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Original Article

Immunoinformatic approach to the design of a novel multi-epitope vaccine against *Leishmania major* fused to human IgG-Fc

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Abstract

Background and purpose: Cutaneous leishmaniasis poses significant health and socioeconomic challenges, making vaccine development a top priority, especially in endemic regions. Cysteine proteases, KMP-11, and HASPB proteins are promising candidates for leishmaniasis vaccine development owing to their immunogenic properties and capacity to provoke robust immune responses, as evidenced by different investigations. This study aimed to design a recombinant chimeric protein (MEV-Fc) vaccine using multi-epitopes from these *Leishmania major* proteins.
Experimental approach: The antigens were subjected to immunoinformatic prediction and screening of HTL, CTL, and B-cell epitopes. The multi-epitope protein was designed with significantly high-scoring epitopes and suitable linkers. Natural adjuvants were then added to enhance immunogenicity. Vaccine potency was innovatively improved by covalently fusing human IgG1 Fc with multi-epitope protein. To investigate how the MEV-Fc vaccine interacts with Toll-like receptors, molecular docking, multi-scale normal mode analysis simulation, and computational immune simulation were employed to study humoral and cellular immune responses.
Findings/Results: The results demonstrated the vaccine's antigenicity, stability, and nontoxicity. The structural validation confirmed the accuracy of the 3D models, indicating robust interactions with TLR2 and TLR4, with binding free energies of -1269.9 and -1128.7 (kcal/mol), respectively. Immune simulation results showed significant increases in IgM and IgG antibody levels following three vaccinations, along with enhanced activation of B cells, helper T cells, and cytotoxic T lymphocytes.
Conclusion and implications: These findings provide novel insights for developing effective candidates for cutaneous leishmaniasis vaccines. However, laboratory experiments are necessary to evaluate its protective effects.

Keywords: Adjuvant; IgG-Fc; Immunodominant epitopes; *Leishmania major*; Subunit vaccine.

INTRODUCTION

Leishmaniasis is a vector-borne infection of different species of the *Leishmania* genus, a dimorphic intracellular parasitic protozoan. The World Health Organization (WHO) has classified this disease as a neglected tropical disease, highlighting its significant impact on health and communities, resulting in a substantial economic burden. Cutaneous leishmaniasis (CL) is the predominant form of the disease and mostly affects the skin, resulting in scarred lesions. About 95% of CL cases are found in the Americas, the Middle East, the Mediterranean basin, and central Asia.

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Globally, the number of new cases is believed to range from 600,000 to 1 million annually, with only around 200,000 cases reported to the WHO. The *Leishmania major* species is responsible for many cases of cutaneous leishmaniasis in Iran (1,2).

Regrettably, despite numerous studies on leishmaniasis, a definitive cure for this zoonotic disease in humans remains elusive. Pentavalent antimony (Sb^{5+}), pentamidine, miltefosine, and amphotericin B are some of the drugs used to combat the disease, but their high cost and adverse effects make their prescriptions uncertain (3,4).

Developing a vaccine for CL would eventually be one of the most efficient methods for managing or possibly eradicating the disease (5). Analysis suggests that a highly efficient vaccination against CL, with a 70% effectiveness rate and 10 years of protection, could potentially prevent between 41,000 and 141,000 CL cases in Latin America (6). Developing an effective vaccination against CL is challenging because of the intricate interplay between the *Leishmania* parasites and the host immune system. Nevertheless, it is worth noting that individuals who have naturally recovered from a CL infection often develop long-lasting antibodies against the specific species that caused the initial infection. This implies that, in principle, developing a vaccine for CL should be feasible. This is also reinforced by the evidence that 'leishmanization' does protect CL. Safety concerns have led to an abandonment of the practice of leishmanization. Clinical trials are currently evaluating several vaccines, but there are currently no approved vaccines for the treatment of human leishmaniasis on the market (7).

Scientists in several fields collaborate closely to design and develop safe and effective vaccines. An optimal vaccination for leishmaniasis should induce durable immunity and, most importantly, promote a well-balanced Th1 and Th2-mediated immune response (8). Considering these challenges, peptide vaccines provide a promising strategy for combating leishmaniasis. They not only elicit potent immune responses but also effectively address the safety issues with live vaccinations (9). Thus, peptide-based interventions offer a viable and safer alternative for managing the devastating impact of leishmaniasis. Immunoinformatics-based methods are a relatively recent strategy that mainly utilizes computational tools to predict a structurally stable and immunogenic peptide-based multi-epitope vaccine in a short amount of time (10). This approach can be employed to design a novel vaccine against leishmaniasis.

Prior research has investigated many surface and intracellular proteins as potential immunodominant antigens for combating leishmaniasis. Among the proteins under investigation, cysteine proteases serve as one of the crucial virulence factors for parasites in mammalian hosts and are promising targets for vaccine development against leishmaniasis (11,12). Kinetoplastid membrane protein-11, or KMP-11, is an immunogenic protein found in all kinetoplastid protozoa and a possible vaccine candidate due to its ability to raise IL-10 levels in mouse models (13). Hydrophilic acylated surface protein B (HASPB) is a Leishmania membrane protein suitable for developing vaccines. It has a high level of immunogenicity, leading to an immediate rise in the production of antibodies against it in the blood of individuals with CL and visceral leishmaniasis (14). The first human trial of ChAd63-KH, which is a replication-defective simian adenovirus expressing a novel synthetic gene encoding the Leishmania proteins KMP-11 and HASPB, has recently shown that it is safe and induces cytokine-producing $CD8^+$ T cells (NCT02894008, NCT03969134) (15). These proteins' ability to trigger immune responses positions them as promising candidates for leishmaniasis vaccine development.

Utilizing Fc fragment fusion protein technology is a very efficient method to enhance the duration of protein and peptide medications inside the body. Important biological molecules such as cell receptors, cytokines, enzymes, and peptide antigens identified in harmful microbes can be conjugated with Fc regions. This enhances the stability of these molecules inside the body and prolongs their lifespan amplifying their impact (16,17). According to research conducted by Alleva *et al*. the fusion of AKS-452 with the Fc fragment resulted in significantly elevated

levels of neutralizing antibodies in mice compared to when Fc was not used. AKS-452 is a biologically engineered vaccine combining an Fc fusion protein of the SARS-CoV-2 viral spike protein receptor binding domain antigen with human IgG1 Fc (SP/RBD-Fc). Clinical development of this vaccine against SARS-CoV-2 is currently underway. The primary goal of the AKS-452 Fc moiety is to augment immunogenicity through two mechanisms: facilitating the uptake of the SP/RBD Ag *via* FcγRs on antigen-presenting cells and prolonging exposure through FcRn recycling (18). The favorable safety and immunogenicity characteristics observed in this phase I study (NCT04681092) provide strong justification for advancing this vaccine to phase II of development (19).

Prior research has shown that multi-epitope vaccines (MEVs) for leishmania, developed by anticipating crucial components recognized by the immune system, provide a certain degree of immunological protection. Nevertheless, there has been little exploration of the potential of using peptide vaccines containing multi-epitope molecules fused to IgG-Fc for combating Leishmania infections. Here, we focused on designing a multi-epitope-based subunit vaccine against *L. major* using three highly immunogenic antigens. Important components of several immunological responses, such as cytotoxic T-lymphocyte (CTL), helper T-lymphocyte (HTL), B cells, RS09 (a synthetic Toll-like receptor-4 (TLR-4) agonist peptide), Pan HLA-DR epitope (PADRE), and human IgG-Fc, are included in this multi-epitope peptide. We hypothesize that conducting an experimental assessment might provide a groundbreaking and immunogenic vaccine that has the potential to provide immunity against leishmaniasis.

