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Epitope-based Vaccine Design Against Common Tree Allergens Using Computational Approaches

Mina Noroozbeygi[1], Mina Mohammad-rezaei[1], Mohammad Hossein Shams[2], Armaghan Nikoosokhan[3], Viana Dayhimi[4], and Mohammad-Ali Assarehzadegan[1]

[1] Department of Immunology, Immunology Research Center, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2] Department of Medical Immunology, Hepatitis Research Center, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran
[3] Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran
[4] Department of Chemistry and Biotechnology, University of Windsor, Windsor, Canada

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ABSTRACT

Allergy-related diseases, driven by type I hypersensitivity reactions, affect over 20% to 25% of the global population and continue to rise. Pollen grains may have allergens that cause hypersensitivity reactions. Pla or 3, Fra e 1, and Bet v 1 are major allergens of pollen grains from the common allergenic trees *Platanus orientalis*, *Fraxinus excelsior*, and *Betula pendula*, respectively, and are employed in this study to design a multi-epitope allergy vaccine using immunoinformatics methods.

The steps after finding the main allergenic epitopes include sequences retrieval, T-cell epitopes selection by IEDB and NetMHCIIpan, B-cell epitopes selection by IEDB, addition of linkers and adjuvant, and construction of multi-epitope vaccine, evaluating antigenicity, allergenicity, and toxicity of vaccine construct, secondary structure prediction, 3-dimensional structure modeling and refinement by GalaxyTBM, validation of the model by ProSA-web, ERRAT, and PROCHECK, and exploring the interaction of the designed molecule with receptor by ClusPro.

The designed vaccine demonstrated favorable properties, including high antigenicity, lack of allergenicity and toxicity, and stable secondary and tertiary structures.

These results suggest that this vaccine has the potential to mitigate allergic responses effectively. However, experimental validation is required to confirm its efficacy and safety.

Keywords: Allergy; Ash; Birch; Immunoinformatics; Sycamore; Vaccine

Corresponding Author: Mohammad-Ali Assarehzadegan, PhD; Department of Immunology, Immunology Research Center, School of Medicine, Iran University of Medical Sciences, Postal code: 1449614535, Tehran, Iran. Tel: (+98 21) 8670 3264, Fax: (+98 21) 8862 2652, Email: assarehma@gmail.com, assareh.ma@iums.ac.ir

*The first and second authors contributed equally to this study

INTRODUCTION

Allergy is a hypersensitivity reaction of the immune system to specific antigens classified as type I (immediate) hypersensitivity.[1] Allergic disorders are one of the most prevalent health issues

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worldwide;according to the World Health Organization (WHO), it is expected to affect nearly half of the global population by 2050,[2] underscoring the need for innovative solutions. Different risk factors for allergic reactions, including patient characteristics and environmental factors, have been described. Among environmental factors, pollen grains from trees, grasses, and weeds play a major role in the onset of allergic reactions in individuals who are atopic.[3]

Traditional management strategies, including allergen avoidance and symptomatic treatments, often fall short in providing long-term relief.20 Current immunotherapy approaches, such as allergen-specific immunotherapy (AIT), have shown promise in addressing the root cause of allergies but face limitations, including high costs and adverse reactions. Moreover, other alternative methods such as production of hypoallergens, and altering allergens into reduced allergenic activity vaccines, have been taken into consideration as allergen-specific immunotherapy methods.

The most widely studied allergenic trees, European ash (*Fraxinus excelsior*), Sycamore (*Platanus orientalis*), and Birch (*Betula pendula*), are found all around the world (Figure 1).[4] *F excelsior* is part of the Oleaceae family, is endemic to the main islands of Britain and most of Europe, Turkey, Caucasus, and northern Iran (Figure 2A).[5] Fra e 1, the major pollen allergen of *F excelsior*, is a protein of the Ole e 1-like family with about 20 kDa.[6] *P orientalis* is found in southwest Asia, southeast Europe, and the USA (Figure 2B).[7] Pla or 3 is a non-glycosylated 18 kDa protein and one of the major allergens of *P orientalis*.[8] Birch trees are part of the Betulaceae family. In Northern and Central Europe, Betulaceae are the most common tree types, and its pollen can cause respiratory allergic reactions (Figure 2C).[9] Bet v 1, which is the allergen affecting over 90% of individuals with birch pollen allergies, is a 17 kDa protein of the pathogenesis-related (PR) protein class 10 family.[10]

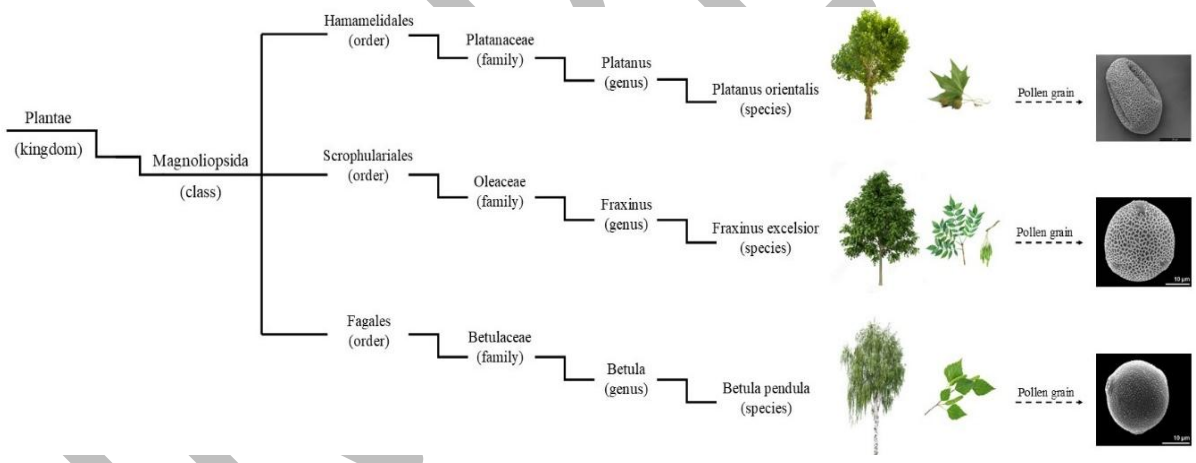
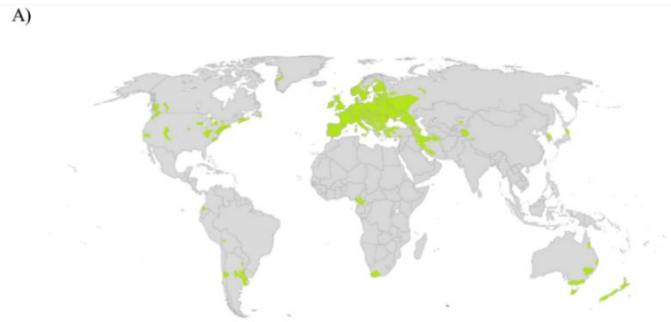


Figure 1. Phylogenetic tree of the plants of interest orders generated based on NCBI taxonomy. Photographs of trees and leaves were obtained from Adobe Stock. The SEM photos of *P orientalis* pollen grain are adopted from reference [11], and *F excelsior* and *B pendula* pollen grains are adopted from PalDat—a palynological database (www.paldat.org).[12,13]



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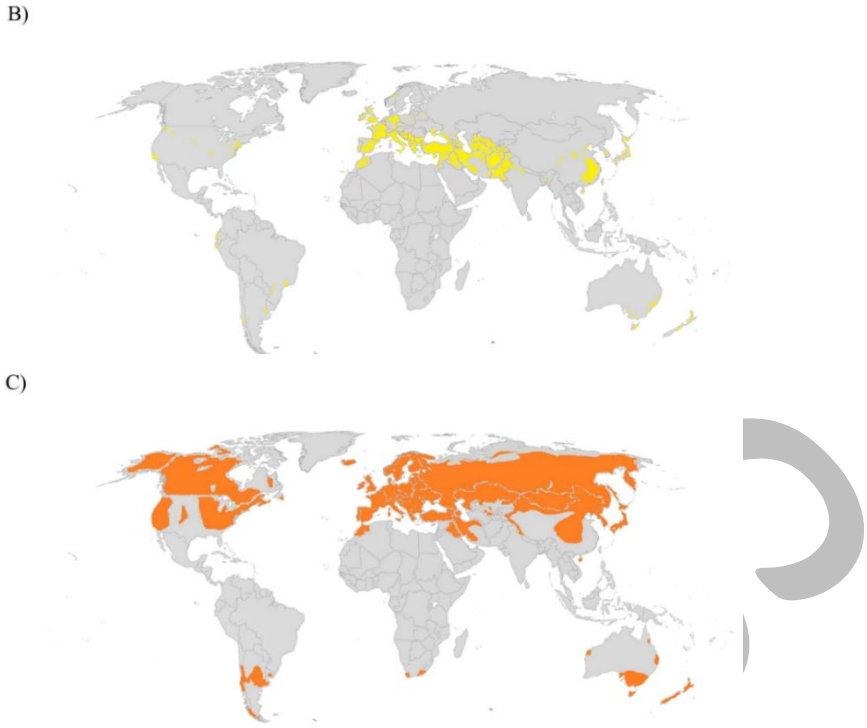

Figure 2. Geographic distribution of (A) *F excelsior*, (B) *P orientalis*, and (C) *B pendula*. The distribution data were extracted using the maps available at www.eol.org and www.plantsoftheworldonline.org/ and references 14–19.

