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Multi-epitope vaccine design against leishmaniasis using IFN-γ inducing epitopes from immunodominant gp46 and gp63 proteins

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ABSTRACT

There is no currently approved human vaccine against leishmaniasis. Utilization of immunogenic antigens and their epitopes capable of enhancing immune responses against leishmaniasis is a crucial step for rational *in silico* vaccine design. The objective of this study was to generate and evaluate a potential vaccine candidate against leishmaniasis, designed by immunodominant proteins from gp46 and gp63 of *Leishmania major*, which can stimulate helper T-lymphocytes (HTL) and cytotoxic T-lymphocytes (CTL). For this aim, the IFN-γ-inducing MHC-I and MHC-II binders were predicted for each examined protein (gp46 and gp63) and connected with appropriate linkers, along with an adjuvant (*Mycobacterium tuberculosis* L7/L12) and a histidine tag. The vaccine's stability, antigenicity, structure, and interaction with the TLR-4 receptor were evaluated *in silico*. The resulting chimeric vaccine was composed of 344 amino acids and had a molecular weight of 35.64 kDa. Physico-chemical properties indicated that it was thermotolerant, soluble, highly antigenic, and non-allergenic. Predictions of the secondary and tertiary structures were made, and further analyses confirmed that the vaccine construct could interact with the human TLR-4 receptor. Virtual immune simulation demonstrated strong stimulation of T-cell responses, particularly by an increase in IFN-γ, following vaccination. In summary, the *in silico* data indicated that the vaccine candidate showed high antigenicity in humans. It was also found to trigger significant levels of clearance mechanisms and other components of the cellular immune profile. Nevertheless, further wet experiments are required to properly assess the efficacy of this multi-epitope vaccine candidate against leishmaniasis.

Abbreviations: **CL**, cutaneous leishmaniasis; **gp46**, glycoprotein 46; **gp63**, glycoprotein 63; **CatB**, cathepsin B-like cysteine protease; **CatL**, cathepsin L-like cysteine protease; **grp78**, glucose-regulated protein 78; **H**, histone; **HSP**, heat shock protein; **rP0**, ribosomal protein 0; **KMP11**, kinetoplastid membrane protein 11; **STI-1**, stress-inducible protein 1; **TSA**, thiol-specific antioxidant; **LeIF**, *Leishmania* elongation-initiation factor; **LACK**, *Leishmania* activated C-kinase antigen; **PSA**, promastigote surface antigen; **IFN-γ**, Interferon gamma; **Th1**, helper Type-I T cell; **NO**, nitric oxide; **ROS**, Reactive oxygen species; **MEVC**, multi-epitope vaccine candidate; MHC, major histocompatibility complex; **HLA**, human leukocyte antigen; **CTL**, cytotoxic T-lymphocyte; **HTL**, Helper T-lymphocyte; **PADRE**, Pan HLA-DR reactive epitope; **ACC**, auto cross-covariance; **MW**, molecular weight; pI, isoelectric pH; **GRAVY**, grand average of hydropathicity; **I-TASSER**, Iterative Threading ASSEmbly Refinement; **3D**, Three-dimensional; **GDT-HA**, global distance test-high accuracy; **RMSD**, root mean square deviation; **IEDB**, Immune epitope database; **TLR**, toll-like receptor; **CAI**, codon adaptation index.

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1. Background

The genus *Leishmania* consists of a heterogeneous, diverse group of tissue flagellates, which can be transmitted by infected phlebotomine sandflies [1–2]. These obligatory intracellular organisms multiply within macrophages, rendering a broad range of clinical manifestations, including cutaneous and mucocutaneous lesions as well as fatal systemic infection [3]. In total, 350 million people in over 100 countries are at risk of *Leishmania* infections [4]. In many Middle Eastern, South American, and North African countries, leishmaniasis may represent as dermal lesions, known as cutaneous leishmaniasis (CL), estimated to be the most common form of the disease [5]. In the Old World, *Leishmania* (L.) *major* and *L. tropica* are two significant species having gerbils and humans as reservoir hosts, respectively [6]. Approximately, CL affects 0.7–1.2 million individuals annually. Several factors are important for effective CL control in tropical and subtropical areas, including inadequate and/or inefficient reservoir and sandfly control strategies, expensive and toxic pentavalent antimonials as the drugs of choice, along with poor treatment adherence by patients [7]. These limitations and the diversity of affected people in endemic countries make immunization as an imperative option to decrease disease burden [8].

For decades, developing a safe and successful vaccine to prevent CL has been a major challenge [9]. The parasite possesses a two-phase life cycle, circulating between amastigotes (in macrophages) and promastigotes (in sandfly gut), each expressing a wide plethora of antigenic proteins [10]. Previously, several antigens were identified and employed in various immunization studies; most of them were described as conserved molecules throughout *Leishmania* species [11–12]. Of the major antigenic molecules, glycoprotein 46 (gp46), glycoprotein 63 (gp63), cathepsin L-like cysteine protease (CatL), cathepsin B-like cysteine protease (CatB), glucose-regulated protein 78 (grp78), histone proteins (H1, H2A, H2B, and H4), heat shock proteins (HSPs; HSP60, HSP70, HSP83 (HSP90), HSP100), ribosomal protein P0 (rP0), kinetoplast membrane protein 11 (KMP11), stress-inducible protein-1 (STI-1), thiol-specific antioxidant (TSA), *Leishmania* elongation initiation factor (LeIF), and *Leishmania* activated C-kinase antigen (LACK) are among the most studied vaccine antigens against CL [13]. The gp46 protein, also known as promastigote surface antigen (PSA-2), has specific leucine-rich repeats and is present in glycolipid-anchored and secretory forms in promastigotes. Previous studies have indicated that gp46 can significantly facilitate parasite attachment to the host cell and invasion to macrophages. Notably, the protein has provided Th1-mediated protection in vaccination studies [14]. Furthermore, the gp63 protein (leishmanolysin) is abundant in promastigotes and, to a lesser extent, in amastigotes, with zinc-dependent metalloprotease activity, and it is the most frequently investigated candidate in *Leishmania* vaccination studies, showing promising results [15–18]. This protein may have critical roles in the prevention of complement-mediated lysis, intra-macrophage survival of amastigotes, and alterations in macrophage signaling and transcription factors[19]. The most powerful immune responses against intracellular amastigotes are elicited by IFN-γ-inducing Type-I helper T-cells (Th1) through reactive oxygen species (ROS) and nitric oxide (NO) upsurges, whereas Th2-mediated cytokines along with humoral responses may favor parasite persistence and devastate the infection course [20]. Accordingly, exploration of parasite virulence factors, including those surface-expressed glycoproteins, benefits us in finding novel immunodominant peptide targets, so-called immunogenic epitopes, and employing them in next-generation vaccine design [21].

