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Precision Immunotherapy for NSCLC: Multi-epitope Peptide Vaccine Targeting VEGF-A, TGF-β and MAGE-A3 Increases Antitumor Key Cytokine Balance

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Abstract

Introduction: Lung cancer remains the leading cause of cancer-related mortality worldwide, underscoring the urgent need for advancements in treatment options. Although, current standard interventions, including surgery, radiotherapy and chemotherapy are widely employed, a significant number of patients experience relapses, highlighting the critical demand for innovative therapeutic strategies. This study was conducted to develop and evaluate a novel multi-epitope peptide vaccine designed from VEGF-A, TGF-β and MAGE-A3 markers, with the aim of enhancing therapeutic efficacy. Lung cancer remains the leading cause of cancer-related mortality worldwide, underscoring the pressing need for more effective therapeutic interventions. Although current standard treatments—including surgery, radiotherapy, and chemotherapy—are widely utilized, a substantial proportion of patients experience disease recurrence, highlighting the necessity for innovative therapeutic strategies. In this study, we developed and evaluated a novel multi-epitope peptide vaccine constructed from VEGF-A, TGF-β, and MAGE-A3 markers, with the objective of enhancing therapeutic efficacy.

Materials and Methods: Optimal epitopes from VEGF-A, TGF-β, and MAGE-A3 were systematic identified and selected, and subsequently conjugated using a KKK linker to form the final multi-epit vaccine construct. Two groups of BALB/c mice were immunized with the peptide at concentrations of mg/mL and 100 mg/mL, following an immunization protocol that included three weekly administratio In the fourth week, spleen tissue was collected from the mice to assess the expression levels of *IFN-γ*, *4*, *IL-6*, *TNF-α*, and *IL-10* cytokine genes, thereby enabling a comprehensive evaluation of immunogenic and functional efficacy of the peptide vaccine.

Results: Bioinformatics evaluations have revealed a promising multi-epitope peptide vaccine,SVRGKGKGQKRKRKKSKKKHHMVKISGGPHISYPPKKKRLESQQTNRRKKRALD.

This peptide notably enhances the expression of key cytokine genes, including *TNF-α*, *IL-6*, *IFN-γ*, *IL-4* and *IL-10* among the group that received the vaccine at a dose of 10. Even more pronounced levels of gene expression were observed at the higher dose of 100.

Conclusion: This multi-epitope peptide demonstrates considerable potential to elicit a robust immune response and effectively target cancer cells. We strongly recommend conducting further supplementary tests to evaluate its efficacy and possible side effects.

Keywords: Multi-epitope Peptide Vaccine, NSCLC, Cytokine genes, Immunotherapy

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, with Non-Small Cell Lung Cancer (NSCLC) accounting for the majority of cases and contributing to significant morbidity and mortality [1]. The heterogeneity of the primary disease, late-stage detection, and the presence of an immunosuppressive tumor microenvironment (TME) often render conventional treatments such as surgery, chemotherapy and radiotherapy, largely ineffective for achieving long-term remission. The TME is a complex and dynamic network comprised of cancer cells, immune cells, stromal cells and soluble factors including cytokines, all of which play crucial roles in tumor progression, immune surveillance, and responses to therapies [2, 3].

Conventional cancer therapies frequently fall short in treating cancer due to the ability of malignant cells to mutate and evade treatment. To overcome these challenges, we must explore innovative therapeutic strategies that harness the immune system's power to precisely target tumor cells. Peptide-based cancer vaccines represent a promising approach, engineered to stimulate robust, tumor-specific immune responses while minimizing side effects. Their success depends on generating robust cytotoxic T lymphocyte (CTL) responses and altering the tumor microenvironment (TME). By controlling immune activation and influencing T cell differentiation and immune cell recruitment, cytokines are essential to this process. Real-time insights into immune dynamics and treatment effectiveness can be obtained by proofing the expression of cytokine genes. In addition to serving as important biomarkers, these cytokines are crucial for assessing treatment success and achieving long-term tumor control. The effectiveness of vaccine-based immunotherapy is shaped by both the genetic characteristics of the tumor and the immune environment, influencing antigen presentation and T-cell activation. Central to this circuitry are cytokine networks and their downstream signaling modules: most prominently JAK-STAT and NF- κB pathways, with integrates signal from tumor and stromal compartments to polarize immunity toward either tumor elimination or immune tolerance. Thus, cytokine transcript profiling and analysis of the genetic status of key signaling nodes offer dynamic, mechanistic biomarkers that can help predict immunotherapy response.

Pivotal cytokines including interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-4 (IL-4), interleukin-6 (IL-6), and interleukin-10 (IL-10) influence tumor progression and therapy resistance in NSCLC. Their profiles within NSCLC will be examined in the upcoming sections [4, 5]. One of the main pro-inflammatory cytokines with a dual role in NSCLC is TNF-α. On the one hand, it triggers vital inflammatory pathways like NF-kB and MAPK, which enhance antigen presentation and draw immune cells to the tumor microenvironment, possibly aiding in the removal of the tumor [6]. Prolonged TNF-α production, however, can increase IL-6, which can support tumor survival and weaken antitumor immunity. Alarmingly, increased concentrations of TNF-α is linked to poor outcomes, metastasis, and resistance to both chemotherapy and immunotherapy, underscoring its pivotal role in immune regulation [7]. The pleiotropic cytokine IL-6, which triggers JAK-STAT3 signalling, is frequently up regulated in NSCLC due to oncogenic drivers such as EGFR and KRAS, as well as chronic inflammatory responses. Sustained activation of the IL-6/STAT3 signalling pathway promotes tumor proliferation, enables immune evasion by inhibiting cytotoxic T lymphocyte activity, and encourages metastasis, thus contributing to a therapy-resistant microenvironment. Treatment strategies for NSCLC are complicated by the association between elevated levels of IL-6 and aggressive tumor behavior as well as poor clinical outcomes [8-10]. Interlukin-4 (IL-4) is an essential Th2 cytokine that fosters an immunosuppressive tumor microenvironment in NSCLC, influencing both prognosis and tumor progression. IL-4 not only suppresses the Th1-mediated cytotoxic immune response but also binds to IL-4Ra, activating key signaling pathways such as JAK1/JAK3-STAT6. This activation drives M2 macrophage polarization, inhibits antigen presentation, and diminishes CTL function. In the context of NSCLC, IL-4 further activates PI3K/AKT and MAPK/ERK pathways, enhancing tumor cell survival, proliferation and resistance to apoptosis. Moreover, indirect interactions with NF-kB and synergistic effects with TGF-β enhance regulatory T cell activity and IL-10 production. Ultimately, this cascade of events shifts the immune balance toward Th2 dominance, contributing to therapeutic resistance [10-12]. Another important immunoregulatory cytokine involved in immune evasion in NSCLC is IL-10. Although IL-10 mainly activates the transcription of anti-inflammatory and immunosuppressive genes via the JAK1/STAT3 axis, there is mounting evidence that is also interferes with other important pathways that are essential to the development of tumors in NSCLC. Variability in cytokine expression and downstream signaling efficacy is influenced by genetic polymorphism in the IL-10 gene and its receptor complex, which also

affects treatments outcomes and NSCLC. IL-10, primarily secreted by regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), inhibits antigen-presenting cell function and effector T cell activation, further compromising vaccine efficacy [13-16]. The promotion of antitumor immunity requires the signature Th1 cytokine interferon-gamma (IFN-γ), which boosts major histocompatibility complex (MHC) expression in NSCLC. This process activates CTLs target and eliminate tumor cells. IFN-γ activates the JAK-STAT1 signaling pathway, improving antigen presentation, inducing apoptosis via FAS signaling, and regulating immune check points such as PD-L1. However, mutations in the IFN-γ pathway like JAK1/2 and STAT1, along with loss of HLA or TAP genes can impair these functions, enabling tumor immune evasion and resistance to immunotherapy. Insufficient levels of IFN-γ restrict the immune system's ability to combat tumors, contributing to resistance against therapies in NSCLC [17-19].

For the creation of successful immunotherapies, it is essential to develop a comprehensive understanding of the cytokine milieu in NSCLC. The regulation of these cytokine pathways not only influences the tumour's immune state but also its capacity to adapt to therapeutic pressure. By integrating tumour genomic profiling with cytokine transcriptomic analysis, we can improve predictions of vaccine responsiveness, uncover immune escape mechanisms, and refine combination strategies. The recent advancements in immunotherapy have led to the emergence of peptide-based vaccines aimed at enhancing tumour-specific immune responses. Mechanistically, these vaccines are designed to stimulate T-cell activation and modify the cytokine environment within the TME to overcome tumour-induced immune suppression [20]. Peptide vaccines have been shown to elicit an improved, dynamic anti-tumour response through the coordinated induction of Th1 and Th2 cytokine profiles in BALB/c mouse models at the preclinical level, suggesting their potential utility as an effective therapeutic modality for low-grade tumors [21].

This study aims to investigate the expression levels of IL-4, IL-10, TNF-α, IFN-γ and IL-6 at two doses of a peptide-based vaccine in mice model using BALB/c mice. Our objective is to determine whether vaccinations influence the immune response in NSCLC, which may yield valuable insights for optimizing immunotherapy strategies targeting NSCLC.

