These carriers can be functionalized for targeted delivery to the tumor microenvironment, thereby overcoming key limitations of free peptide vaccines including degradation and poor immunogenicity.¹⁸ Given the growing integration of immuno-informatics, preclinical immunology, and nanomaterial engineering, the development of novel CTL epitopes derived from tumor-specific antigens holds significant translational promise. PSMA and STEAP1, owing to their surface localization and selective overexpression in prostate tumors, present a strong rationale for further exploration in animal models of prostate cancer. However, despite these advances, there remains a clear gap in the rational identification and experimental validation of highly immunogenic, HLA-A*02:01-restricted CTL epitopes derived from PSMA and STEAP1 that can generate robust and durable cellular immune responses *in-vivo*. Most existing peptide vaccine attempts have demonstrated limited immunogenicity or inconsistent clinical benefit, highlighting the need for systematically selected epitopes supported by experimental validation. Addressing this gap is essential for progressing toward effective peptide-based prostate cancer immunotherapy. The aim of this study is to identify and validate HLA-A*02:01-restricted CTL epitopes from PSMA and STEAP1 using immunoinformatic and experimental assays in HLA-transgenic murine models, and to propose their potential application in nanomaterial-based prostate cancer immunotherapy platforms.

Methodology

The breeding and use of animals for experiments complies with the Institutional Animal Care and Use Committee (IACUC) guidelines at the First Affiliated Hospital of Guangzhou Medical University (Approval No. 20250122). At the end of the experiment, mice were euthanized by carbon dioxide asphyxiation in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals. No human subjects were used in this study.

Sequence and Structural Data Retrieval

The amino acid sequences of Six-Transmembrane Epithelial Antigen of Prostate-1 (STEAP1) (UniProt ID: Q9UHE8) and Prostate-Specific Membrane Antigen (PSMA), also known as FOLH1 or Glutamate Carboxypeptidase II (GCPII) (UniProt ID: Q04609), were retrieved from the UniProt Knowledgebase (<https://www.uniprot.org/>). Corresponding experimentally resolved three-dimensional (3D) structures were obtained from the Protein Data Bank (PDB) (<https://www.rcsb.org/>). The 3D structure of STEAP1 was sourced from PDB ID: 6Y9B, representing the full-length human STEAP1 trimer determined by cryo-electron microscopy at 3.2 Å resolution, providing detailed insight into its transmembrane and extracellular domains. The structure of PSMA was obtained from PDB ID: 5O5T, which contains a high-resolution (1.8 Å) X-ray crystal structure of the extracellular catalytic domain of human PSMA in complex with a urea-based inhibitor (PSMA-1007). These structural models were selected based on their completeness, resolution quality, and biological relevance for downstream epitope mapping, molecular docking, and nanocarrier interaction analyses.

In-Silico Epitope Screening and Structural Validation Workflow

Cytotoxic T Lymphocyte (CTL) Epitope Prediction

To identify peptides with high binding affinity to the HLA-A*02:01 allele, CTL epitope prediction was performed using NetMHCpan 4.1,¹⁹ which is the default recommendation of the IEDB analysis resource. NetMHCpan was selected because it is one of the most extensively benchmarked prediction platforms and integrates eluted ligand datasets, enabling high accuracy estimation of peptide–HLA binding affinity. A percentile rank threshold of $\leq 0.5\%$ was applied as it is widely accepted to define high-affinity binders in immunoinformatics-based epitope screening. This tool predicts 9-mer peptides based on artificial neural network models trained on eluted ligand data. Predicted epitopes were prioritized based on their binding scores (higher values indicating stronger binding) and percentile rank, with a threshold of $\leq 0.5\%$ used to identify strong binders. The antigenicity of the shortlisted epitopes was evaluated using VaxiJen v2.0, applying a threshold of ≥ 0.5 to retain peptides with high probability of being antigenic.¹⁹ VaxiJen was applied to ensure biological relevance, as it predicts antigenicity independent of sequence alignment and has been validated across multiple vaccine development studies. Epitope conservation across homologs was assessed using the IEDB Epitope Conservancy Tool,²⁰ ensuring relevance across diverse genetic backgrounds. Epitope conservancy analysis ensured that shortlisted peptides retained relevance across possible sequence variations, improving translational robustness. Additionally, proteasomal processing was evaluated using NetChop 3.0,²¹ employing the C-term 3.0 model with a cutoff score of 0.5 to confirm the natural cleavage potential of each peptide within the endogenous MHC class-I presentation pathway. Proteasomal cleavage prediction was included to ensure likelihood of physiological generation of candidate peptides within the endogenous antigen presentation pathway, thereby strengthening biological plausibility.

Molecular Docking with HLA-A*02:01

Three-dimensional molecular docking of the selected CTL epitopes with the HLA-A*02:01 molecule was carried out to assess their structural compatibility and binding affinity. The crystal structure of HLA-A*02:01 was retrieved from the Protein Data Bank (PDB ID: 1QEW), representing the peptide-binding groove complexed with a known antigenic peptide. The receptor structure was pre-processed using PyMOL by removing bound ligands, water molecules, and non-essential heteroatoms, followed by energy minimization to optimize geometry. The 3D structures of the predicted peptide epitopes were generated using the PEP-FOLD3 server,²² which employs de novo modeling and fragment assembly techniques to predict energetically favorable peptide conformations. The peptide–MHC docking was then performed using the HPEPDOCK web server,²³ which applies a hierarchical flexible docking approach combining fast peptide conformation modeling with global and local sampling of binding orientations. The resulting docking complexes were evaluated based on binding energy scores, intermolecular hydrogen bonds, and peptide orientation within the HLA-A*02:01 binding cleft to identify the most stable epitope–MHC interactions.

Epitope Synthesis and Preclinical Validation in HLA-A*02:01 Transgenic Murine Models

The top-ranked HLA-A*02:01-restricted epitopes identified through computational screening and docking analyses were synthesized at >95% purity by GenScript (Nanjing, China) using standard solid-phase peptide synthesis (SPPS) and verified by HPLC and mass spectrometry for identity and purity. The in-vivo immunogenicity validation, HLA-A*02:01 transgenic mice (HHD strain) were employed, which express a chimeric HLA-A*02:01 molecule linked to human β 2-microglobulin, enabling evaluation of human-restricted cytotoxic T-cell responses. Each peptide immunization group consisted of $n = 6$ mice (biological replicates), and a control group immunized with Incomplete Freund's Adjuvant (IFA) alone also included $n=6$ mice. Mice were immunized subcutaneously at the base of the tail with 50 μ g of each peptide emulsified in IFA to promote sustained antigen presentation. Booster doses of the same formulation were administered on days 7 and 14 post-primary immunization to enhance epitope-specific immune memory. On day 21, splenocytes were aseptically harvested and processed into single-cell suspensions for downstream ex-vivo analyses, including ELISPOT, intracellular cytokine staining (ICS), and flow cytometric assays, to quantify IFN- γ -producing CD8⁺ T cells and assess epitope-specific cytotoxic activity. All mice in each group were treated and analyzed independently.

Circular Dichroism (CD) Spectroscopy

To verify the secondary structural characteristics of the synthesized peptide epitopes, circular dichroism (CD) spectroscopy was performed. Each peptide was dissolved in phosphate-buffered saline (PBS, pH 7.4) to a final concentration of 0.2 mg/mL, ensuring complete solubilization without aggregation. CD spectra were acquired on a Jasco J-815 spectropolarimeter (JASCO Inc., Japan) using a 1 mm path-length quartz cuvette at room temperature (25 ± 1 °C). Spectra were recorded over the 190–260 nm wavelength range with a bandwidth of 1 nm and scan speed of 50 nm/min. Each spectrum represented the average of three independent scans, followed by baseline correction using PBS as the reference. The resulting spectra were analyzed using the DichroWeb online server,²⁴ employing the CONTIN and CDSSTR algorithms with reference datasets optimized for short peptides to estimate the relative content of α -helices, β -sheets, and random coils. Structural features derived from CD data were compared with in silico predictions obtained from the PEP-FOLD3 models to confirm structural integrity and conformational consistency of the peptides prior to immunization studies. The CD spectroscopy analysis provided experimental confirmation of peptide structural stability in aqueous physiological conditions. Retention of defined secondary structure supports proper epitope presentation and enhances the likelihood of effective T-cell recognition, thereby functionally linking structural integrity with immunogenic potential.