Traditionally, vaccine engineering and production pipelines demand appropriate animal models, specialized biomolecular equipment, expert researchers, long-term experiments, and follow-up [22]. In the recent millennium, the rapidly progressing trend in computer sciences has dramatically revolutionized the utilization of computer-based data for medical and biological purposes. This ongoing flow of information will expand our understanding of host-parasite interaction and contribute to the research on developing vaccines for zoonotic parasitic diseases [23–25]. With the aim of such modalities, the discovery of novel antigenic proteins, specific B- and T-cell epitopes, and subsequent development of multi-component or multi-epitope vaccines can be much easier, saving time and money [26]. Therefore, *in silico* exploration of vaccine candidate antigens and their immunodominant epitope regions can provide significant insights for future vaccinology studies. In the present study, novel T-cell epitopes capable of inducing IFN-γ were predicted in *L. major* gp46 and gp63 proteins, to develop a novel multi-epitope vaccine candidate (MEVC) against leishmaniasis by different specific web servers.

2. Methods

2.1. gp46 and gp63 amino acid sequence retrieval

For this purpose, the UniProt Knowledge Base (https://www.uniprot.org/) as a free resource of protein sequences was used to acquire the amino acid sequences of gp46 (accession: Q4Q6B6) and gp63 (accession: P08148) [27].

2.2. Prediction of IFN-γ inducing epitopes

Through mhcii tool of IEDB server (http://tools.iedb.org/mhcii/), a recommended method was used to forecast major histocompatibility complex (MHC) class II epitopes, against seemingly protective human leukocyte antigen (HLA) alleles for CL (DRB1*15:01, DRB1*15:02, DRB1*15:03, DRB1*15:04, DRB1*15:05, DRB1*16:01, DRB1*16:02, DRB1*16:03, DRB1*16:04, DRB1*16:05, DPB1*04:01) [28]. For both proteins, these helper T-lymphocytes (HTL) epitopes with lower percentile ranks were subsequently screened in terms of antigenicity, IFN-γ induction and IL-4 induction, by using VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), IFNepitope (http://crdd.osdd.net/raghava/ifnepitope/) and IL4pred (hybrid approach) (https://crdd.osdd.net/raghava/il4pred/) web servers, respectively.

Regarding MHC-I binders against HLA reference set alleles, mhci tool of IEDB (http://tools.iedb.org/mhci/) [28] was used with recommended method [29]. The best epitopes were screened in terms of immunogenicity and IFN-γ induction using the immunogenicity tool in the IEDB server (http://tools.iedb.org/immunogenicity/) and IFNepitope server.

2.3. Design and assemblage of the multi-epitope vaccine construct

Strictly-screened epitopes were finally included in the final vaccine sequence using specific linkers such as EAAAK (adjuvant), GPGPG (HTL epitopes), and AAY (CTL epitopes). In the current study, *Mycobacterium tuberculosis* L7/L12 (accession no: P9WHE3) was used as an immune enhancer. Moreover, “AKFVAAWTLKAAA” (PADRE sequence) was embedded N-terminally after the adjuvant sequence, and it was adjoined with the designed vaccine candidate sequence using the cathepsin S cleavable linker (PMGLP). Of note, a histidine tag (6 × His) was placed C-terminally for purification purposes.

2.4. Allergenic, antigenic, solubility and physico-chemical properties

Two web servers were used to evaluate allergenicity, including AllergenFP v1.0 and AllerTOP v2.0 (https://www.ddg-pharmfac.net/AllerTOP/). AllerTOP performs an 85.3 % prediction by transforming the amino acid sequences into integral vectors with equivalent lengths [30]. Also, a novel alignment-independent, descriptor-based fingerprint method is used by AllergenFP v1.0 using physico-chemical and structural properties, making a prediction with 88 % accuracy [31].

In the following, antigenicity was forecasted by the VaxiJen server, depending on the proteins chemical nature, with 70–89 % accuracy [32]. Regarding protein solubility, Protein-Sol server was used, available at https://protein-sol.manchester.ac.uk/, with a threshold of 0.45 [33,34]. In the next step, major physico-chemical characteristics of the engineered MEVC were predicted using the ExPASy ProtParam web tool, available at https://web.expasy.org/protparam/ [35].

2.5. Secondary and tertiary structure prediction

The GOR IV server (https://npsa-prabi.ibcp.fr/NPSA/npsa_gor4.html) was employed to predict the secondary structure of the vaccine model. This server provides information on the distribution and proportions of residues in various secondary structures, such as the alpha helix, extended strand, and random coil [36]. In the following, the Iterative Threading ASSEmbly Refinement (I-TASSER) server, available at https://zhanggroup.org/I-TASSER/, was used for three-dimensional (3D) modeling for the submitted MEVC. It employs different 3D templates for the homology modeling of the input sequence and finally provides top-five 3D models with different C-scores. The C-score is a confidence index used to evaluate the reliability of predictions. It ranges from −5 to 2 and models with higher C-scores are reliably predicted [37].