MATERIALS AND METHODS

Selection and sequence retrieval of L. major antigenic proteins

The amino acid sequences of the main *L. major* antigens, specifically Cpa (Q4QD68), HASPB (Q25326), and KMP-11 (E9AF45), were retrieved from the UniProt (Universal Protein Resource) database (https://www.uniprot.org/uniprot/). To assess the antigenic characteristics of these three proteins, we used the VaxiJen server v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with a threshold value set at 0.5 (20). As previously mentioned, these proteins have been identified as immunogenic, highly conserved, and promising candidates for vaccine development based on past immunological investigations using animal models or computational *in-silico* prediction approaches (13,14).

Prediction of CTL, HTL, and linear B-cell epitopes

We used the IEDB (Immuno Epitope Database and Analysis) MHC I server (21), available at http://tools.iedb.org/mhci/, to predict the CTL epitopes for the selected *L. major* antigen. We used the IEDB-recommended epitope predictor 2023.09 (NetMHCpan EL 4.1) as the prediction method. In addition, we used the Class I Immunogenicity tool (http://tools.iedb.org/immunogenicity/) to predict the immunogenicity. The immunogenicity of a peptide MHC complex can be predicted by this tool using amino acid properties and their position within the peptide. For further analysis, we chose CTL epitopes with the lowest percentile rank (percentile rank of < 3%) and an immunoscore greater than zero. We used the IEDB MHCII server 2, available at http://tools.iedb.org/mhcii/, to predict HTL epitopes for the selected antigen. The IEDB-recommended epitope predictor (NetMHCpan EL 4.1, version 2023.09) was used for our prediction methods. We chose the epitopes with the lowest percentile rank (percentile rank of < 3%). The IFN epitope server (http://crdd.osdd.net/raghava/ifnepitope/) was also used to test these epitopes for their ability to trigger a Th1-type immune response, which is marked by the production of interferon-gamma (IFN-γ) (22). The B-cell epitopes of the screened *L. major* proteins were predicted using the ABCpred server (http://crdd.osdd.net/raghava/abcpred/) and BepiPred 2.0 (https://services.healthtech.dtu.dk/services/BepiPred-2.0/) (23,24). The threshold in these screenings was set at 0.80 and 0.6, respectively. The epitopes were screened

individually based on their anticipated antigenicity using the Vaxijen 2.0 server, allergenicity using the AllergenFP v1.0 server (https://ddg-pharmfac.net/AllergenFP/) (25), and toxicity using ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) (26). The epitopes predicted to be antigenic, non-allergenic, and non-toxic were considered immunodominant epitopes to construct the MEV.

Construction of the MEV-Fc

The vaccine subunit was constructed by incorporating adjuvants, CTL, HTL, and B-cell epitopes joined together by particular linkers to provide sufficient spatial separation of epitopes *in vivo*. The EAAAK linker was used to connect the adjuvants and CTL. The intra-CTL, intra-HTL, and B-cell epitopes were connected using AAY, GPGPG, and KK, respectively. These linkers help prevent junctional immunogenicity, maintain the structural integrity of each epitope, and optimize their presentation to the immune system. The adjuvants employed to improve the immunogenicity of the vaccine construct were RS09 and the PADRE peptide. To enhance the immunogenicity of the vaccine and prolong its duration in the body, we included the sequence of the human immunoglobulin IgG Fc fragment (UniProt ID: P01857) by joining it to the C-terminus of the MEV using the KK linker. Consequently, we obtained the complete sequence of the vaccine construct.

Evaluation of physical and chemical properties of the vaccine

We used bioinformatic analysis tools to evaluate the properties of MEV-Fc. Initially, we used the ProtParam tool from the ExPASy database, accessible at http://web.expasy.org/protparam/, to analyze the physicochemical characteristics of the vaccine. After that, we performed immunogenicity analyses using the VaxiJen v2.0 server and the AntigenPro module of the SCRATCH protein predictor (27). The SOLpro server, accessible at http://scratch.proteomics.ics.uci.edu/ was then used to predict solubility. The server exhibited an overall accuracy of 74.15% (28). Ultimately, we conducted allergenicity and toxicity analyses with AllergenFP v1.0 and the ToxinPred server, respectively.

Structure prediction and validation of the vaccine

We applied the PSIPRED 4.0 server (http://bioinf.cs.ucl.ac.uk/psipred/) to predict the secondary structural elements of the final vaccine, and we used the online web server I-TASSER to predict the tertiary structure (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The I-TASSER is an automated methodology with a hierarchical protocol for protein structure prediction and structure-based function annotation. I-TASSER starts with the target protein's amino acid sequence and employs multiple threading alignments and iterative structural assembly simulations to generate full-length atomic structural models, subsequently refining them at the atomic level (29,30). The refined model was acquired from the GalaxyRefine server. The objective of protein structure refinement is to enhance the accuracy of template-based protein models through conformational sampling, ultimately bringing them closer to their natural state (31). To evaluate the accuracy of the built models, we used the ProSA web server (https://prosa.services.came.sbg.ac.at/prosa.php), the Ramachandran plot, and the ERRAT server, accessible at https://saves.mbi.ucla.edu/. These methods aid in assessing the correctness and quality of the protein's three-dimensional structure.

Molecular docking of MEV-Fc with immune receptors (human TLRs-2 and 4)

TLR are crucial components of cellular immune responses that detect small molecular motifs conserved within a class of microbes, called pathogen-associated molecular patterns, and trigger innate immune responses against infections. Research revealed that Leishmania components directly activated TLR-2. Other experimental studies have shown that the absence of TLR-4 leads to increased parasite proliferation and slower healing of skin lesions caused by *L. major* infections. This suggests that TLR-4 plays a possible role in promoting immunity against Leishmania (32).

The ClusPro web server was used to conduct vaccine-receptor docking to assess the binding affinity between the vaccine and the TLR-2 and TLR-4 receptors. Next, we analyzed the interaction surfaces of the complexes obtained using PDBsum, accessible at http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/. Ultimately, we used PyMOL software to generate visual representations of the complexes and conducted analysis using Ligplot+ software.

Molecular dynamics simulations

In this study a molecular dynamic simulation was conducted to assess the stability of our most optimal complex, *i.e.* TLR with the constructed vaccine, using the iMOD server (https://imods.iqf.csic.es/). This online server utilizes enhanced normal mode analysis (NMA) to assess several dynamic attributes of biomolecules, such as protein flexibility and stability, through an intra-atomic force field. Normal mode analysis is a computational technique to study the vibrational movements of a system (a protein for instance) near its equilibrium, revealing its inherent dynamic behaviors. This research focused on analyzing several metrics, such as main chain deformities, B factor, eigenvalues, covariance factor, and elastic network model, to evaluate protein-protein complexes generated through *in-silico* methods (33).

Immune simulation

We used the C-ImmSim server, available at https://kraken.iac.rm.cnr.it/C-IMMSIM/index.php, to forecast the capacity of MEV-Fc to induce the generation of specific antibodies and diverse cytokines by immune cells. This server can predict the immunological response of B lymphocytes and T lymphocytes, including Th1 and Th2 cells (34).