The overall work flow of this research is schematically illustrated in the Graphical Abstract. Initially, computer-based methods were employed to screen and select the most immunogenic regions from VEGF-A, TGF-β, and MAGE-A3. Bioinformatics tools were utilized to predict their physicochemical characteristics and cytokine-inducing potential. Following selection, the chosen epitopes were linked using lysine (KKK) linkers to generate a single multi-epitope peptide. Subsequently, structural modeling and molecular dynamics simulations were conducted to evaluate the stability, flexibility, and intermolecular interactions of the designed peptide. The synthetic peptide was then incorporated into a nanoliposomal-alum delivery system, with subsequent verification of its biocompatible, encapsulation efficacy, and capacity to stimulate immune responses. Following this, BALB/c mice were immunized with two different vaccine doses (10 mg/ml and 100 mg/ml). Post-vaccination, splenic tissues were harvested, and the mRNA expression levels of key cytokine genes (IFN-γ, IL-6, TNF-α, IL-4, and IL-10) were quantified via real-time PCR to assess immune activation. Statistical and comparative analyses were subsequently performed to evaluate the immune response changed with different doses and confirm the peptide's efficacy. The Graphical Abstract provides a schematic overview of this integrated workflow, illustrating the progression from in-silico design to experimental validation and immune profiling.

2. Materials and Methods

Bioinformatic data analysis

We utilized the VaxinPAD server (https://webs.iiitd.edu.in/raghava/vaxinpad/protein.php), an advanced hybrid SVM-based model with an impressive 95% accuracy to identify immunomodulatory peptides. After compiling the selected peptides into the FASTA format, we submitted them to the Prediction and Protein-based Adjuvant Vaccines panel of the VaxinPAD program, using the default settings for comprehensive analysis. This process provided essential physicochemical properties, including molecular weight, hydrophobicity, hydrophilicity, steric hindrance, charge, solvation and isoelectric point. To ensure the effectiveness of these peptides we assessed hematological toxicity using the HemoPI server (https://webs.iiitd.edu.in/raghava/hemopi/team.php), which predicts red blood cell hemolysis with approximately 95% accuracy. Scores above 0.5 indicate hemolytic potential, with melittin, serving as a positive control at a score of 0.8. Additionally, to enhance the safety and efficacy of our immunomodulatory peptides, we evaluate their potential for blood brain barrier (BBB) penetration using the "predict" mode of the B3Pred program, which demonstrates an impressive accuracy of 85.08% (https://webs.iiitd.edu.in/raghava/b3pred/predict.php)

. We also identified immune adjuvant peptides with antifungal and antituberculosis properties using the AntiFP (https://webs.iiitd.edu.in/raghava/antifp/predict3.php) and AntiTbPred (https://webs.iiitd.edu.in/raghava/antitbpred/predict3.php) servers, with accuracies of 83.33% and 76.56%, respectively. Then, we employed AntiCP 2.0 to selected peptides predicted to function as anticancer peptides (ACPs), with an accuracy of approximately 71% (https://webs.iiitd.edu.in/raghava/anticp/submission.php). The functionality of the selected targets was validated through the NCBI and NTXpred servers (https://webs.iiitd.edu.in/raghava/ntxpred/submission.html).

Examining some properties of peptide targets

The iMODs server serves as a vital resource for conducting molecular dynamics (MD) simulation studies, enabling researchers to analyze the complete sequence of each target individually. By calculating molecular motion and stability with high accuracy and reliability using default parameters, this sophisticated system delivers robust results. The server employs normal mode analysis (NMA) to effectively predict the collective functional motions of biomacromolecules within a two-dimensional internal coordinate framework. Comprehensive analyses of the sequences of each selected target, performed in PDB format, yielded valuable insights. Among its features, the server generates detailed diagrams of B-factors, deformation, eigenvalues, covariance matrix, and elastic network, thereby enhancing our understanding of molecular behavior in significant ways. Examining the Immune cell stimulation by in-silico prediction

Identifying cytokine-inducing peptide targets is essential for advancing immunotherapy, and there are several reliable tools available for this purpose, including *IL2*Pred, *IL4*pred, *IL6*Pred, *IL10*Pred, *IL13*Pred, *TNF*epitope and *IFN*epitope. These servers allow researchers to assess whether specific peptide targets can effectively stimulate immune system cells. For example, *IL2*Pred employs a sophisticated random forest-based machine learning approach, achieving a commendable accuracy of 73.25% in predicting peptide inducers for Interleukin 2 (http: //webs.iiitd.edu.in/raghava/il2pred). In a similar vein, *IL4*pred utilizes an SVM-based classifier that successfully identifies interlukin-4 inducing peptides with an accuracy of 75.76%, utilizing the default settings in its virtual screening mode (http:// webs.iiitd.edu.in/raghava/il4pred/design.php).
Predicting the ability of designed peptides to induce interleukin-6 using *IL6*Pred, applies a random forest-based method that attains an accuracy of 75.79% (http: //webs.iiitd.edu.in/ raghava/ il6pred/predict3.php). Furthermore, IL10Pred is an advanced SVM model, delivering an impressive accuracy of 78.42 in predicting the potential induction of interlukin-10 cytokine. (http: //webs.iiitd.edu.in/raghava/il10pred/predict3.php).
Then, the *TNF*-αepitope web server effectively forecasts the impact of designed epitopes on *TNF-α* induction (https:// webs.iiitd.edu.in/raghava/tnfepitope/). The *IFN*epitope server is highly effective for identifying interferon-gamma-inducing peptides, achieving a notable accuracy of 82.10% (http://webs.iiitd.edu.in/raghava/ifnepitop/predict.php). In addition, we evaluated the potential induction of *IL-13* by utilizing targets designed through the *IL13*Pred program, which employs the extreme gradient boosting probability (XGB) method and reaches an accuracy of around 71%. Moreover, the capacity of the target peptide to stimulate B cells was investigated using the LBtope server. Finally. we utilized peptide targets from the CTLPred web server, setting a cut-off of 0.5, and anything above this value is regarded as a potential stimulus for T-lymphocyte cells (http: //webs.iiitd.edu.in/raghava/ctlpred/).

Experimental analysis

Vaccine design and immunization

Finally, we selected three peptide sequences for MAGE-A3/VEGF-A /TGF-β2, connected by KKK linkers. The designed peptide sequence SVRGKGKGQKRKRKKSKKKHHMVKISGGPHIS YPPKKKRLESQQTNRRKKRALD was synthesized using solid-phase methods. For its delivery, the peptide was integrated into cationic nanoliposomes formulated with DOSPA and DOPE in a 3:1 molar ratio. Following centrifugation, excess unencapsulated peptide was removed through PBS washes. The loading efficacy was subsequently determined spectrophotometrically by measuring absorbance at 280 nm using a NanoDrop system. Then, BALB/c mice were subjected to subcutaneous injections of peptide-loaded nanoliposomes at two distinct concentrations. All animal procedures were conducted in accordance with institutional ethical standards and approved by the animal Ethics Committee (IR.IAU.TNB.REC.1403.142).Group A received a dosage of 10 mg/ml of the peptide vaccine, while group B was administered a higher dosage of 100 mg/ml. The vaccine doses of 10 mg/ml and 100 mg/ml represent the final concentrations of the complete nanoliposomal-alum-peptide complex, not the peptide alone. These concentrations were determined

experimentally to ensure an optimal balance between immunogenicity, physicochemical stability, and injection safety. The two doses were chosen to provide a 10-fold differences, allowing clear assessment of dose-response relationship. Higher concentrations were avoided due to liposomal aggregation, poor bioavailability, increased viscosity and reduced syringeability, while lower doses induced minimal immune activation and they did not produce antibody titers in pilot tests. These doses represent biologically relevant, physically stable, and ethically justified concentrations for immunization in the BALB/c model. The immunizations were carried out weekly over a period of three weeks. One week following the final injection, spleen tissues were collected and transferred to the laboratory, where samples were either placed in RNA Later solution and frozen at -80˚C until RNA isolation. Subsequently, spleen cells were harvested for further immunological evaluation. Following this, we evaluated the gene expression of *IFN-γ*, *TNF-α*, *IL-4*, *IL-6* and *IL-10* to determine the vaccine's effectiveness in eliciting an immune response.

Realtime PCR

In order to extract the RNA, the procedure was executed according to the protocol. First, 750µl of Trizol solution was added. After pipeting the cells, followed by 5 minutes incubation, 200µl of chloroform was added to the mixture. The tubes were shaken completely for 15 seconds and incubated for 3 minute at room temperature. Then, the samples were spun for 12 minutes (Eppendorf, Germany). In the next step, 400µl of the upper phase were transferred to the new micro tube and 400µl 0f 96% ethanol was added to the mixture. Then the Mixture transferred to the spin column and spun again. Next, the supernatant was removed and 700µl of PW was added and spun and the supernatant was removed. This process was repeated once more time. Afterwards, the spin columns were transferred to the new micro tube. 50µl of DEPC-treated water was added to each well. After 3-minute incubation, the samples were spun to extract RNA from the column. Subsequently, the RNA concentration was determined using a nanodrop device and the OD260/OD280 ratio were quantitatively checked to ensure the absence of contamination. To further ensure the quality of the extracted RNA amount, RNA was run on a 1.5% agarose gel. cDNA synthesis was performed with 7 µg RNA using a ParsTous kit (Iran) as well with 1 microliter of DNase I enzyme, 1 microliter of buffer (OligoT) and 1 microliter of RT enzyme, with the following reaction conditions: 25˚C for 5 minutes, 42˚C for 60 minutes and 70˚C for 5 minutes in Thermo cycler. For the next step:1µl of the first-strand cDNA was placed in 24 µl of reaction mixture containing 1U Taq polymerase and its buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs, and 0.4 pmol each of *GAPDH* (As Housekeeping gene) or cytokines specific oligonucleotide primers. The conditions for the PCR reactions were initial denaturation at 95˚C for 5 minutes, followed by 30 cycles of denaturation at 94˚C for 45 seconds, annealing at 60˚C for 45 seconds, extension at 72˚C for 1 minutes, and a final extension at 72˚C for 10 minutes. The PCR products were visualized by Gel electrophoresis to assess the correct product length.