Traditional management strategies, including allergen avoidance and symptomatic treatments, often fall short in providing long-term relief.[20] Current immunotherapy approaches, such as allergen-specific immunotherapy (AIT), have shown promise in addressing the root cause of allergies but face limitations, including high costs and adverse reactions.[21] Moreover, other alternative methods such as production of hypoallergens,[22] and altering allergens into reduced allergenic activity vaccines,[23] have been taken into consideration as allergen-specific immunotherapy methods.

Peptide-based epitope vaccines offer a novel, targeted strategy to combat allergies. These synthetic vaccines are composed of selected short peptide fragments representing immunodominant epitopes that elicit precise immune responses.[24] Peptide-based vaccines are water-soluble and can maintain stability under simple conditions.[25] Since these vaccines contain only a small chain of peptides, they are less likely to cause allergic or autoimmune reactions.[24] Recent advances in immunoinformatic methods have revolutionized vaccine development, enabling the identification of epitopes with high immunogenicity and minimal toxicity. Currently, an epitope-based vaccine with a certain allergen's immunodominant T- and B-cell epitopes has been used for dealing with allergic disorders.[26,27] Such vaccines have several advantages, including providing more long-term immunity, preventing adverse immunological reactions, and lower price. Epitope-based peptide vaccine design methods have been used for developing vaccines against a variety of disorders.[28,29]

This study aims to design a multi-epitope allergy vaccine using immunoinformatics tools that were based on the T- and B-cell epitopes from major tree allergens Pla or 3, Fra e 1, and Bet v 1. By integrating epitopes with robust linkers and adjuvants, we aim to develop a vaccine candidate with optimized antigenicity, stability, and safety for further experimental evaluation.

MATERIALS AND METHODS

Sequence Retrieval

The Pla or 3, Fra e 1, and Bet v 1 allergens were acquired from the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee

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(http://allergen.org/), and their sequences were retrieved from the protein database of NCBI (http://www.ncbi.nlm.nih.gov/) with the accession numbers of ABY21307, AAQ83588, and CAB02156, respectively.

Epitope Prediction and Selection

B-cell Epitope Prediction; The Immune Epitope Database Analysis Resource (IEDB-AR) server was employed to predict linear B-cell epitopes. Potential linear B-cell epitopes were identified and predicted using the BepiPred online server.[30] The recognition of B-cell epitopes is influenced by different parameters, such as hydrophilicity, polarity, and antigenic propensity of polypeptide chains, which are associated with the location of continuous epitopes. Moreover, flexibility, accessibility, turns, and exposed surface are also significant features for B-cell epitope identification. The key parameters were analyzed using multiple prediction tools: Karplus and Schulz flexibility prediction,[31] Parker hydrophilicity index,[32] Emini surface accessibility prediction,[33] Kolaskar and Tongaonkar antigenicity scale,[34] and Chou and Fashman β-turn analysis.[35] The most immunogenic epitopes were prioritized for further processing.

T-cell Epitope Prediction: Linear T-cell epitopes were predicted by applying IEDB and NetMHCIIpan servers. The IEDB T-Cell Epitope Prediction Tools were employed for determining elements with high binding affinity to MHC class II molecules, focusing on alleles associated with widespread immune responses. IC_{50} values were calculated using the Stabilized Matrix Method (SMM).[36–38] Moreover, NetMHCIIpan provided complementary predictions. The NetMHCII-2.2 server at https://www.cbs.dtu.dk/services/NetMHCII-2.2/ was used to predict the binding of peptides to class II MHC.[39] Peptides with $IC_{50} \leq 500$ nM were considered strong binders, while those between 500 and 1000 nM were regarded as moderate binders. Furthermore, peptides showing a percentile rank ≤10% were considered as potential high-affinity binders. The top epitopes with the highest affinity and immunogenicity were selected for vaccine construction.

Population Coverage Analysis

The global population coverage of the selected MHC class II epitopes was assessed using the IEDB Population Coverage tool (https://tools.iedb.org/population/). The HLA alleles corresponding to the predicted T-cell epitopes were submitted to estimate the fraction of individuals expected to respond to these epitopes based on allele frequency data across different ethnic and geographic regions. Default parameters were applied for analysis, and results were expressed as total population coverage (%).

Multi-epitope Vaccine (MEV) Construction

In order to build the multi-epitope vaccine construct, the total top ten candidates of helper T lymphocyte (HTL) and B-cell lymphocyte (BCL) epitopes from the previous step (epitope selection) were linked through GPGPG and KK amino acid linkers, respectively. Moreover, to enhance vaccine efficacy, the TLR-4 agonist adjuvant RS04 (GLQQVLL) was fused to the N-terminal and C-terminal of the completed vaccine construct via EAAAK linkers. The complete vaccine sequence was composed of 186 amino acids.

Physicochemical Properties of MEV

The ExPASy ProtParam Tool at https:// web.expasy. org/protparam/ was used to predict key physicochemical properties of the vaccine construct, including molecular weight, theoretical isoelectric point (pI), hydropathicity, instability index, and estimated half-life in mammalian cells, yeast, and *E coli*.[40]

Secondary Structure Prediction

PSIPRED (http://bioinfcs.ucl.ac.uk/psipred/), a secondary structure prediction tool, was used to analyze α-helices, β-strands, and random coils in the final vaccine sequence by examining sequence segments through the Protein Data Bank (PDB) library (http://www.rcsb.org/) and identifying conserved substructures.[41] The proportion of structural features was graphically represented for further evaluation.

3D Structure Modeling and Validation

It has been established that 3-dimensional protein structures can provide a great deal of information about the biological functions of a protein. The 3D structure of the vaccine construct was modeled using GalaxyTBM, a template-based modeling server. The GalaxyTBM server at https://galaxy.seoklab.org/ processed the final amino acid sequence of the multi-epitope vaccine protein in the FASTA format to predict the 3D structure of the protein construct. The structure was refined using GalaxyRefine to optimize lateral chain positioning and global conformation. The refinement method has been tested in

CASP10.[42] Three servers were employed to verify the overall quality of the generated models: Protein Structural Analysis tools (PROSA-web) at https://prosa.services.came.sbg.ac.at/prosa.php evaluated structural energetics to ensure residue interactions were energetically favorable.[43] The model's incorrect statistics of bad non-bonded interactions were detected by the ERRAT server at https://saves.mbi.ucla.edu/.[44] The PROCHECK server at https://saves.mbi.ucla.edu/ assessed stereochemical properties using Ramachandran plots, categorizing residues into favorable, allowed, and disallowed regions.[45,46]

Allergenicity, Antigenicity, and Toxicity Assessment

The AlgPred server at https://webs.iiitd.edu.in/raghava/algpred/index.html is used to ensure the construct lacked allergenic potential.[47] The ANTIGENpro server at https://scratch.proteomics.ics.uci.edu/ was used, which provided an antigenicity score for immunogenic potential. Toxicity was evaluated by ToxinPred at https://webs.iiitd.edu.in/raghava/toxinpred/index.html to confirm the absence of toxic peptides.[48]

Molecular Docking Analysis

Molecular docking, utilizing ClusPro at https://cluspro.bu.edu/login.php, was employed to predict the interaction of the vaccine adjuvant with its receptor, TLR-4. The ClusPro server is based on PIPER and the implementation of the FFT correlation method.[49–52] The docking analysis determined binding conformations, cluster size, and the lowest-energy configurations. Interaction residues and bond lengths were analyzed to confirm receptor binding.