Our simulation accounted for the suggested minimum interval of 28 days between the first and subsequent doses of most vaccinations (35). Hence, we established the simulation settings with the following steps. Three injections, with a 28-day interval between each injection; a random seed value of 12,345; a simulation volume of 50; and a total of 1,050 simulation steps. The other settings were maintained at their default levels.

Codon adaptation and in-silico cloning

To assess the vaccine's high-level expression in *E. coli* strain K12, the vaccine sequence was codon optimized using the Java Codon Adaptation Tool (JCAT; http://www.jcat.de/). In addition, the optimized cDNA sequence generated by the JCat tool was modified to include restriction sites for the enzymes *Xho*I and *Nde*I. This modification was made to aid in the process of cloning. The modified sequence, containing restriction sites, was then incorporated into the *E. coli* pET28-a(+) plasmid vector using the restriction cloning module of the SnapGene software.

RESULTS

Selection and construction of immuno-dominant epitopes

The amino acid sequences of three *L. major* proteins, namely Cpa, HASPB, and KMP-11, were obtained from the UniProt database in FASTA format. These sequences were then analyzed to design a MEV for leishmaniasis. These proteins were chosen based on prior research that identified them as promising vaccine candidates, either through evaluation in experimental animal models or in-silico prediction methods. In line with the data retrieved *via* literature mining, the VaxiJen server's antigenicity analysis confirmed that all the selected proteins can serve as potential antigens (Table 1).

Table 1. Antigenicity score of the selected proteins.

Protein	UniProt ID	VaxiJen score
Cpa	Q4QD68	0.6795 (Probable antigen)
HASPB	Q25326	1.2205 (Probable antigen)
KMP-11	E9AF45	0.6018 (Probable antigen)

Table 2. Final selection of HTL, CTL, linear B-cell epitope.

CTL epitopes						
Epitope sequence	**Protein**	**Percentile rank (IEDB MHC I server)**	**Immunoscore (class I immunogenicity tool)**	**Allergenicity (AllergenFP servers)**	**Toxicity (ToxinPred server)**	**Antigenicity (VaxiJen server)**
GEFGARISGY	Cpa	0.05	0.16606	Non-allergen	Non-toxic	2.3423
FLFAIVVTI	Cpa	0.11	0.30335	Non-allergen	Non-toxic	0.8731
RAKPPYWIV	Cpa	0.01	0.16926	Non-allergen	Non-toxic	0.6363
CPKEDGHAP	HASPB	1.5	0.13441	Non-allergen	Non-toxic	1.4560
DPKDDGFTP	HASPB	2.6	0.1273	Non-allergen	Non-toxic	2.3719
KMHEHSEHFK	KMP-11	0.07	0.1716	Non-allergen	Non-toxic	0.7862
TTYEEFSAKL	KMP-11	0.14	0.06234	Non-allergen	Non-toxic	0.6506
EMKEHYEKF	KMP-11	0.05	0.01727	Non-allergen	Non-toxic	0.6295
HTL epitope						
Epitope sequence	**Protein**	**Percentile rank (IEDB MHC II server)**	**IFN-γ inducer score (IFNepitope server)**	**Allergenicity (AllergenFP servers)**	**Toxicity (ToxinPred server)**	**Antigenicity (VaxiJen server)**
VDNFIASAHYGRFKE	Cpa	0.36	0.0755	Non-allergen	Non-toxic	0.9505
MQTAYFLNTHNPHAH	Cpa	0.35	4	Non-allergen	Non-toxic	0.6321
SRKSAGGRANEYDPK	HASPB	2.1	0.3940	Non-allergen	Non-toxic	1.3986
HYEKFERMIKEHTEK	KMP-11	1.3	0.34859	Non-allergen	Non-toxic	0.6107
Linear B-cell epitope						
Epitope sequence	**Protein**	**Score (ABCpred server)**		**Allergenicity (AllergenFP servers)**	**Toxicity (ToxinPred server)**	**Antigenicity (VaxiJen server)**
RFKERHGKSFGEDADE	Cpa	0.88		Non-allergen	Non-toxic	1.4586
SAGGTSPPCHDKGEFG	Cpa	0.88		Non-allergen	Non-toxic	0.9028
VGEGGKGNEDGNDDQP	HASPB	0.94		Non-allergen	Non-toxic	2.1180
KSAGGRANEYDPKDDG	HASPB	0.93		Non-allergen	Non-toxic	1.4126
KEHYEKFERMIKEHTE	KMP-11	0.88		Non-allergen	Non-toxic	0.6037

HTL, Helper T-lymphocyte; CTL, cytotoxic T-lymphocyte; IFN, interferon.

Epitope prediction was performed and the chosen epitopes were filtered based on several factors, such as antigenicity, allergenicity, toxicity, immunogenicity (for CTL epitopes), and the ability to induce an IFN-γ response (for HTL epitopes). Table 2 displays the chosen epitopes in detail.

The MEV was designed using 8 CTLs, 4 HTLs, and 5 B-cell epitopes obtained from the three proteins, following strict filtering criteria. The CTL, HTL, and linear B-cell epitopes that were predicted and screened were linked using AAY, GPGPG, and KK, respectively. The RS09 and PADRE sequence (adjuvants) were joined using EAAAK linkers, while the IgG Fc fragment (PDB ID: P01857) sequences were fused using KK linkers. Figure 1A and B display a schematic design of the resultant protein molecule called MEV-Fc, and the amino acid sequence.

Physiochemical characterizations, antigenicity, allergenicity, and toxicity prediction of the designed MEV-Fc

The ProtParam server was used to compute the physicochemical characteristics of the designed MEV-Fc (Table 3). It consists of 522 amino acids and has a molecular weight of around 57.8 kDa. The molecule's isoelectric point is around 8.21, which represents the pH value at which it is electrically neutral. The half-life refers to the duration required for half of the protein to degrade inside the cell after its production. The findings indicate that the MEV has an instability value of less than 40 which categorizes this protein as stable.

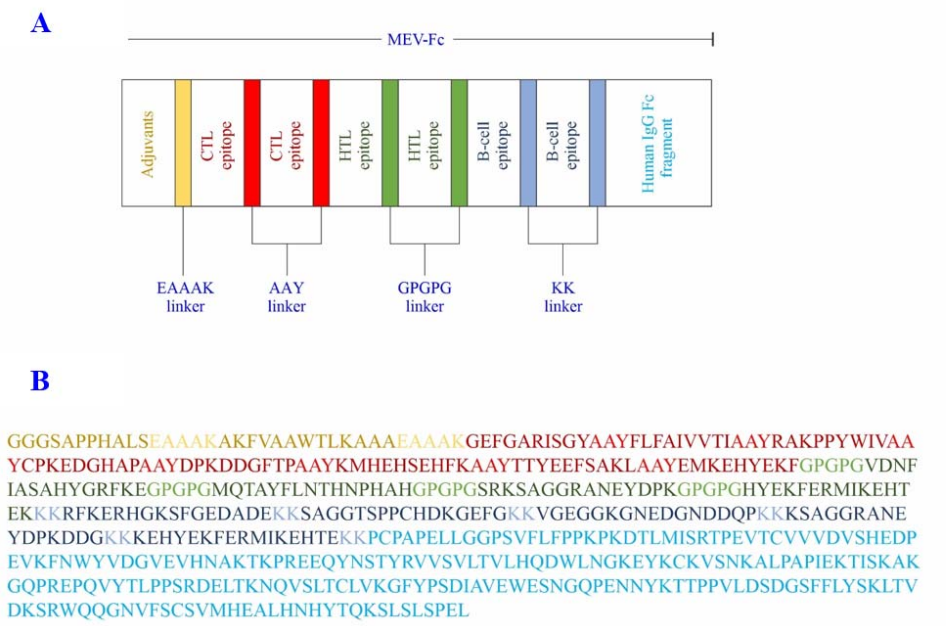

Fig. 1. (A) Schematic diagram of the vaccine (MEV-Fc). (B) MEV-Fc amino acid sequence. MEV-FC, Multi-epitope Vaccine-Fc fragment; HTL, Helper T-lymphocyte; CTL, cytotoxic T-lymphocyte.

