



# Immunoinformatics Approach to Design a Novel Subunit Vaccine Against Visceral Leishmaniasis

Alireza Lari<sup>1</sup> · Niloofar Lari<sup>1</sup> · Atefeh Biabangard<sup>2</sup>

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## Abstract

Visceral leishmaniasis (VL) infection is mostly caused by *Leishmania donovani* and affects countries worldwide. Despite the need for a safe and effective vaccine against leishmaniasis due to the increased drug resistance, however, no vaccine has yet been licensed for clinical use. This study revolves around the immunoinformatics approach to design a multi-epitope vaccine against VL infection. In this case, the proteome of *L. donovani* has been investigated, and three host non-homologous and antigenic extracellular secretory proteins have been identified as potential vaccine candidates with low transmembrane helices ( $\leq 1$ ). The multi-epitope subunit vaccine construct consists of T-cell (cytotoxic T-lymphocyte (CTL) and helper T-lymphocyte (HTL)) epitopes accompanied by appropriate adjuvant and linkers. A 372-amino acid vaccine construct has been established with specific characteristics, such as soluble, stable, antigenic, non-allergenic, non-toxic, and non-host homologous. Besides, the tertiary structure of the designed vaccine was modeled and validated. Also, the stability and affinity of the vaccine- TLR4 complex were confirmed by using molecular docking and molecular dynamics (MD) simulation. In addition, in silico immunization assay showed the efficiency of this candidate vaccine to stimulate an effective immune response. Furthermore, the refined vaccine was optimized and cloned in the pET28a (+) vector, and its successful expression was confirmed virtually. However, the experimental validation is required to verify the multi-epitope vaccine efficacy against VL infection.

**Keywords** Visceral leishmaniasis (VL) · Hypothetical proteins (HPs) · Subunit vaccine · Immunoinformatics

## Introduction

Leishmaniasis is a vector-borne disease caused by infection with *Leishmania* parasites that predominantly affects the world's most vulnerable and poorest populations. *Leishmania* parasites are transmitted to humans by the bite of infected female sandflies (Alvar et al. 2006). The clinical manifestations of leishmaniasis depend on the causative *Leishmania* species. There are mainly three forms of

leishmaniasis, including cutaneous, mucocutaneous, and visceral. The latest estimate of the World Health Organization (WHO) in 2019 demonstrated that more than 90% of the new cases of visceral leishmaniasis (VL) were reported in ten countries, including Brazil, Ethiopia, Eritrea, India, Iraq, Kenya, Nepal, Somalia, South Sudan, and Sudan (<https://www.who.int/news-room/fact-sheets/detail/leishmaniasis>). The VL, caused by *Leishmania donovani* and *Leishmania infantum*, is the most severe form of the disease. Also, it can lead to death if not treated. The mortality rate in VL is higher than in other types of disease. This issue is related to the emergence of resistance in the *Leishmania* strains against most antibiotics (Moore et al. 2010).

Various VL vaccines have been tested in animal models, such as the fucose mannose ligand (FML)-saponin (Santos et al. 2003), *Leishmania* excreted/secreted antigens (Lemesre et al. 2005), and liposome-based vaccines (Afrin et al. 1997; Sharma et al. 2006). Although vaccines are the most effective way to prevent diseases, there is still no approved vaccine against human VL (Kedzierski et al. 2006). This situation is

✉ Niloofar Lari  
nlari2010@gmail.com

Alireza Lari  
alir.Lari@gmail.com

Atefeh Biabangard  
at.biabangard@mail.um.ac.ir

<sup>1</sup> Department of Biochemistry, Faculty of Science, Payame Noor University, Tehran, Iran

<sup>2</sup> Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

due to the pathogen antigenic variation and gene polymorphism (Ghorbani et al. 2018; Kumar et al. 2014). One of the computational methods for detecting protective antigens by screening the proteome of a pathogen is reverse vaccination (RV), which is highly recommended for vaccine development (Rappuoli 2000). Therefore, researchers' have focused on selecting of the appropriate pathogenic antigen in designing vaccines. Immunoinformatics approaches are cost-effective methods used for designing a peptide-based vaccine. This vaccine contains the antigen part of pathogens that can induce the immunogenicity and affect the vaccine efficacy (Bertholet et al. 2009). Thus, developing a novel multi-epitope subunit vaccine by predicting T-cell epitopes (Patronov et al. 2013) and employing computational tools are efficient ways to terminate the virulence of this pathogen.

Some proteins in the proteome of *Leishmania* have been identified as unknown/hypothetical proteins (HPs). These proteins were potentially utilized as drug targets or vaccine candidates (Sinha et al. 2017). Since some HPs substantially affect the pathogenesis of leishmaniasis, some of these proteins are used in a recombinant vaccine against infection with *Leishmania* parasites (Duarte et al. 2017; Martins et al. 2016; Ribeiro et al. 2018). Besides, the membrane and secretory proteins play a crucial role in stimulating a host-specific immune response (Khatoon et al. 2019). The extracellular secretory proteins of *Leishmania* contain several antigenic components and induce an immune response (John et al. 2012; Khan et al. 2020). Therefore, these proteins are very helpful for novel vaccine development.