RESULTS

T-cell Epitope Prediction

T-cell epitopes with high binding affinity to MHC class II molecules were identified for the Pla or 3, Fra e 1, and Bet v 1 allergens using IEDB and NetMHCIIpan servers. IC_{50} values and allele associations were determined, with selected epitopes demonstrating strong immunogenicity and coverage across multiple alleles. For instance, the Pla or 3 epitope LTPCLTYLRSGGAVA displayed high binding affinity to HLA-DRB1*01:01 and other common alleles. A comprehensive list of epitopes, their binding affinities, and associated MHC class II alleles is presented in Table 1 and Table 2.

Population Coverage Analysis

The IEDB population coverage analysis indicated that the selected MHC class II epitopes collectively cover over 80% of the global population. These findings demonstrate the global immunogenic potential of the designed construct, supporting its suitability as a broadly applicable vaccine candidate.

B-cell Epitope Prediction

B-cell epitopes were predicted and validated using BepiPred and other complementary tools, assessing features such as hydrophilicity, surface accessibility, flexibility, β-turn propensity, and antigenicity. Key epitopes, including 60ALNNDAKTTPDRQ72 for Pla or 3, 44CRDKENG50 for Fra e 1, 67YVKDRVDEV75 and 130KAEQVKA136 for Bet v 1, were selected for vaccine construction due to their high immunogenic scores and favorable physicochemical properties. Validation results for selected epitopes are summarized in Table 3.

Multi-epitope Vaccine (MEV) Construction

The final vaccine construct, consisting of 186 amino acids, incorporated top-ranked T-cell and B-cell epitopes linked with GPGPG and KK linkers, respectively. The TLR-4 agonist adjuvant RS04 was added to both the N-terminal and C-terminal of the MEV structure using EAAAK linkers for enhanced immunogenicity and stability. A schematic diagram of the final vaccine construct is shown in Figure 3.

Table 1. The immunodominant epitope selected from Pla or 3, Fra e 1, and Bet v 1 allergens via IEDB-AR server, according to IC_{50}. The selected epitopes for vaccine construct are bold.

Allergen name	Start	End	smm_align_ic50	Epitopes	Allele
Pla or 3	1	14	70.00	**MAFSRVAKLACLLL**	HLA-DRB1*01:01
			89.00		HLA-DRB1*11:01
			183.00		HLA-DRB1*07:01
			430.00		HLA-DQA1*01:02/DQB1*06:02
			70.00		HLA-DRB1*01:01
	41	54	13.00	LTYLRSGGAVAPAC	HLA-DQA1*05:01/DQB1*03:01
			22.00		HLA-DRB1*01:01
			250.00		HLA-DRB1*09:01
			332.00		HLA-DRB1*15:01
			446.00		HLA-DRB1*04:01
	37	51	18.00	**LTPCLTYLRSGGAVA**	HLA-DRB1*01:01
			145.00		HLA-DRB1*15:01
			138.00		HLA-DQA1*05:01/DQB1*03:01
			185.00		HLA-DRB1*09:01
	41	57	38.00	LTYLRSGGAVAPACCNG	HLA-DQA1*05:01/DQB1*03:01
			50.00		HLA-DRB1*01:01
			437.00		HLA-DRB1*15:01
	85	97	30.00	ISGIQLGNAASLA	HLA-DRB1*01:01
			37.00		HLA-DRB1*13:02
			77.00		HLA-DQA1*05:01/DQB1*03:01
			141.00		HLA-DRB1*09:01
			187.00		HLA-DRB1*07:01
	9	23	70.00	LACLLLACMVATAPH	HLA-DRB1*01:01
			120.00		HLA-DQA1*01:02/DQB1*06:02
			167.00		HLA-DQA1*05:01/DQB1*03:01
			403.00		HLA-DQA1*05:01/DQB1*03:01
	40	54	13.00	CLTYLRSGGAVAPAC	HLA-DQA1*05:01/DQB1*03:01
			23.00		HLA-DRB1*01:01
			257.00		HLA-DRB1*09:01
	43	58	39.00	YLRSGGAVAPACCNGV	HLA-DQA1*05:01/DQB1*03:01
			92.00		HLA-DRB1*01:01
Fra e 1	30	43	9.00	**KLSEFITGASVRLQ**	HLA-DRB1*01:01
			23.00		HLA-DRB1*07:01
			59.00		HLA-DRB1*09:01
			111.00		HLA-DQA1*05:01/DQB1*03:01
			156.00		HLA-DRB1*13:02
			158.00		HLA-DQA1*01:02/DQB1*06:02
			178.00		HLA-DRB1*04:01
			186.00		HLA-DRB5*01:01
	74	89	89.00	HKNEFCEITLLSSGRK	HLA-DRB5*01:01
			406.00		HLA-DPA1*03:01/DPB1*04:02

Table 1. Continued...

Allergen name	Start	End	smm_align_ic50	Epitopes	Allele
	28	43	27.00	ITKLSEFITGASVRLQ	HLA-DRB1*01:01
			69.00		HLA-DRB1*07:01
			174.00		HLA-DRB1*09:01
			316.00		HLA-DQA1*05:01/DQB1*03:01
			431.00		HLA-DRB1*04:01
			457.00		HLA-DQA1*01:02/DQB1*06:02
			460.00		HLA-DRB1*13:02
	102	116	56.00	PSLKFILNTVNGTTR	HLA-DRB1*01:01
			73.00		HLA-DRB1*04:05
			123.00		HLA-DRB1*13:02
			161.00		HLA-DRB1*04:01
			197.00		HLA-DRB1*11:01
			430.00		HLA-DRB1*08:02
	104	118	69.00	**LKFILNTVNGTTRTI**	HLA-DRB1*01:01
			123.00		HLA-DRB1*13:02
			142.00		HLA-DRB5*01:01
			174.00		HLA-DRB1*04:05
			219.00		HLA-DRB1*04:01
Bet v 1	17	32	28.00	**ARLFKAFILDGDNLFP**	HLA-DRB3*01:01
			48.00		HLA-DRB1*03:01
			202.00		HLA-DRB1*15:01
			213.00		HLA-DQA1*05:01/DQB1*02:01
			261.00		HLA-DRB1*04:05
	142	155	15.00	ETLLRAVESYLLAH	HLA-DRB1*01:01
			23.00		HLA-DRB1*07:01
			38.00		HLA-DRB1*15:01
			95.00		HLA-DRB1*09:01
			120.00		HLA-DRB5*01:01
			173.00		HLA-DRB1*04:01
			250.00		HLA-DPA1*02:01/DPB1*01:01
	64	78	68.00	PFKYVKDRVDEVDHT	HLA-DRB3*01:01
			176.00		HLA-DRB1*03:01
	9	26	183.00	ETTSVIPAARLFKAFILD	HLA-DRB1*15:01
			272.00		HLA-DQA1*01:02/DQB1*06:02
	110	124	48.00	DGGSILKISNKYHTK	HLA-DRB1*11:01
			91.00		HLA-DRB1*15:01
			183.00		HLA-DRB5*01:01
			227.00		HLA-DRB1*08:02
			368.00		HLA-DRB1*12:01

Table 2. The immunodominant epitope selected from Pla or 3, Fra e 1, and Bet v 1 allergens via NetMHCII-2.2 server, according to their affinity. The selected epitopes for vaccine construct are bold.