2.6. Tertiary model refinement and validations

Tertiary model refinement was performed by mild or aggressive quality improvement through the GalaxyRefine web server. Several parameters are provided as output, encompassing Rama favored, Clash score, root mean square deviation (RMSD), MolProbity, global distance test-high accuracy (GDT-HA), and Poor rotamers [38]. In the following, the quality of the rehashing process was validated, in comparison with crude models, using Ramachandran plot analysis; for this aim, the PROCHECK tool of the SAVES v6.0 server was used [39]. Another tool for validation of the refinement process was Prosa-Web, where a z-score is assigned to overall model quality [40].

2.7. Molecular docking by LightDock

For this aim, the tertiary structure of the human TLR-4/MD2 molecule (Accession No.: 3FXI) was retrieved *via* the RCSB (https://www.rcsb.org). Next, an artificial intelligence-powered service, "LightDock", available at https://server.lightdock.org/, which is based on the Glowworm Swarm Optimization (GSO) algorithm, was used to dock the MEVC designed and engineered in the present study (as a ligand) and human TLR-4 (as a receptor). The framework is written in the Python programming language and Rust for selected high computation-intensive parts of the framework. The server output includes a cluster of top-ranked results, from which the best docking pose is chosen for visualization [41–43].

2.8. Simulation of immune responses

The C-ImmSim online server, which can be accessed at https://150.146.2.1/C-IMMSIM/index.php, was used to predict the virtual immune simulation process. The predictions were based on a position-specific scoring matrix (PSSM) for machine learning methods, and the output indicated stimulation in three regions: bone marrow, thymus, and lymph node. The simulation was conducted using default parameters, including a random seed of 12345, a simulation volume of 10, and 100 simulation steps [44].

2.9. Safety prediction, codon optimization and in silico cloning

For this aim, the BLAST online tool of the UniProtKB server (https://www.uniprot.org/blast) was utilized in order to discriminate likely similar regions between selected vaccine sequences and the respective organism. In this study, the human proteome was defined as target and identity rates over 35 % mean homologous proteins with the human proteome [45–47].

Efficient protein expression is important for subunit vaccine production. Accordingly, reverse translation of the designed vaccine sequence was done by an online tool (https://www.bioinformatics.org/sms2/rev_trans.html), and subsequent codon optimization by the JCat server (http://www.jcat.de/). The JCat server evaluates various crucial characteristics of the DNA sequence that play a significant role in the expression of chimeric proteins in their respective hosts. These include properties like GC content and the codon adaptation index (CAI). Hence, in the present study, codon optimization was done for expression in the *E. coli* K12 strain [48,49]. Next, the NEBcutter 2 server (https://nc2.neb.com/NEBcutter2/) was employed to evaluate the presence of cutting sites for restriction enzymes within codon-adapted vaccine sequences. Ultimately, the cutting sites of Eco53KI (5′-OH) and EcoRV (3′-OH) were added. Moreover, the Shine-Dalgarno sequence (AGGAGG) was added before the start codon to improve the yield of expression. The *in-silico* cloning process of the selected final multimeric vaccine models was accomplished using SnapGene® v6.2.2. standalone software (https://www.snapgene.com).

3. Results

3.1. Selected human MHC-binders and engineering the MEVC

Following strict prediction and screening procedures applied to the *L. major* gp46 and gp63 as potent vaccine candidate antigens against leishmaniasis, six CTL epitopes ($gp46_{3\text{-}11}$, $gp46_{39\text{-}47}$, $gp46_{13\text{-}21}$, $gp63_{25\text{-}33}$, $gp63_{30\text{-}38}$, and $gp63_{33\text{-}42}$) (Supplementary Table 1) and five HTL epitopes ($gp46_{161\text{-}175}$, $gp46_{160\text{-}174}$, $gp46_{270\text{-}284}$, $gp46_{271\text{-}285}$, and $gp63_{14\text{-}28}$) (Supplementary Table 2) were finally highlighted for designing a novel multi-epitope vaccine model using appropriate linkers (AAY, GPGPG, and EAAAK) along with 50S ribosomal L7/L12 (Locus RL7_MYCTU) as adjuvant (N-terminal) and a histidine tag (C-terminal), as described previously (Fig. 1).

3.2. Prediction of antigenicity, allergenicity, solubility and physico-chemical properties

The designed vaccine model was shown to be antigenic, with 0.5829 on the VaxiJen server. Moreover, it was approved as non-allergenic by the AllergenFP v1.0 and AllerTOP v2.0 servers. The protein-Sol web server demonstrated above-threshold solubility for the designed protein (score: 0.502). The protein molecular weight (MW) was estimated to be 35643.23 Dalton with a pI of 6.22. The number of negatively charged (n = 32) and positively charged (n = 31) residues was relatively equal. The estimated half-life for this protein was shown to be 30 h (mammalian reticulocytes, *in vitro*). The vaccine model designed in the present study was predicted to be stable (17.94), highly thermotolerant (87.65), and hydrophobic (GRAVY: 0.312).