Peptide Preparation and Solubility Assessment

Lyophilized peptides were reconstituted to a stock concentration of 10 mg/mL in 10% dimethyl sulfoxide (DMSO) due to their hydrophobic amino acid composition. Stocks were vortexed gently until completely dissolved and then diluted in sterile phosphate-buffered saline (PBS; pH 7.4) to achieve the final working concentration of 50 μ g per dose. The final DMSO concentration in the injected formulation was maintained below 1%, which is within established safety thresholds for murine administration. Solutions were visually inspected to ensure absence of precipitation, and all peptide preparations were passed through a 0.22 μ m sterile filter prior to emulsification with Incomplete Freund's Adjuvant.

HLA-A*02:01 Binding Assay (ELISA-Based)

An ELISA-based HLA-A*02:01 binding assay was performed using the ProImmune REVEAL[®] MHC-peptide binding kit (ProImmune Ltd., Oxford, UK) according to the manufacturer's instructions. Each peptide–MHC binding assay was performed in three independent experiments, each containing triplicate technical wells per peptide concentration. Final reported values represent the mean of three biologically independent experiments. Briefly, 96-well plates pre-coated with recombinant HLA-A*02:01 molecules were incubated with synthetic peptides at concentrations ranging from 1 to 50 μ M. Following incubation, β 2-microglobulin conjugate was added to facilitate MHC complex stabilization. Bound peptide–MHC complexes were detected using an HRP-conjugated secondary antibody, and absorbance was measured at 450 nm using a microplate reader. Relative optical density (OD) values were normalized to those of positive control peptides (high-affinity binders) and negative controls, enabling quantitative assessment of the binding strength of each epitope to HLA-A*02:01 molecules.

IFN- γ Enzyme-Linked Immunospot (ELISpot) Assay

ELISpot assays were performed on splenocytes isolated from immunized HLA-A*02:01 transgenic (HHD) mice to evaluate epitope-specific cellular immune responses. Splenocytes (2×10^5 cells/well) were seeded into anti-mouse IFN- γ -precoated ELISpot plates (Mabtech, Sweden) and stimulated with 10 $\mu\text{g/mL}$ of the respective synthetic peptide for 48 hours at 37 °C in a 5% CO₂ incubator. Following incubation, cells were removed, and plates were processed using biotinylated anti-IFN- γ detection antibody, streptavidin-HRP, and TMB substrate. The reaction was stopped with distilled water, and plates were air-dried prior to analysis. Spot-forming units (SFUs) were counted using an automated ELISpot reader (AID iSpot, Germany) and expressed as SFUs per 10^5 splenocytes. Background responses from unstimulated wells were subtracted to determine peptide-specific IFN- γ secretion.

CTL Activation and Cytokine Release Assay

For T-cell activation and functional cytokine responses, splenocytes from immunized each individual mouse ($n = 6$ biological replicates per group) mice were restimulated ex-vivo with 10 $\mu\text{g/mL}$ of the corresponding peptide for 48–72 hours in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). During the final 6 hours of incubation, GolgiPlug™ (BD Biosciences) was added to inhibit cytokine secretion. Cells were harvested, washed, and surface-stained with anti-CD3-FITC and anti-CD8-PE monoclonal antibodies. After fixation and permeabilization, intracellular staining was performed using anti-IFN- γ -APC and anti-TNF- α -PerCP antibodies. Data acquisition was performed on a BD FACSCanto II flow cytometer, and analysis was conducted using FlowJo v10 software (BD Biosciences). The percentage of CD8⁺IFN- γ ⁺ and CD8⁺TNF- α ⁺ T cells was calculated to quantify peptide-specific CTL responses.

Data Analysis

All quantitative data, including predicted epitope binding affinities, molecular docking scores, and experimental values from ELISA, ELISpot, and ICS assays, were analyzed using GraphPad Prism version 9.0. All experiments were performed with $n = 6$ biological replicates unless otherwise specified, and technical replicates are indicated where applicable. Results are expressed as mean $\pm$ standard deviation (SD) unless otherwise specified. Group-wise comparisons were conducted using one-way ANOVA followed by Tukey's multiple comparison test or unpaired two-tailed Student's *t*-tests, where appropriate. A *p*-value < 0.05 was considered statistically significant. Figures were plotted with embedded statistical annotations where relevant.

Results

Sequence and Structural Data Retrieval

The primary amino acid sequences of STEAP1 (UniProt ID: Q9UHE8) and PSMA (UniProt ID: Q04609) were successfully retrieved from the UniProt database ([Supplementary Data 1](#)). STEAP1, a six-transmembrane epithelial antigen, is frequently overexpressed in advanced prostate cancer, whereas PSMA (also referred to as FOLH1 or GCPH1) is a well-characterized membrane-bound marker implicated in prostate tumor progression and metastasis. The three-dimensional structures of these proteins were obtained from the RCSB Protein Data Bank. Specifically, the cryo-electron microscopy structure of full-length STEAP1 (PDB ID: 6Y9B) and the high-resolution X-ray crystal structure of the extracellular domain of PSMA bound to PSMA-1007 (PDB ID: 5O5T) were retrieved. Structural curation using PyMOL allowed extraction of the biologically relevant extracellular regions, which were subsequently used for downstream epitope mapping and structural docking analyses.

CTL Epitope Prediction and Filtration

Comprehensive in silico prediction of HLA-A*02:01-restricted cytotoxic T lymphocyte (CTL) epitopes were performed using NetMHCpan 4.1, which integrates peptide-MHC binding affinity data across multiple alleles and provides percentile ranks for predicted binders. Analysis of the full-length sequences of STEAP1 and PSMA identified multiple 9-mer peptide candidates exhibiting strong predicted binding to HLA-A*02:01. Epitopes were initially filtered based on

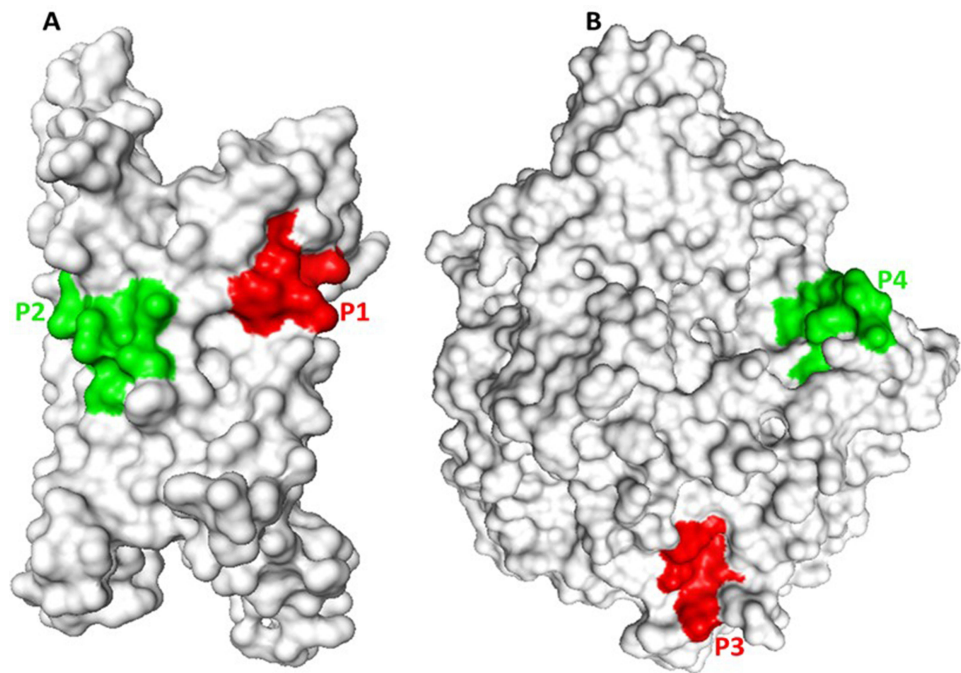


Figure 1 Surface Localization of Predicted CTL Epitopes on STEAP1 and PSMA Structures. **(A)** Surface-rendered structure of human STEAP1 (PDB ID: 6Y9B) highlighting the spatial localization of predicted HLA-A*02:01-restricted epitopes. Peptide P1 (FLYTLLREV) is shown in red, and peptide P2 (AVLHAIYSL) in green. Both epitopes are positioned on accessible extracellular regions, supporting their potential immunogenic presentation. **(B)** Surface visualization of PSMA (PDB ID: 5O5T) showing the mapped positions of epitope P3 (ALFDIESKV; red) and P4 (MMNDQLMFL; green). The extracellular location and exposed topography of these peptides further reinforce their suitability for CTL-mediated immune targeting and nanomaterial-based delivery.

