Biologia (2022) 77:277–289
https://doi.org/10.1007/s11756-021-00934-3

ORIGINAL ARTICLE

Engineering a multi-epitope vaccine candidate against *Leishmania infantum* using comprehensive Immunoinformatics methods

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Received: 18 August 2021 / Accepted: 9 October 2021 / Published online: 29 November 2021

Abstract

Visceral leishmaniasis (VL) is a severe disease with particular endemicity in over 80 countries worldwide. There is no approved human vaccine against VL in the market. This study was aimed at designing and evaluation of a multimeric vaccine candidate against *Leishmania infantum* through utilization of helper T lymphocyte (HTL) and cytotoxic T lymphocyte (CTL) immunodominant proteins from histone H1, KMP11, LACK and LeIF antigens. Top-ranked mouse MHC-I, MHC-II binders and CTL epitopes were predicted and joined together via spacers. Also, a TLR-4 agonist (RS-09 synthetic protein) and His-tag were added to the N- and C-terminal of the vaccine sequence, respectively. The final chimeric vaccine had a length of 184 amino acids with a molecular weight of 18.99 kDa. Physico-chemical features showed a soluble, highly-antigenic and non-allergenic candidate. Secondary and tertiary structures were predicted, and subsequent analyses confirmed the construct stability that was capable to properly interact with TLR-4/MD2 receptor. Immunoinformatics simulation displayed potent stimulation of T cell immune responses, with particular rise in IFN-γ, upon vaccination with the proposed multi-epitope candidate. In conclusion, immunoinformatics data demonstrated a highly antigenic vaccine candidate in mouse, which could develop considerable levels clearance mechanisms and other components of cellular immune profile, and can be directed for VL prophylactic purposes.

Keywords Chimeric vaccine · Immunoinformatics · *Leishmania infantum*

Abbreviations

CL	Cutaneous Leishmaniasis
VL	Visceral Leishmaniasis
MHC	Major Histocompatibility Complex
TCR	T cell receptor
APCs	Antigen-presenting cells
ROS	Reactive oxygen species
KMP11	Kinetoplastid membrane protein 11
LACK	*Leishmania*-activated C-kinase
LeIF	*Leishmania* elongation initiation factor

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NCBI	National Center for Biotechnology Information
IEDB	Immune Epitope Database
HTL	Helper T lymphocyte
CTL	Cytotoxic T lymphocyte
ANN	Artificial neural network
SVM	Support vector machine
TLR-4	Toll-like receptor-4
ACC	Auto cross covariance
MW	Molecular weight
pI	isoelectric pH
GRAVY	Grand average of hydropathicity
SOPMA	Self-Optimized Prediction Method with Alignment
LOMETS	Local meta-threading server
GDT-HA	Global distance test-high accuracy
RMSD	Root mean square deviation
PI	Protrusion index
RCSB	Research Collaboratory for Structural Bioinformatics
CAI	Codon adaptation index
PPRs	Pattern recognition receptors
PAMPs	Pathogen-associated molecular patterns
LPS	Lipopolysaccharide
DC	Dendritic cell
RSV	Respiratory Syncytial Virus
MD-2	Myeloid differentiation factor-2

Introduction

Leishmaniases are a family of diseases caused by *Leishmania* protozoan parasites, having different clinical outcomes, from ulcerative cutaneous lesions to life-threatening generalized disease (Desjeux 2004; Meeting WECotCotL, Organization WH 2010). The *Leishmania* agents are transmitted via female phlebotomine sand flies, with a substantial impact on 350 million individuals in 88 countries worldwide (Akhoundi et al. 2016; Maroli et al. 2013). Visceral leishmaniasis (VL) or kala-azar is a neglected disease with *Leishmania donovani* and *Leishmania infantum* as the causative agents, and based on estimated disease burden demonstrates remarkable morbidity and mortality amongst the tropical infectious diseases (Desjeux et al. 2013; Ready 2014). Current VL treatment options are confined to pentavalent antimonials, paramomycin, miltefosine and amphotericin B; however, they are costly and toxic, require long-term administration, have side effects with the progressive risk of drug resistance phenomenon (Freitas-Junior et al. 2012; Solano-Gallego et al. 2017). As well, control strategies on reservoir hosts and vectors are not entirely applicable in endemic areas (Valero and Uriarte 2020). Alternatively, developing vaccines is a safer option for appropriate VL control, comparable to other therapeutic regimens, which elicit durable immunity against the infection (Iborra et al. 2018).

Nowadays, high-throughput vaccine development is facilitated via a close cooperation among immunologists, molecular biologists and chemical engineers (Parvizpour et al. 2020). Our understanding of the host-pathogen interplay has improved with the discovery of newer technologies, opening unprecedented avenues into the field of rational vaccine design (Khatoon et al. 2017). Such outstanding advances include major histocompatibility complex (MHC) molecule structure and restriction, the nature of antigen presentation, T cell receptor (TCR) elucidation as well as identification of cytokines (Djaoud and Parham 2020). An efficacious vaccine candidate would be able to strongly stimulate IFN-γ-releasing Th-1 cells, through antigen presenting cells (APCs), with subsequent activation of macrophages leading to upsurge in reactive oxygen species (ROS) and nitric oxide to combat intracellular amastigotes (Rodrigues et al. 2016). Within this interaction, various antigenic peptides of the pathogen are provided on the surface of APCs by different allelic forms of MHC molecules. Therefore, proper antigen presentation via MHC molecules is a crucial step, which could be exploited to enhance cell-mediated immunotherapies and multi-epitope-based vaccination platforms (Mahida et al. 2015; Matsumura et al. 1992).

Immunoinformatics approach is a novel way to characterize immunogenic B- and T cell epitopes of a particular antigenic molecule for accurate design and engineering of a multi-epitope subunit vaccine model, which could be directed towards activation of host's humoral and cellular responses (Parvizpour et al. 2020). The vaccine design field for *Leishmania* parasites focuses on all three forms of the disease due to the conserved molecules in all involved species (Kumari et al. 2008). In the early twentieth century, "leishmanization" was developed as the first applicable procedure in the history of vaccination against leishmaniasis, however, it was subsequently forbidden to be used in human models for safety concerns (Noazin et al. 2008). As well, the immunity conferred by first-generation vaccines such as those in Iran CL and Sudan VL was not significant regarding cutaneous leishmaniasis (CL) and VL, respectively (Rafati et al. 2005; Singh and Sundar 2012). However, next-generation vaccines including DNA and subunit vaccines opened new doors towards improved vaccine design and delivery against leishmaniasis (Singh and Sundar 2012). In this context, several multicomponent vaccines including KSAC, Leish110-f, Leish111-f and the putative Q protein have been shown to provide protective immunity against VL due to *L. infantum*; however, no such human vaccine have passed the trials towards international markets to date (Chakravarty et al. 2011; Goto et al. 2011; Joshi et al. 2014; Molano et al. 2003).