3.3. Secondary structure and homology modelling

The GOR IV online tool for secondary structure predictions revealed that the alpha helix (n = 193; 56.10 %), random coils (127; 36.92 %), and extended strands (24; 6.98 %) are the three most frequent secondary structures in the designed vaccine model, respectively (Fig. 2**A**). In the following, a homology modeling prediction by the I-TASSER server was used to illustrate the 3D model of the engineered MEVC. A high C-score usually supports a more reliable predicted model. On this basis, model number 1 (C-score: −2.13; estimated TM-score: 0.46 ± 0.15; estimated EMSD: 11.5 ± 4.5 Å) was selected

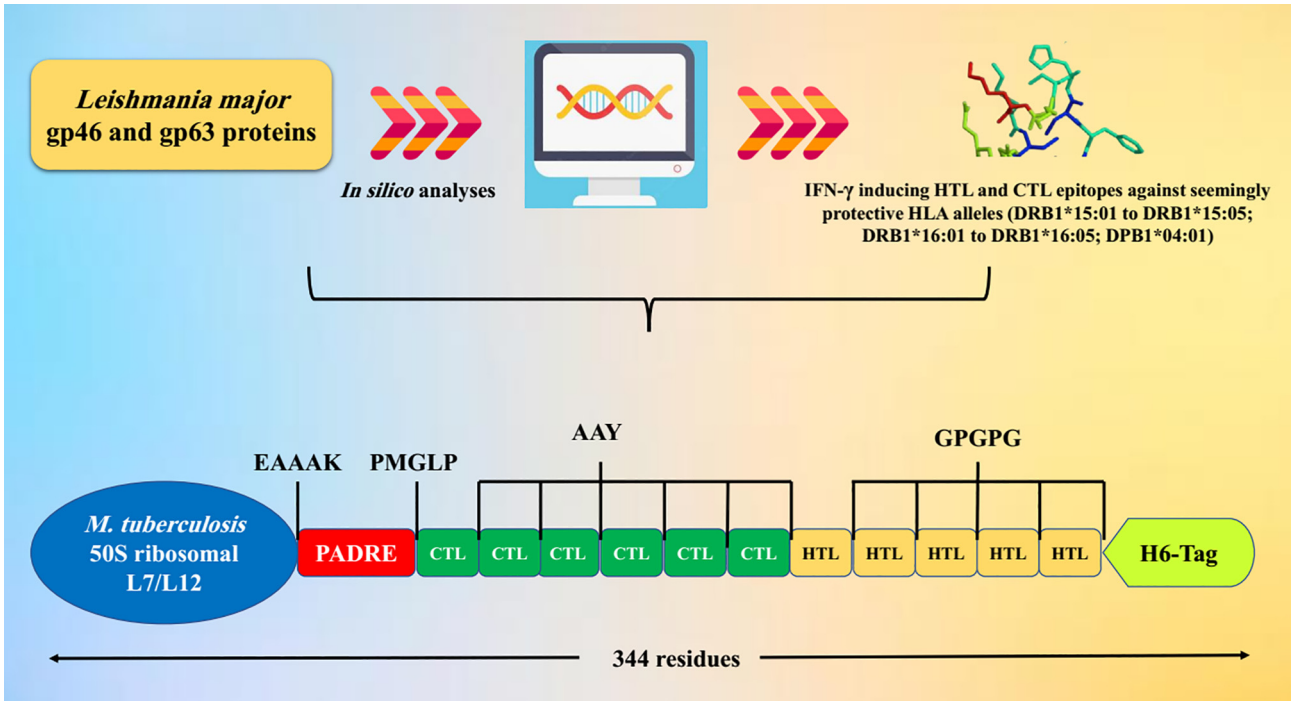

Fig. 1. Schematic illustration of multi-epitope vaccine construct and *in silico* analyses.

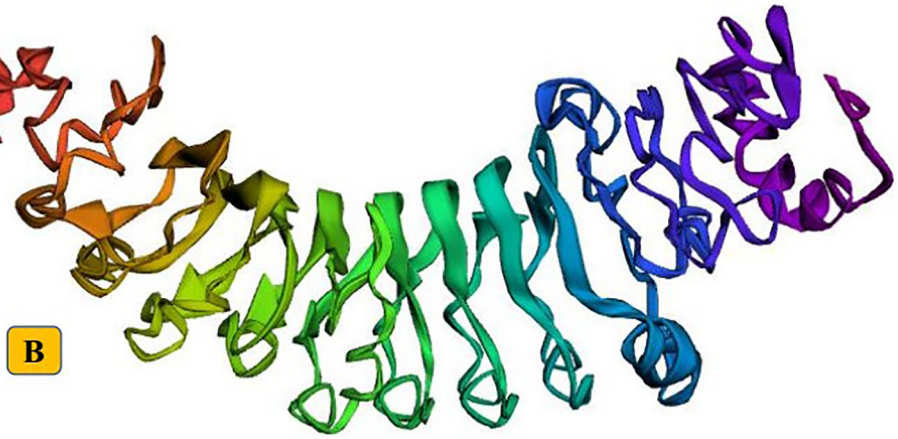

Fig. 2. Secondary (**A**) and tertiary (**B**) structure analysis of the designed vaccine candidate using GOR IV and I-TASSER web servers, respectively.

among the top-five predicted models by the I-TASSER web server for further refinement and analysis (Fig. 2**B**).

3.4. Tertiary model refinement and validation

Refinement was performed using the GalaxyRefine server for structural relaxations of the designed 3D construct. Accordingly, five refined models were provided by this server, among which the best one (model #4) was selected with the following parameters: GDT-HA of 0.9128, RMSD of 0.513, MolProbity of 3.176, clash score of 42.0, poor rotamers of 2.5 and rama favored of 77.2. The quality improvement of the refined models in comparison with the crude models was confirmed by the PROCHECK and Prosa-Web online tools. Ramachandran plot analysis by PROCHECK showed the percentage of residue allocation in the crude and refined models was as follows: 44.8 % vs. 63.8 % (in the most favored regions), 42.1 % vs. 29.7 % (in additional allowed regions), 9.3 % vs. 2.8 % (in generously allowed regions), and 3.8 % vs. 33.8 % (in disallowed regions) (Fig. 3). In addition, the estimated Z-score using the Prosa-Web server for the crude model was −2.76, which was enhanced to −3.58 in the refined model.