Allergen name	Possition	Epitopes	Affinity(nM)	%Rank	Allele
Pla or 3	37	**LTPCLTYLRSGGAVA**	94.5	8.00	DRB1_0404
			23.2	1.90	DRB1_1501
			5.8	0.90	DRB1_0101
			211.2	6.50	DRB1_0802
			104.3	6.00	DRB1_1602
	10	ACLLLACMVATAPHA	375.1	0.07	DRB1_0403
			20.5	7.50	DRB3_0301
	84	SISGIQLGNAASLAG	105.6	9.50	DRB1_0901
			22.1	4.50	DRB1_1302
			7.0	1.30	DRB3_0301
	71	RQAACGCLKTASTSI	5.50	0.94	DRB1_0701
Fra e 1	32	SEFITGASVRLQCRD	7.2	1.40	DRB1_0101
			60.0	3.00	DRB1_0401
			72.5	10.00	DRB1_0701
			12.2	0.30	DRB1_0901
			80.9	4.50	DRB1_1602
			51.6	4.00	DRB3_0202
			23.9	6.00	DRB4_0103
	104	**LKFILNTVNGTTRTI**	24.7	8.50	DRB1_0101
			72.9	4.00	DRB1_0401
			473.2	0.40	DRB1_0403
			54.7	4.00	DRB1_0404
			218.3	7.00	DRB1_0802
			20.9	4.50	DRB1_1302
			6.2	0.40	DRB3_0202
			7.9	1.70	DRB3_0301
	75	KNEFCEITLLSSGRK	54.4	2.50	DRB1_0401
			30.8	7.50	DRB4_0103
			4.1	0.25	DRB5_0101
	15	QGQVYCDTCRARFIT	112.6	5.00	DRB1_0301
Bet v 1	142	**ETLLRAVESYLLAHS**	8.5	2.00	DRB1_0101
			133.4	7.50	DRB1_0401
			33.0	5.00	DRB1_0701
			70.5	1.20	DRB1_0802
			65.6	5.50	DRB1_0901
			15.9	2.50	DRB1_1001
			3.6	0.04	DRB1_1501
	61	EGFPFKYVKDRVDEV	2335.2	1.10	DRB1_0103
			60.5	2.50	DRB1_0801
			32.5	1.90	DRB3_0101

Table 2. Continued...

Allergen name	Possition	Epitopes	Affinity(nM)	%Rank	Allele
	17	ARLFKAFILDGDNLF	132.9	6.00	DRB1_0301
			54.7	2.50	DRB1_0401
			854.5	7.00	DRB1_0402
			732.1	3.00	DRB1_0403
			32.1	1.10	DRB1_0405
			30.6	4.50	DRB1_0701
			7.9	0.50	DRB1_1001
			21.2	1.70	DRB1_1501
			4.2	0.08	DRB3_0101
			35.7	1.60	DRB4_0101
	100	SNEIKIVATPDGGSI	29.3	1.50	DRB1_0404
			39.6	0.40	DRB1_0802

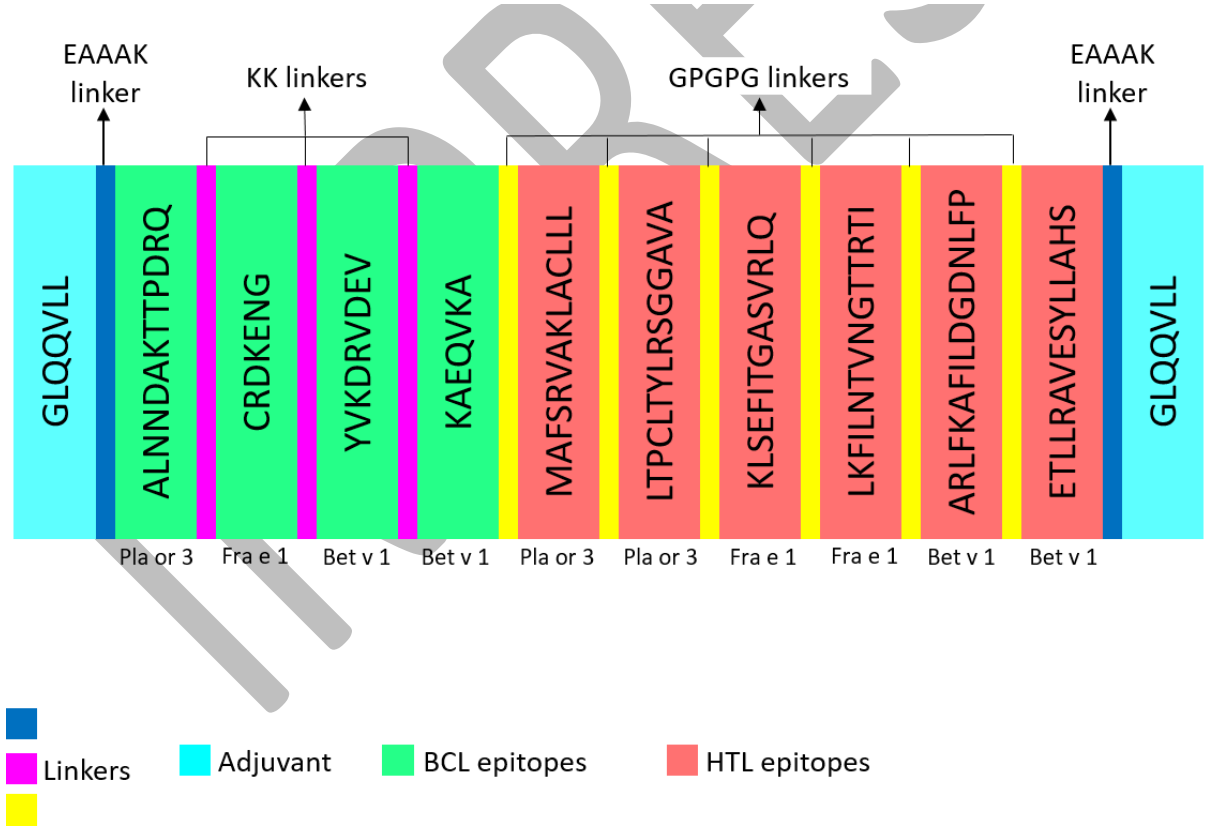

Figure 3. Schematic diagram of vaccine construct consists of TLR-4 agonist and high immunogenic epitopes of Pla or 3, Fra e 1, and Bet v 1 allergens, fused together by appropriate linkers (EAAAK, GPGPG, KK).

Table 1. Surface accessibility, hydrophilicity, flexibility, beta turn and antigenicity prediction score for each residue of B cell epitopes from Pla or 3, Fra e 1, and Bet v 1 allergens via IEDB-AR server.

Allergen name	B-cell epitope	Emini surface accessibility score for each residue	Parker hydrophilicity score for each residue	Karplus and Schulz flexibility score for each residue	Chou and Fashman beta turn score for each residue	Kolaskar and Tongaonkar antigenicity score for each residue
Pla or 3		threshold=1.000	threshold=1.525	threshold=0.983	threshold=0.984	threshold=1.069
	^{60}A	1.124	2.086	0.998	1.063	1.008
	^{61}L	2.528	2.7	1.007	1.049	1.006
	^{62}N	1.277	3.529	1.027	1.071	0.961
	^{63}N	2.528	3.529	1.049	1.071	0.961
	^{64}D	4.424	3.971	1.065	1.114	0.939
	^{65}A	3.97	6.029	1.071	1.167	0.89
	^{66}K	3.817	5.329	1.073	1.161	0.931
	^{67}T	3.817	5.757	1.067	1.147	0.944
	^{68}T	7.401	4.929	1.062	1.074	0.945
	^{69}P	6.409	5.486	1.054	1.12	0.938
	^{70}D	4.486	4.971	1.04	1.07	0.957
	^{71}R	3.14	4.529	1.019	1.027	0.979
	^{72}Q	1.089	3.986	0.99	1.06	1.051
Fra e 1		threshold=1.000	threshold=1.483	threshold=1.000	threshold=0.992	threshold=1.028
	^{44}C	1.207	3.186	0.973	1.019	1.031
	^{45}R	2.535	3.7	1.007	0.989	1.028
	^{46}D	2.354	6.014	1.038	1.127	0.96
	^{47}K	4.345	5.971	1.072	1.21	0.94
	^{48}E	3.705	7.2	1.092	1.249	0.862
	^{49}N	1.647	6.071	1.097	1.184	0.935
	^{50}G	1.188	5.386	1.085	1.113	0.941
Bet v 1		threshold=1.000	threshold=1.871	threshold=1.010	threshold=0.986	threshold=1.020
	^{67}Y	1.493	1.243	0.979	0.911	1.093
	^{68}V	3.377	1.543	0.993	1.034	1.061
	^{69}K	1.253	2.329	1.01	0.953	1.033
	^{70}D	1.336	2.943	1.019	0.939	1.075
	^{71}R	3.116	4.329	1.029	1.003	1.066
	^{72}V	1.157	4.329	1.03	0.946	1.022
	^{73}D	1.157	4.943	1.025	0.946	1.022
	^{74}E	0.804	3.814	1.012	1.01	1.013
	^{75}V	1.562	3.957	1.0	0.937	1.047
	^{130}K	1.729	3.971	1.015	0.797	1.028
	^{131}A	0.741	3.143	1.024	0.733	1.068
	^{132}E	1.996	2.843	1.029	0.771	1.079
	^{133}Q	1.008	3.671	1.029	0.794	1.034
	^{134}V	1.338	3.786	1.027	0.854	1.046
	^{135}K	1.545	4.3	1.036	0.904	1.026
	^{136}A	1.545	4.3	1.043	0.904	1.026

Physicochemical Properties of MEV

Physicochemical properties, including molecular weight, half-life, instability index, pI (theoretical isoelectric point value), and hydropathicity, are predicted by ExPASy ProtParam Tool (Table 4). The results confirm the vaccine construct's stability, hydrophilicity, and suitability for further testing.