Table 3. Physicochemical characteristics of multi-epitope vaccine-Fc fragment predicted by the ProtParam server.

Number of amino acids	Molecular weight (Dalton)	Theoretical isoelectric point	Half-life	Aliphatic index	GRAVY	Instability
522	57778.79	8.21	30 h (mammalian reticulocytes, *in vitro*) > 20 h (yeast, *in vivo*) > 10 h (*E. coli*, *in vivo*).	51.84	-0.828	32.48 (Stable)

GRAVY, Grand average of hydropathy.

The MEV-Fc protein had an aliphatic index greater than 50%, demonstrating its stability against heat. GRAVY's index determines a protein's hydrophobicity/hydrophilicity by dividing the total hydropathy values of all its components by its sequence length. According to the GRAVY value, the protein had a negative value, indicating its hydrophilic nature.

One crucial factor in vaccine design is ensuring that the designed vaccines can induce an immunological response, either *via* a humoral and/or cell-mediated immune response, against the specific targeted parasite. The computational data indicates that our vaccine indicates an antigenic property with probability scores of 0.765 and 0.946 as predicted by VaxiJen v2.0 and AntigenPro servers, respectively.

Importantly, MEV-Fc is predicted to be non-allergenic and non-toxic suggesting its favorable safety profile. This MEV-Fc has desirable characteristics such as stability, solubility, safety, and the capacity to induce an immunological response, which positions it as a promising candidate for a peptide vaccine.

Structure prediction and validation of the MEV-Fc

The PSIPRED server predicted that the 522-residue chimeric protein had 18% alpha helix, 35.25% beta-sheet, and 46.75% coils, as shown in Fig. S1 (available at: https://github.com/mahfadaie/RPS-Supplementary). We used the I-TASSER server to predict the tertiary structure of MEV-Fc and then employed the PyMOL software to

visualize the generated model (Fig. 2A). After the 3D model was generated using I-TASSER modeling, it was subjected to further refinement and analysis using the SAVES v6.0 server. This server provided information about the Ramachandran plot. According to the findings, 86.1% of the residues were located in the favored region, 12.1% in the allowed region, and 1.8% in the disallowed region (Fig. 2B). The energy plot and Z-score plot were also generated using the ProSA web server. The calculated Z-score (-5.3) was within the usual range for X-ray crystal structures (Fig. 2C). Most of the residues in the model structure had low energy values, according to the energy plot (Fig. 2D). The ERRAT server reported an overall quality factor of 76.07, which is above the commonly recognized criterion of 50 for an acceptable model. Hence, we may certainly regard the tertiary structure of MEV-Fc as a dependable and precise model.

Analysis of docking interactions of the MEV-Fc with TLRs

We used a robust tool ClusPro 2.0 to acquire insights into the vaccine interaction with TLR-2 and TLR-4 two important components for developing immunity against *Leishmania*. Upon analyzing the interaction between the vaccine and TLR-2, we obtained 10 distinct models. Our analysis revealed that one model had 23 hydrogen bonds and 7 salt-bridge interactions, making it distinct from the others (Fig. 3A, Table S1, available at: https://github.com/mahfadaie/RPS-Supplementary). The binding free energy, which measures the strength of the bond, was determined to be -1260.9 kcal/mol, showing a strong interaction between MEV-Fc and TLR-2. To gain a deeper understanding of this interaction, Ligplot+ was employed to provide a clear two-dimensional depiction of their bonding (Fig. 3B).

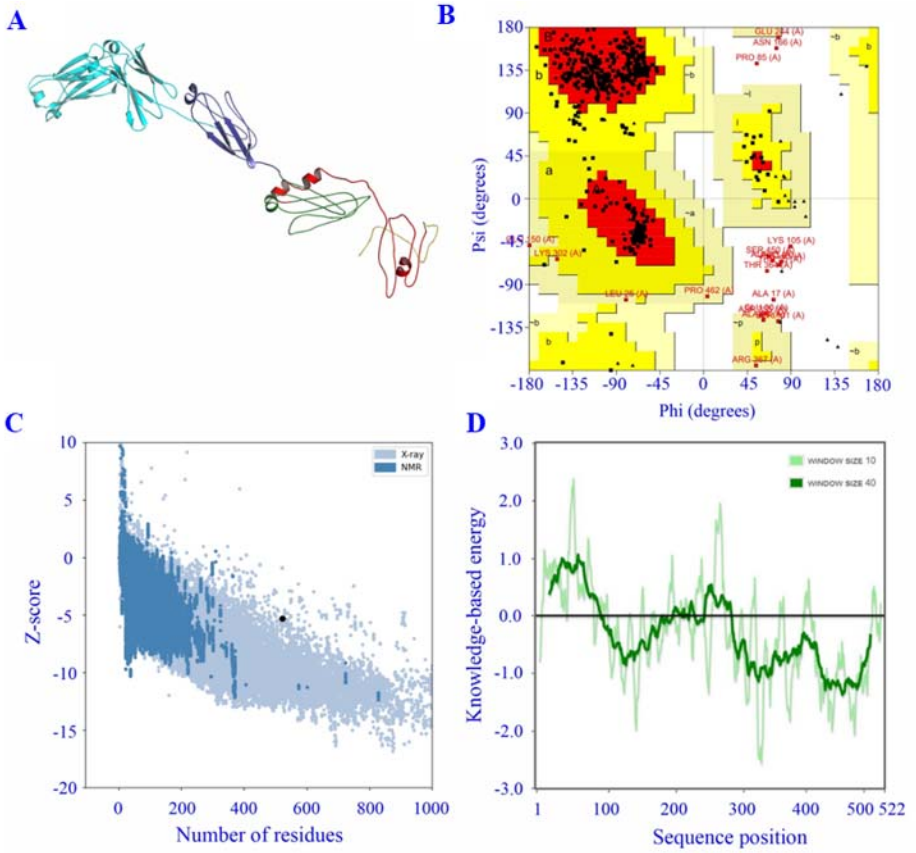

Fig. 2. 3D structure model prediction and validation. (A) Visualization of a 3D model for the multi-epitope vaccine-Fc fragment using PyMOL software; (B) the Ramachandran plot depicts the distribution of amino acid residues in areas that are favored (red color), allowed (yellow/faint yellow color), and disallowed (white color); (C) Z-score plot for the predicted 3D model using ProSA-web; (D) energy plot for each residue in the predicted model.