Primers for *IFN-γ*, *TNF-α*, *IL-4*, *IL-6* and *IL-10* genes and *GAPDH* were constructed using Perl primer and primer3 software, as indicated in [Table 1]. Their specificity for cDNA was verified in the BLAST database. Additionally, the thermodynamic results of designed primers were checked using Gene Runner Software to confirm the specificity of achieved primers. Primer sequences, size of achieved reaction products and T_m (melting temperature) were carefully checked and assessed.

Table 1. Primer Sequence of Target Genes

Gene	Forward (5´ to 3´) Reverse (3´ to 5´)	Tm	Length	%CG	Self complementarity	Self 3' complementarity
INF-γ	F: CAGCAACAGCAAGGCGAAAAAGG R: TTTCCGCTTCCTGAGGCTGGAT	63.70 64.0	23 22	52.17 54	2 6	0 4
IL-6	F: TACCACTTCACAAGTCGGAGGC R:CTGCAAGTGCATCATCGTTGTTC	62.24 61.21	22 23	54 48	5 6	2 2
TNF-α	F: GGTGCCTATGTCTCAGCCTCTT R: GCCATAGAACTGATGAGAGGGAG	61.88 59.99	22 23	54 52.17	3 4	0 0
IL-10	F: CGGGAAGACAATAACTGCACCC R: CGGTTAGCAGTATGTTGTCCAGC	61.51 61.77	22 23	54.55 52.17	4 2	1 2

IL-4.	F: ATCATCGGCATTTTGAACGAGGTC R: ACCTTGGAAGCCCTACAGACGA	61.98 63.08	24 22	45.83 54.55	3 4	2 0
GAPDH	F: GGGAGCCAAAAGGGTCATCA R: AGTGATGGCATGGACTGTGG	59.96 60.03	20 20	55 55	3 5	2 0

The expression of *GAPDH* and cytokine genes: *IFN-γ*, *IL-6*, *TNF-α*, *IL-10*, and *IL-4* were assessed with SYBR Green qPCR Master Mix (Applied Biosystems by ThermoFisher Scientific, USA) and StepOne Plus Real-Time PCR system (Applied Biosystems by ThermoFisher Scientific, USA). Reactions were carried out with 5µl of SYBR Green PCR Master Mix, 1 µl of each primer (F/R), 2 µl of cDNA and also 11 µl of H_2O. The relative mRNA expression levels were normalized to GAPDH using the comparative $\Delta\Delta C_T$ method.

After each run, a melting curve analysis was performed to verify the specificity of the PCR reaction. The size of the expected RT-PCR product was confirmed by Gel Red-Stained 2.0% agarose gel electrophoresis. The Procedure was repeated twice independently to ensure the reproducibility of the results. To establish the levels of given transcript in the studied samples, standard curves were generated using C_t (value where amplification curve crosses the threshold line) and the thermal profile was based on the manufacturer′s instructions regarding the specific annealing temperature of the primers.

All data were calibrated against the average ΔΔCt of the control samples. The results were reported as fold change with respect to controls. No template control (NTC) was used in all runs. Relative expression of selected genes was calculated by the comparative cycle threshold method (ΔΔCt). Standard curves were constructed and relative gene expression data were generated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All data were presented as the mean± standard error of the mean using SPSS software (version 22). Differences between the two compared groups were assessed with the Independent Sample T-test, in accordance with the data's normality. The effects of the peptide vaccine on gene expression in mice serum were also evaluated through Odds ratio (OR) and corresponding 95% confidence intervals. A *P*-value of ≤ 0.05 was considered statistically significant, highlighting the robustness of our results.

3. Results

Peptide target design by Bioinformatic Tools

The assessment of selected epitopes for the VEGF-A, TGF-β2, and MAGE-A3 proteins was conducted using sophisticated bioinformatic web tools, that is illustrated in Figure 1.

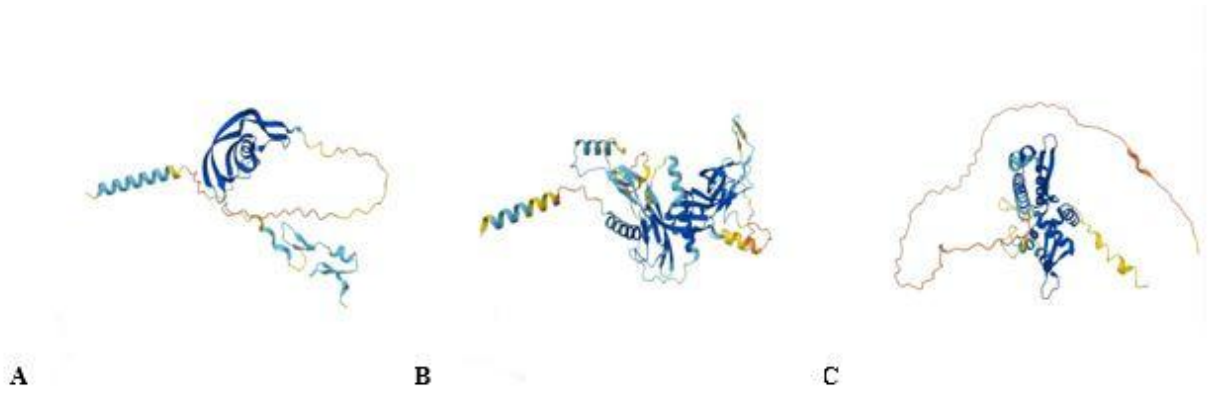


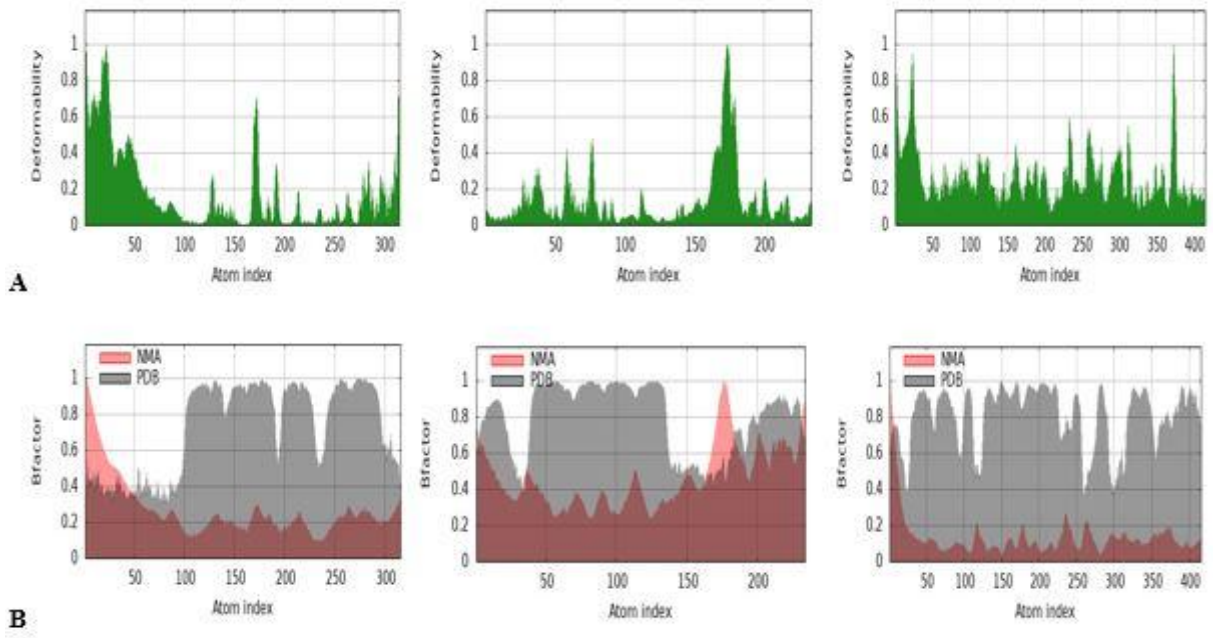
Figure 1. 3D structure of A: VEGF-A, B: TGF-β, C: MAGE-A3

This thorough analysis allowed us to identify sequences with the highest overlap among various isoforms, leading to the selection of sequences that met defined and functionally advantageous criteria. Firstly, these sequences were carefully selected to reside outside of restricted regions, thereby avoiding areas susceptible to acetylation, phosphorylation, and ubiquitination. They are strategically located within favourable regions of the proteins, encompassing essential functional domains,

active sites, and binding sites, thereby ensuring their capacity to effectively interfere with their respective targets. In addition, the selected peptide sequences are positioned for optimal surface accessibility, significantly enhancing their potential efficacy. Each selected sequence for VEGF-A, TGF-β2, and MAGE-A3 exhibits favorable tolerability, stability under various conditions, high immunogenicity, and minimal side effects due to controlled immune system stimulation. Notably, they also display promising antifungal and anti-tubercular properties. Ultimately, we have synthesized these valuable peptides into a single 54-mer multi-epitope peptide target: (SVRGKGKGQKRKRKKSKKKHHMVKISGGPHI SYPPKKKRLESQQTNRRKKRALD) interconnected by three lysine (KKK) residue, establishing a promising avenue for targeted therapeutics.