Secondary Structure Prediction

PSIPRED was used to generate the secondary structure of the final vaccine construct and revealed the secondary structure composition as 47.3% α-helices, 9.6% β-strands, and 43.01% random coils. Graphical representation of the secondary structure features is shown in Figure 4, highlighting the structural integrity and flexibility of the construct.

3D Structure Modeling and Validation

The 3D structure of the vaccine was modeled using GalaxyTBM and refined with GalaxyRefine. PROCHECK, ERRAT, and ProSA-web were used to validate the models.

Five models have been developed based on the refinement of the predicted GalaxyWeb vaccine model. Model 1 had the best structural features, and the refined 3D model is shown in Figure 5. The model then was used for further validation (Figure 6).

The *Z* score of the model in ProSA-web was −3.5, indicating favorable residue interactions (Figure 6A). The amino acid residues of the model are located in 3 regions according to Ramachandran plot analysis: (1) 91.6% of residues were in the most favorable regions (expected to have over 90%), (2) the allowed region has a number of residues, and (3) no residues in disallowed regions (Figure 6B). The overall quality score of 80.58 in ERRAT confirming reliable structural accuracy (Figure 6C). Collectively, these validation scores confirm the structural stability of the predicted tertiary model, providing confidence for subsequent analyses.

Table 4. Physicochemical properties of the designed MEV were determined by ExPASy.

Multi-epitope vaccine properties	Value
Molecule weight (Da)	19 368.49
Half-life (mammalian reticulocytes, in vitro)	30 hours
Half-life (yeast, in vivo)	>20 hours
Half-life (*E coli*, in vivo)	>10 hours
Total number of negatively charged residues (Asp + Glu)	15
Total number of positively charged residues (Arg + Lys)	26
Grand average of hydropathicity (GRAVY)	−0.216
Instability index	24.33
Theoretical pI	9.77

Arg: arginine; Asp: aspartic acid; Glu: glutamic acid; Lys: lysine; MEV: multi-epitope vaccine; pI: isoelectric point.

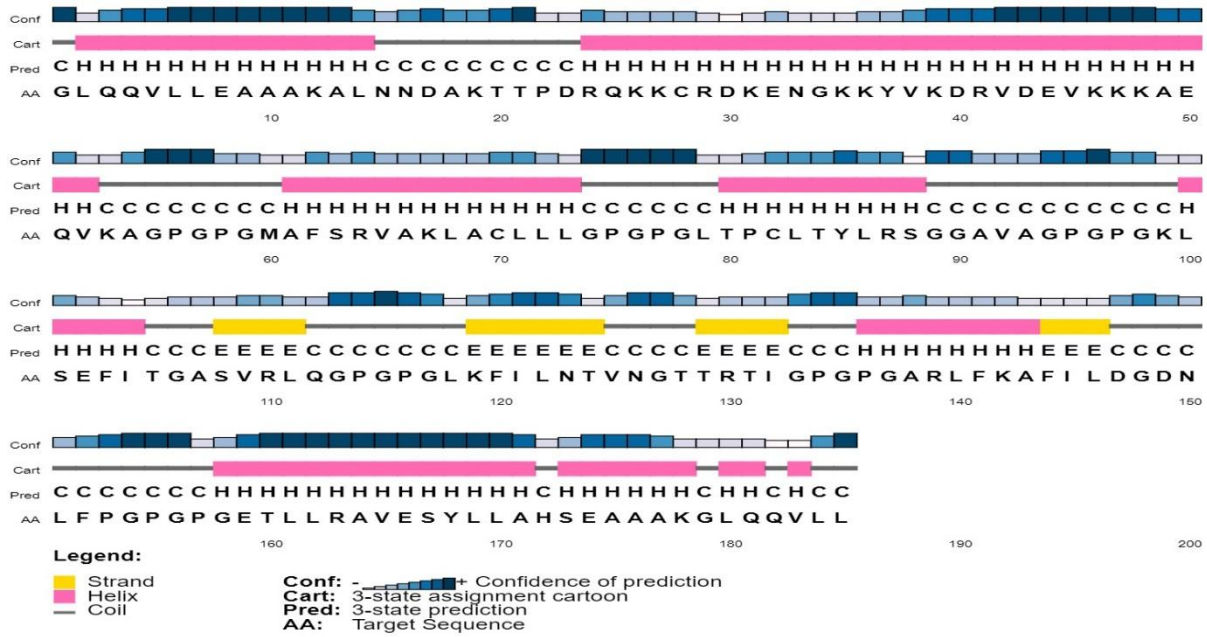

Figure 4. Graphical representation of the secondary structure features. The α-helix residues are shown in pink, the β-strand residues are shown in yellow, and the coil residues are shown in grey. The predicted secondary structure indicates that the final multi-epitope peptide vaccine constitutes 47.3% α-helix, 9.6% β-strand, and 43.01% coil.

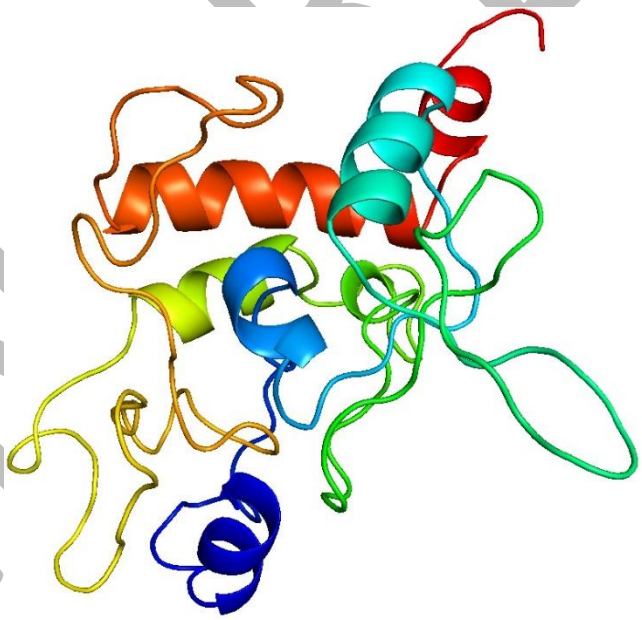

Figure 5. 3D structure of vaccine construct in cartoon model. The structure is represented in ribbon format using a rainbow color gradient, where blue corresponds to the N-terminal region and red to the C-terminal region. This color scheme illustrates the residue order along the polypeptide chain. α-helices, β-strands, and loops are shown in their respective secondary-structure conformations.