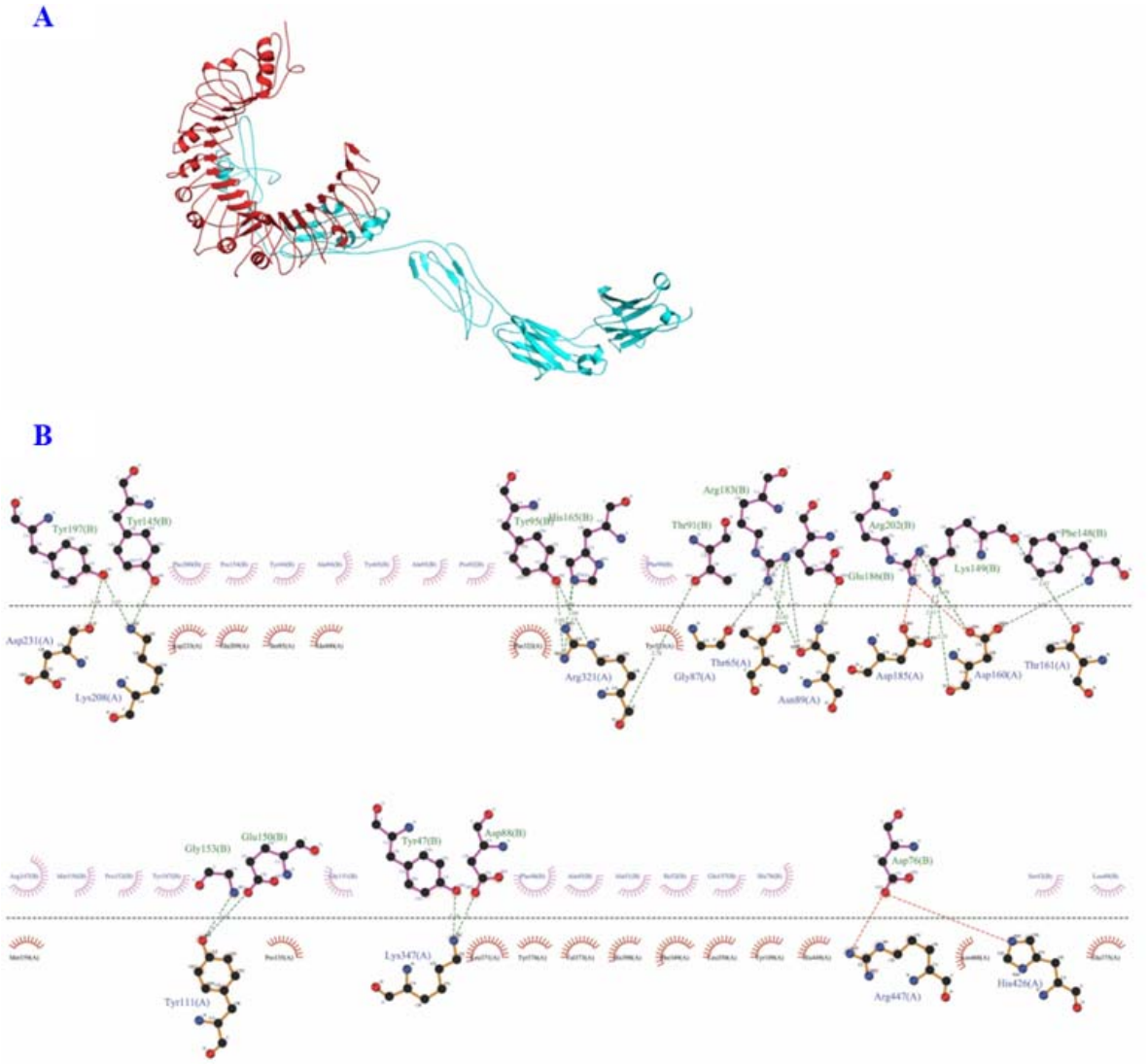

Fig. 3. Molecular docking interaction analysis of the vaccine and TLR-2. (A) The outcome visualization of molecular docking of the vaccine structures (cyan) and TLR-2 receptor (red) derived from the ClusPro; (B) more investigation into the multi-epitope vaccine-Fc fragment-TLR2 complex interactions and the generation of two-dimensional pictures of these complexes using the Ligplot+ visualization program. TLR2, Toll-like receptor 2.

Similarly, we analyzed the interaction between our vaccine and TLR4. A total of 10 distinct models were obtained. One model exhibited a remarkable 28 hydrogen bonds and 13 salt-bridge interactions, as seen in Fig. 4A and Table S2 (available at: https://github.com/mahfadaie/RPS-Supplementary). Again, the binding free energy was -1128.7 kcal/mol, demonstrating a strong interaction between MEV-Fc and TLR4. We used Ligplot+ once again to provide a clear, two-dimensional depiction of the intricate relationship in question (Fig. 4B).

Interpretation of molecular dynamic simulations between the MEV-Fc and TLRs

The best docking model achieved by the TLR2-vaccine complex was further analyzed by NMA-assisted molecular dynamic simulation. The complexes' deformability graphs demonstrated the extent to which the corresponding molecule could deform, as indicated by the peaks (Fig. 5A). The eigenvalue (Fig. 5B) represents the energy needed to distort the structure. The TLR-2 vaccine complex has an eigenvalue of around 2.851×10^{-7}. The variance (Fig. 5C) exhibits an

inverse relationship with the eigenvalue. The B factor (Fig. 5D) was determined using NMA, a method that assesses protein flexibility by analyzing atomic displacement parameters (36). The covariance matrix (Fig. 5E) shows the degree of correlation between pairs of residues, where red indicates a positive correlation, white: is no correlation, and blue: is a negative correlation. The elastic network model (Fig. 5F) calculates springs between corresponding atom pairs, with dots in the graph representing each spring's rigidity or flexibility. The color gradient indicates stiffness, with darker colors suggesting stiffer springs. For TR4, similar results were achieved (Fig. S2, available at: https://github.com/mahfadaie/RPS-Supplementary).

Immune simulation

We employed C-ImmSim to forecast the immune response of mice to the MEV-Fc vaccine. The results of this analysis are illustrated in Fig. 6A-F. The findings indicated that the levels of IgM and IgG antibodies showed a progressive rise after three vaccine injections (Fig. 6A), suggesting that mice exhibited an immune response by producing antibodies. The activity of B-cells, which are responsible for antibody production, increased progressively with each injection, reaching its highest level after the last injection (Fig. 6B). As shown in Fig. 6C, there was a notable increase in both the total and memory populations of helper T cells. The number of activated helper T cells also saw a substantial rise, reaching its highest point following the third vaccination (Fig. 6D). The quantity of functional CTL progressively rose with each vaccination (Fig. 6E). Levels of IFN-γ and IL-2, the molecules involved in immunological signaling, markedly rose in response to antigen stimulation (Fig. 6F). The findings suggest that the MEV-Fc vaccine can elicit a robust and diverse immunological response in mice, including the production of antibodies, activation of B-cells, helper T cells, cytotoxic T cells, and the release of cytokines.