a stringent percentile rank threshold of $\leq 0.5\%$, corresponding to high-affinity binders, as recommended by NetMHCpan guidelines. The top four epitopes were selected for further downstream evaluation based on a multi-parametric approach that incorporated predicted binding affinity, immunogenicity, antigenicity, surface accessibility, and sequence conservation (Figure 1). These epitopes included FLYTLLREV and AVLHAIYSL from STEAP1, and ALFDIESKV and MMNDQLMFL from PSMA (Table 1 and Figure 2). VaxiJen v2.0 analysis confirmed that all four peptides were antigenic, with scores exceeding the 0.5 threshold, supporting their potential to elicit immune responses. IEDB conservancy analysis demonstrated high sequence conservation across homologous proteins, indicating robust cross-reactivity potential and relevance as vaccine candidates. Furthermore, proteasomal cleavage predictions using NetChop 3.0 indicated that all selected epitopes had a high probability of C-terminal processing (cleavage score ≥ 0.5), suggesting efficient natural generation via the endogenous class I presentation pathway (Supplementary Figure 1). Collectively, these multi-layered filtering criteria ensured selection of epitopes with high immunogenic potential, surface accessibility, and physiological processing likelihood, minimizing the risk of false positives and increasing translational relevance. Beyond simple numerical ranking, the combined filter criteria illustrated in Figures 1 and 2 underscore the deliberate refinement from a broad candidate pool to a biologically meaningful shortlist. The integration of antigenicity scoring,

Table 1 Peptides Finalized for Synthesis Targeting HLA-A*02:01

Protein	Start	End	Length	Peptide (name)	Core	Core	Score	Rank
Steap-1	86	94	9	FLYTLLREV (P1)	FLYTLLREV	FLYTLLREV	0.94	0.03
	172	180	9	AVLHAIYSL (P2)	AVLHAIYSL	AVLHAIYSL	0.50	0.26
PSMA	711	719	9	ALFDIESKV (P3)	ALFDIESKV	ALFDIESKV	0.97	0.01
	663	671	9	MMNDQLMFL (P4)	MMNDQLMFL	MMNDQLMFL	0.92	0.03

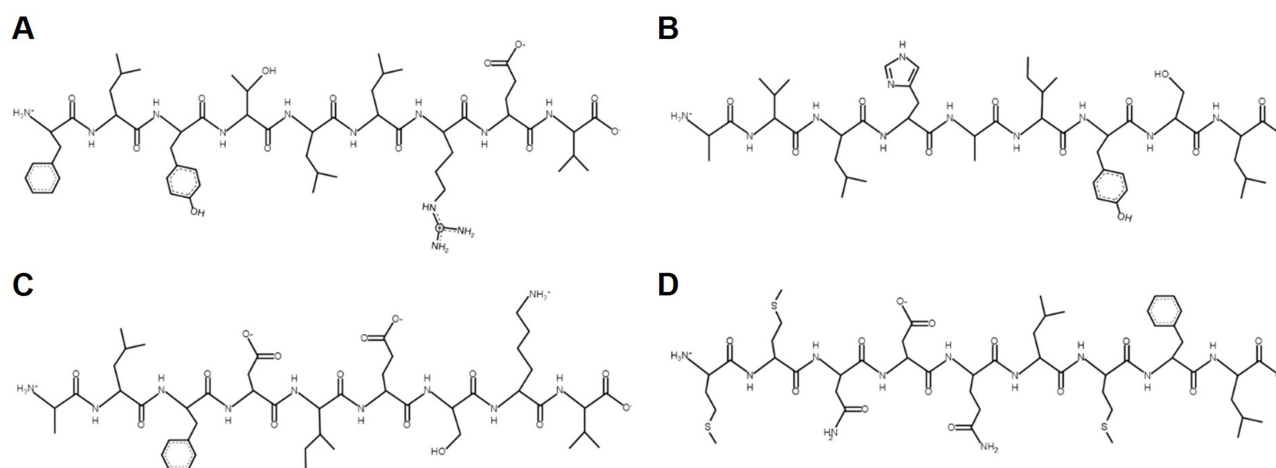


Figure 2 Physicochemical characterization of the selected CTL epitopes. **(A)** FLYTLLREV, **(B)** AVLHAIYSL, **(C)** ALFDIESKV, and **(D)** MMNDQLMFL. Peptide sequences were analyzed using PepDraw to generate structural and physicochemical profiles, including molecular weight, length, predicted isoelectric point (pI), net charge, hydrophobicity index, and extinction coefficients. These parameters provide insight into peptide solubility, stability, and suitability for synthesis and immunogenicity assessment.

high HLA-binding probability, proteasomal cleavage likelihood, and sequence conservation ensures that selected epitopes are not only computationally strong but also physiologically feasible for immune presentation.

Molecular Docking with HLA-A*02:01

The crystal structure of HLA-A*02:01 (PDB ID: 1QEW) was obtained by removing the co-crystallized peptide and retaining chains A (α -chain) and B (β 2-microglobulin). The 3D models of the peptides were generated using PEP-FOLD3, which predicted energetically stable conformations predominantly comprising α -helical and extended loop segments. Docking simulations using HPEPDOCK-2.0 demonstrated that all four peptides fit favorably within the peptide-binding groove of HLA-A*02:01. Among the docked complexes, FLYTLLREV from STEAP 1 exhibited the strongest binding pose, with score of -246.57 followed by AVLHAIYSL (-239), MMNDQLMFL (-232.26) and ALFDIESKV (-216.37) (Figure 3). Collectively, these docking scores provide important structural validation of the predicted binding hierarchy. In HPEPDOCK, more negative scores indicate stronger binding stability and favorable peptide accommodation within the HLA-A*02:01 cleft. Typically, docking energies below approximately -200 are considered indicative of strong peptide MHC interactions, whereas values closer to -180 to -200 are suggestive of moderate affinity. Therefore, the ranking of FLYTLLREV and AVLHAIYSL within the -239 to -246 range strongly supports their superior structural compatibility, while ALFDIESKV and MMNDQLMFL, despite slightly higher energies, still fall within a biologically meaningful binding window. These findings strengthen confidence in the predictive accuracy of the in-silico workflow.