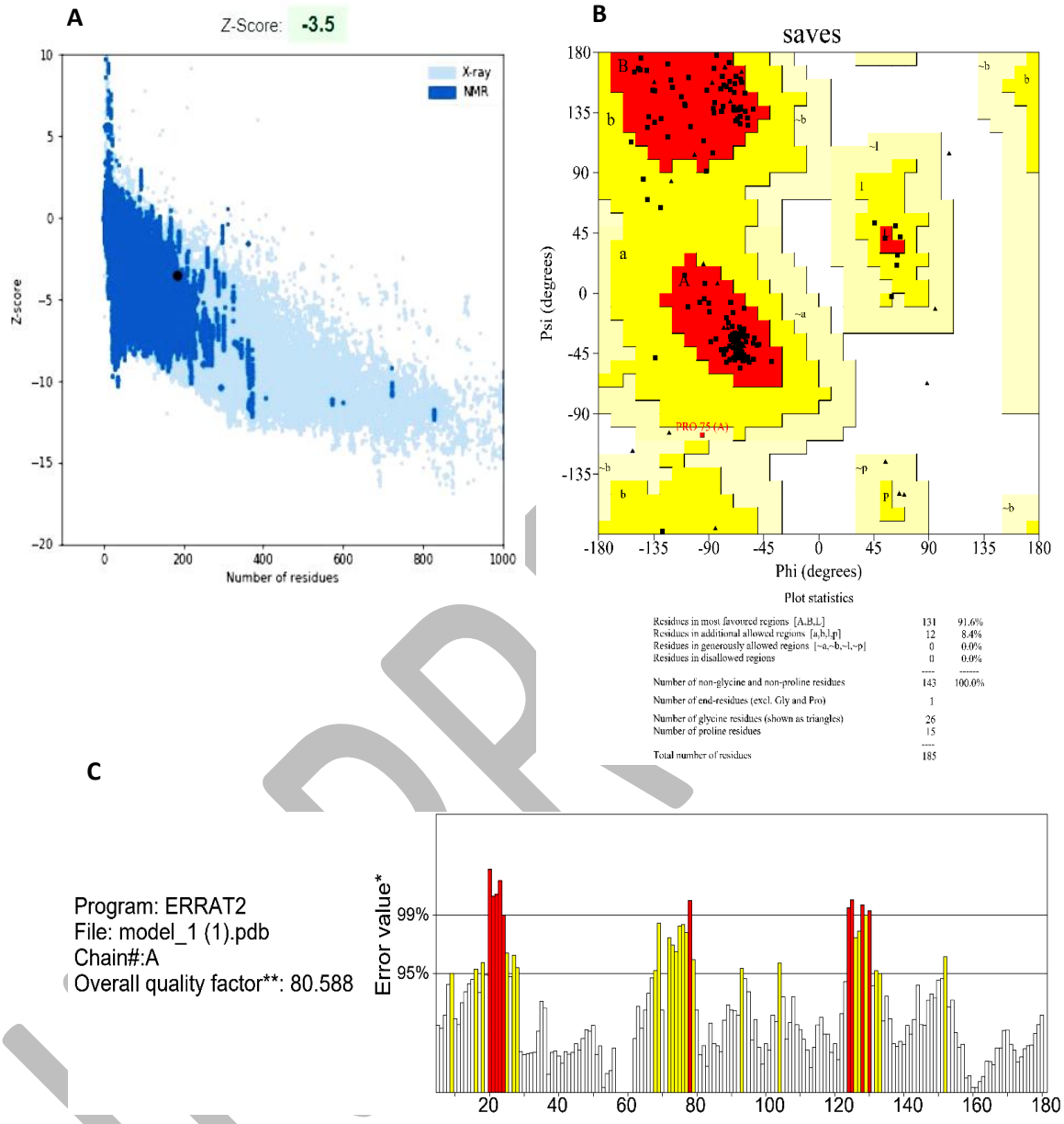

Figure 6. The validation of the vaccine construct. (A) ProSA-web analysis showing the overall quality of the predicted 3D model with a Z-score of −3.5, which falls within the range of experimentally determined structures of comparable size (light blue: NMR; dark blue: X-ray). (B) Ramachandran plot generated by PROCHECK indicating that 91.6% of residues are in the most favored regions (red), 8.4% in additionally allowed regions (yellow), and none in disallowed regions, confirming good stereochemical quality of the model. (C) ERRAT quality factor plot displaying an overall score of 80.58, indicating reliable non-bonded interactions. Yellow regions correspond to residues with acceptable error values (<95%), while red bars highlight segments with higher local error (>95%), often located in flexible or loop areas.

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Allergenicity, Antigenicity, and Toxicity Assessment

The ANTIGENpro server evaluates the antigenicity of the multi-epitope vaccine construct and assigns a score of 0.778, confirming strong antigenic potential. In addition, the vaccine was classified as non-allergenic by AlgPred. The vaccine must not have potential toxicity. ToxinPred analysis indicated no toxic peptides, confirming the vaccine's safety profile.

Molecular Docking Analysis

Docking studies using ClusPro revealed strong interactions between the vaccine construct and TLR-4. The best docked model was chosen based on the knowledge of binding sites between TLR4 (PDB ID: T43f) and the vaccine. The lowest-energy binding configuration had a score of −1042.6, indicating a stable interaction. Detailed interaction analysis is shown in Table 5, with the docked complex visualized in Figure 7.

Table 5. Analysis of the interaction between vaccine construct residues and TLR-4 and MD2 molecules in the docked complex.

MD2	MEV	Bond length, Å	TLR chain B	Vaccine	Bond length, Å
Arg90	Gln181	1.7	Lys560	Gln112	1.7
Val82	Glu174	2.7	Gln112	Asp17	2.2
Asn86	Val92	2.0	Asp580	Arg130	1.8
NA	Arg87	2.2	Glu485	Lys142	1.8
NA	NA	NA	Gln507	Glu159	2.1
NA	NA	NA	Ser482	NA	NA

Arg: arginine; Asn: asparagine; Asp: aspartic acid; Gln: glutamine; Glu: glutamic acid; Lys: lysine; MD2: myeloid differentiation factor 2; Ser: serine; TLR: Toll-like receptor; Val: valine.

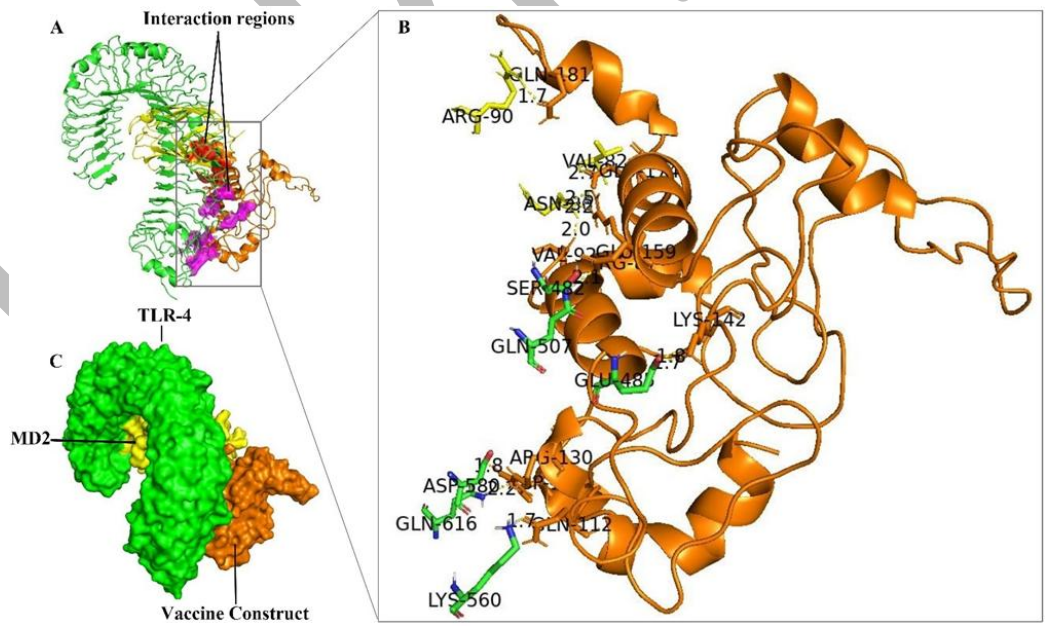

Figure 7. Docked complex of vaccine constructs with human TLR4 and MD2: (A) cartoon model of vaccine construct, interaction of MD2 and TLR-4 chain b with vaccine construct depicted with red and magenta colors, respectively. (B) The cartoon depictions of the complex are illustrated using the PyMol software. Interacting residues are labeled, and interatomic distances are provided in Table 5. (C) Space-filling model of vaccine construct in orange color, TLR4 receptor in green, and MD2 in yellow color.

The docking binding energy is −1042.6, indicating a strong and stable interaction. The docking scores of known TLR4 agonists, including LPS (PDB ID: 3FXI), monophosphoryl lipid A (MPLA; PDB ID: 2E59), and Eritoran (PDB ID: 2Z65), are −980, −1080, and −1020, respectively. When compared with the predicted affinity of the vaccine construct, it was found to be within the same range (Figure 8). This comparable energy profile suggests that the designed construct can effectively engage the TLR4 receptor complex, supporting its potential to activate innate immune signaling through TLR4-mediated pathways.