Molecular Dynamics simulation of Target peptides

Our thorough analysis of each protein target's sequence in PDB format has provided valuable insights into residue correlation, atomic connectivity, and a stability of complex structures observed during our molecular dynamics (MD) simulations utilizing the advanced the iMODs approach. This methodology effectively characterizes macromolecular functional motions by assessing relative motion amplitude frequencies and deformation vectors, opening understanding of molecular plasticity in a cellular context. It underscores the flexibility of the molecular structure at each residue, which is vital for its functional dynamics. Our findings reveal the B-factor derived from the PDB file, calculated using normal mode analysis (NMA), effectively linking the mobility of the NMA-bound complex to PDB scores and reflecting the root mean square deviation (RMSD). Although some intermediate NMR models may not include a B-factor. Additionally, the analysis highlights the distinct stiffness of movements within protein, with lower values indicating a reduced energy requirement for shape modification. This insight is essential for understanding material behavior, as deformability signifies notable conformational peaks in proteins, typically representing flexible regions with higher values. We also present the variance of normal modes, emphasizing their inverse relationship with eigenvalue. Each mode corresponds to a unique eigenvalue that represents molecular stiffness; lower eigenvalues suggest less energy is required to deform a stable structure. The use of color-coded bars effectively clarifies the distinction between individual (red) and cumulative (green) variances. Lastly, our examination of the coupling covariance matrix among various pairs illuminates the nature of their motions, whether correlated (red), uncorrelated (white), or anti correlated (blue). Our depiction of the elastic lattice model illustrates the connections between pair of atoms via springs, with each point representing a linkage. The color gradient indicating stiffness, darker gray for stiffer springs adds an intuitive and visually engaging element to the interpretation of these molecular interactions as seen in Figure 2.



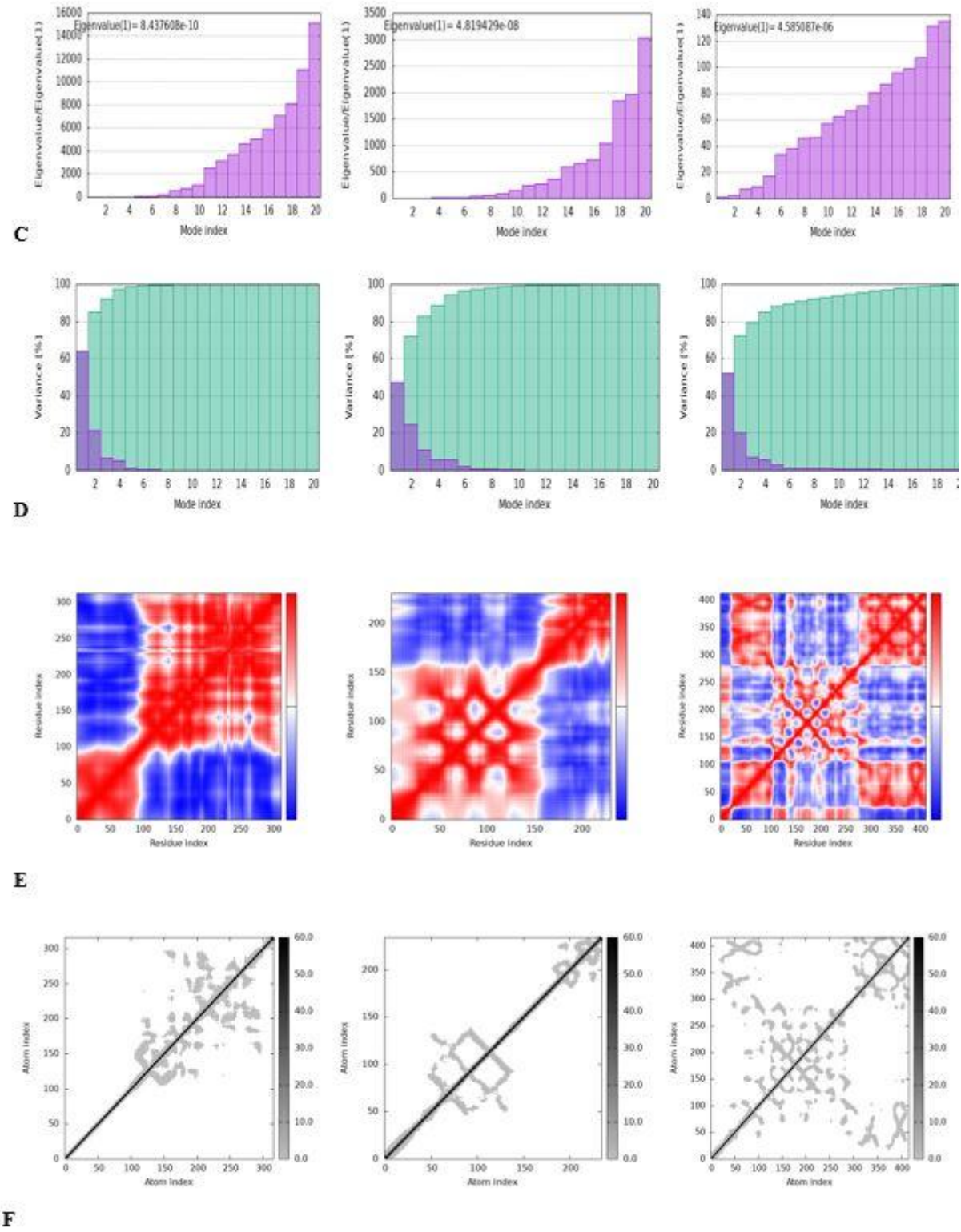


Figure 2. Molecular dynamics simulation of the MAGE-A3 (left), VEGF-A (middle), TGF-β (right)

A) Deformability, B) B-factor, C) Eigenvalues, D) Variance, E) Covariance matrix, F) Elastic network map

Immune cell stimulation by in-silico prediction

Base on Table 2, Th1 cytokines, including IFN-γ, TNF-α and IL-2, function in tandem with Th2 cytokines such as IL-4, IL-10 and IL-13 to orchestrate the immune response. Importantly, IL-6 is a key regulator of the Th1/Th2 balance as it inhibits Th1 differentiation while promoting Th2 induction [22]. As shown in Table 2t2, our result demonstrates that the designed peptide acts as a mild inducer of IL-2, IFN-γ, IL-4, IL-6 and IL-13, contributing to a well-balanced Th1/Th2 response. The data presented in the accompanying table highlights that each peptide developed in this study selectively stimulates a certain type of cytokines. By strategically linking three peptides, we can create a synergistic effect that enhances the overall stimulation of a broader range of cytokines. If one peptide does not effectively activate the target immune cell, the complementary peptide can compensate for this deficiency, promoting a balanced Th1/Th2 ratio. In addition, our advanced bioinformatics analyses indicate that this multi–epitope peptide effective at engaging immune cells and stimulating both B and T lymphocytes. As a cell-penetrating peptide (CPP), it represents a promising candidate for targeting intracellular tumor cells. This peptide's exceptional ability to penetrate cellular membranes establishes it as a reliable carrier, capable of transporting larger macromolecules, such as proteins, across barriers. Notably, our in-silico findings suggest that this peptide may also traverse the blood-brain barrier (BBB), enabling it to enter cells and access intercellular spaces, thereby facilitating stimulation of immune cells. Importantly, this peptide can penetrate cells, reach the nucleus, and inhibit DNA synthesis, thereby disrupting the process of cell division.

Table2. Immune cell induction and penetration of the target peptides

Target	Sequence	*IL2*	*IL4*	*IL6*	*IL10*	*IL13*	*IFN-γ*	B-cell	T-cell	*TNF-a*	CCP	BBBP Peptide
VEGF-A	SVRGKGK GQKRKRK KS	×	✓	✓	×	×	×	✓	✓	×	✓	✓
MAGE-A3	HHMVKIS GGPHISYP P	×	✓	×	×	×	✓	×	✓	×	✓	✓
TGF-β	RLESQQTN RRKKRAL D	✓	×	✓	×	✓	×	✓	✓	×	✓	×

RNA quality and expression level of reference gene

For qPCR reactions, only samples of the appropriate quality and quantity were used. At 260 and 280 nm (A260/280) and 260 and 230 nm (A260/230), the absorbance values for every RNA sample were within the ideal ranges of 2.0–2.2 and 1.8–2.2, respectively. Gel electrophoresis confirmed the integrity of the RNA, revealing distinct bands related to rRNA (28s and 18s) along with 5s band which confirmed the quality of RNA that were shown in Figure 3. Importantly, no genomic DNA contamination was observed in any of the runs, and the negative control samples further validated this finding. The expression levels of the reference gene (*GAPDH*) were almost similar in all cases which are critical criteria for reference gene.

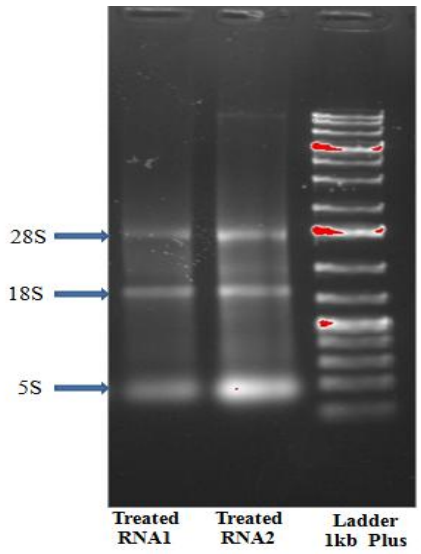


Figure 3. Agarose gel electrophoresis of RNA. The 5S, 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA samples with 1kb Ladder.