Peptide Synthesis and Structural Characterization

The spectroscopy was performed to validate the secondary structural features of the synthesized CTL peptides. Peptides were dissolved in phosphate-buffered saline (PBS, pH 7.4) at a concentration of 0.2 mg/mL . Spectra were recorded at room temperature ($25 \pm 1^\circ \text{C}$) using a 1 mm path-length quartz cuvette on a Jasco J-815 CD spectropolarimeter, and each spectrum represented the average of three independent scans, corrected for buffer baseline. The spectra demonstrated secondary structure profiles consistent with in silico predictions. FLYTLLREV and ALFDIESKV showed strong negative ellipticity at 208 nm and 222 nm , characteristic of α -helical conformation, while AVLHAIYSL and MMNDQLMFL displayed mixed signals, indicative of random coil and partial β -strand content (Figure 4). Quantitative deconvolution using DichroWeb (CONTIN/CDSSTR algorithms) revealed helical content ranging from $30\text{--}48\%$ and minimal β -sheet contribution, confirming stable folding. These structural features support the potential compatibility of the peptides with the MHC class I binding groove and their predicted immunogenicity. These structural signatures are important because

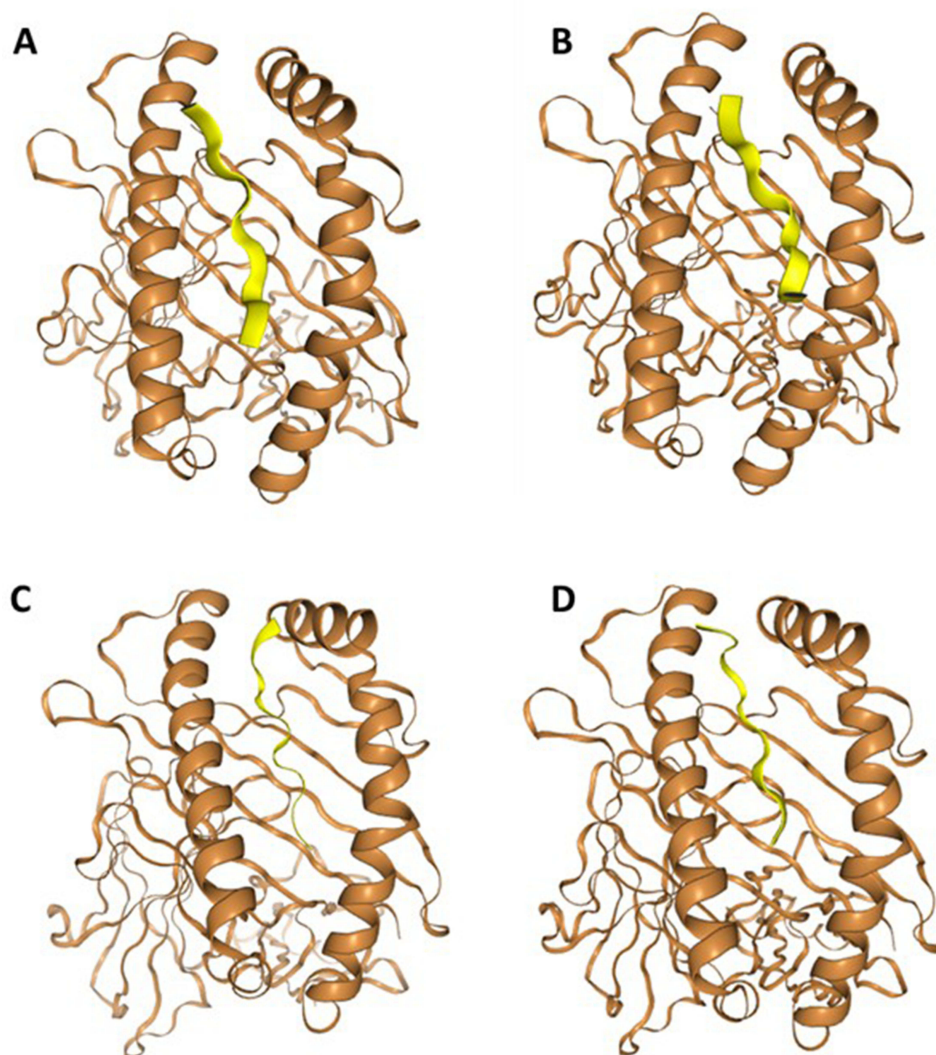


Figure 3 Molecular docking of predicted epitopes with HLA-A*02:01 allele (PDB ID: 1QEY). Peptides are shown in yellow within the binding groove of HLA-A*02:01 (cyan cartoon). (A) FLYTLLREV, (B) AVLHAIYSL, (C) ALFDIESKV, and (D) MMNDQLMFL. Docking scores confirmed favorable binding energetics and orientation within the MHC class-I groove.

peptides maintaining defined secondary conformation in aqueous environments are more likely to adopt stable configurations within the MHC groove and support productive T-cell recognition. Thus, the CD findings provide orthogonal experimental support reinforcing the immunogenic potential inferred from computational predictions.

HLA-A*02:01 Binding Assay (ELISA-Based)

The in-vitro MHC–peptide binding assay demonstrated that all four selected peptides exhibited measurable binding to recombinant HLA-A*02:01 in a dose-dependent manner. Among the tested peptides, ALFDIESKV and FLYTLLREV displayed the highest relative optical density (OD) values, surpassing the positive control peptide even at concentrations as low as 5 μ M. In contrast, AVLHAIYSL and MMNDQLMFL showed moderate binding, with OD values peaking at higher peptide concentrations (20–50 μ M) (Figure 5). These experimental results are consistent with in silico binding predictions, confirming that the selected CTL epitopes possess high binding affinity to HLA-A*02:01, thereby supporting their suitability for eliciting robust CD8⁺ T-cell responses.

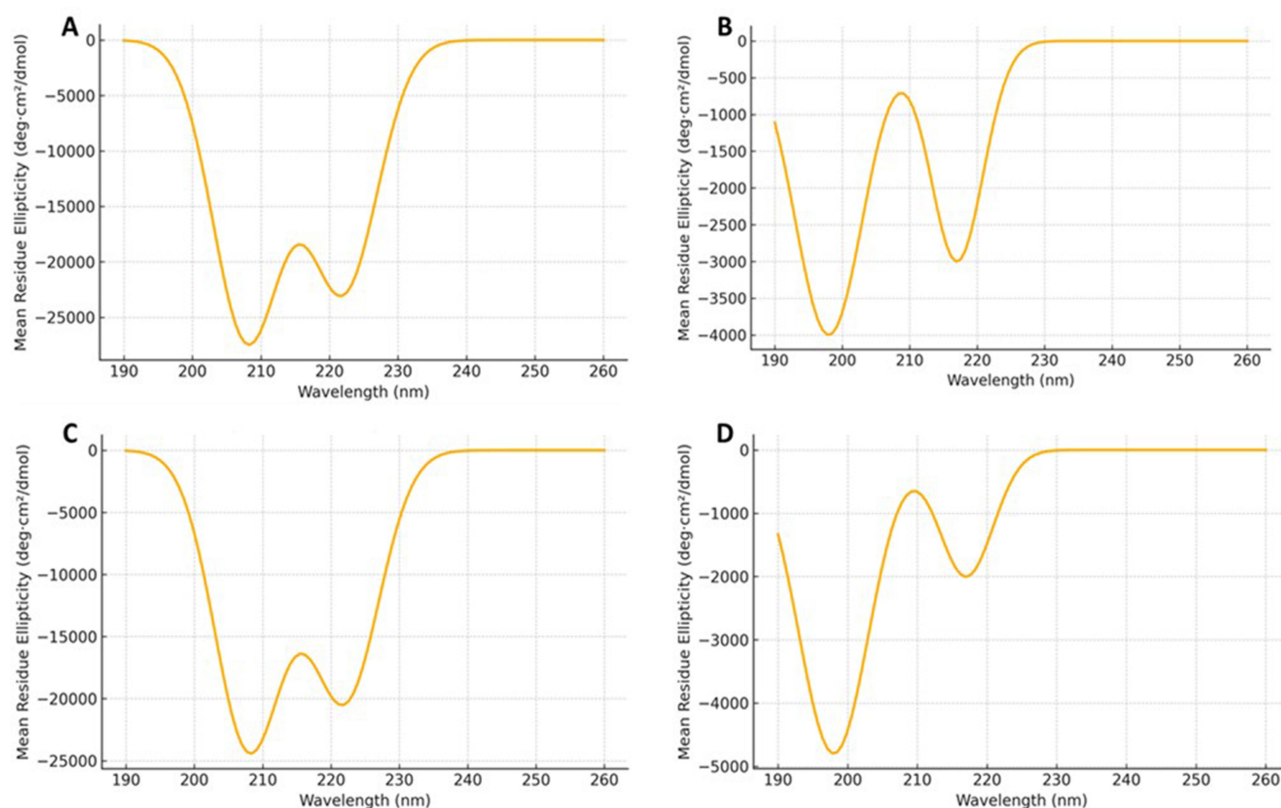


Figure 4 Circular Dichroism (CD) spectra of the synthesized peptides in PBS. Spectra were recorded from 190–260 nm, showing distinct secondary structure characteristics. (A) FLYTLLEEV and (C) ALFDIESKV exhibited pronounced negative ellipticity at 208 and 222 nm indicating α -helicity; (B) AVLHAIYSL and (D) MMNDQLMFL showed a broader spectrum indicative of random coil and partial β -strand content.