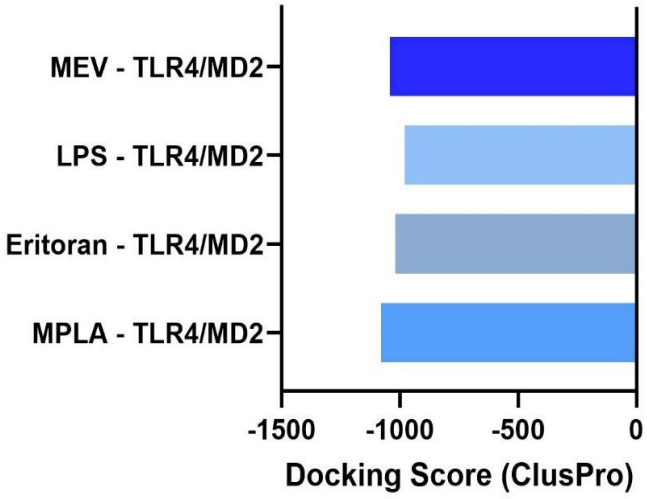

Figure 8. Comparative analysis of docking scores for the designed vaccine construct and known TLR4 agonists. The MEV–TLR4/MD2 complex exhibited a ClusPro docking score of −1042.6, which is comparable to the scores of established TLR4 ligands such as MPLA (−1080), Eritoran (−1020), and an LPS fragment (−980). All values fall within the typical range reported for high-affinity TLR4–ligand interactions (−900 to −1150). The close similarity of docking energies suggests that the designed construct forms a stable and potentially functional interaction with the TLR4/MD2 receptor complex.

DISCUSSION

Allergies remain a significant health challenge, affecting nearly one-third of the population in Western countries and causing considerable morbidity.[53] Current treatments, including allergen avoidance and symptomatic therapies, often provide only temporary relief.[54] Allergen-specific immunotherapy (AIT) offers a promising long-term solution but is associated with drawbacks such as high costs, variable efficacy, and potential adverse reactions.[55–57] On this matter, different types of allergy vaccines, including vaccines based on T- and B-cell epitopes, can be engineered. This study addresses these limitations by designing a novel multi-epitope vaccine targeting allergens from *Platanus orientalis* (sycamore), *Fraxinus excelsior* (ash), and *Betula pendula* (birch) using immunoinformatics approaches. Epitope vaccines, or synthetic peptide-based vaccines, are subunit vaccines made from peptides, in which peptides act as antigenic epitopes and cause immune responses.[58] The main goal of immunoinformatics studies is to increase the quality of experimental studies and have revolutionized vaccine design. In fact, the main advantage of in silico methods is to select fully analyzed vaccine candidates and work on these candidates in the next steps.[59]

The multi-epitope vaccine developed in this study combines immunodominant T-cell and B-cell epitopes from Pla or 3, Fra e 1, and Bet v 1 allergens. The inclusion of robust linkers (GPGPG, KK, and EAAAK) and the TLR-4 agonist adjuvant enhances its immunogenic potential while maintaining structural stability. The vaccine construct demonstrated several favorable properties: High antigenicity and TLR-4 binding affinity were confirmed through computational analyses, indicating the potential of the vaccine to elicit strong immune responses. The vaccine is non-allergenic and non-toxic, as predicted by AlgPred and

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ToxinPred. Physicochemical properties such as the instability index, half-life, and hydropathicity suggest that the vaccine is stable and hydrophilic, making it suitable for further experimental evaluation. The structural validation of the modeled vaccine construct demonstrated that its stereochemical and energetic parameters fall within the acceptable range for high-quality protein models. The ProSA *Z*-score (−3.5) was consistent with the scores of experimentally solved proteins of comparable length (−2 to −12), indicating a reliable folding pattern. The ERRAT overall quality factor of 80.59 is above the generally accepted threshold of 50, suggesting favorable non-bonded atomic interactions and minimal local errors. PROCHECK Ramachandran analysis showed 91.0% of residues in the most favored regions and 8.4% in allowed regions, exceeding the 90% benchmark commonly used for well-refined models. In allergic reactions, specific IgE antibodies are produced against epitopes of allergens, causing the symptoms. Increased production of T_H2 cell cytokines such as IL-4 and IL-13 can induce IgE synthesis in B cells. In order to prevent the production of IgE, it is possible to help the improvement of allergen-specific IgG and specific IgA by a vaccine as an immunotherapy approach.[60] Thus, T-cell epitopes that are presented by MHC-II molecules can induce specific T-cell immune responses, producing the subclasses of immunoglobulin other than IgE.

Peptide-based vaccines offer significant advantages, such as precise targeting of immune responses, ease of synthesis, and reduced risk of adverse reactions. These features position the designed vaccine as a promising candidate for combating tree pollen allergies. However, there are also drawbacks, such as false-positive and false-negative reactions, MHC restriction, low immunogenicity, the need for adjuvants, etc.[61] This research demonstrates the power of immunoinformatics tools in vaccine design, streamlining the identification and validation of immunodominant epitopes while minimizing experimental costs. By focusing on conserved allergenic regions, the vaccine reduces the likelihood of resistance and cross-reactivity, enhancing its applicability across diverse populations. The use of TLR-4 agonists as adjuvants further strengthens the vaccine's design by linking innate and adaptive immunity. TLR-4 activation triggers pro-inflammatory cytokine production via the NF-κB signaling pathway, promoting robust immune responses. The docking analysis confirmed stable interactions between the vaccine construct and TLR-4, underscoring its potential efficacy.

The designed multi-epitope construct has the potential to modulate both humoral and cellular immune responses by simultaneously engaging B and T lymphocytes. The selected epitopes include sequences capable of activating helper T cells as well as B-cell epitopes that can induce antibody production, thereby promoting strong immune activation. The inclusion of the TLR4 agonist adjuvant further amplifies this response by stimulating innate immune receptors and enhancing antigen presentation. TLR4 engagement activates the NF-κB signaling pathway, leading to the secretion of pro-inflammatory and T_H1-associated cytokines such as IFN-γ and IL-12.[62,63] These cytokines promote a shift from T_H2-dominated allergic responses toward a T_H1-biased profile, which contributes to reduced IgE production and increased allergen-specific IgG and IgA antibodies.[64] Although the vaccine structure may not induce a robust T_{reg} response comparable to natural tolerance mechanisms, it is expected to enhance the T_H1 axis and suppress IgE-driven hypersensitivity. Nevertheless, these mechanisms require further validation, particularly via in vitro assays assessing cytokine secretion and T-cell polarization.

It is worth noting that the linkers have several advantages in epitope-based vaccines, including improving stability, increasing biological activity, increasing expression efficiency, preventing the production of neo-epitopes, facilitating MHC-II binding epitope presentation, and the process of the immune system.[65,66] The EAAAK linker is an α-helix-forming linker; thus, its structure is rigid and stable, with intrasegmental hydrogen bonding and a densely packed backbone.[67] The GPGPG linkers are capable of inducing T helper cells and serve as a tool in breaking the junctional immunogenicity and restoring the immunogenicity of each epitope.[68]

Using TLR-ligand-based adjuvants in vaccines helps to activate immune cells, induce protective immune responses, and link innate and adaptive immunity.[69] TLRs are a class of innate immune receptors that have a major role in recognizing PAMPs (pathogen-associated molecular patterns). The binding of PAMPs to the domain of TLRs, which are extracellular, establishes signaling pathways leading to the stimulation of innate immune responses.[70] TLR-4 is a transmembrane protein and part of a group of proteins called the Toll-like receptor family, belonging to the pattern recognition

receptor (PRR) family. The outcome of its activation will be the intracellular NF-κB signaling pathway and the induction of pro-inflammatory cytokines involved in the activation of the innate immune system.[71] TLRs are responsible for recognizing PAMPs, mainly expressed on pathogens, and inducing the production of cytokines required for effective immunity. Most myeloid cells express TLR-4 in addition to high levels of CD14, facilitating TLR-4 activation by LPS (lipopolysaccharide).[72] Stimulating by LPS induces a series of interactions with several proteins making up the TLR4 complex. Binding of LPS to the LBP protein initiates LPS recognition, and the complex transfers LPS to CD14. The binding of CD14 to the LPS-LBP complex enhances the transfer of LPS to the MD-2 protein, which is associated with the extracellular domain of TLR4. LPS binding will increase the dimerization of TLR4/MD-2. Changes in conformation of TLR4 induce the intracellular adapter proteins containing the TIR domain recruitment necessary for the activation of the downstream signaling pathways.[73] Comparative docking analysis provided further insight into the potential interaction strength of the designed vaccine construct with the TLR4/MD2 complex. The calculated binding energy was found to be within the same range as that of well-characterized TLR4 agonists such as MPLA and Eritoran, as shown in Figure 8. This close similarity indicates that the vaccine construct is capable of establishing a stable and biologically relevant interaction with the receptor complex. Considering that TLR4 agonists such as MPLA are known to promote T_H1-skewed immune responses through MyD88- and TRIF-dependent signaling,[74] the comparable binding affinity of our construct suggests a similar potential for triggering innate immune activation. Such engagement may enhance antigen presentation and cytokine production, ultimately facilitating a balanced adaptive immune response.[75] The comparable docking energies also reinforce the structural integrity and surface accessibility of the adjuvant-linked epitope regions within the construct, which are crucial for receptor recognition and downstream immune stimulation.