3.5. Molecular docking analysis using human TLR-4

Based on the LightDock server output for the protein–protein docking, the top-ranked docked conformation with a score = 64.238, Swarm = 384, and Glowworm = 64 was selected for visualization and evaluation of the interactions. The non-bonded contacts (n = 226) such as the Van der Waals forces, hydrophobic forces, etc. constituted the majority of interactions between the designed vaccine and human TLR-4. The vaccine-receptor interactions have been illustrated in detail in Fig. 4.

3.6. Simulation of the immune responses

Using the C-ImmSim web server, two major immune-related parameters, including cytokine induction and Th cell population per state (cells per mm^3), were evaluated for the designed vaccine candidate. Based on the cytokines, a considerable upsurge in IFN-γ induction, ranging from 400000 ng/ml to 450000 ng/ml, was predicted. Also, IL-2, another Th1-specific cytokine, was highly induced (over 250000 ng/ml). With respect to the Th cell population type, the highest number of specific memory T cells were induced (over 400 per mm^3) by this vaccine candidate (Fig. 5**A**).

3.7. Vaccine safety and in silico cloning

Based on the BLASTp tool output of the UniProtKB server, the novel vaccine sequence had no homology with the human proteome, hence it was considered safe for human use. There were no cutting sites for *EcoRV* and *Eco53KI* in the final sequence; hence, they were selected along with the Shine-Dalgarno sequence and start/stop codons. The reverse-translated vaccine model underwent additional codon optimization using the JCat web tool to improve its expression in the *E. coli* K12 strain. The GC content and CAI value of the vaccine sequence were 65.01 and 0.58, respectively, which were improved to 54.74 and 1.0 after optimization. Ultimately, the final vaccine sequence was ligated into the pET28a(+) plasmid using SnapGene® v6.2.2. software, and the total length of the formed clone was 5040 bp (Fig. 5**B**).

4. Discussion

A proper vaccine against leishmaniasis would actually enhance the leishmanicidal activity of macrophages, hence decreasing the parasite load in the lesion site [50]. Since leishmanization in the 1940 s, *Leishmania* vaccine design has been dramatically evolved, along with advances in molecular biology [51]. Second and third generation vaccines such as Leishmune (fucose-mannose ligand), [52] Leish-Tec (Adenoviral-expressing *L. donovani* A2 protein) [53], the gp63 DNA vaccine [54], as well as Leish-111f recombinant vaccine (LeIF, LmSTI-1, and TSA) in Brazil, Peru, and the USA [55] are promising examples of efficient immunization against leishmaniasis. Peptide-based immunogenic constructs, discovered by a number of *in silico* (B- and T-cell epitope prediction, protein localization, conservation analysis, etc.) and *in vitro* methods (bio-panning assay, peptide-binding assay), constitute the next-generation vaccines capable of efficiently targeting the appropriate classes of immune cells in order to yield more effective immunity against a given pathogen [56]. This approach seems to be highly stable and reproducible, with decreased

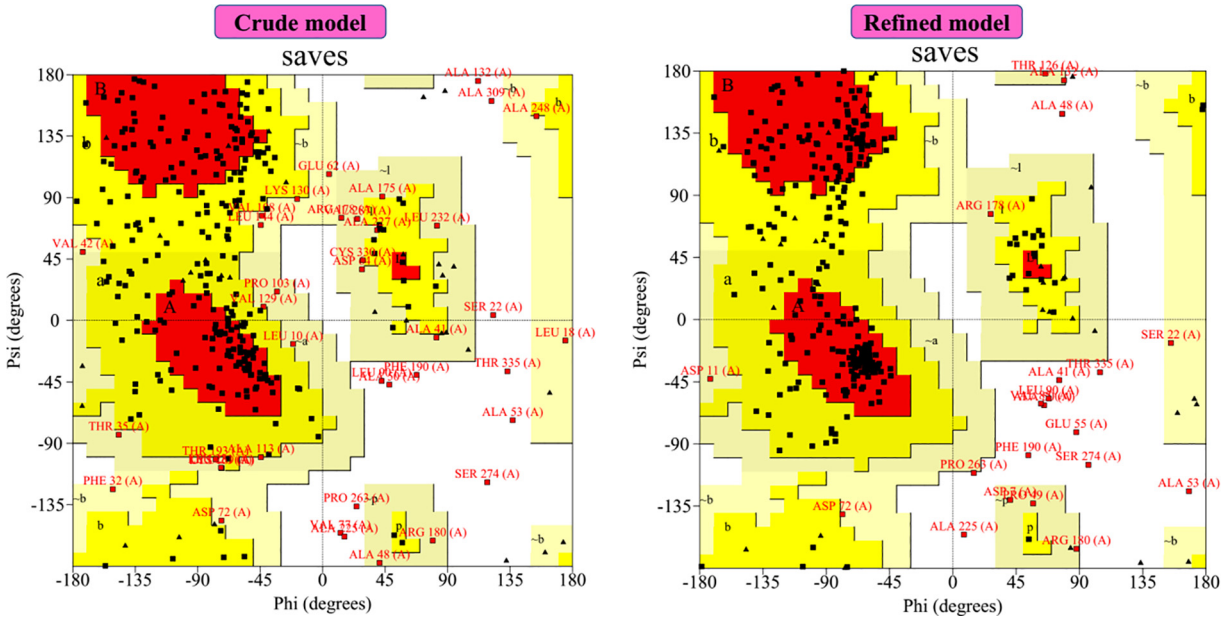

Fig. 3. The validation of the 3D vaccine model before and after refinement, using Ramachandran plot analysis by PROCHECK web tool.