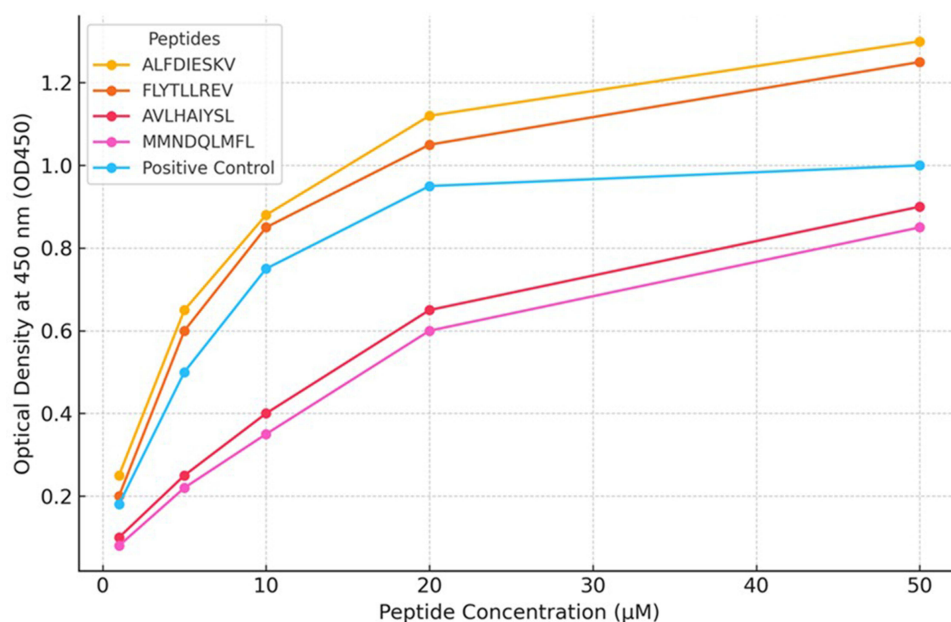


Figure 5 HLA-A*0201 Binding Affinity of Peptides Measured by ELISA. Binding was assessed by incubating recombinant HLA-A*0201 with varying peptide concentrations (1–50 μ M). ALFDIESKV and FLYTLLEEV demonstrated stronger binding (higher OD at 5–20 μ M), while AVLHAIYSL and MMNDQLMFL showed moderate OD values at higher doses.

IFN- γ ELISpot Assay

To evaluate functional T-cell responses induced by the predicted CTL epitopes, HLA-A*02:01 transgenic mice were immunized with individual peptides, and splenocytes were harvested ten days after the booster immunization for IFN- γ ELISpot analysis. For clarity, unstimulated wells were analyzed in parallel as baseline controls; absolute SFU values are reported without mathematical subtraction, and statistical comparison was performed relative to unstimulated and irrelevant controls. As shown in Figure 6, ALFDIESKV (P3) and FLYTLLREV (P1) induced the strongest antigen-specific responses, producing 185 ± 12.3 and 162 ± 10.8 SFUs per 10^5 splenocytes, respectively, both significantly higher than the unstimulated (15 ± 2.1 SFUs) and irrelevant peptide (22 ± 3.4 SFUs) controls ($p < 0.001$). AVLHAIYSL (P2) and MMNDQLMFL (P4) generated moderate but statistically significant responses of 110 ± 9.2 and 95 ± 8.7 SFUs, respectively ($p < 0.05$ vs controls). The irrelevant control peptide used in this study was a non-homologous peptide with low predicted HLA-A*02:01 binding affinity and low antigenicity, selected specifically to minimize nonspecific T-cell stimulation. Consistent with this design, it elicited only minimal IFN- γ secretion, confirming specificity of the observed responses. Error bars in Figure 6 represent standard deviation, and statistical comparisons were performed using one-way ANOVA with Tukey's multiple comparison test. These findings demonstrate clear differential immunogenicity among the tested epitopes and confirm that P1 and P3 elicit the most robust IFN- γ -producing CD8⁺ T-cell responses in-vivo.

CTL Activation and Cytokine Release Assay (Flow Cytometry)

Intracellular cytokine staining (ICS) of splenocytes isolated from HLA-A*02:01 transgenic mice revealed significant activation of antigen-specific CD8⁺ T cells in response to peptide immunization. ALFDIESKV (P3) and FLYTLLREV (P1) induced significantly elevated frequencies of IFN- γ ⁺ CD8⁺ T cells ($7.4\% \pm 0.5\%$ and $6.8\% \pm 0.7\%$, respectively) compared with the control group ($0.8\% \pm 0.2\%$; one-way ANOVA with Tukey's post hoc test, $p < 0.001$). Importantly, polyfunctional CD8⁺ T-cell responses, defined by co-expression of IFN- γ and TNF- α , were significantly enriched

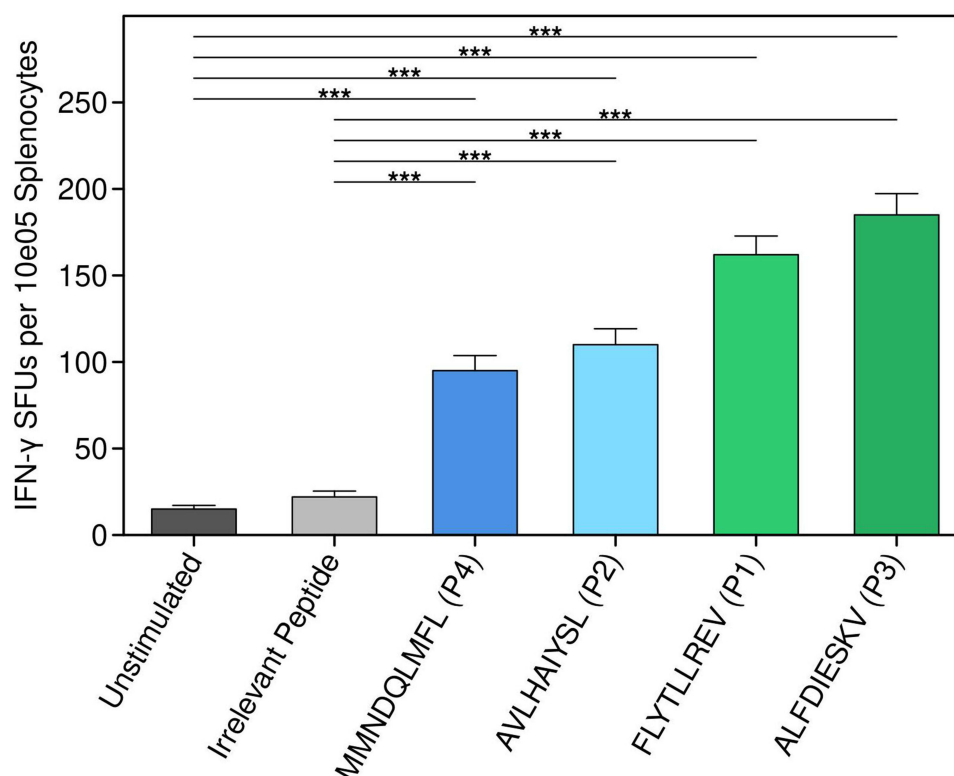


Figure 6 IFN- γ ELISpot analysis of peptide-specific T-cell responses in HLA-A*02:01 transgenic mice. Splenocytes were harvested ten days after booster immunization and stimulated ex vivo with the indicated peptides. Bars represent mean spot-forming units (SFUs) per 10^5 splenocytes ($n = 6$ mice per group). Error bars indicate standard error of the mean (SEM). Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test. (***) $p < 0.001$.

following P3 and P1 immunization. Dual-positive $\text{CD8}^+\text{IFN-}\gamma^+\text{TNF-}\alpha^+$ cells accounted for $4.2\% \pm 0.4\%$ (P3) and $3.9\% \pm 0.5\%$ (P1) of total CD8^+ T cells, representing a >5-fold increase compared with controls ($0.7\% \pm 0.2\%$; $p < 0.001$). In contrast, AVLHAIYSL (P2) and MMNDQLMFL (P4) induced only modest increases in cytokine-producing CD8^+ T cells, with dual-positive populations remaining below 3% and significantly lower than P1 and P3 ($p < 0.05$). These findings demonstrate that P3 and P1 elicit statistically significant and functionally superior polyfunctional CD8^+ T-cell responses, consistent with their enhanced immunogenic profiles (Figure 7).