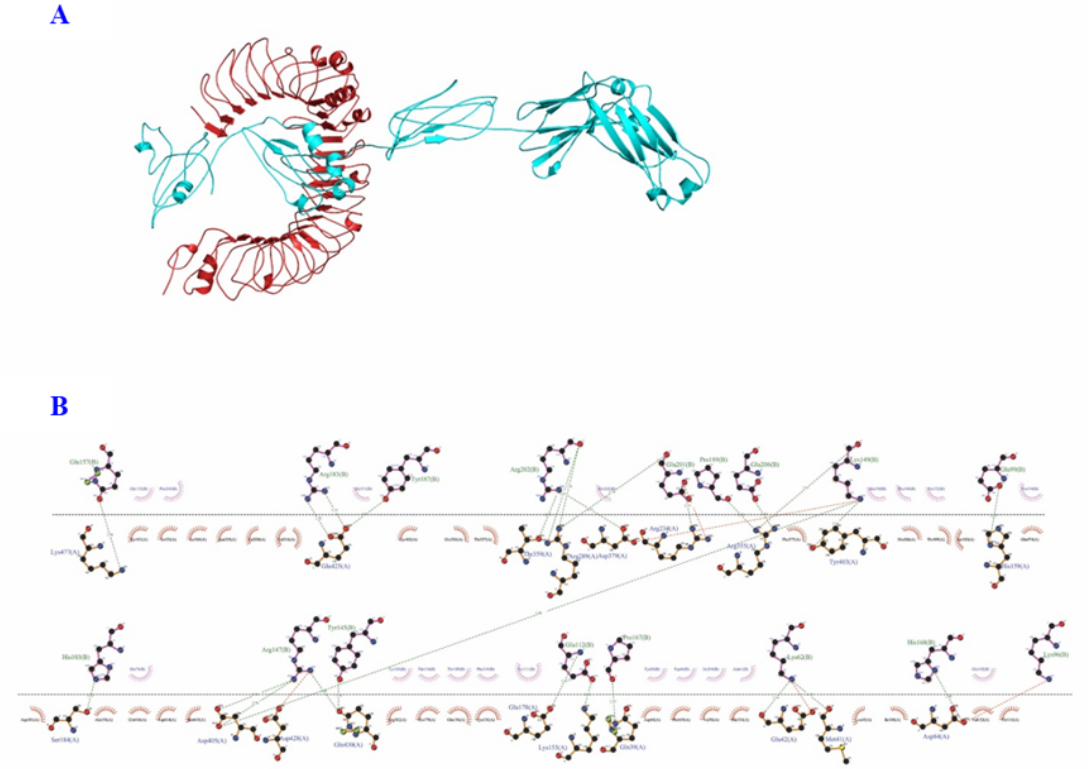

Fig. 4. Molecular docking interaction analysis of the vaccine and TLR-4. (A) The outcome visualization of molecular docking of the vaccine structures (cyan) and TLR-4 receptor (red) derived from the ClusPro; (B) more investigation into the multi-epitope vaccine-Fc fragment-TLR2 complex interactions and the generation of two-dimensional pictures of these complexes using the Ligplot+ visualization program. TLR4, Toll-like receptor 4.

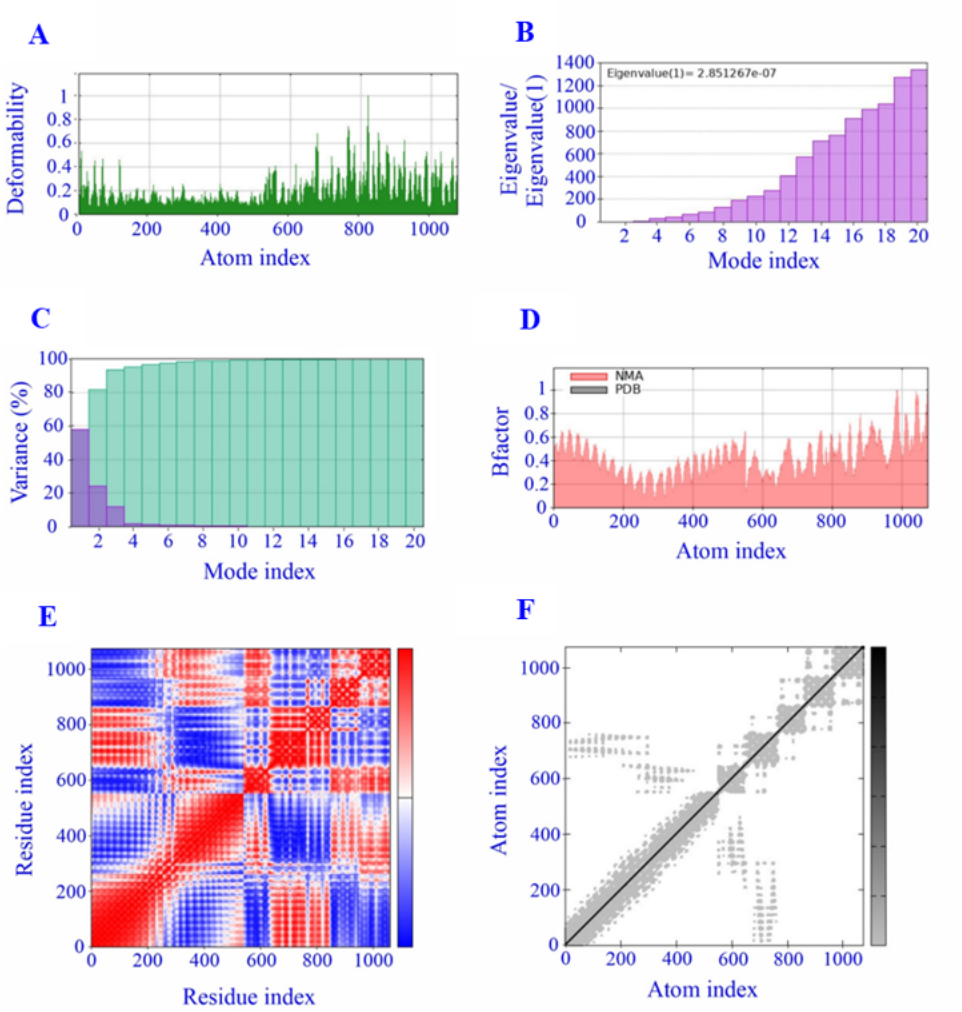

Fig. 5. The findings of the molecular dynamics simulation of MEV-Fc-TLR2. (A) Deformability analysis; (B) the simulation's eigenvalues; (C) variance plots display individual variances in red and cumulative variances in green; (D) comparison of B-factors; (E) covariance map, illustrating movements that are correlated (red), uncorrelated (white), and anticorrelated (blue); (F) the depiction of an elastic network, with darker gray regions suggesting more stiffness.

Codon optimization and in-silico cloning for expression of MEV-Fc

The JCat tool was used to optimize the codons in the vaccine protein's amino acid sequence to address the issue of codon bias. The tool generated a reverse-translated cDNA sequence with an optimized codon adaptation index (CAI) value of one. Using a gene's codon sequence, CAI provides a quantitative way to forecast the gene's expression level. The CAI value is within the acceptable range of 0.8-1.0, indicating a high level of gene expression in the host organism, *E. coli* K12. Furthermore, the improved sequence exhibited a GC content of 49.94%, suggesting favorable codon optimization. To achieve optimal gene expression, a GC content ranging from 35% to 70% is necessary (37). The restriction sites for the *Nde*I and *Xho*I enzymes were introduced at the 5' and 3' of the sequence ends. Subsequently, the modified sequence was inserted into the pET28-a(+) vector. The cloned insert, seen in purple, is positioned between the designated restriction sites inside the vector. At both ends of the cloned sequence, there is a stretch of six histidine residues that aid in the purification of the recombinant vaccine protein after it has been produced (Fig. 7). These results point to a likelihood that the *E. coli* expression system will effectively express the optimized sequence.