Previously, several *Leishmania* antigens were used as vaccine candidates. Histone H1 includes several basic proteins involved in nucleosome stabilization with high immunogenicity being overexpressed in amastigote and promastigote stages of *L. major* and *L. infantum* (Carmelo et al. 2002; Galanti et al. 1998; Requena et al. 2000). Kinetoplastid membrane protein 11 (KMP11) is a highly-conserved potent immunogen in *Leishmania*, expressed in both developmental stages, and the only antigen being detected by the sera of asymptomatic subjects (Fuertes et al. 1999; Jardim et al. 1995). The *Leishmania*-activated C-kinase (LACK) antigen, expressed in both amastigote and promastigote forms, is the most investigated component in DNA vaccine candidates against both CL and VL (Nagill and Kaur 2011). Also, *Leishmania* elongation initiation factor (LeIF) is a virulence factor expressed in both stages of all species of *Leishmania* (Cordin et al. 2006), being involved in transcription, translation, RNA export and degradation, pre-mRNA splicing and ribosomal biogenesis (Fuller-Pace 1994). The present in silico study was aimed at identification of immunogenic epitopes of four antigenic compounds from *L. infantum*, i.e. histone H1, KMP11, LACK and LeIF, in order to assemble a novel multi-epitope vaccine candidate to be used against VL.

Materials and methods

Retrieval of protein sequences

The amino acid sequences of *L. infantum* histone H1 (Accession no. ABB22792), KMP11 (Accession no. CAC9544709.1), LACK (Accession no. ALP73427.1) and LeIF (Accession no. XP_001466106.1) were gathered in FASTA format from the National Center for Biotechnology Information (NCBI), available at http://www.ncbi.nlm.nih.gov (Sayers et al. 2021).

MHC-I binding epitope prediction and screening

In this study, 3 web servers were used to predict potential MHC-I binding peptides: Immune Epitope Database (IEDB), ProPred-I and SYFPEITHI. High affinity epitopes to mouse MHC-I alleles, including H2-Db, H2-Dd, H2-Kb, H2-Kd, H2-Kk and H2-Ld were recognized as immunodominant epitopes.

The ProPred-I server (http://crdd.osdd.net/raghava//propred1/) identifies MHC-I promiscuous regions in an antigen based on matrices for 47 MHC class-I alleles, with the highest accuracy of 75% at 4% threshold using default settings (Singh and Raghava 2003). In IEDB server a percentile rank is given to each epitope, having inverse correlation with affinity. Epitope prediction was done using 10-mer length and IEDB recommended method 2020.04 (NetMHCpan EL 4.0) (http://tools.iedb.org/mhci/) (Kim et al. 2012). Also, SYFPEITHI server was used to predict decamer peptides against the selected mouse MHC-I alleles (http://www.syfpeithi.de/) (Rammensee et al. 1999). At final step, potent overlapping peptides among three web servers were selected and assessed regarding antigenicity and allergenicity using VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) and AllerTOP v2.0 (https://www.ddg-pharmfac.net/AllerTOP/) servers, respectively. One epitope with highest antigenic index without allergenicity was ultimately selected from each antigen to be embedded in the final vaccine construct.

MHC-II binding epitope prediction and screening

Helper T lymphocyte (HTL) epitopes were predicted using IEDB, RANKPEP and MHCPred v2.0 servers, against 5 mouse MHC-II alleles, i.e. H2-IAb, H2-IAd, H2-IAs, H2-IEd and H2-IEb.

The MHC-II binding prediction tool of IEDB server, available at (http://tools.iedb.org/mhcii/), was utilized to predict 15-mer binders using NN-align 2.3 (NetMHCII 2.3) method (Kim et al. 2012). Another web server, RANKPEP (http://imed.med.ucm.es/Tools/rankpep.html), makes predictions of epitopes (9-mer) on the basis of position-specific scoring matrix (PSSM) with threshold percentage of 4% (Reche et al. 2002). MHCPred V.2.0 (http://www.ddg-pharmfac.net/mhcpred/MHCPred/) was also employed for prediction of MHC-II binding epitopes (Vakili et al. 2018). Finally, shared, high-ranked epitopes predicted by above servers were selected to evaluate their antigenicity and allergenicity using VaxiJen v2.0 and AllerTOP v2.0 servers. Only 1 antigenic, non-allergenic epitope was selected from each antigen to be included in the vaccine sequence.

Prediction and screening of cytotoxic T lymphocyte (CTL) epitopes

In order to predict CTL epitopes, the CTLpred server (http://crdd.osdd.net/raghava/ctlpred/) with combined method was employed, applying artificial neural network (ANN) and support vector machine (SVM) algorithms (Bhasin and Raghava 2007). Furthermore, defined epitopes were screened in terms of antigenicity, allergenicity and toxicity using VaxiJen v2.0, AllerTOP v2.0 and ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) servers, respectively. For each antigen, only 1 peptide having highest antigenic score and without allergenic and toxic traits was selected for the vaccine model construction.

Design and assemblage of the multi-epitope vaccine model

The final multi-epitope vaccine model was engineered and assembled using the qualified MHC-binders and CTL epitopes for each candidate antigen. Hence, 12 immunogenic epitopes were accurately predicted in silico and screened using different web servers. These epitopic regions were linked together with separate linkers or spacers. The CTL and MHC-I epitopes were connected by "AAY" linker, while HTL epitopes were linked by GPGPG spacers. Moreover, the vaccine immunogenicity was enhanced by addition of a toll-like receptor 4 (TLR-4) agonist peptide (RS-09; sequence: APPHALS) to the N-terminal of the vaccine sequence with the aid of EAAAK linker. Notably, a 6×histidine (6×His) residue was considered at the C-terminal of the designed sequence for further protein purification purpose.

Evaluation of allergenicity, antigenicity, solubility and physico-chemical properties

Two web servers evaluated the allergenicity of the designed vaccine model: AllerTOP v2.0 (accuracy: 85.3%) and AllergenFP v1.0 (accuracy: 88%). The AllerTOP server (https://www.ddg-pharmfac.net/AllerTOP/) transforms protein sequences into the integral equal-length vectors based on auto cross covariance (ACC) (Dimitrov et al. 2014a). On the other hand, AllergenFP server (https://ddg-pharmfac.net/AllergenFP/) predicts allergens by a new alignment-free, descriptor-based fingerprint approach based on structural and physico-chemical characteristics (Dimitrov et al. 2014b).

The protein antigenicity was predicted using two servers: VaxiJen v2.0 (accuracy: 70–89%) and ANTIGENpro (accuracy: 82%). The VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) makes predictions based on ACC transformation of the submitted sequence into uniform vectors, which is truly relied on the chemical nature of proteins (Doytchinova and Flower 2007). Also, ANTIGENpro (http://scratch.proteomics.ics.uci.edu/) is a pathogen-independent, alignment-free tool of SCRATCH protein predictor server (Magnan et al. 2010).