Effect of peptide vaccine on IFN-γ, IL-6, TNF-α, IL-10, and IL-4 genes profiling

As shown in Figure 4, The Real-Time PCR analysis of lung cancer cell lines illustrated the effectiveness of the peptide vaccine. To evaluate its immunomodulatory potential, we measured the mRNA expression levels of key cytokines (*IFN-γ*, *IL-6*, *TNF-α*, *IL-10*, and *IL-4*) in splenocytes from immunized BALB/c mice. We conducted this assessment using qRT-PCR after treating the mice with two concentrations of the vaccine (10 mg/ml and 100 mg/ml).

IFN-γ (Interferon gamma): *IFN-γ* expression rose dramatically with increasing concentrations, showing a notable elevation at 10 mg/ml and an even larger fold change at 100 mg/ml.

A significant dose-dependent increase in *IFN-γ* was observed, with the 100 mg/ml group showing the largest fold change (~28), while the 10 mg/ml dose exhibited a moderate increase (~12). Strong Th1-type immune activation is suggested by this pattern, particularly at elevated antigen doses, which are essential for triggering CTL responses against tumor cells. The vaccine effectively stimulated cell-mediated immunity at both doses, especially at the higher concentration, with a statistically significant difference between 10 mg/ml and 100 mg/ml ($P < 0.05$).

IL-6 (Interleukin 6): At the 10 mg/ml dose, *IL-6* exhibited a moderate increase, with a fold change of 2.8. In contrast, at the 100 mg/ml dose, *IL-6* expression was significantly elevated, demonstrating a fold change of 7.2. This dose-dependent increase suggests that higher doses of the peptide vaccine stimulate a more pronounced inflammatory response through *IL-6*. The difference between these two doses was statistically significant ($P < 0.05$). However, the absence of a further increase in comparison to other cytokines at the 100 mg/ml dose indicates that *IL-6* induction may reach a plateau or be tightly regulated to prevent excessive inflammation.

TNF-α (Tumor Necrosis Factor-α): At the dosage of 10 mg/ml, *TNF-α* levels increased by a factor of 16, while at 100 mg/ml, they rose to approximately 24.5, indicating a remarkable inflammatory response. The *TNF-α* gene expression was notably higher in the 10 mg/ml group ($p < 0.05$), Although the 100 mg/ml group exhibited a slightly higher average *TNF-α* expression, this difference was not statistically significant, possibly due to limited number of replicates.

IL-4 (Interleukin-4): *IL-4* expression increased with both doses of the vaccine, exhibiting a fold change of 17 at 10 mg/ml and 22 at 100 mg/ml, which indicates a dose-dependent enhancement of Th2-type immune responses. The difference in expression at the 10 mg/ml dose was statistically significant ($P< 0.05$), whereas the 100 mg/ml group revealed high variability, as indicated by the wide error bars. At the 10 mg/ml dose, *IL-4* exhibited the highest expression among all measured cytokines.

IL-10 (Interleukin-10): *IL-10* expression was elevated at both doses, with a fold change of 7 at 10 mg/ml and 12 at 100 mg/ml. Analysis of *IL-10* gene expression

revealed a statistically significant increase in the group treated with 10 mg/ml of the peptide vaccine ($P<$ 0.05), indicating a consistent up regulation of this immunoregulatory cytokine at lower antigenic dose. In contrast, although the 100 mg/ml group showed a higher average fold change, the effect was not significant, likely due to greater inter-individual variability. In addition, *IL-10*, a regulatory cytokine with immunosuppressive activity, was elevated modestly, but significantly less than the pro-inflammatory cytokines. The fold change for *IL-10* is somewhat similar to that observed for *IL-4* and *IL-6*.

The group-by group comparison of mice receiving 10 doses of the vaccine reveals that the expression of cytokine genes follows this sequence: *IL-6*, then *10*, followed by *IFN-γ*, and finally TNF-α, with the highest absolute expression observed in *IL-4*. In contrast, mice that received the vaccine at a dose of 100 mg exhibit a different pattern of cytokine gene expression, ranked as IL-6 first, followed by *IL-10*, then *IL-4*, and lastly *TNF-α*. In this group, *IFN-γ* demonstrates the highest absolute expression.

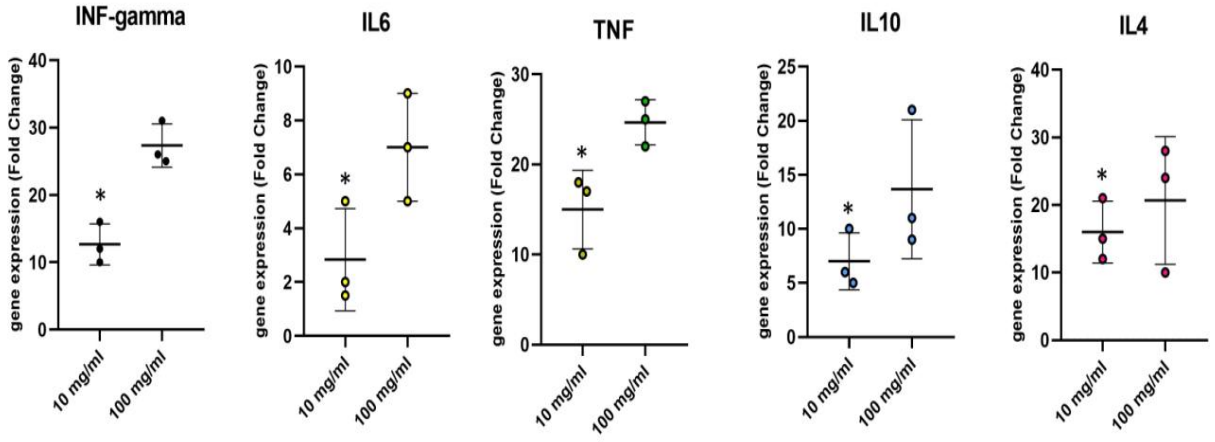


Figure 4. Results of quantitative Real-Time PCR analyses of *IFN-γ* , *IL-6*, *TNF-a*, *IL-10* and *IL-4* in mice spleen tissue cells after exposure to the serum of mice treated with peptide targets. All Cytokine genes were up-regulated in both doses. The comparative analysis indicated that the 100 mg/ml group exhibited higher expression levels than the 10 mg/ml group. While no significant differences were detected in 100 mg/ml dose group, all cytokines at the 10 mg/ml dose showed significant increases. Gene expression levels were compared using a Two-way ANOVA, with results presented as mean ± SEM, and significant differences denoted by an asterisk (*). Each group consisted of three biological replicates, and all data were analyzed using SPSS software, with statistical significance defined as $P<0.05$.

4. Discussion

Within the rising incidence of lung cancer, targeting cytokines in cancer cells presents a promising therapeutic approach. This strategy not only has the potential to improve cancer treatment outcomes but also underscores the importance of investigating cytokine-related genes within the field of cancer research. This work serves as a complementary extension to our previous study that explored the role of key cytokine genes in vaccinated mice. The 54-mer multi-epitope peptide vaccine was designed and synthesized using advanced bioinformatics software and incorporates epitopes from VEGF-A, TGF-β, and MAGE-A3, formulated with alum and encapsulated within a nanoliposomal platform, to elicit an immune response against tumor cells in the spleen tissue of a BALB/c mouse model.We tested two concentrations (10 and 100 mg/ml) of the target peptide to analyze the effects on the expression of important cytokine genes: *IFN-γ*, *IL-4*, *IL-6*, *TNF-α* and *IL-10*. Our analysis of real-time PCR indicated a dose-dependent increase in the expression of all five cytokines at the high dose. To stimulate a stronger immune response, the dose of 100 mg/ml was greater than the minimal immune threshold, which led to higher cytokine expression and stronger immunogenicity. This would indicate that higher antigen loads potentiate immune activation, probably via an enhanced antigen uptake and processing by dendritic or other antigen presenting cells. Tumor-specific antigens were efficiently cross-presented via MHC class I and class II, and induced

the activation of $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T helper (Th) cells. These activated Th1 cells and CTLs secreted increased amounts of *IFN-γ*, *TNF-α* and *IL-6* which are essential for antitumor immunity. Notably, *IFN-γ* promotes antigen presentation in addition to immune cell attraction, while *TNF-α* induces the apoptosis of tumor cells and *IL-6* supports immune cell proliferation. This combination leads to a favourable microenvironment for efficient tumor control.