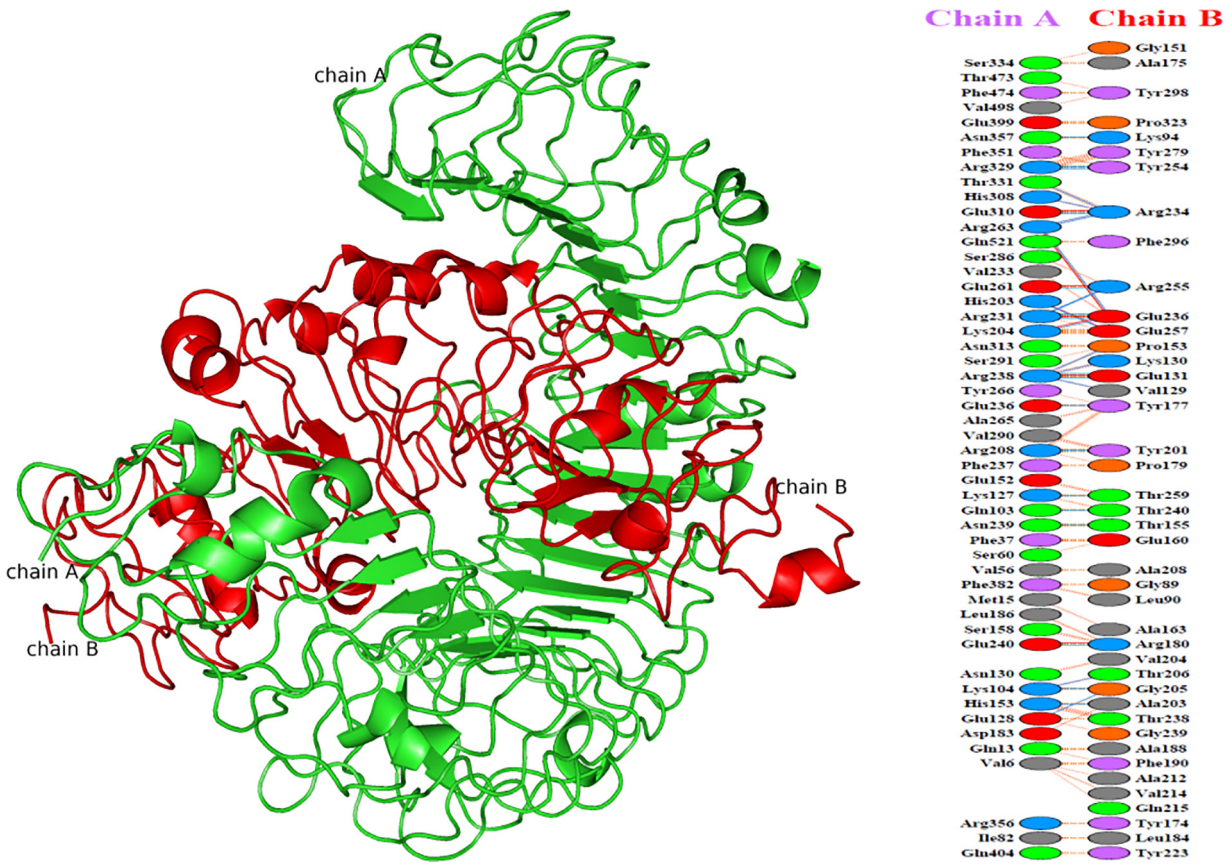

Fig. 4. The representation of the whole docked complexes (vaccine-TLR4) and their respective residue-by-residue interactions.

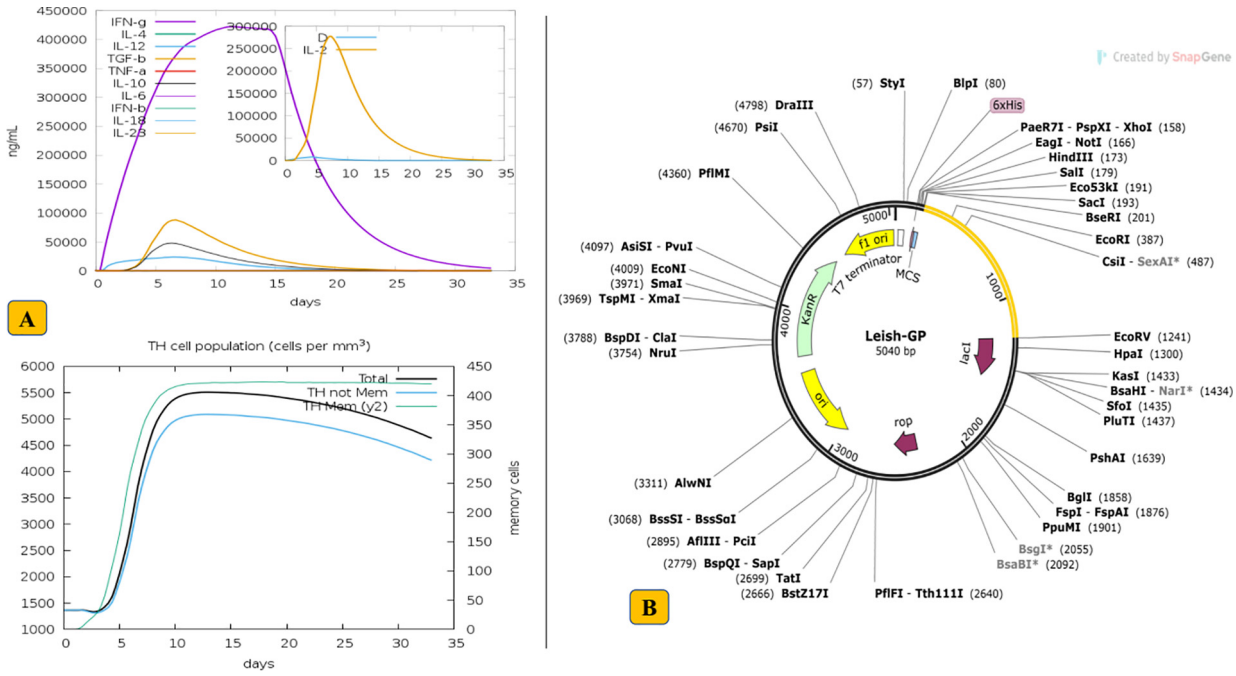

Fig. 5. Immune simulation profile (Th cell population and cytokines) (**A**) and *In-silico* cloning of the final vaccine construct into the pET28a(+) vector using SnapGene 6.2.2 (**B**).

antigen complexity and lower costs for mass production. In this sense, the KSAC polyprotein has shown protection against *L. major* lesion formation in BALB/c mice [57].