ProtParam server was used for physico-chemical prediction, being available at https://web.expasy.org/protparam/. This server computes several parameters comprising molecular weight (MW), theoretical isoelectric pH (pI), amino acid composition, in vitro and in vivo protein half-life, aliphatic index, instability index and the grand average of hydropathicity (GRAVY) score (Gasteiger et al. 2005). The Protein-Sol server (https://protein-sol.manchester.ac.uk/) was also employed for the prediction of protein solubility. Protein scores over 0.45 are considered as highly soluble (Hebditch et al. 2017).

Secondary and tertiary structure prediction

The Self-Optimized Prediction Method with Alignment (SOPMA) (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) is a web server to predict the secondary structure of a given protein (Geourjon and Deleage 1995). Next, the LOMETS (LOcal MEta-Threading-Server, version 3) web server (https://zhanglab.ccmb.med.umich.edu/LOMETS/) was used for homology modelling with full-length atomic models constructed by template-based fragment assembly simulations based on the results from ten threading programs, including FUGUE, HHsearch, MUSTER, PPA, PROSPECT2, SAM-T02, SPARKSX, SP3, FFAS, and PRC (Wu and Zhang 2007).

Refinement of the 3D model and validations

Refinement was done using GalaxyRefine server, available at http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE. For this purpose, the high-ranked 3D model predicted by LOMETS was submitted to GalaxyRefine server for further rehashing and mild/aggressive quality improvement. The output is provided by several parameters, including global distance test-high accuracy (GDT-HA), root mean square deviation (RMSD), MolProbity, Clash score, Poor rotamers and Rama favored (Heo et al. 2013). Subsequently, three web servers validated the refinement process: ERRAT (https://saves.mbi.ucla.edu/) (Colovos and Yeates 1993), Prosa-Web (https://prosa.services.came.sbg.ac.at/prosa.php) (Wiederstein and Sippl 2007) and MolProbity (http://molprobity.biochem.duke.edu/) (Majid and Andleeb 2019).

Conformational B cell epitope prediction

ElliPro of the IEDB server (http://tools.iedb.org/ellipro/) is one of the well-known online tools for conformational B cell epitope prediction, which employs a significant AUC score of 0.732, using default options of 6 Å max distance and 0.5-min score. The prediction of epitopes occurs in a three-step process: calculation of neighbor residue clustering, residual protrusion index (PI) and protein shape. Of note, high-score resides may be associated with improved solvent accessibility (Ponomarenko et al. 2008).

Vaccine protein disulfide engineering

The geometric conformation and total stability of a protein could be improved by disulfide bond formation. Hence, DbD2 server (http://cptweb.cpt.wayne.edu/DbD2/index.php) was used to evaluate the possibility of the occurrence of such bonds in the chimeric protein, via making cysteine mutations in those residues located at the highly-mobile area of the vaccine sequence (Craig and Dombkowski 2013).

Molecular interaction between the vaccine model and TLR-4 ligand

At first, the 3D structure of the receptor, TLR4/MD2 (Accession no. 3FXI) was retrieved from the PDB database of Research Collaboratory for Structural Bioinformatics (RCSB) (https://www.rcsb.org). ClusPro 2.0 protein-protein docking server was used to predict the binding affinity between the designed vaccine model and the examined receptor with default settings (Kozakov et al. 2010). The output of the server is provided as a top-rank cluster, among which the best docking pose is selected for visualization.

Codon optimization and in silico cloning

High-level protein expression in *E. coli* is a vital step for subunit vaccine production. Thus, reverse translation and codon optimization were performed by reverse translate tool of Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/rev_trans.html) and JCat server (http://www.jcat.de/), respectively. Several key features of a DNA sequence such as codon adaptation index (CAI) and GC content are appraised using JCat, which play a major role in efficient expression of a chimeric protein in the respective host. In this section, codon optimization was done for better expression in *E. coli* K12 strain. At final stage, the suitable restriction sites of *Eco53KI* and *EcoRV* restriction enzymes were added to the 5′ and 3′-OH of the codon adapted vaccine sequence.

Immune simulation

The virtual immune simulation profile provoked by the finally approved vaccine model was predicted using C-ImmSim online server, available at http://150.146.2.1/C-IMMSIM/index.php. These predictions are premised on PSSM for machine learning methods. The output implies to three stimulated regions including bone marrow, thymus and lymph node (Rapin et al. 2010). This computer-aided simulation was accomplished using default parameters with random seed 12,345, simulation volume 10 and simulation steps 100. The output was compared to simulation data from A2 protein of *L. chagasi* (Accession No: GQ290460) as a representative immunogenic protein (Coelho et al. 2003).

Results

Prediction of MHC-binders and CTL epitopes

Highly antigenic, non-allergenic MHC-I binding epitopes (Supplementary File 1) and MHC-II binding peptides (Supplementary File 2) were selected for each examined *L. infantum* protein through a multi-step process using multiple web servers. Moreover, CTL epitopes with highest antigenic score and without allergenic and toxic features were included in the final vaccine construct (Supplementary File 3).

Engineering and assemblage of the multi-epitope peptide vaccine

Based on the shared, high-ranked, antigenic CTL, HTL and MHC-I binding epitopes, totally 12 epitopic regions were selected from candidate proteins, which subsequently linked with appropriate linkers. Moreover, a TLR-4 agonist (RS-09 peptide) as a potent adjuvant and a histidine tag for purification were respectively added to the N- and C-terminal of the final vaccine sequence. Therefore, a 184 amino acid multi-epitope peptide vaccine was designed and assembled. A schematic representation of the vaccine design procedure, the employed web servers and the final vaccine construct are provided in Fig. 1.

Prediction of physico-chemical, solubility, antigenicity and allergenicity characteristics

The protein had an 18.99 kDa MW (immunogenic) with an alkaline theoretical pI (8.77). It was considered as stable (instability index: 34.91), relatively thermotolerant (aliphatic index: 66.20) and hydrophobic in nature (GRAVY score: 0.138). Other physico-chemical details of the vaccine model are provided in Table 1. Based on Protein-Sol server, the protein was soluble (0.562), while it was non-allergenic in nature based on AllerTOP and AllergenFP outputs. Furthermore, the probability of the whole vaccine antigenicity was calculated to be 0.7845 (threshold 0.5) by VaxiJen v2.0 server and 0.8546 by ANTIGENpro server, showing proper antigenic properties.

Secondary structure and homology modelling

SOPMA results for secondary structure prediction unleashed that 90 (48.91%) residues are alpha helix, 59 (32.07%) are random coil, 26 (14.13%) are extended strand and 9 (4.89%) are beta-turn (Fig. 2A). Ten 3D templates were generated by threading servers of LOMETS, among which 5a9q8 template had a good alignment based on normalized Z-score of 3.50. Also, the first model generated by the server was used for further refinement and validations (Fig. 2B).