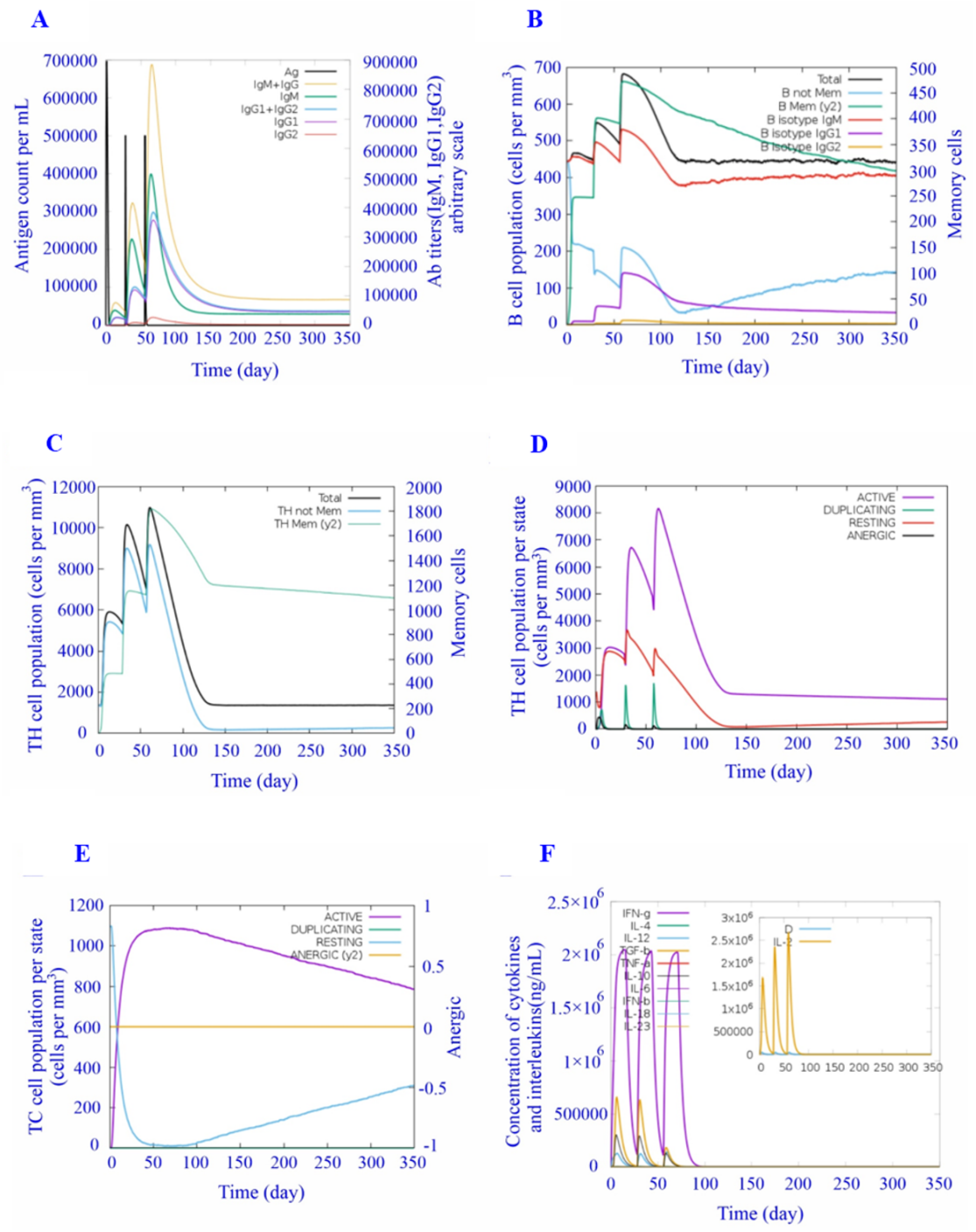

Fig. 6. C-ImmSim server-based immune simulation. (A) Immunoglobulin levels in various states following antigen stimulation; (B) various B-cell subtype distributions in response to antigen stimulation; (C) CD4 HTL generation in response to antigen stimulation; (D) distribution of CD4 HTL according to their activation status following antigen stimulation; (E) the total (active and resting) cytotoxic T lymphocyte count; (F) cytokine production profile following antigen stimulation. CT, cytotoxic T cells; HTL, Helper T-lymphocyte.

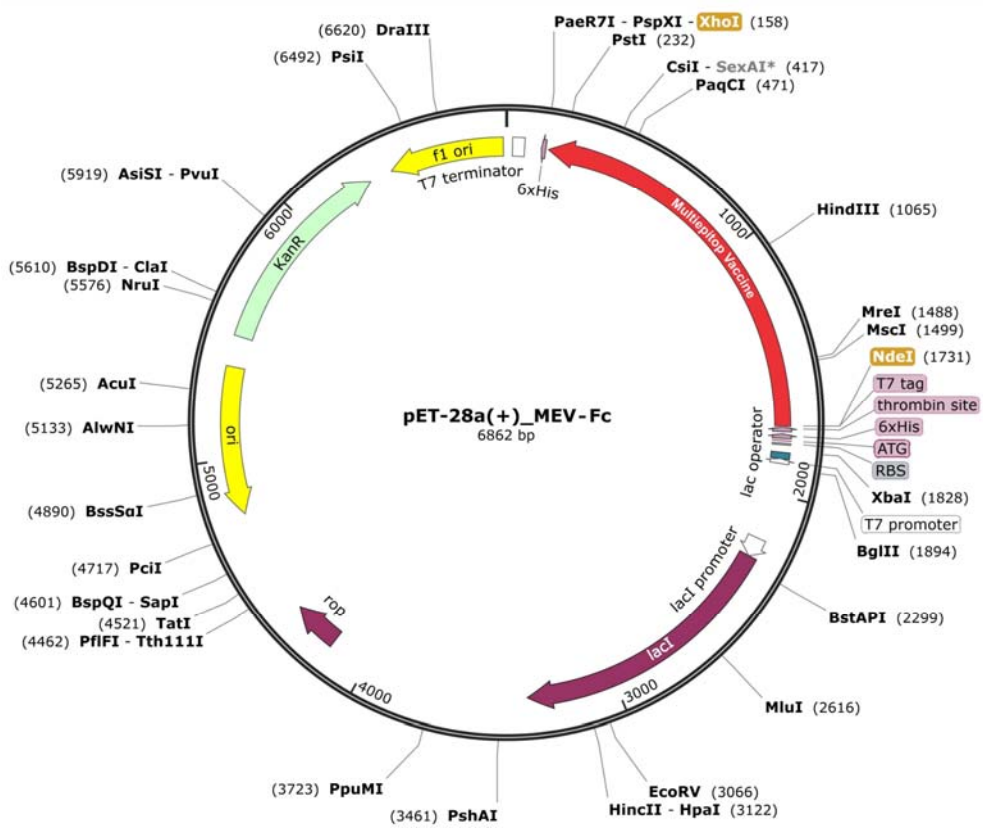

Fig. 7. *In silico* cloning. The MEV-Fc sequence (highlighted in red) was successfully inserted into the pET28-a(+) expression vector at the *Nde*I and *Xho*I restriction endonuclease cleavage sites. MEV-Fc, Multi-epitope vaccine-Fc fragment.