As mentioned earlier, *Leishmania* possesses a diverse array of antigenic compounds that can be employed in rational vaccine design studies [58]. The surface glycoprotein gp46 is expressed in most para-

site species, and reactive cells were shown to produce higher levels of IFN-γ, conferring natural immunity [14]. It has also been demonstrated to elicit significant immune responses against *L. amazonensis* [59] and *L. major* [60] experimentally. Also, leishmanolysin, gp63, is known as a surface-expressed, critical virulence factor with degrees of immunogenicity; for example, it has shown remarkable immunity in mice vaccinated with gp63-embedded cationic liposomes [61] and gp63-expressing *Salmonella typhimurium* [54]. Based on the immunogenic capacity of both proteins, immunodominant HLA-binding and IFN-γ-inducing epitopes were spotted in the gp46 and gp63 proteins of *L. major*, and the potency of a novel MEVC using those epitopes was evaluated by comprehensive *in silico* approaches.

At the first step, extensive epitope prediction and screening were performed using seemingly protective HLA alleles, which were studied previously. Olivo-Diaz *et al.* (2004) demonstrated that resistance to localized cutaneous leishmaniasis can be facilitated in patients with the DPB1*04:01 allele group [62]. Furthermore, De Vrij *et al.* (2021) demonstrated that individuals with variants of the DRB1*15 and DRB1*16 allele groups had a reduced susceptibility to leishmaniasis, irrespective of the method used for HLA-typing [63]. Notably, B-cell epitopes were excluded from the prediction step since humoral immune responses and higher levels of specific antibodies fail to provide protection against *Leishmania* infections and may be strong predictors of parasite persistence [64]. Additionally, those CD_8^+, IFN-γ-inducing T cell epitopes were predicted being involved in parasite killing and lesion recovery [64]. A crucial part of the multi-epitope vaccine design is the utilization of appropriate linkers. Linkers or spacers are responsible for the flexibility of the polyprotein, its proper folding, and the segregation of functional domains, rendering a more stable protein structure [65]. The “AAY” linker, used to connect CTL epitopes in the current study, increases epitope partitioning and presentation by making the C-termini of CTL epitopes more accessible for binding [66]. Also, a glycine-rich linker, “GPGPG”, not only enhances the construct solubility but also provides flexibility, high accessibility, and free activity for adjacent domains. Of note, these epitopes are capable of inducing HTL responses [67]. Consistent with our study, Khatoon *et al.* (2017) [68] and Shams *et al.* (2021)[25] employed AAY and GPGPG spacers to adjoin the epitope fragments of multi-epitope to be used against visceral leishmaniasis. In the following, we used a rigid, non-flexible linker, “EAAAK”, after the adjuvant sequence in order to prevent possible interaction with the rest of the vaccine sequence and prevent the formation of neo-epitopes [69]. Adjuvants are known as innate immune boosters or catalysts, and their utilization is implicated by the type of immune response required to be elicited. There are a wide range of genetic adjuvants that can be embedded in the MEVCs [70]. In the present study, the 50S ribosomal L7/L12 of *M. tuberculosis* was embedded as an immune enhancer. In fact, it functions as a strong activator of TLR-4, triggering the Toll/IL-1R domain-containing adaptor-inducing IFN-beta (TRIF) and MyD88 pathways. It can also polarize T CD_8^+ and T CD_4^+ cells to release IFN-γ, which could be essential for parasite clearance during leishmaniasis [71]. In other words, TLR4 activation may lead to the release of Th1-mediated cytokines (IFN-γ and IL-12) and assist in combating *Leishmania* parasites [21]. We also embedded the PADRE sequence into the vaccine sequence since it can potentially bind to most HLA-DR molecules, effectively induce T CD_4^+ cells, and is clinically safe for human use as well [72]. Furthermore, due to the primary role of cathepsin S in MHC-II antigen presentation pathways in human skin, the PMGLP cleavable linker was used in the vaccine sequence [73].

Our designed MEVC possessed good antigenicity but lacked allergenicity, with a MW of 35.64 kDa and a final length of 344 residues. An ideal vaccine should have appropriate physico-chemical properties and also elicit a robust immune response. On this basis, the ProtParam web server was used for physico-chemical evaluation. The output showed that the vaccine candidate was hydrophobic (GRAVY: 0.312), highly thermotolerant (high aliphatic index value), and stable in laboratory settings (instability index < 40). This vaccine was confirmed to be soluble in nature, having a solubility score of 0.502. Vaccine solubility helps in better interaction and outcome in a fluid-based milieu in the host’s body [74]. In contrast with previous multi-epitope vaccine design studies against *L. major* by Rabienia *et al.* (2020) [75] and against *L. donovani* by Khatoon *et al.* (2017), [68] helices (56.1 %) were the most plentiful secondary structures in our designed vaccine model, as evidenced by the GOR IV secondary structure prediction web server. Next, the 3D structure of the designed MEVC was predicted using the I-TASSER server and rehashed for global and regional structural relaxations. In the following, the Ramachandran plot analysis and Z-score estimation showed satisfactory improvement, with most residues tightly clustered in the most favored and additional allowed regions in Ramachandran plots.