Refinement and validations of the tertiary modeled structure

The chosen 3D model was refined using GalaxyRefine server. This server introduced five rehashed models among which model number two was the best one, having GDT-HA

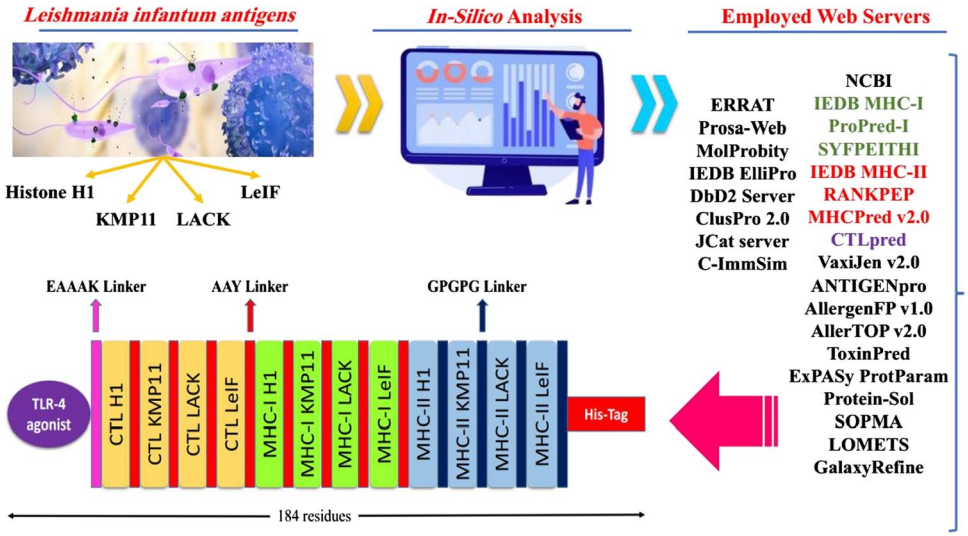

Fig. 1 Schematic representation of the multi-epitope vaccine design, used web servers and the final vaccine construct (184 residues). Those servers highlighted in green, red and purple were used for the prediction of MHC-I binding, MHC-II binding and cytotoxic T lymphocyte epitopes, respectively

Table 1 Physico-chemical assessment of the vaccine construct

Parameter	Result
Number of amino acids	184
Molecular weight (MW)	18.99 kDa
Positive residues (Arg + Lys)	17
Negative residues (Asp + Glu)	11
Theoretical isoelectric point (pI)	8.77
Extinction coefficient (at 280 nm in H_2O)	29,045 M^{-1} cm^{-1}
Estimated half-life (mammalian reticulocytes, in vitro)	4.4 h
Estimated half-life (Yeast cells, in vivo)	>20 h
Estimated half-life (*Escherichia coli*, in vivo)	>10 h
Instability index	34.91
Aliphatic index	66.20
Grand average of hydropathicity (GRAVY)	0.138

of 0.9688, RMSD of 0.383, MolProbity of 2.371, Clash score of 21.5, Poor rotamers of 0.0 and Rama favored of 90.1. Based on Prosa-Web and ERRAT outputs, the quality of the model was shown to be improved from −5.93 Z-score and 70.45 quality factor in crude model to −6.02 Z-score and 75.00 quality factor in refined model. Moreover, MolProbity server result demonstrated that 80.2% of residues were in favored regions, while 96.7% of residues were located in allowed areas. After refinement, 90.1% and 98.4% of all residues were allocated to the favored and allowed regions, respectively (Fig. 3).

Prediction of conformational B cell epitopes

Based on ElliPro tool of the IEDB server, six conformational B cell epitopes were predicted in the refined vaccine sequence with the following scores: 1) 26 residues (0.834), 2) 16 residues (0.736), 3) 12 residues (0.669), 4) 6 residues (0.645), 5) 19 residues (0.587) and 6) 27 residues (0.559) (Fig. 4).

Vaccine protein disulfide engineering

In total, DbD2 server showed that 24 residues possessed the potential to establish disulfide bonds. However, chi3 and B-factor energy (Kcal/mol) screening of the residues elucidated that only one residue pair (GLY 100 – ALA 160) could actually satisfy the formation of disulfide bonds, if mutated to cysteine. Screening of the residues was done on the basis of <2.5 energy value and − 87 to +97 chi3 value.

Molecular docking with TLR-4

The ClusPro 2.0 server was used for molecular docking procedure of the vaccine model with TLR4/MD2 receptor. First rank and most populated docking cluster (50 members) with the highest binding score (−940.6) was chosen for the visualization of the binding conformation between both examined molecules. As depicted in Fig. 5, the designed vaccine peptide has interactions with chains A (red) and C (yellow) of the receptor complex.

Codon optimization and in silico cloning

Reverse translation of the protein sequence into the DNA sequence was done using the reverse translate tool of the Sequence Manipulation Suite. In the following, the sequence was submitted to the JCat server for codon optimization and improved expression level in *E. coli* K12 strain. The CAI value and GC% of the initially submitted sequence were 0.56 and 61.77, respectively, whereas these were improved in the codon optimized sequence as 1.0