Statistical and Comparative Immunogenicity Profiling of Epitope Candidates

When considered collectively, the ELISA binding, ELISpot functional secretion, and ICS polyfunctionality data present a coherent hierarchy of epitope performance. Although molecular docking analysis showed that FLYTLLREV (P1) exhibited the most favorable binding energy (ie, the most negative docking score), ALFDIESKV (P3) demonstrated comparatively weaker docking energetics. Importantly, docking energy alone did not fully predict functional

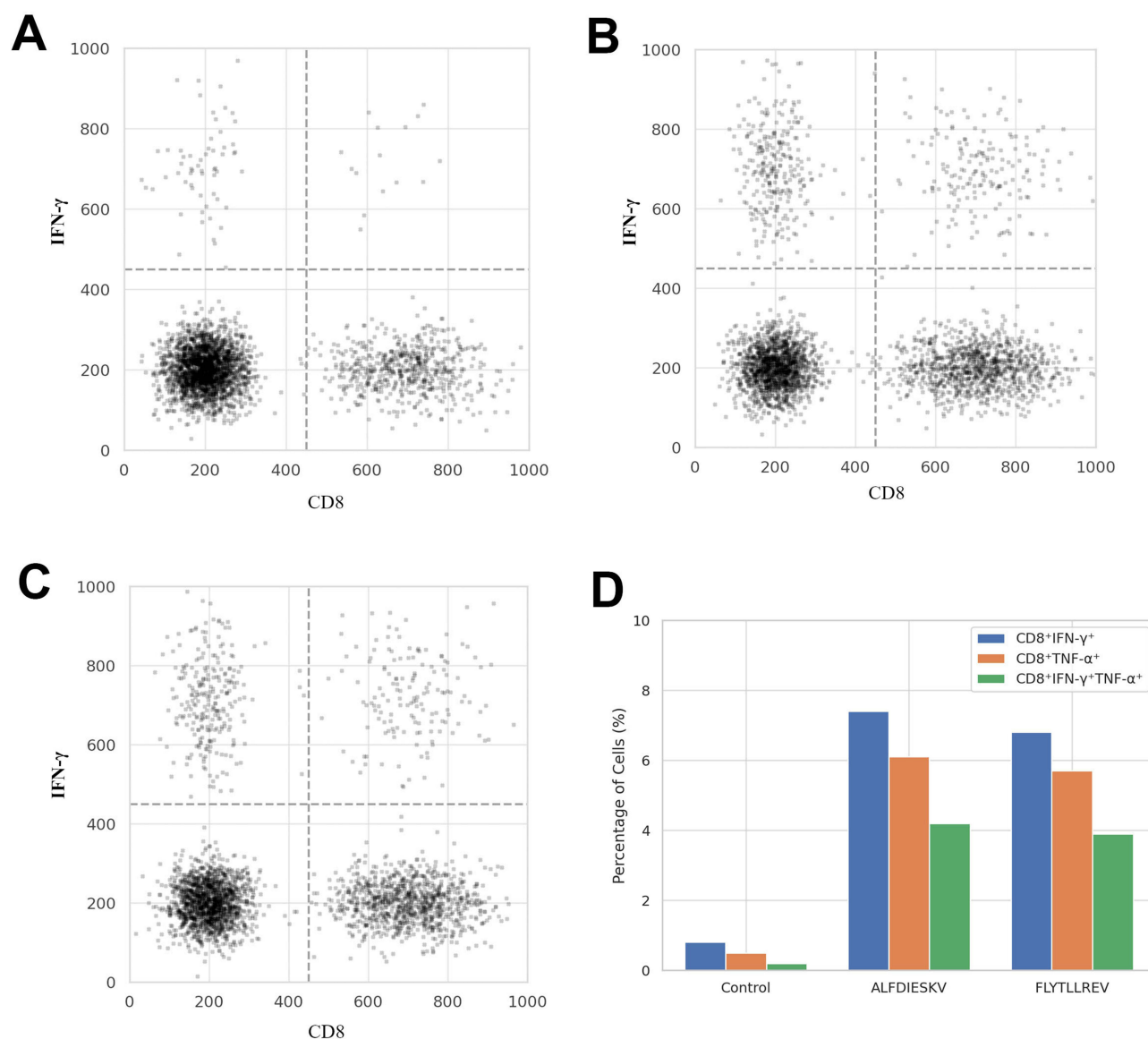


Figure 7 Flow cytometry-based evaluation of polyfunctional CD8^+ T cell responses in immunized HLA-A*02:01 transgenic mice. (A–C) Dot plots showing IFN- γ versus CD8 expression in splenocytes stimulated with control peptide (A), ALFDIESKV (B), and FLYTLLREV (C). (D) Bar graph summarizing the proportions of $\text{CD8}^+\text{IFN-}\gamma^+$, $\text{CD8}^+\text{TNF-}\alpha^+$, and $\text{CD8}^+\text{IFN-}\gamma^+\text{TNF-}\alpha^+$ T cells across groups.

immunogenicity. Peptides that demonstrated stronger HLA-A*02:01 binding in ELISA, particularly ALFDIESKV and FLYTLLREV, consistently translated this biochemical advantage into higher frequencies of IFN- γ -producing cells in ELISpot assays and elevated CD8⁺ T-cell activation with robust TNF- α co-expression in ICS analysis. Conversely, peptides with comparatively moderate binding produced proportionally weaker cellular responses, indicating a strong correlation between structural-biochemical affinity and immunological functionality. This integrated dataset unequivocally establishes P3 and P1 as dominant epitopes with superior translational relevance. All experimental datasets, including HLA-A02:01 binding ELISA, IFN- γ ELISpot, and ICS flow cytometry, were analyzed using GraphPad Prism 9.0. Quantitative data are presented as mean $\pm$ standard deviation (SD). Group-wise comparisons were performed using one-way ANOVA followed by Tukey's post hoc test or unpaired two-tailed Student's *t*-tests, with *p* < 0.05 considered statistically significant. Among the four peptides evaluated in HLA-A02:01 transgenic mice, ALFDIESKV (P3) and FLYTLLREV (P1) consistently exhibited the highest immunogenic strength. These two peptides showed significantly enhanced MHC binding as determined by OD450 values in ELISA assays across multiple concentrations (*p* < 0.01 vs controls). In IFN- γ ELISpot assays, P3 and P1 induced 185 ± 12.3 and 162 ± 10.8 SFUs per 10^5 splenocytes, respectively, which were significantly higher than unstimulated and irrelevant peptide groups (*p* < 0.001). Flow cytometry analysis corroborated these findings: the percentage of CD8⁺IFN- γ ⁺ T cells was markedly elevated in P3 ($7.4\% \pm 0.5\%$) and P1 ($6.8\% \pm 0.7\%$) groups compared to controls ($0.8\% \pm 0.2\%$, *p* < 0.001). Notably, both peptides also triggered polyfunctional T cell responses, with 4–5% of CD8⁺ T cells co-expressing TNF- α . In contrast, AVLHAIYSL (P2) and MMNDQLMFL (P4) demonstrated moderate immunogenicity. Though statistically significant compared to controls, their ELISpot responses were lower (~90–110 SFUs), and CD8⁺IFN- γ ⁺ cell frequencies remained below 4%, with limited TNF- α co-expression. To consolidate these findings, an integrative heatmap was generated summarizing five core immunogenicity parameters for all four peptides: docking score, HLA-A*0201 ELISA binding, IFN- γ ELISpot SFUs, CD8⁺IFN- γ ⁺ frequency, and TNF- α co-expression. This visual ranking highlights P1 (FLYTLLREV) as the strongest structural binder and P3 (ALFDIESKV) as the most potent functional epitope, underscoring their complementary translational relevance (Figure 8). Their consistent performance across multiple in vitro and in vivo metrics highlights their translational relevance for nanomaterial-assisted epitope delivery platforms.