In the following, a protein–protein docking analysis was performed using the LightDock server. The server is a complete open-access framework that utilizes Swarm Intelligence, a family of artificial intelligence algorithms inspired by natural emergent systems. These algorithms enable more efficient searches in complex spaces and provide a flexible docking platform [40]. Based on the score, Swarm, and Glowworm values, the top-ranked docked conformation possessed the most populated cluster, demonstrating higher interactions between the vaccine model and immune receptor. Using the C-ImmSim web server, the vaccine candidate demonstrated extensive cell-mediated immune induction, as evidenced by high numbers of memory T-cells and elicited Th1-type cytokines (IFN-γ and IL-2). Since immunity against *Leishmania* represents, higher levels of IFN-γ are suggestive of protective immune responses, as evidenced in the simulated immune profile in our study. Also, helper T cells, particularly Th1, play a critical role in combating *Leishmania* parasites and favor protection [76]; on this basis, they were adequately elicited upon vaccination using this novel vaccine candidate, with subsequent production of Th memory cells. Such immune excitement may partly arise from the immunogenic nature of the adjuvants used as potent TLR-4 agonists, *i.e.*, *M. tuberculosis*. 50S ribosomal L7/L12 (Locus RL7_MYCTU). The vaccine candidate showed no homology to the human proteome; it was considered safe for human use. Ultimately, improvements in transcriptional and translational efficiency were done using codon optimization, directed towards high-level expression yield of the recombinant proteins. In the final step, we had a successful virtual cloning of the vaccine into the pET28a(+) vector.

In the literature, some studies have performed immunoinformatics-based predictions to design, engineer, and evaluate different MEVCs against leishmaniases. In a study by Hashemzadeh *et al.* (2019), three *L. infantum* proteins, gp63, KMP-11, and HSP-70 were targeted for B- and T-cell epitope prediction, and a 45.9 kDa polyprotein was designed using GGGGS and GSGSGS linkers, connected to two adjuvants (*M. tuberculosis* RpfE and RpfBG G5 domain) [77]. However, this study lacked secondary structure analysis, molecular docking, and vaccine immune profile evaluation. Rabienia *et al.* (2020) designed a 27.17 kDa MEVC using B-cell, T-cell, and IFN-γ Inducing epitopes HASPB and KMP-11 from *L. major*, with GDGDG linker and profilin (adjuvant). the vaccine and TLR-11 receptor showed stable interaction, and no immune stimulation was done [78]. Another study by Yadav *et al.* (2020) designed a 71 kDa, stable, and hydrophilic multi-component vaccine candidate using B- and T-cell epitopes derived from three *L. donovani* HyP proteins, prevailed by random coils, using AAY and KK spacers [75]. Ropon-Palacios *et al.* (2019) investigated the *in silico* binding of a novel multi-component vaccine (32.5 kDa), designed by four conserved epitopes from Latin American species such as *L. panamensis, L. mexicana*, *L. braziliensis*, and *L. guyanensis*, with the TLR4/MD2 receptor complex, showing a stable interaction [79]. Altogether, the next-generation vaccine design process against leishmaniasis demands the utilization of immunogenic epitopes derived from potent antigenic molecules, with subsequent *in vitro* and *in vivo* confir-

mation using wet laboratory experiments. Our study met some limitations, including **i**the lack of molecular dynamic simulation studies between the vaccine and the immune receptor due to lack of access to high-throughput computer systems; and **ii**) the absence of wet experiments on the safety and efficacy of the designed vaccine construct against challenge with *Leishmania* spp. in animal models. In the future, it would be beneficial to conduct *in vivo* preclinical and clinical experiments to better assess this novel vaccine candidate against *Leishmania* infections.

5. Conclusion

The safety and rational design nature of the multi-epitope vaccines have led researchers to engineer more robust, stable, and efficient vaccine candidates against many pathogens and cancers. Hence, this novel field of immunoinformatics saves time and experimental resources and deserves further exploration. The aim of the present study was to design a novel MEVC against leishmaniasis through the use of IFN-γ. Inducing T-cell epitopes derived from two highly immunogenic vaccine candidate antigens of L. major (gp46 and gp63) are arranged together by different linkers (GPGPG, AAY, EAAAK, PMGLP) and a TLR-4 agonist as adjuvant (M. tuberculosis 50S ribosomal L7/L12). As a final word, the engineered vaccine model in the present study demonstrated antigenic, allergenic, solubility, safety, and physicochemical properties. Moreover, adequate binding scores and members were predicted between the vaccine candidate and the human TLR-4 receptor, along with robust cell-mediated immune stimulation. It is finally noteworthy that *in vitro* and *in vivo* experiments are required to validate the efficacy of the proposed MEVC against leishmaniasis.

CRediT authorship contribution statement

Amir Dehghani: Formal analysis, Writing – original draft. **Mina Mamizadeh:** Software, Writing – original draft. **Atena Karimi:** Investigation, Methodology, Project administration. **Seyyed Amir Hosseini:** Investigation, Methodology, Software. **Davood Siamian:** Investigation, Methodology, Resources, Software. **Morteza Shams:** Supervision, Validation, Project administration, Writing – review & editing. **Shadan Ghiabi:** Formal analysis, Validation, Writing – original draft. **Gholam Basati:** Validation, Visualization, Writing – review & editing. **Amir Abaszadeh:** Formal analysis, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and material

Data is available from the corresponding author on reasonable request.

Ethical approval

This research was ethically approved by the ethics committee of Ilam University of Medical Sciences (Code No.: IR.MEDILAM.REC.1402.162).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgeb.2024.100355.

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