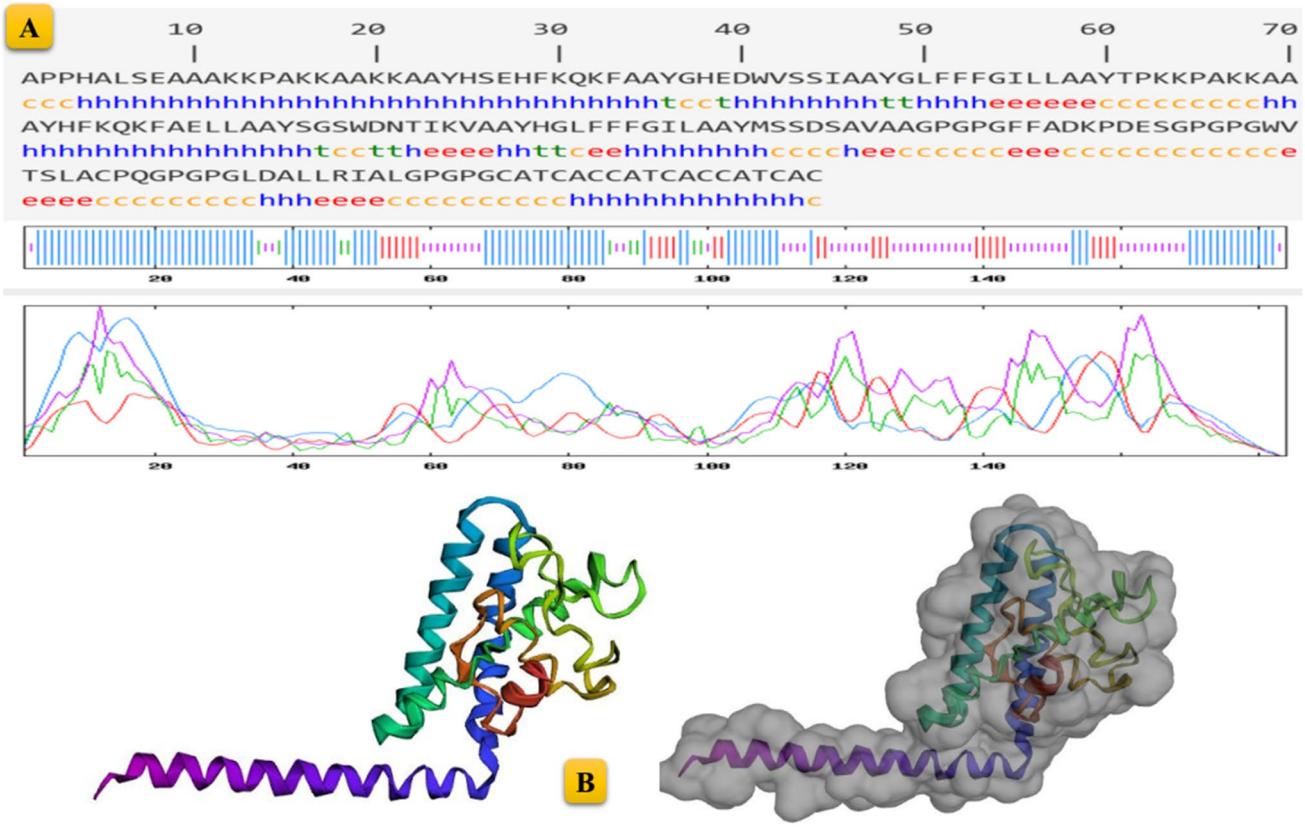

Fig. 2 (A) SOPMA server sequence-based (above) and graphical (below) secondary structure prediction, with Blue (Hh), orange (Cc), Red (Ee) and green (Tt) are alpha helix, random coil, extended strand and beta turn, respectively; (B) 3D model of the final vaccine construct shown as ribbon and surface predicted by LOMETS server

and 55.07, correspondingly. These obtained results suggest that the expression of enhanced DNA sequence of the vaccine is maximum in the selected host.

Immune simulation profile

In silico evaluation of the immune response profile of the vaccine candidate revealed the generation of appropriate immunity against *L. infantum* infection (Fig. 6). In comparison with *L. chagasi* A2 protein, our vaccine model showed elevated active and presenting macrophage population (about 120 cells/mm^3), progressively high levels of helper T memory cells (over 5500 cells/mm^3 for up to 30 days) and relatively higher IFN-γ levels (over 400,000 ng/ml). Regarding A2 protein immune profile, no helper T memory cells were observed, while cytotoxic T cells showed a more rapid upsurge than our vaccine model but the persistence of such population was proportionately equal between the two proteins (Supplementary File 4).

Discussion

Vaccination is an outstanding preventive strategy for rapid, affordable and efficient improvement of the public health and infectious diseases control (Yaqub et al. 2014). Current subunit vaccines, with special emphasis on multi-epitope platforms, which harbor particular immunogenic components of a given pathogen, are more focused than whole organism and/or attenuated vaccines (Parvizpour et al. 2020). This approach is facilitated using bioinformatics and immunoinformatics web servers and/or standalone programs, which could efficiently discover the novel vaccine targets, i.e. immunogenic B- and T cell epitopes, from a huge bulk of genomic and proteomic information and direct them towards rational vaccine design (Goumari et al. 2020). In current study we exploited their immunodominant epitopes from histone H1, KMP11, LACK and LeIF proteins to develop a multi-epitope vaccine model using immunoinformatics web servers.

Since *L. infantum* is an intracellular parasite, Th-1 immune profile play a critical role to control the expansion of