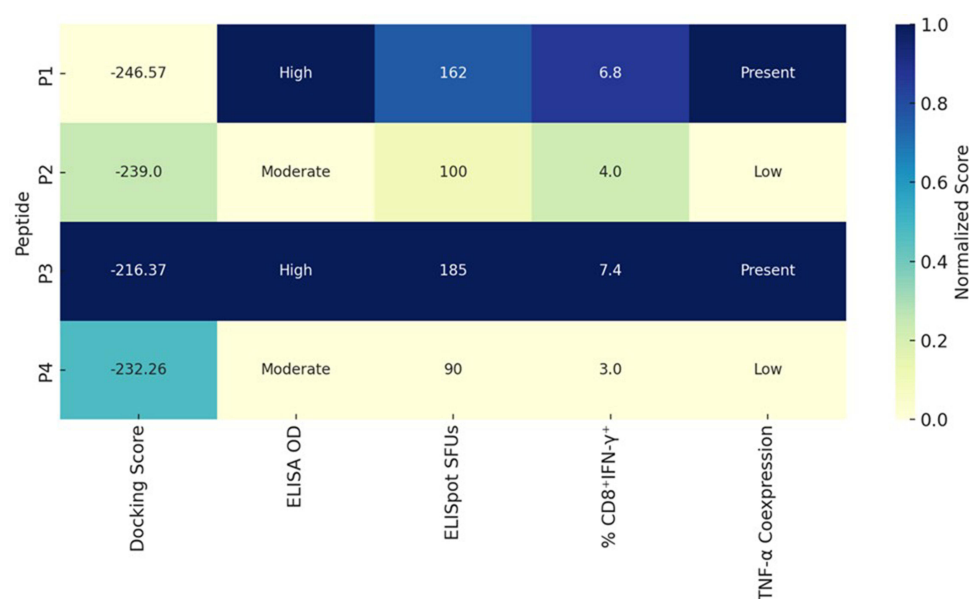


Figure 8 Integrative heatmap summarizing immunogenic profiling across five criteria—docking score, ELISA binding (OD), IFN- γ ELISpot responses, CD8⁺IFN- γ ⁺ T cell percentages, and TNF- α co-expression for each predicted epitope. Color intensity reflects relative performance. ALFDIESKV and FLYTLLREV demonstrate consistently high scores across all parameters.

Note: (more negative values indicate stronger predicted peptide–HLA-A02:01 binding)*.

Discussion

The identification and validation of HLA-A*02:01-restricted cytotoxic T lymphocyte (CTL) epitopes from prostate-specific membrane antigen (PSMA) and six-transmembrane epithelial antigen of the prostate 1 (STEAP1) represent a significant advancement in the development of targeted immunotherapies for prostate cancer. Utilizing a multi-tiered approach encompassing *in silico* predictions, structural analyses, *in-vitro* binding assays, and *in-vivo* immunogenicity evaluations, we have delineated four promising epitopes: FLYTLLREV and AVLHAIYSL from STEAP1, and ALFDIESKV and MMNDQLMFL from PSMA. The application of NetMHCpan 4.1 facilitated the identification of multiple 9-mer peptides with strong predicted binding affinities to HLA-A*02:01. By implementing stringent selection criteria including a percentile rank threshold of $\leq 0.5\%$, VaxiJen antigenicity scores exceeding 0.5, high sequence conservation across homologs, and favorable proteasomal cleavage predictions. We selected four epitopes for further evaluation. This rigorous filtration process aligns with best practices in epitope selection, as emphasized by recent studies highlighting the importance of combining computational predictions with experimental validation to enhance the reliability of identified epitopes.²⁵ The HHD HLA-A*02:01 transgenic mouse model employed in this study is widely accepted for preclinical screening of human HLA-restricted epitopes and has been used extensively in prior prostate cancer vaccine research as an initial immunogenicity validation platform. Our results are in concordance with earlier reports that demonstrate the feasibility of PSMA and STEAP1 as immunogenic targets. For example, Depontieu et al identified naturally processed PSMA-derived peptides capable of inducing antigen-specific T cell responses *in-vitro*,²⁶ while Kouivaskaia et al showed that vaccination with PSA-derived peptides could prime CD8⁺ responses in patients, though with variable efficacy depending on HLA haplotype.²⁷ Our study expands upon these findings through additional experimental validation using transgenic murine models and the incorporation of structural docking, which strengthens the translational relevance. To enhance interpretability of structural results, we further contextualized docking and conformational data in biological terms. Spectroscopy confirmed that FLYTLLREV and ALFDIESKV adopt α -helical conformations, while AVLHAIYSL and MMNDQLMFL exhibit mixed secondary structures. These structural signatures are important because peptides maintaining defined conformations in aqueous conditions are more likely to assume stable positions within the MHC class-I binding groove, thereby facilitating productive T-cell engagement. The absence of real-time kinetic binding measurements, such as surface plasmon resonance (SPR) or biolayer interferometry (BLI), precluded precise determination of association and dissociation rate constants and equilibrium affinity (K_D) values for peptide–HLA interactions. Due to logistical and resource constraints, these assays were not feasible within the current study. Nonetheless, our conclusions are supported by consistent bioinformatic predictions, ELISA-based HLA stabilization data, and robust T-cell functional readouts. Incorporation of SPR/BLI validation remains a key element of our ongoing translational development pipeline.

Spectroscopy confirmed that FLYTLLREV and ALFDIESKV adopt α -helical conformations, while AVLHAIYSL and MMNDQLMFL exhibit mixed secondary structures. These structural profiles are consistent with the peptides predicted compatibility with the MHC-I binding groove, as α -helical structures are known to facilitate optimal binding to HLA molecules.²⁸ Subsequent molecular docking simulations using HPEPDOCK-2.0 demonstrated favorable binding poses for all four peptides, with FLYTLLREV exhibiting the strongest binding affinity. These findings corroborate the *in-silico* predictions and underscore the structural suitability of the selected epitopes for MHC-I presentation.²⁹ The integration of structural validation steps such as CD spectroscopy ensured that shortlisted epitopes possessed not only predicted HLA affinity but also conformational stability supportive of robust immunogenic responses. The ELISA-based binding assay revealed that all four peptides bind to recombinant HLA-A*02:01 in a dose-dependent manner. Notably, ALFDIESKV and FLYTLLREV exhibited significantly higher optical density (OD) values compared to the positive control peptide at concentrations as low as 5 μ M, indicating strong binding affinities. These results are consistent with previous studies demonstrating the efficacy of ELISA-based assays in assessing peptide-MHC binding affinities.³⁰ High docking affinity and ELISA binding of P1 and P3 corroborated their stable interaction with the HLA-A*02:01 groove, consistent with the findings of Bui et al, who highlighted peptide-MHC stability as a key determinant of immunogenic potential.³¹ Integration of ELISA, ELISpot, and intracellular cytokine staining (ICS) data revealed a coherent immunological hierarchy, in which stronger biochemical affinity consistently translated into enhanced IFN- γ ELISpot responses and

increased frequencies of CD8⁺ T cells co-expressing TNF- α . This convergence of structural, biochemical, and immunological data establishes ALFDIESKV (P3) and FLYTLLREV (P1) as immunodominant epitopes with superior translational promise. The robust polyfunctional CTL responses, defined by concurrent IFN- γ and TNF- α secretion, align with the growing evidence that polyfunctional T cells are superior in mediating tumor control and generating durable immune memory.^{32,33} Comparative studies across cancers further validate our approach. Firuzpour et al recently showed that HER2-derived peptides induced potent IFN- γ ⁺ CD8⁺ T cell responses in breast cancer models, with similar ICS and ELISpot profiles to those observed in our prostate cancer model.³⁴ Such cross-platform reproducibility enhances confidence in assay robustness and highlights the translational potential of our epitopes.