DISCUSSION

Leishmaniasis is classified as one of the most neglected tropical diseases and mostly impacts those living in impoverished regions of developing nations. *L. major* primarily causes zoonotic cutaneous leishmaniasis in Iran accounting for a substantial number of cases, most of which occur in rural regions (38,39). Developing a vaccine against cutaneous leishmaniasis is important because of its significant health and socio-economic consequences, especially in endemic areas such as Iran. The disease causes profound skin lesions and scarring, resulting in psychological and social ostracism. Ongoing research and novel techniques are crucial for successfully overcoming the hurdles in leishmania vaccine development, such as the complicated life cycle of the parasite and the need for a well-balanced immune response (40,41). Utilizing bioinformatics enables the expedited progress of vaccine development *via* the ability to computationally assess potential candidates before experimental confirmation results in a reduction in both time and expense (42). In the current study bioinformatics approaches were employed to design a promising multi-epitope vaccine derived from the proteins Cpa, HASPB, and KMP-11. These proteins were chosen based on their high immunogenicity and ability to stimulate immune responses. The designed vaccine is anticipated to offer some protection against *L. major*.

Cutaneous leishmaniasis elicits an intricate immune response that includes cellular and humoral components. T-cell-mediated immune responses play a prominent role in combating *Leishmania* parasites. When activated, T cells undergo differentiation into effector T cells, which include cytotoxic T cells ($CD8^+$) and HTCs ($CD4^+$) (43). Effector T cells secrete

cytokines and other chemicals that help eliminate intracellular parasites. Long-term immunity against Leishmania is achieved by the action of several subsets of memory T cells, which produce the cytokines IL-2, IL-4, and IFN-γ (44). In addition, humoral immunity also contributes to the defense against leishmaniasis. B cells produce antibodies that can attach to Leishmania parasites and enhance their elimination by activating the complement system or promoting antibody-dependent cellular cytotoxicity (45). The interplay between cellular and humoral immunity is crucial for producing a thorough immune response against cutaneous leishmaniasis.

To elicit a strong immune response against cutaneous leishmaniasis, our vaccine combined epitopes from both B and T cell antigens. In particular, it contained 8 CTL epitopes and 4 HTL epitopes derived from the aforementioned Leishmania parasite antigens. The CTL epitopes significantly contribute to $CD8^+$ T lymphocyte activation which is responsible for detecting and eliminating infected host cells harboring intracellular parasites. Simultaneously, four HTL epitopes activate $CD4^+$ T cells, which coordinate the immune response by producing cytokines (especially INF-γ) that boost macrophage activation and facilitate the elimination of *L. major* parasites. In addition, our vaccine formula included 5 B-cell epitopes that stimulate the generation of targeted antibodies capable of neutralization, opsonization, and activation of the complement system. This strategic combination of B and T cell epitopes was designed to offer broad immune protection against cutaneous leishmaniasis by engaging both immune arms. The designed vaccine in this study included PADRE and RS09 peptides, as well as human IgG-Fc, to provide particular immunological benefits. PADRE binds to HLA-DR molecules and acts as a universal T cell epitope, allowing for strong activation of HTCs across diverse human populations (46). This ensures a consistent and potent T-cell response that is essential for fighting against Leishmania parasites. RS09 functions as an agonist for TLR-4, which is an essential component of the innate immune system (47). TLR-4 recognition improves the vaccine's immunogenicity against Leishmania infection by inducing both innate and acquired immune responses (48). In addition, the incorporation of a human IgG-Fc fragment improves antigen uptake by antigen-presenting cells *via* Fc receptors and extends the vaccine's lifetime, thereby promoting effective T-cell presentation and increasing antibody responses (18,49). This plan aims to optimize humoral and cellular immunity against cutaneous leishmaniasis by employing human IgG-Fc, RS09, and PADRE. This could enhance vaccine effectiveness and provide long-term protection.

Data analysis has shown that the designed vaccine is safe, non-allergenic, and highly antigenic. Even at high temperatures, the vaccine remains effective because of its excellent thermal stability. Furthermore, its hydrophilicity allows for easier administration and formulation.

The 3D model of the protein vaccine was predicted using the I-TASSER server. The accuracy of this structure was checked using the z-score (-5.3), which confirmed that our model falls within the usual range when compared to high-resolution structures. The Ramachandran plot analysis also showed that a large portion (86.1%) of the amino acid residues is located in energetically favorable regions proving that the model is correct. The 3D model provides precise amino acid coordinates essential for analyzing the dynamics and interactions of the vaccine with other molecules. To understand how the vaccine interacted with TLRs molecular docking and molecular dynamic simulations were conducted. We specifically investigated TLR2 and TLR4 due to their crucial role in the immune response against cutaneous leishmaniasis (50). The results indicated the existence of an atomistic interaction interface between MEV-Fc and TLR2/TLR4, suggesting a substantial binding affinity and the vaccine's ability to efficiently activate these receptors. This reaction is critical for inducing a strong immune response, which improves the vaccine's chances of protecting against the disease.

The immune simulation of our vaccine demonstrated comprehensive and robust immune activation, which is critical for combating cutaneous leishmaniasis. The data

demonstrated the production of both IgM and IgG antibodies, indicating a robust humoral immune response. Furthermore, we observed a significant cellular response involving T cells, characterized by the secretion of important cytokines like IL-2 and IFN-γ. These cytokines play a crucial role in controlling the infection (44). In comparison with the research conducted by Margaroni *et al*. which indicated that a multi-epitope chimeric protein elicited an elevation in IgM and IgG antibodies, activation of memory $CD4^+$ and $CD8^+$ T cells, and a Th1-type response marked by IFN-γ production, our vaccination strategy has yielded markedly superior outcomes (51). Our *in-silico* immune simulations resulted in a several-fold increase in the production of both IgM and IgG antibodies, along with enhanced activation of memory T cells. Furthermore, our formulation elicited significantly elevated levels of IFN-γ and IL-2, signifying a more vigorous and efficient immune response.

In the current study, the JCat tool was applied to optimize the codons of our vaccine, aiming to improve its expression in *E. coli*. The findings demonstrated a GC content of 49.94% and a CAI of 1, suggesting a high probability of successful and efficient protein production in the *E. coli* expression system. It is crucial to experimentally confirm the immunogenicity and safety of the vaccine candidates designed in this investigation. To achieve this, the recombinant polyepitope vaccine must first be produced, purified, and subsequently evaluated for immunogenicity and effectiveness in animal models. This will determine whether the vaccine is protective against cutaneous leishmaniasis caused by *L. major*.

CONCLUSION

In summary, bioinformatic approaches were used to design a multi-epitope vaccine (MEV-Fc) for combating cutaneous leishmaniasis. This vaccine included the immunogenic proteins Cpa, HASPB, and KMP-11 as well as 8 CTL epitopes, 4 HTL epitopes, and 5 B-cell epitopes to help stimulate both cellular and humoral immunity. PADRE, RS09, and human IgG-Fc were added to enhance the vaccine's effectiveness. MEV-Fc could elicit a strong and comprehensive immunological response, resulting in elevated levels of Th1-type cytokines such as IFN-γ and IL-2. The analyses showed the non-toxicity, stability, and antigenicity of the vaccine. The structural validation demonstrated accurate 3D modeling with strong interactions with TLR2 and TLR4 which are necessary for triggering robust immune responses. The promising results indicated that MEV-Fc has the potential to be an effective vaccine candidate for preventing cutaneous leishmaniasis caused by *L. major*. However, experimental validation is necessary to ensure the effective induction of protective, long-lasting immunity while minimizing potential toxicity.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

M. Fadaie contributed to the methodology, investigation, writing, reviewing, editing of the original draft, and the design and preparation of the figures. H. Khanahmad and Z. Shahmoradi were responsible for conceptualization, funding acquisition, review and editing, supervision, and project administration. All authors read and approved the finalized article.

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