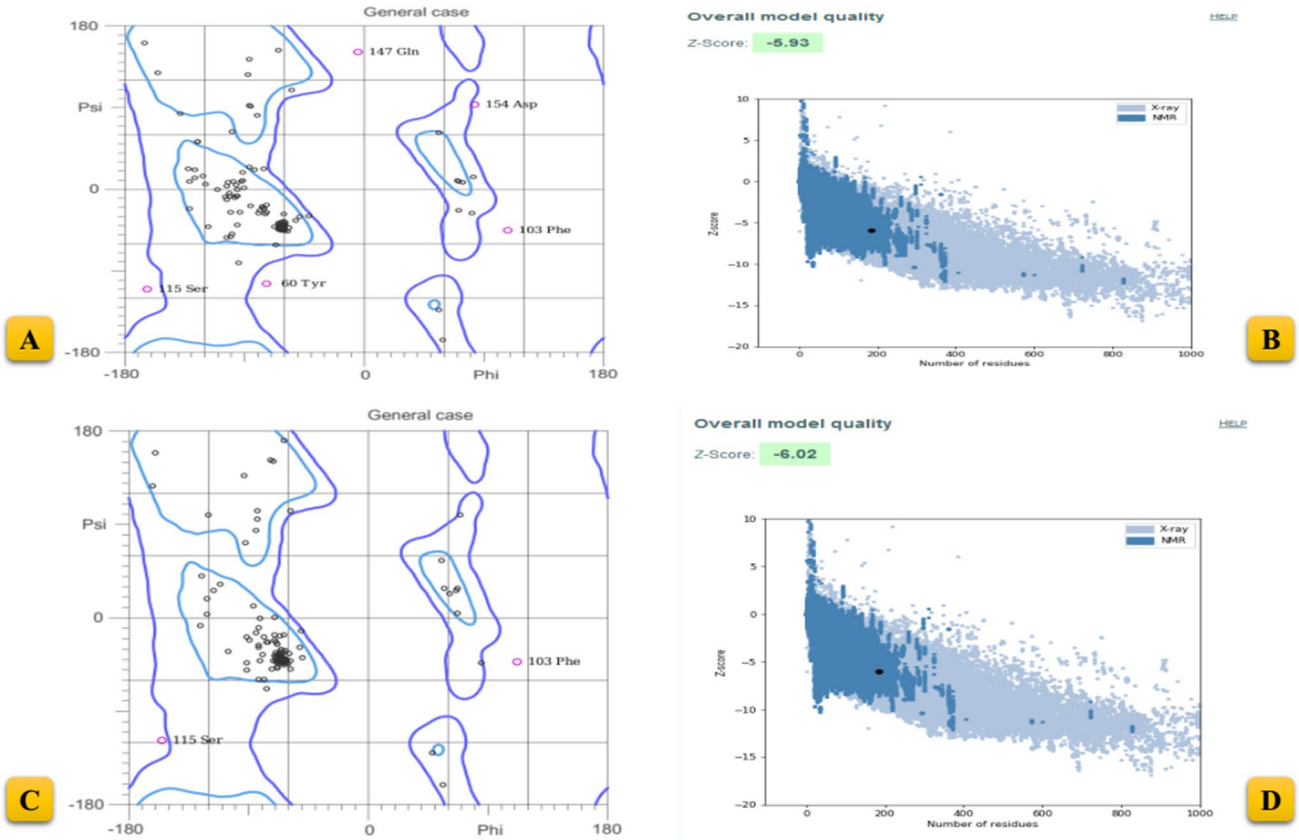

Fig. 3 Confirmation of the quality of refined model provided by the GalaxyRefine server. (A) Based on MolProbity results for crude vaccine model, 80.2% of residues were in favored regions, while 96.7% of residues were located in allowed areas. (B) The overall crude model quality by Prosa-Web server was a Z-score of −5.93. (C) After refinement, 90.1% and 98.4% of all residues were allocated to the favored and allowed regions, respectively, as illustrated in MolProbity server. (D) The refined vaccine model showed improvement in quality as demonstrated by a Prosa-Web Z-score of −6.02

the infection, while neutralizing antibodies are less involved in protection. Accordingly, vaccine candidates composed of only specific T cell epitopes are more in focus (Joshi et al. 2014); thus in the present study we discovered, screened and selected only T cell epitopes from the above-mentioned protein targets (Histone H1, KMP11, LACK and LeIF). In details, HTL epitopes were predicted against various alleles of mouse MHC-I (H2-Db, H2-Dd, H2-Kb, H2-Kd, H2-Kk and H2-Ld) and MHC-II (H2-IAb, H2-IAd, H2-IAs, H2-IEd and H2-IEb) molecules, using cross-validating approach via multiple bioinformatics servers. Next, shared epitopes were selected and screened regarding antigenicity and allergenicity. As well, CTL-specific epitopic regions of each examined antigen were predicted and screened in terms of antigenicity, allergenicity and toxicity. Ultimately, high-scored, antigenic, non-allergenic and non-toxic peptides were chosen to assemble a multi-epitope vaccine construct using specific linkers, i.e. "AAY" (CTL), "GPGPG" (HTL) and "EAAAK" (adjuvant). In general, linkers are crucial in producing flexible molecules (extended conformation), proper folding of proteins and separation of functional domains, hence rendering the protein structure more stable (Dong et al. 2020).

Adjuvants act as innate immune catalysts, which initiate focal inflammatory sites that is relieved by antigen-TLR interactions. In this sense, the aluminum-based adjuvants are mostly the first choice for the majority of vaccines, however, they represent various side effects (Shanmugam et al. 2012). TLRs are a group of pattern recognition receptors (PPRs), specialized for the detection of pathogen-associated molecular patterns (PAMPs) (Coffman et al. 2010; Suresh and Mosser 2013). Recently, novel synthetic peptides mimicking the interaction of bacterial lipopolysaccharide (LPS) with TLR-4 receptor, can substantially balance signal transduction pathways, likewise those triggered by the adjuvants. Hence, they could be recognized as LPS mimotopes having particular association with TLR-4 receptor, so they're called TLR-4 agonists (Shanmugam et al. 2012). The potential low immunogenicity of the multi-epitope vaccine was obviated by means of adding a TLR-4 agonist peptide (RS-09; sequence: APPHALS), as a natural adjuvant, to the N-terminal of the designed sequence. Upon binding to TLR-4,

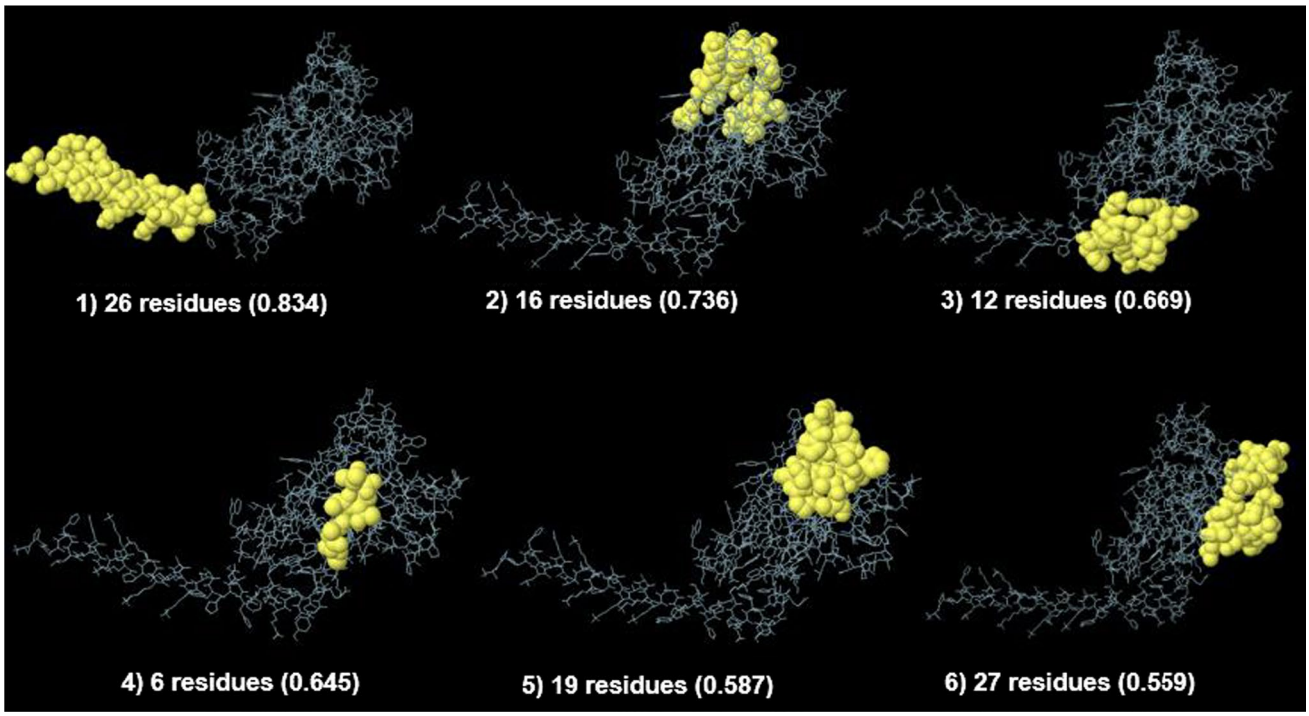

Fig. 4 Predicted conformational B cell epitopes of the chimeric vaccine by ElliPro tool of IEDB analysis Resource. Length and score of each epitope are given in parentheses