Comparative analyses reveal both similarities and differences with previous studies, reflecting variations in experimental models, vaccine platforms, and tumor microenvironments. For example, Chen et al. (2018) and Kumar et al. (2019) showed a robust induction of *IFN-γ* and *TNF-α* in murine lung and melanoma models, correlating strongly with enhanced cytotoxic T lymphocyte (CTL) activity and tumor regression, which aligns with our observed potent Th1 response. However, their studies reported minimal *IL-4* and *IL-10* changes, suggesting a more polarized Th1 response compared to the mixed cytokine profile we observed. In contrast, Peng et al. (2020) observed that the administration of a chitosan-encapsulated peptide-DNA vaccine in murine models challenged with bacterial pathogens led to elevated levels of *IL-4*, *IL-6* and *IFN-γ* alongside *TNF-α* and other cytokines. This finding highlights the dual role of these cytokines in modulating inflammation and preventing excessive immune activation. This parallels our findings and suggests that peptide vaccines can induce feedback regulatory mechanisms to fine-tune immune responses and reduce potential immunopathology [23]. Interestingly, as highlighted by Mahaki et al. (2021), peptide- based vaccines can effectively elevated *IFN-γ*, *IL-2*, *IL-12* and *TNF-α* while concurrently inhibiting *IL-4* or *IL-6*. This finding emphasizes the potential of peptide vaccine to enhance CTL responses without activating regulatory cytokine activity, thereby preventing unwanted immunosuppression and promoting a strong antitumor immune response [24]. Such variability is further exemplified in studies by Rašková et al. (2022), where *IL-6*'s role oscillates between promoting inflammation and supporting tumor progression, underscoring the cytokine's pleiotropic nature [25]. Furthermore, clinical investigations summarized by Crotty (2014) and Abbas et al. (2013) reinforce that successful peptide vaccines typically promote Th1-skewed immunity characterized by *IFN-γ* and *TNF-α* production, correlating with improved patient outcomes. However, raised *IL-10* might dampen immunity in some contexts (i.e., tolerogenic tumours), whilst still allowing for inflammation to be better controlled and therefore long-term responses maintained. Hence the *IL-10* rise after vaccination is a relatively invariant regulatory response, but the salient effect of this depends upon the immunological context. [26, 27]. Moreover, the paradoxical role of *IFN-γ* is worth noting. While essential for antitumor activity, excessive or prolonged *IFN-γ* expression as reported in melanoma TriVax vaccine studies (2011) may contribute to T cell exhaustion or immune-related adverse events. Our data's moderate yet significant *IFN-γ* increase suggests an immunological balance potentially optimized by the vaccine dose [28]. In addition, Hunter et al. (2015) and Grivennikov et al. in 2010 emphasized that *IL-6* plays vital role in supporting immune activation during acute responses. Consequently, elevated *IL-6* levels during peptide vaccination should be regarded as indicative of an effective immune response [29, 30]. Consistent with our findings, Iglesia et al. (2020) demonstrated that high-dose peptide vaccination led to early cytokine expression, facilitating efficient priming of cytotoxic T lymphocytes (CTLs) and reduced $CD8^+$ dependency. Vaccine dose concentration is consistent with prior reports indicating that antigen dose critically modulates cytokine profiles and downstream adaptive immunity [31]. Gong et al. (2021) examined the efficacy of MP3RT peptide vaccine in a wild type and humanized murine model to evaluate its immunogenic potential. The increased levels of *IFN-γ*, *TNF-α* and *IL-12* by immunized mice with MP3RT revealed a strong Th1-polarized immune response that was associated with high titres of MP3RT-specific IgG antibodies. This study, however, did not investigate Th2 derived cytokines: *IL4*, *IL-6* and *IL-10*. A comparative analysis further showed that although wild-type mice had overall higher cytokine levels, humanized mice displayed improved protective efficacy. The protective efficacy was associated with increased T cell responses rather than antibody levels, indicating that cellular immunity is the predominant mechanism of protection [32]. Lian et al. (2024) studied a personalized neoantigen-derived peptide vaccine combined with Poly-ICLC in an NSCLC mouse model. The combination produced a potent Th1 response involving an increased level of *IFN-γ* and *TNF-α*, which was indeed accompanied by massive T cell infiltration into tumor tissue. Reduced tumor growth and better control of the tumors were associated with these immunological changes. The study emphasizes the applicability of neoepitope vaccination using an adjuvant to enhance anti-tumor immunity [33].

Below is a detailed description and explanation of each of the factors:

Immunotherapy represents a promising primary

treatment modality for cancer. Current anti-cancer vaccination strategies are increasingly incorporating immunotherapeutic components, such as cytokines and immunomodulatory agents, to enhance overall efficacy. In light of the rising incidence of lung cancer, innovative therapies, such as peptide-based vaccines, demonstrate significant functional potential. By utilizing peptides derived from tumors, these vaccines elicit a robust immune response, enabling the body to detect and eradicate tumor cells. Additionally, they aim to enhance localized inflammation, thereby improving therapeutic effectiveness while minimizing adverse effects. Although they offer advantages such as ease of synthesis and a reduced risk of carcinogenicity, challenges such as insufficient immune activation and tumor-induced immune suppression persist. This limitation can be addressed by incorporating alum as an adjuvant, which enhances inflammation and strengthens the overall immune response [34–36].

Hence, the tripeptide construct was administered alongside Alum and nanoliposomes, both recognized for potentiate immune responses through distinct mechanisms. Alum, an aluminum-based adjuvant, induces local inflammation at the injection site, which facilitates the recruitment of immune cells and activates the innate immune system. This inflammatory response supports antigen-presenting cells (APCs), including dendritic cells and macrophages, in processing and presenting antigens to T cells. Primarily, Alum skews the immune response towards Th2 by activating the NLRP3 inflammasome, promoting the maturation of dendritic cells, and guiding $CD4^+$ T cells. While it is chiefly recognized for promoting Th2 responses, Alum can also facilitate broader immune activation depending on the design and delivery of the antigen. Its pro-inflammatory effects elevate the expression of MHC class II and co-stimulatory molecules (CD80/CD86) on dendritic cells, thereby improving antigen presentation and likely increasing levels of *IL-4* and *IL-10*. When combined with nanoparticle carriers, Alum effectively activates dendritic cells and improves cross-presentation to $CD8^+$ cytotoxic T lymphocytes. This robust activation of the adaptive immune system underscores our choice of Alum adjuvant. The co-administration of Alum and the tripeptide construct may further amplify both innate and adaptive immune responses [37-40].

Traditionally, most peptide based trials have focused on a single antigen, which restricts their overall effectiveness. However, multi-epitope vaccines represent a noteworthy advancement in cancer immunotherapy. The multi-epitope peptide construct, which is relatively long (54 amino acids), is processed by antigen-presenting cells into smaller epitopes that are compatible with MHC-I binding, facilitating CTL activation, supported by the observed *IFN-γ* elevation. Additionally, the native peptide length is optimal for MHC-II presentation, promoting $CD4^+$ T helper activation and cytokine responses. This simultaneous engagement of both MHC pathways activates various T-cell subsets, thereby improving both humoral and cellular immune responses while effectively reducing the risk of tumor evasion. We selected three marker epitopes, each targeting distinct mechanisms and pathways, creating a synergistic effect that enhances overall efficacy. This approach leverages positive feedback loop of the acquired immune system, leading to a strengthened response against lung cancer. Hence, targeting multiple peptides offers better tumor control than monotherapies [22, 41].

We observed that administering the multi-epitope peptide, led to an increase in *TNF-α* expression, with the 100 mg/ml dose was more effective at consistently upregulating *TNF-α*. This cytokine is a central pro-inflammatory cytokine that facilitates communication between immune cells. The activation of macrophages and dendritic cells leads to the secretion of *TNF-α*, which serves as a critical early signal, enhancing vascular permeability and facilitating the migration of immune cells to the injection site. Moreover, Increase peptide concentrations not only amplify antigen uptake but also support more efficient cross-presentation and sustained T cell activation, resulting in a heightened inflammatory cytokine environment. The elevated levels of *TNF-α* observed at both dose aligns with its well-established role in enhancing anti-tumor immunity through the induction of apoptotic pathways in tumor cells, the recruitment of cytotoxic immune cells, the stimulation of Th1 responses, and the improvement of antigen presentation [6, 42]. The peptide vaccine also trigger the activation of critical intracellular pathways such as NF-κB and STAT3, which drive the transcription of *TNF-α* and *IL-6* genes, concurrently enhancing MAPK signaling in both antigen-presenting cells and T-helper cells [43]. Elevated *TNF-α* levels following vaccination serve as a strong indicators of vaccine efficacy, reflecting immune system activation. In cancer vaccine studies, particularly in murine models like BALB/c mice, increased *TNF-α* expression indicates the initiation of antitumor immunity and is often accompanied by the upregulation of other important cytokines, such as *IFN-γ*. It is important to recognize, however, *TNF-α*'s role in cancer is context-dependent. While it signifies a rapid and coordinated innate response, which is consistent with *TNF-α*'s established function in initiating acute inflammation, enhancing vascular

permeability, facilitating leukocyte recruitment, and inducing apoptosis in target cells, chronic *TNF-α* exposure within the tumor microenvironment may also contribute to tumor progression through mechanisms like increased angiogenesis, metastasis, and immune evasion [6]. Thus, while *TNF-α* is a key marker of immune activation post-vaccination, its expression must be carefully regulated to avoid potential protumorigenic effects, especially in therapeutic cancer vaccine contexts [24].

In the context of cancer immunotherapy, particularly peptide-based vaccines, interleukin-6 (*IL-6*) plays a crucial role as a pro-inflammatory cytokine involved in early immune responses. Historically, it has been treated with caution due to its association with tumor progression and immunosuppression under chronic conditions. However, recent insights suggest a more complex role for *IL-6*: in the controlled, acute inflammatory environment induced by therapeutic vaccination, it may actually enhance anti-tumor immune responses [44].