The designed MEV represents a promising candidate for the development of next-generation allergen-specific immunotherapy. In contrast to conventional approaches such as subcutaneous or sublingual allergen immunotherapy that rely on crude allergen extracts, peptide-based vaccines are composed of epitopes with minimized allergenic potential and improved safety profiles. This design enables the induction of immune tolerance through precise modulation of allergen-specific T- and B-cell responses while reducing the risk of IgE-mediated side effects. Regarding practical translation, several delivery routes could be considered, including sublingual, intranasal, and transcutaneous administration. Particularly, sublingual and intranasal immunization pathways activate mucosal immune tissues, promoting the generation of allergen-specific IgG and IgA antibodies that compete with IgE for allergen binding. These routes are non-invasive, enhance patient compliance, and have demonstrated efficacy in other allergy vaccine studies. Therefore, with appropriate formulation and adjuvant optimization, the designed epitope-based construct may serve as a safer, more standardized, and patient-friendly alternative to current allergen immunotherapy methods. Cross-reactivity among homologous allergens represents a primary challenge in the design and efficacy of allergen-specific immunotherapy.[76] Many tree pollen allergens, particularly Pla or 3 from *Platanus orientalis* and Bet v 1 from *Betula pendula*, share structural and sequence homology that can lead to IgE cross-reactivity and polysensitization in allergic individuals.[77] This phenomenon complicates diagnosis and treatment, as patients sensitized to one allergen may exhibit clinical reactivity to several unrelated pollen sources due to shared epitopes. The current MEV integrates immunodominant regions from Pla or 3, Fra e 1, and Bet v 1, allowing for a more comprehensive immune modulation across related allergen families. By focusing on conserved but immunogenic epitopes and excluding highly IgE-reactive sequences, this approach may reduce unwanted cross-linking of IgE and mitigate adverse reactions associated with extract-based therapies. Furthermore, the rational inclusion of epitopes from cross-reactive allergens contributes to a broader, yet safer, desensitization profile suitable for patients exhibiting multi-pollen sensitivities.

To contextualize the novelty of our designed construct, we compared its key structural and immunological characteristics with previously reported multi-epitope vaccine studies (Table 6). Unlike earlier approaches that primarily incorporated mixed T-cell epitopes without structural reinforcement, the present vaccine design integrates immunodominant T- and B-cell epitopes from three major allergenic tree pollen proteins and employs a dual-terminal RS04 TLR-4 agonist to enhance innate and adaptive immune

activation. The selection of EAAAK, GPGPG, and KK linkers contributed to improved domain separation and preserved epitope immunogenicity. Notably, the vaccine exhibited higher predicted antigenicity, complete absence of allergenicity and toxicity, and demonstrated a stronger binding affinity to TLR-4 (−1042.6) compared to previously reported constructs, suggesting a potentially more efficient immunomodulatory profile.

Table 6. Comparative analysis of multi-epitope allergy vaccine studies.

Study	Target allergen(s)/ disease	No. and type of epitopes used	Linkers used	Adjuvant used	Antigenicity/ allergenicity outcome	Docking target and score	Notes/distinction
Present study	Pla or 3, Fra e 1, Bet v 1 (Tree-pollen allergy)	HTL + B-cell epitopes (Total length: 186 aa)	EAAAK, GPGPG, KK	RS04 (TLR-4 agonist)	Antigenic; Non-allergenic, Non-toxic	TLR-4: −1042.6 (stable, strong binding)	Novel integration of three major tree allergens with dual-terminal TLR-4 agonist strategy
Samad et al, 2022	Respiratory allergy (general allergen vaccine framework)	Mixed T-cell epitopes	Not specified clearly	Not specified	Antigenic; Non-allergenic	Not evaluated against TLR-4	Multi-epitope design reported; lacks TLR-based immunostimulation
Arya and Arora, 2021	Viral vaccine model (immune-activating multi-epitope construct)	CTL + HTL epitopes	Short Gly-rich linkers	Freund-type adjuvant (in vivo)	Antigenic; Non-allergenic	Docking performed to MHC but not TLR-4	Demonstrates experimental validation potential; not allergy-focused
Rahman et al, 2024 (Yak Milk Allergy Vaccine)	Food allergy (IgE-mediated)	B-cell + T-cell epitope fusion	Standard peptide linkers	Adjuvant included in silico	Antigenic; Non-allergenic predicted	Docking performed; receptor not TLR-4	Closest model to allergy field, but lacks structural refinement level of present study

CTL: cytotoxic T lymphocyte; HTL: helper T lymphocyte; IgE: immunoglobulin E; MHC: major histocompatibility complex; TLR: Toll-like receptor.

Despite promising results, this study has several limitations. Although in silico methods provide valuable insights, in vitro and in vivo experimental validation is essential to confirm the efficacy and safety of the vaccine in biological systems. There are many potential variabilities in human populations and MHC polymorphism that may affect the efficacy across different genetic backgrounds, necessitating broader population studies. In addition, this study has not focused on different adjuvant efficacy. Although TLR-4 was chosen for its immunostimulatory properties, additional studies comparing different adjuvants could identify optimal formulations. Future in vitro experiments should include peptide synthesis, IgE-binding assays using sera from allergic patients, basophil activation tests, and cytokine profiling to assess T_H1/T_H2 polarization and potential induction of IL-10–secreting T_{regs}. In parallel, in vivo studies using animal models are required to evaluate the ability of the vaccine to reduce allergen-specific IgE, enhance IgG2a/IgA production, and suppress allergic inflammation. Additionally, exploring alternative adjuvants and delivery systems, such as nanoparticles, may further enhance the vaccine's effectiveness.

Although the present study demonstrates promising immunological and structural properties for the designed

multi-epitope vaccine, it is important to acknowledge the inherent limitations of in silico methodologies. Epitope prediction algorithms rely on available training datasets and model assumptions, which may not fully capture the complexity of human immune responses or HLA polymorphism across diverse populations. Similarly, protein structure modeling and refinement tools generate the most energetically favorable conformations, yet the actual tertiary structure of the expressed construct may differ under physiological conditions due to folding dynamics, post-translational modifications, or molecular crowding. Moreover, docking simulations evaluate receptor–ligand interactions under simplified static conditions and therefore may not entirely reflect the dynamic nature of TLR-4 engagement in vivo. Consequently, while computational predictions provide valuable preliminary insights and significantly reduce exploratory experimental burden, in vitro and in vivo validation—including protein expression, antigen presentation assays, cytokine profiling, and allergenicity assessment—are essential to confirm the vaccine's safety, stability, and immunological efficacy.

Future experimental validation will be required to confirm the structural stability, immunogenicity, and safety of the designed multi-epitope vaccine. In the next phase, the vaccine sequence will be synthesized and expressed in an appropriate expression system (e.g., *E coli* BL21). Purified protein will then be evaluated through in vitro assays, including ELISA-based IgE binding analysis, lymphocyte proliferation assays, cytokine profiling, and basophil activation tests to assess allergic reactivity and T-cell responses. Following these tests, in vivo studies in animal models of allergic sensitization (such as murine models) will be conducted to examine immune polarization (T_H1/T_H2 balance), antibody isotypes (IgE/IgG/IgA), and clinical symptom reduction. These steps will help verify whether the predicted immunological effects translate into physiological efficacy and safety.

In this study, we tried to design a multiple-epitope vaccine targeting common tree pollen allergens. The integration of T-cell and B-cell epitopes, adjuvants, and optimized linkers highlights the advantages of immunoinformatics in advancing vaccine development. While experimental validation is necessary, this work lays a strong foundation for developing safe and effective allergy vaccines, addressing a growing public health challenge.

STATEMENT OF ETHICS

Ethical approval is not applicable. This study is a bioinformatics analysis based on publicly available datasets and did not involve any direct experiments on human participants or animal subjects.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

The authors declare that no artificial intelligence (AI) tools, large language models (LLMs), or AI-assisted technologies were utilized in the writing, editing, or preparation of this manuscript.

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