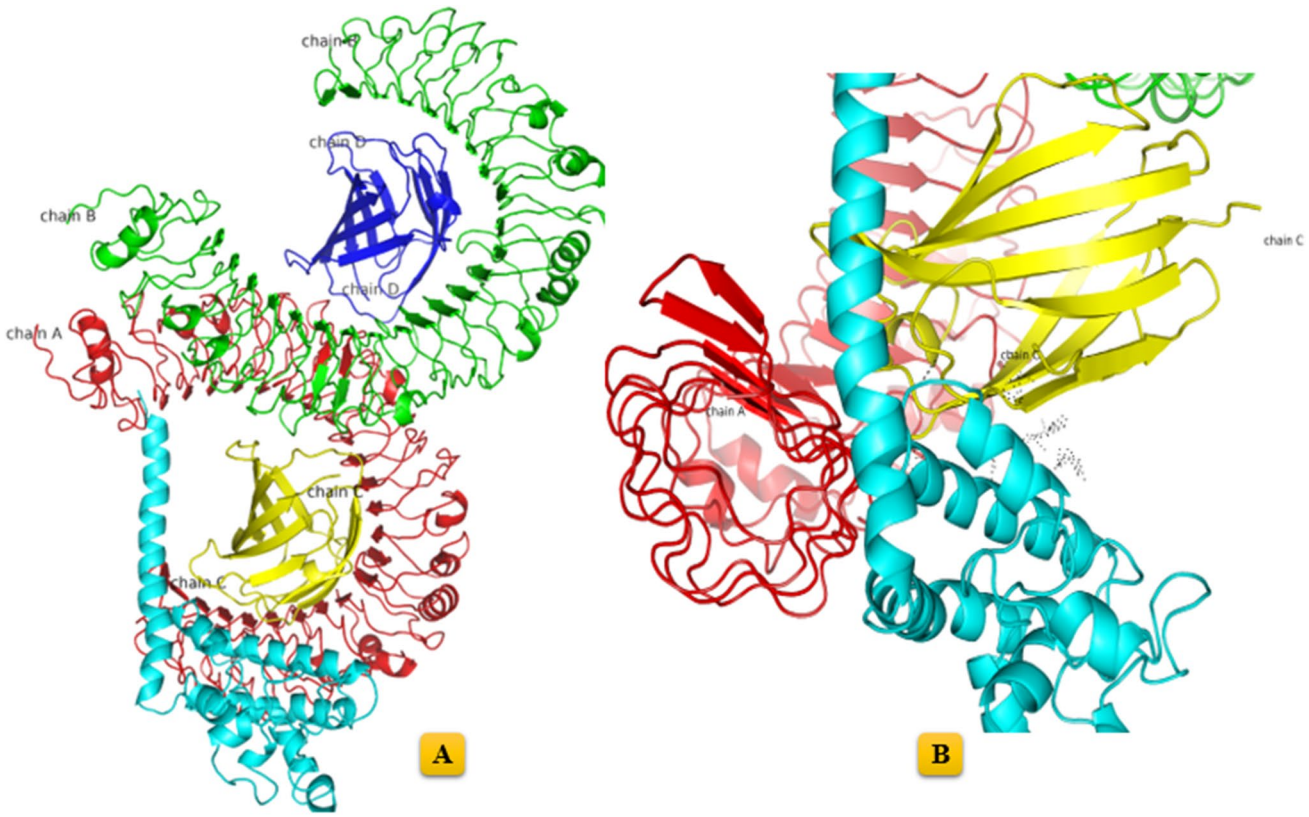

Fig. 5 The binding conformation of the designed multi-epitope vaccine and TLR-4/MD-2 receptor complex. The designed peptide is in association with chains A (red) and C (yellow) of the receptor

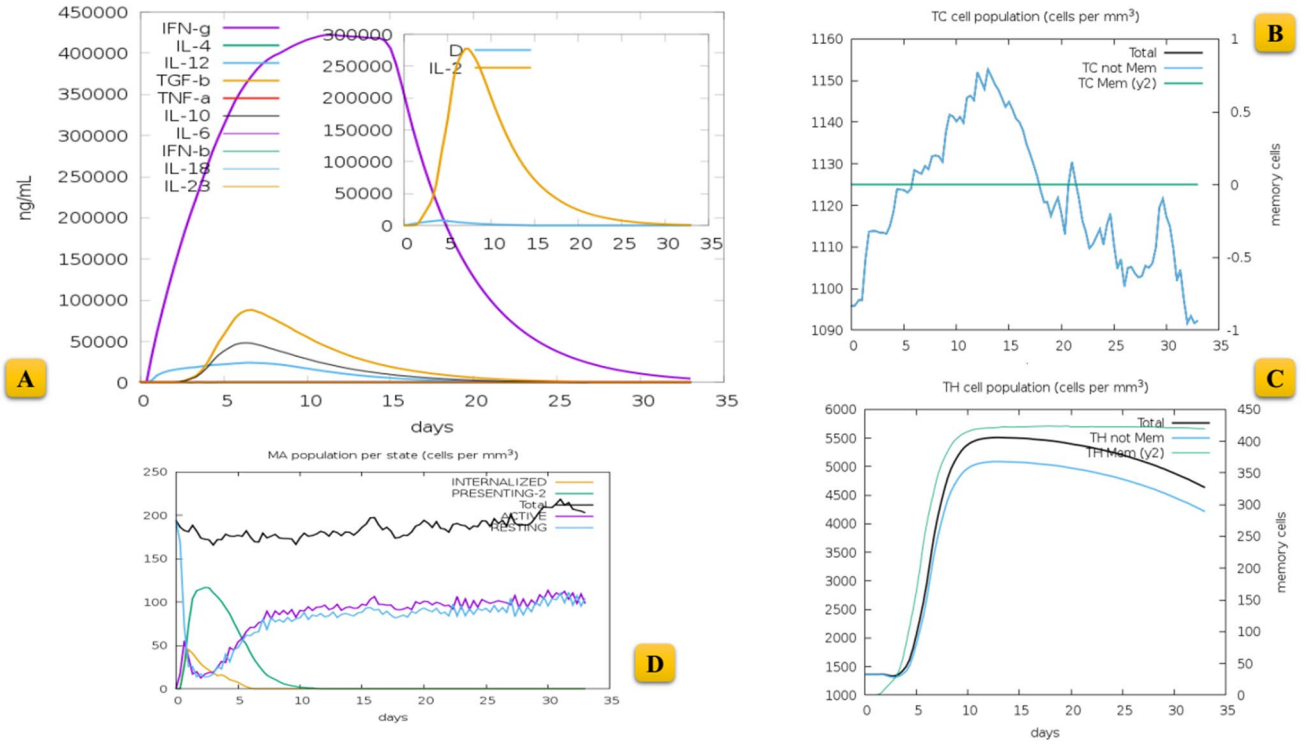

Fig. 6 The output of in silico immune simulation with the subunit vaccine provided by C-ImmSim server. (A) Level of cytokines (ng/ml) induced by the vaccine injection. The insert plot shows the IL-2 level with the Simpson index, D indicated by the dotted line, (B) T cytotoxic cell population per state (cells/mm^3) upon antigen injection, (C) T helper cell population per state (cells/mm^3) after antigen injection. (D) Macrophage population per state (cells/mm^3) after antigen injection

this peptide activates dendritic cells (DCs), leading to the polarization of naïve T CD_4^+ and CD_8^+ cells and subsequent secretion of IFN-γ and IL-2. Such a Th-1 immune profile is absolutely essential for the parasite clearance; hence, immunization against VL using natural adjuvants would be more effective (Vakili et al. 2018). Of note, a 6×His sequence was applied in the C-terminal of the designed vaccine model to improve the purification process of the respective vaccine protein.