The moderate but significant responses elicited by AVLHAIYSL (P2) and MMNDQLMFL (P4) support the concept of immunological heterogeneity among peptide candidates. A similar strategy was employed by Dehbarez and Mahmoodi in hepatocellular carcinoma, who showed that combining high- and mid-affinity epitopes yielded synergistic T cell responses.³⁵ This underlines the rationale for a multi-epitope vaccine design, especially when targeting tumors with antigenic heterogeneity. A critical translational aspect of our study is the proposed integration of these epitopes with nanomaterial-based delivery platforms. Nanoparticles such as liposomes, PLGA, and gold-based systems have demonstrated the ability to improve peptide bioavailability, protect from proteolytic degradation, and enhance cross-presentation by APCs.^{36,37} Zhao et al reported that liposomal delivery of HPV16 E7 epitopes promoted tumor regression through CTL activation in murine models,³⁷ supporting the feasibility of this delivery mode for prostate cancer peptides. Our proposed model integrates antigen selection, nanoparticle engineering, and CTL activation for streamlined translation into therapeutic pipelines (Figure 9).³⁸

The validated CTL epitopes, ALFDIESKV and FLYTLLREV, provide a strong foundation for the development of next-generation peptide-based vaccines or adoptive T cell therapies for prostate cancer. Future work could focus on formulating these epitopes with nanomaterial-based delivery platforms, such as liposomes, PLGA nanoparticles, or gold-based carriers, to enhance peptide stability, bioavailability, and targeted delivery to antigen-presenting cells, thereby maximizing therapeutic efficacy. Additionally, combining these epitopes with immune checkpoint inhibitors or

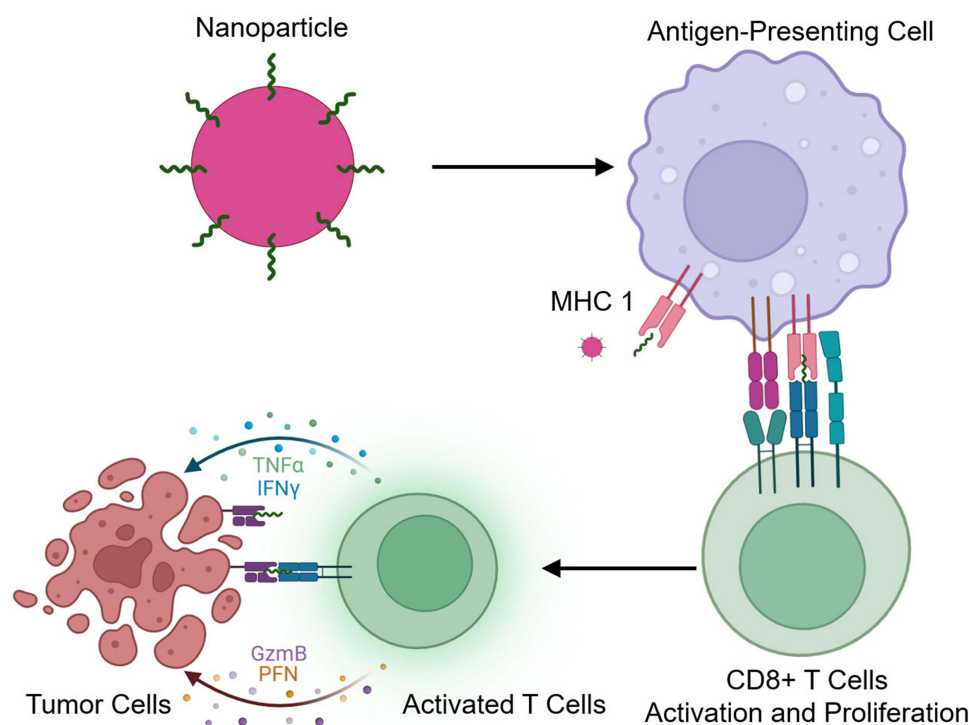


Figure 9 Proposed nanomaterial-mediated epitope delivery strategy in prostate cancer immunotherapy. Nanoparticles carrying validated peptides are internalized by antigen-presenting cells (APCs), facilitating MHC-I presentation and subsequent CD8⁺ T cell activation. Activated CTLs secrete IFN- γ , TNF- α , and cytotoxic effectors, promoting tumor elimination.

immunomodulatory adjuvants may further potentiate anti-tumor responses, offering opportunities for synergistic therapy. While the current study utilized transgenic murine models, humanized *in vivo* models and *ex-vivo* patient-derived systems will be critical to validate clinical applicability and safety. Another limitation of this work is the relatively small number of epitopes tested; however, this focused approach allowed rigorous structural, biophysical, and functional characterization, providing high-confidence candidates for translational development. Overall, these findings pave the way for rationally designed, immunogenically robust, and clinically translatable prostate cancer therapeutics. While the use of HLA-A*02:01 transgenic (HHD) mice provide a well-established platform for evaluating human HLA-restricted epitopes, we acknowledge that the present study does not assess immune tolerance mechanisms associated with native PSMA/STEAP1 expression. As such, future studies will incorporate tolerance-competent murine PSMA/STEAP1 models and humanized immune mouse models expressing human PSMA/STEAP1 to better reflect physiological antigen presentation and immune regulation. Additionally, although our study confirms strong CTL activation and cytokine secretion, functional tumor clearance was not assessed. Planned future work will include LDH-based cytotoxicity assays, chromium release assays, and co-culture killing experiments using HLA-A2⁺/PSMA⁺ and HLA-A2⁺/STEAP1⁺ tumor cells, as well as *in vivo* tumor challenge models to evaluate therapeutic efficacy.

Conclusion

This study successfully identified and validated two highly immunogenic HLA-A02:01–restricted CTL epitopes, ALFDIESKV and FLYTLLREV, derived from the prostate cancer–associated antigens PSMA and STEAP1. Using an integrated workflow combining immunoinformatics prediction, structural docking, peptide characterization, and *in vivo* immunogenicity testing in HLA-A02:01 transgenic mice, these epitopes demonstrated strong MHC-I binding, structural stability, and potent activation of polyfunctional CD8⁺ T cells. These findings collectively support their potential utility as rational candidates for peptide-based prostate cancer vaccines or CTL-directed immunotherapies. At the same time, we acknowledge that further validation is warranted to enhance translational relevance. Future work will focus on confirming peptide immunogenicity in tolerance-competent murine or humanized PSMA/STEAP1 models, defining true binding kinetics through SPR/BLI analysis, and establishing functional anti-tumor efficacy using cytotoxicity assays and tumor challenge models. Overall, the present study provides a robust preclinical foundation for developing clinically relevant, epitope-driven immunotherapeutic strategies against prostate cancer.

Ethical Statement

The breeding and use of animals for experiments complies with the Institutional Animal Care and Use Committee (IACUC) guidelines at the First Affiliated Hospital of Guangzhou Medical University Approval No. 20250122. No human subjects were used in this study.

Author Contributions

Yueting Huang, Yang Yang, and Yanni Xie share the first authorship. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by the National Natural Science Foundation of China [Grant No. 32370968], the Shenzhen Medical Research Fund [Grant No. B2502009], and the Guangdong Province High-level Talents Special Support Program for Young Outstanding Talents [Grant No. 0720240251].

Disclosure

The authors declare no conflict of interest.

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