Our findings support this perspective, as *IL-6* demonstrated a moderate yet statistically significant increase of approximately 6.5-fold in the high-dose group, coupled with notable inter-individual variability, which should be viewed positively. Although the change in *IL-6* were less pronounced compared to other cytokines, the observed levels following peptide vaccine administration indicate a successful initiation of early inflammation, an essential step for antigen processing and adaptive immune activation. This is particularly relevant in vaccine models where *IL-6* functions as a pro-inflammatory signal rather than a chronic suppressor. This likely reflects the multifaceted role of *IL-6*, which not only contributes to acute-phase responses but also mediates long-term immune activation and regulation by promoting Th17 polarization, B cell maturation, and immune memory development. *IL-6*, with its context-dependent functions, may facilitate T cell differentiation or support tumor-associated inflammation, necessitating careful interpretation in vaccine design. It also facilitates dendritic cell maturation and enhances the proliferation of $CD8^+$ cytotoxic T lymphocyte, particularly in conjunction with *IFN-γ*, known for its anti-tumor effects. Thus the combination of elevated *IFN-γ* and *IL-10* alongside *IL-6* fosters a robust anti-tumor immune response. Furthermore, *IL-6* plays a role in reshaping the tumor microenvironment in suppressive tumors such as lung cancer by downregulating regulatory T cells and enhancing dendritic cell function [44, 45].

Interferon- γ (*IFN-γ*) is another crucial cytokine that was assessed that can activate macrophages into their lethal M1 form and enhances the production of nitric oxide and free radicals. It increases the expression of MHC I/II and co-stimulatory molecules, including CD80 and CD86, which are vital for effective antigen presentation. Additionally, it promotes the differentiation of Th1 cells and elevates $CD8^+$ T cell levels, thereby strengthening the anti-tumor immune response. Furthermore, *IFN-γ* matures dendritic cells, enhancing their ability to present antigens to T cells, and boosts the production of antibodies with strong phagocytic capabilities while facilitating class switching to IgG. It also reduces the function of regulatory T cells, preventing their formation and directing the immune response toward a Th1 profile. Moreover, it inhibits angiogenesis, which is a critical factor in cancer defense. In summary, elevated levels of *IFN-γ* indicate a more robust immune response against cancer, providing both direct cytotoxic effects and a significant role in immune memory [46]. The substantial increase in *IFN-γ* observed in the 100 mg/ml dose group in comparison with other assessed cytokines, highlights a key aspect of the immune response triggered by the vaccine. The synergy between IFN-γ and TNF-α further enhances the effectiveness of this immune activation. The data reveals that the increased expression of IFN-γ, in relation to TNF-α, signifies the successful activation of the Th1 response and the recruitment of cytotoxic T cells. This compelling evidence confirms that the peptide vaccine effectively induces cellular immunity. Notably, the expression of *IFN-γ* was significantly higher than that of *IL-6*, reinforcing the dominance of the Th1 pathway. While *IL-6* levels also rose, this occurred in a moderate and controlled manner, indicating that an effective immune response against the tumor was initiated without excessive inflammation. This suggests that *IFN-γ* plays a suppressive role on *IL-6* levels. In addition, our findings demonstrate that the highest expression levels in mice receiving the peptide at a dose of 100 mg/ml corresponded to the peak absolute levels of *IFN-γ* and *TNF-α*, respectively. These results validate that the increase in *TNF-α*, in conjunction with *IFN-γ*, signifies a robust Th1-based immune response characterized by the activation of cytotoxic T lymphocytes (CTLs) and the targeted destruction of tumor cells through both inflammation and direct cytotoxicity. The consistency and low variability of *IFN-γ* across subjects suggest reliable immune stimulation [46-48].

The *IL-10* level was elevated in the group receiving the 100 dose vaccine compared to the 10 dose group; however, the 100-dose group displayed greater variability among the mice, whereas 10 mg/ml dose elicit a more uniform and reproducible regulatory

immune response. The increase in *IL-10* likely serves as a physiological feedback mechanism to alleviate excessive inflammation and maintaining immune balance. In contrast to the primarily pro-inflammatory cytokines *IL-6* and *TNF-α*, *IL-10* functions as a regulatory and anti-inflammatory cytokine with a more nuanced role in immune homeostasis. It is produced by regulatory T cells, M2 macrophages, Th2 cells, and occasionally dendritic cells under inflammatory stress to prevent tissue damage. *IL-10* suppresses the production of *IL-6* and *TNF-α*, down regulates co-stimulatory molecules, and inhibits the activity of T cells and antigen presenting cells to prevent excessive inflammation [13, 14]. The simultaneous expression of *IL-10*, *TNF-α* and *IFN-γ* indicates a coordinated activation of both inflammatory and regulatory responses. The peptide vaccine likely stimulated a robust immune response, leading to an increase in *IL-10* levels to safeguard against tissue damage. This highlights the body's ability to engage regulatory pathways that mitigate the risk of cytokine storms and autoimmunity while also promoting anti-tumor immunity.

IL-10 produced by Tregs may dampen effective anti-tumor responses, possibly due to the influence of *TGF-β*, although its effect is less significant than that of the dominant Th1 response [49]. The high *IFN-γ*/*IL-10* ratio, reveals a pro-inflammatory and tumor-fighting environment that correlates with better outcomes in immunotherapy and reduced tumor progression. The vaccine's focus on inducing a Th1-dominant phenotype is vital for overcoming challenges posed by solid tumors like NSCLC, where immune evasion is prevalent. While elevated levels of *IL-4* and *IL-10* could raise concerns about Th1-mediated cytotoxicity, it is essential to recognize that these cytokines also contribute to maintaining immune tolerance and preventing autoimmunity, indicating that the immune response is activated and finely regulated to avoid excessive tissue damage.

Furthermore, the lower dose of 10 mg/ml of *IL-4* exhibited the highest gene expression among the cytokines tested. This suggests that spleen cells respond more effectively to this dose by activating *IL-4* related signaling pathways, likely including the STAT6 pathway, with is crucial for *IL-4*'s Function [50]. Additionally, the alum adjuvant may contribute to the increased *IL-4* expression observed at this lower vaccine dose. Conversely, the higher dose of 100 mg/ml did not result in a significant increase in *IL-4* expression. This phenomenon may be attributed to several factors: 1. Feedback inhibition: at elevated doses, the immune system may activate negative feedback mechanisms, such as the up regulation of *IL-10*, which can down regulate cytokine expression and prevent overstimulation. 2. Receptor Saturation: At high cytokine concentrations, *IL-4* receptors may become saturated, limiting their capacity to transmit signals even with increased stimulus. 3. Reduced Sensitivity: Spleen cells from vaccinated mice may exhibit decreased responsiveness to higher doses, potentially due to desensitization or a threshold effect, where maximum activation occurs at lower doses and higher concentrations do not lead to a significant increase in gene expression.

The increase in *IL-4* expression within both dosage groups indicates a balanced immune response rather than a reduction in vaccine efficacy. In the 100 mg/ml peptide vaccine group, the significant rise in *IFN-γ* levels is noteworthy, as *IL-4* does not inhibit CTL activation and can enhance Th1 responses when paired with *IFN-γ*, thereby potentially improving the vaccine's antitumor effects. The concurrent increase in both *IL-4* and *IFN-γ* suggests a multifunctional immune response that enhances vaccine efficacy and immunological memory. Although pro-inflammatory cytokines such as *IFN-γ* and *TNF-α* may show less reduced levels at higher doses, the variability in *IL-4* responses could reflect the distinct immune profiles of spleen cells from vaccinated mice. *IL-4* plays a crucial role in the Th2 immune response, promoting anti-inflammatory functions without diminishing CTL activity and modulates dendritic cell function. In addition, factors such as tolerance may influence cytokine expression following vaccination. *IL-4* is known to enhance antibody production and can lead to isotype switching to IgG1 and IgE during periods of inflammation. In the context of cancer treatment, *IL-4* supports B-cell activation and humoral immunity [50]. Proper regulation of *IL-4* expression is essential for maintaining immune balance and preventing excessive tissue damage. Its increase following vaccination may signify an effective immune response that targets tumor cells while minimizing adverse effects, which is critical for achieving therapeutic efficacy [48].

Here, we explore the genetics of immune pathways related to the relevant cytokines:

In mice receiving vaccination, nanoliposomes enhance the uptake of antigens by antigen presenting cells, thereby facilitating MHC class I cross-presentation to $CD8^+$ T cells and MHC class II cross-presentation to $CD4^+$ T cells [51]. Furthermore, interactions between the liposomal surface and innate immune receptors, combined with the immune-stimulatory properties of alum, activate pattern recognition receptors. This triggers transcriptional regulators such as NF-κB and MAPKs (p38, JNK, ERK), leading to the rapid