Along with the ability to elicit strong cell-mediated immune responses, an appropriate vaccine candidate should, also, possess admissible physico-chemical features during production steps. Based on bioinformatics analysis, the MW of the vaccine protein was 18.99 kDa with pI of 8.77, showing that the molecule is a potent immunogen (> 5–10 kDa) and it is relatively alkaline in nature. Moreover, this protein was proven to be stable (instability index: 34.91), hydrophobic in nature (GRAVY score: 0.138) and relatively thermotolerant (aliphatic index: 66.20). Additionally, the multi-epitope vaccine was highly soluble, antigenic and non-allergenic, which could be expressed efficiently in *E. coli* (K12 strain) after codon optimization. In the following secondary structure prediction results by SOPMA server showed that there were 48.91% alpha helix, 32.07% random coil, 14.13% extended strand and 4.89% beta turn in the sequence. Also, the 3D structure was modelled by LOMETS server, which needed refinement to yield a high-quality tertiary structure for docking process. Hence, GalaxyRefine server was used, which provided five refined models, among which the model with GDT-HA of 0.9688, RMSD of 0.383, MolProbity of 2.371, Clash score of 21.5, Poor rotamers of 0.0 and Rama favored of 90.1 was selected. After refinement, also, three web servers (ERRAT, Prosa-Web and MolProbity) confirmed the quality of the rehashed model. In the following the interaction between the refined vaccine model as a ligand and TLR-4 as a receptor was performed using ClusPro 2.0 server. As mentioned above, a small peptide (RS-09) as the TLR-4 agonist was added to the N-terminal of the vaccine sequence, which interacts with this receptor. The interplay between TLR-4 and its ligands has not been clearly elucidated (Wang et al. 2016); for instance, some ligands such as LPS, fusion protein of Respiratory Syncytial Virus (RSV) and *Chlamydia pneumonia* heat shock protein 60 bind to the receptor via a co-receptor named myeloid differentiation factor-2 (MD-2) (Peri and Piazza 2012; Rallabhandi et al. 2006; Wang et al. 2016). An internal pocket containing hydrophobic and positively-charged residues is formed by MD-2 co-receptor, which adequately fits amphipathic and negatively-charged agnostic

molecules such as LPS (Park and Lee 2013). The findings of the molecular docking process showed that chains A (red) and C (yellow) of the receptor complex are in association with the multimeric vaccine. Finally, the immune response profile of the engineered multi-epitope vaccine was simulated using C-ImmSim server and compared with that of *L. chagasi* A2 protein. Our multi-epitope vaccine showed some advantages, including potent memory development in helper T cell population, relatively higher IFN-γ and active/presenting macrophage populations. Regarding A2 protein, no helper T memory cell was elicited and only a rapid upsurge was observed in cytotoxic T cell population, in comparison to our vaccine model, which both persisted equally.

Previously, some studies employed immunoinformatics approach to design and evaluate multi-epitope vaccine candidates against *Leishmania* parasites. Hashemzadeh et al. (2020) selected B- and T cell epitopes from GP63, KMP-11 and HSP-70 proteins of *L. infantum* and designed a multi-epitope vaccine construct using GGGGS and GSGSGS linkers and RpfE and RpfBG G5 domain of *Mycobacterium tuberculosis* as adjuvant. In contrast to our study, only preliminary *in-silico* analyses were done for the 45.9 kDa candidate without secondary structure prediction, protein disulfide engineering, molecular docking and evaluation of immunological profile (Hashemzadeh et al. 2020). Another study by Rabienia et al. (2020) utilized B- and T cell as well as IFN-γ inducing epitopes of *L. major* KMP-11 and HASPB to engineer a multi-epitope vaccine candidate (27.17 kDa), adjoined by GDGDG linker and profilin as adjuvant. Docking results demonstrated that profilin adequately binds well to TLR11 and the vaccine/receptor interaction is stable (Rabienia et al. 2020). Ropón-Palacios et al. (2019) exerted a somehow different strategy to develop a highly-efficacious vaccine candidate possessing 4 conserved epitopes among important *Leishmania* spp. in Latin America, including *L. braziliensis*, *L. mexicana*, *L. panamensis* and *L. guyanensis*. They utilized AAY and GPGP linkers to connect epitopes and 50S ribosomal protein L7/L12 as adjuvant. The 32.5 kDa vaccine candidate stably coupled with TLR4/MD2 receptor complex with strong H bond and hydrophobic interaction (Ropón-Palacios et al. 2019). In our study, we performed immune simulation to predict the immunological profile of the designed vaccine candidate upon injection, while such analysis was not performed in above studies. Moreover, α-helix was the most prominent secondary structure in our candidate, similar to the latter study, whereas random coils were most frequent in Rabienia et al. study. Notably, the MW of our vaccine candidate (about 19 kDa) was lower than that of previously-mentioned studies, which could be beneficial for future wet lab experiments such as purification purposes.

Conclusion

The gradual increase in the development of multi-epitope peptide vaccines is, to a great extent, due to their safety and rational design, which saves experimental resources and time. The present computer-aided study was aimed at accurate prediction of immunodominant CTL and HTL epitopes of four candidate proteins of *L. infantum* (Histone H1, KMP11, LACK and LeIF) to engineer and organize a multi-epitope-based subunit vaccine against VL. In total, the designed vaccine model showed acceptable structural, physico-chemical, antigenicity, allergenicity, solubility characteristics. As well, molecular docking with the TLR-4 receptor and the immune profile elicited by the vaccine candidate added more to the novelty and acceptability of this in silico designed molecular vaccine. However, in vitro and in vivo immunological studies are needed to confirm the efficacy of this maiden multi-epitope vaccine.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11756-021-00934-3.

Authors' contributions M. Shams and S. A. Shariatzadeh conceived the study protocol; M. Shams, M. Fatollahzadeh and H. Majidiani performed the bioinformatics analyses; A. Asghari assisted to prepare the revised version and improved the discussion; H. Irannejad performed the molecular docking process; M. Shams and H. Nourmohammadi drafted the manuscript. All authors read and approved the final version of the manuscript.

Funding This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and material Data are available from the corresponding author on reasonable request.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare none.

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