transcription of pro-inflammatory cytokine genes like *TNF* and *IL-6*. *TNF-α* further amplifies inflammatory gene expression through TNFR-mediated feedback on NF-κB, while *IL-6* engages the gp130/JAK/STAT3 pathway to promote the survival, proliferation, and differentiation of T and B lymphocytes, effectively linking innate activation to adaptive immunity [52]. The pro-inflammatory environment established during this early phase facilitates the secretion of *IL-12* by activated dendritic cells, which in turn drives the differentiation of naïve $CD4^+$ T cells into the Th1 lineage through STAT4 activation. This process leads to a robust production of *IFN-γ* by both Th1 cells and $CD8^+$ cytotoxic T lymphocytes. In response, *IFN-γ* enhances Th1 polarization via STAT1 activation and increases the expression of MHC class I and II, thereby improving antigen presentation and establishing a positive feedback loop that bolsters effective antitumor responses. With the 100 mg/ml vaccine, the prolonged availability of antigen and more robust receptor interactions further amplify these effects [19]. Additionally, *IL-4* signaling activates the JAK1/JAK3-STAT6 pathway, which induces GATA3. GATA3 then remodels the chromatin at the *IL-4*/*IL-13* locus, stabilizing Th2 differentiation and enhancing humoral immunity. This Th2 bias occurs concurrently with Th1 activation, resulting in a mixed cytokine profile [12]. *IL-6* acts as a multifunctional mediator that links innate and adaptive responses and plays a role in shaping the response through the activation of STAT3, which can influence both cell differentiation and the induction of regulatory pathways. Additionally, *IL-6* interacts with the MAPK and PI3K/AKT pathways during acute inflammation, supports antitumor immunity, and in some cases, may assist tumors in evading the immune system. IL-10 binds to its receptor complex (IL-10R1 and IL-10R2), activating JAK1 and TYK2, which phosphorylate STAT3 to promote the expression of anti-inflammatory genes. The increased expression of *IL-10* likely serves as an innate regulatory mechanism that engages the STAT3 pathway to inhibit NF-κB activity in antigen-presenting cells. Such a negative feedback loop is crucial within the tumor microenvironment, where uncontrolled pro-inflammatory signaling can lead to tissue damage. Additionally IL-10 influences pathways such as MAPK/ERK, and TGF-β/SMAD, thereby enhancing inflammation suppression through mechanisms like SOCS inhibition of the JAK/STAT pathway Notably, *IL-10* affects the balance between pro and anti-inflammatory responses in the tumors microenvironment by modulating the PI3K/AKT signaling cascade, improving tumor cell survival and proliferation [53].

The cytokine signature demonstrates the simultaneous activation of various transcriptional hubs: NF-κB stimulates *TNF* and *IL-6*, STAT1/STAT4 regulates *IFN-γ*, STAT6 controls *IL-4*, and STAT3 governs both *IL-6* and *IL-10* from different perspectives. The interplay among these pathways stabilizes the mixed Th1/Th2 regulatory profile. This pattern is consistent with the dual nature of the vaccine formulation, where the liposomal carrier enhances cytotoxic and Th1-type immunity, while the alum promotes the Th2 and regulatory balance. The peptide vaccine is designed to activate multiple pathways concurrently, especially JAK/STAT, NF-κB, and the MAPK/ERK. This coordinated activation generates a complex immune network capable of not only destroying tumor cells but also preventing uncontrolled inflammatory responses. This coordinated transcriptional activation suggests that the vaccine does not merely steer the immune response towards a single pathway; rather, it fosters a multifaceted immune activation. Such a balanced and genetically coordinated cytokine profile could prove valuable in cancer immunotherapy, where both effective immune responses and precise regulation are essential for achieving long-term therapeutic efficacy [54].

The observed divergence between computational immunogenicity predictions [**Table2**] and actual cytokine upregulation can be attributed to a confluence of immunological, methodological, and formulation-specific factors. *In-silico* analyses indicated that some epitopes had limited immune stimulatory potential based on MHC class II binding affinity and epitope profiling, yet the *in-vivo* results demonstrated significant cytokine gene upregulation. First, this discrepancy can be explained by the fact that computational prediction tools primarily assess the theoretical binding capacity of peptides to MHC molecules, relying on algorithms trained on existing epitope datasets and structural motifs. However, these models do not fully capture the complexities of antigen processing, T-cell receptor engagement, co-stimulatory signaling, and the immunological microenvironment. Consequently, a peptide that appears non-stimulatory in computational analyses may still elicit a robust immune response in physiological conditions. Second, *in-vivo* administrations of combined multi-epitope peptides construct introduces the possibility of synergistic or antagonistic interactions between epitopes that are not considered by prediction algorithms analyzing them in isolation. Epitopes predicted to be weakly immunogenic may perform better when co-administered, benefiting from enhanced processing or T-cell priming. This can amplify antigen presentation and cytokine signaling pathways, resulting in

heightened immune activation that computational models do not anticipate. Third, the biological context is also crucial; BALB/c mice typically exhibit a Th2-skewed immune response, characterized by elevated expression of *IL-4*, *IL-10*. Moreover, the spleen, being a secondary lymphoid organ, rich in dendritic cells, macrophages, T cells, and B cells, provides an interactive environment that facilitates complex multi-pathway activation beyond what *in-silico* models can predict. Finally, understanding cytokine expression dynamics is essential for grasping immune responses. Early-response cytokines such as *TNF-α* and *IL-6*, reach peak levels within hours, while regulatory or Th1-associated cytokines like *IL-10* and *IFN-γ* are expressed at a later stage. Static *in-silico* models do not effectively capture the dynamic regulation and interactions of these cytokines in cell cultures, resulting in a disconnect between computational predictions and actual experimental data. Therefore, while these models serve as useful tools for initial screening of candidate epitopes, they cannot replace the need for comprehensive *in-vivo* validation to truly evaluate the immunogenic potential and functional relevance of peptide-based constructs [55-57].

We propose that a peptide vaccine can enhance cytokine gene expression and improve immune responses by generating specific antibodies that target and eliminate cancer cells. Administration of different doses of the peptide construct demonstrated significant activation of cytokine genes, underscoring the sensitivity of cancer cells to this approach. An important immunological threshold was identified, indicating the vaccine's effectiveness and the necessity of optimizing the antigenic load for improved therapeutic outcomes. In addition, successful cancer vaccines should stimulate a strong Th1-skewed immune response to support the CTL response, while also maintaining balanced immune regulation to avoid adverse effects. Our vaccine activates both pro-inflammatory and regulatory pathways, leading to a vigorous yet appropriately balanced immune response necessary for long-term immunity with low toxicity. The higher levels of *IL-4* and *IL-10* likely function as a negative-feedback mechanism to temper inflammatory processes, ensuring that the immune response effectively targets tumor cells without inducing cytotoxicity in normal tissues.

Taken together, these observations indicate that peptide vaccines do not simply elicit a unilateral Th1 response; rather, they generate a dynamic, context-dependent cytokine network. Our results align with and extend the existing literature, suggesting that effective cancer vaccination requires a carefully calibrated cytokine environment that balances antitumor immunity with immune regulation to maximize therapeutic benefit while minimizing adverse effects. Nonetheless, current research in peptide therapy encounters challenges due the reliance on single animal models. In the 100-dose vaccine group, although overall cytokine expression increased, the data regarding interleukins 4, 6, and 10 was inconsistent. This may be attributed to sample variability or high doses causing strong immune responses in some mice while leading to T cell exhaustion in others. Such variability increases data dispersion and reduces statistical significance. Given the limited sample sizes, significant differences can only be detected if the data are highly consistent. To make meaningful advancements, it is essential to conduct comprehensive studies that explore additional key factors, including *IL-2*, *IL-12*, TGF-β, Treg, $CD4^+/CD8^+$ markers, as well as perforin and granzyme A/B. Furthermore, the reliability of gene expression assays can be influenced by various factors such as cell culture conditions, which underscores the importance of employing diverse methodologies for accurate assessment. An overly focus on a limited number of cytokines risks overlooking other critical proteins that govern immune cell response, potentially resulting in less effective therapies. Consequently, it is imperative to advocate for a comprehensive approach that targets multiple signaling pathways to enhance the immune responses. Ongoing research is vital for a thorough understanding of cytokine genes in lung cancer and for developing effective therapies, which require substantial investments of time and resources. Future studies should explore whether modification in dosing or the application of alternative adjuvant strategies can improve outcomes while avoiding immune tolerance and excessive inflammation. Additionally, investigating the long-term durability of immune responses and their correlation with clinical outcomes will be critical for practical applications in treatment and focus on correlating these cytokine profiles with functional outcomes such as tumor regression, T cell cytotoxicity, and immune memory formation.

5. Conclusion

In summary, the multi-epitope peptide vaccine demonstrates substantial potential for inducing a well-balanced immune response through regulatory modulation and Th1 activation. The high levels of *IL-6*, *TNF-α*, and *IFN-γ* reflect a strong Th1 response, which is essential for attracting cytotoxic T lymphocytes and promoting tumor cell elimination. At the same time, the expression of *IL-4* and *IL-10* indicates a counterbalancing regulatory mechanism that supports tissue integrity and helps manage excessive inflammation. This balance

between immune activation and suppression underscores the intentional design of the vaccine, which aims to enhance antitumor immunity while minimizing the risk of autoimmunity. The transient increase in *TNF-α* and *IL-6*, along with elevated *IFN-γ* levels and a cytokine milieu biased toward Th1 responses, collectively establishes an immunological environment conducive to tumor regression. Overall, the prudent selection of VEGF-A, TGF-β, and MAGE-A3 epitopes is supported by these findings, reinforcing the translational potential of the vaccine. Future efforts should focus on further optimizing this immunoregulatory balance to improve therapeutic outcomes, thereby laying the groundwork for continued research and eventual clinical application in lung cancer immunotherapy.

Ethical Considerations

Compliance with ethical guidelines

All animal procedures were approved by the Committee of Animal Ethics of the North Tehran Branch of Islamic Azad University (IR.IAU.TNB.REC.1403.142). All the experiments were performed and reported according to relevant guidelines and regulations of the ethics committee of the Pasteur Institute of Iran. All methods are reported by ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments.

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Author's contributions

(I) Conception and design: M.H, A.J, V.M,Provision of study: M.H, S.J, A.J, and M.E, (IV) Collection, and assembly of data: M.H, A.J, and V.M, (V) Data analysis and interpretation: M.H, S.J, A.J, and M.E, (VI) Manuscript writing and final approval of manuscript: M.H, S.J, A.J, and M.E

Conflict of interest

Not applicable.

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