Several recent studies have investigated the secretory or membrane proteins of *Leishmania* using computational tools (Khan et al. 2020; Khatoon et al. 2017, 2019; Vakili et al. 2018). In these studies, a multi-epitope vaccine was designed against leishmaniasis. The aim of this study was to pinpoint the potential T-cell epitopes from three secretory HPs of the genome of *L. donovani* that can be further joined through proper linkers and adjuvant to design a multi-epitope subunit vaccine. Then, the designed vaccine's physicochemical properties, antigenicity, and allergenicity were predicted by in silico approaches. The secondary and tertiary structures of the designed vaccine were also predicted and validated. Molecular docking and molecular dynamics simulation have been performed to confirm the binding interaction and stability of the docked complex. Finally, in silico cloning into the pET28a (+) vector has been performed to confirm the expression efficiency of the final vaccine construct. The proposed vaccine is also expected to be effective in stimulating humoral and cellular immunity against VL infection.

## Methodology

### The Collection of Hypothetical Secretory Proteins in *Leishmania donovani*

In this study, protein sequences of *L. donovani* were derived from the TriTrypDB (<http://tritrypdb.org/tritrypdb/>) to identify new HP candidates for vaccine design. The amino acid sequences of three HPs of *L. donovani* (*LDBPK\_120390*, *LDBPK\_300860*, and *LDBPK\_312610*) have been chosen as secretory proteins. This procedure has been accomplished by predicting the score derived from the SignalP (Armenteros et al. 2019) server and evaluating the combined prediction of SecretomeP (Bendtsen et al. 2004) and TMHMM 2.0 (Krogh et al. 2001). Besides, CELLO2GO (Yu et al. 2014) and PSORTb v3.0 (Yu et al. 2010) have been employed to identify the subcellular localization of hypothetical *L. donovani* proteins. Also, the antigenic property has been measured for these HP sequences using VaxiJen 2.0 (Doytchinova et al. 2007).

### The Prediction of T-cell (Cytotoxic T Lymphocyte and Helper T Lymphocyte) Epitopes

Cytotoxic T lymphocytes (CTLs) play a crucial role in host defense against foreign antigens. Thus, it is essential to predict CTL epitopes for the vaccine design. The mentioned HP sequences of *L. donovani* were submitted to the NetCTL 1.2 (<http://www.cbs.dtu.dk/services/NetCTL/>) server to predict CTLs. In this case, the threshold value was set at 0.75. This server allows prediction of CTL epitopes based on MHC-I binding peptides, proteasomal C-terminal cleavage, and Transporter Associated with Antigen Processing (TAP)-associated efficiency with antigen processing (Larsen et al. 2007).

Since the helper T cells had significant influences on adaptive immunity, the IEDB database (<http://tools.iedb.org/mhcii/>) has been utilized to predict helper T lymphocyte (HTL) epitopes based on their receptor binding affinity (Vita et al. 2019). In this case, epitopes with the lowest percentile rank represent a high affinity of MHC-II. Also, the 15-mer epitopes in mouse alleles were retrieved for all HP sequences in FASTA format.

### Designing a Multi-epitope Vaccine Construct

In this study, the predicted epitopes have been combined to construct a multi-epitope subunit vaccine. This procedure has been performed using suitable linkers and adjuvants. Besides, the selected CTLs and HTLs epitopes were linked via AAY and GPGPG linkers to connect various components

of a multi-epitope vaccine construct. Also, the EAAAK linker has been considered to add the *Mycobacterium tuberculosis* 50 S ribosomal L7/L12 (UniProt ID: P9WHE3.1) to the N-terminal sequence as an adjuvant (Lee et al. 2014). Thus, the immunogenicity of the vaccine has been enhanced.

### The Prediction of Antigenicity, Allergenicity, and Toxicity

Antigenicity is defined as the ability of a chemical structure to produce an immune response. In general, it is recognized as an antigen. Besides, allergenicity is characterized as the ability of small antigens in creating immune-mediated and allergic reactions. In this case, these two properties were identified as significant factors affecting vaccine development. Also, ANTIGENpro (Magnan et al. 2010) and Allertop 2.0 (Dimitrov et al. 2014) servers have been measured the antigenic and allergenic properties of the designed vaccine protein. In addition, the toxicity of the designed vaccine construct has been checked by the ToxinPred (Gupta et al. 2013) server.

### The Analyses of Physicochemical Properties and Solubility Prediction

ExPASy ProtParam tool (<https://web.expasy.org/protparam/>) has been used to calculate a set of physicochemical parameters such as theoretical isoelectric point (pI), amino acid composition, in vitro and in vivo half-life, instability, aliphatic index, molecular weight (MW), and grand average of hydropathicity (GRAVY) for the subunit vaccine protein (Gasteiger et al. 2005). Also, the Protein-Sol (<https://proteinsol.manchester.ac.uk/>) server has been employed to determine the solubility of the designed vaccine protein. This server predicts the vaccine solubility based on properties like isoelectric pH and amino acid compositions. In this case, protein scored  $\geq 0.45$  is expected to be soluble compared to the average solubility of *E. coli* proteins (Hebditch et al. 2017).

Furthermore, the NCBI BlastP has been considered to compare the whole multi-epitope vaccine sequence with *Homo sapiens* proteome for preventing autoimmunity. In this case, non-host homologous proteins were screened with default parameters (Mahram et al. 2015).

### The Prediction and Validation of Multiple-Epitope Vaccine Structures

PSIPRED 4.0 (Buchan and Jones 2019) online web server has been considered to predict the secondary structure of the multi-epitope subunit vaccine construct. Besides, the homology modeling tool in the RaptorX (Kallberg et al. 2012) server has been utilized to prepare the tertiary model of the

subunit vaccine protein. Also, the Galaxy Refine (Heo et al. 2013) server has been employed to refine the 3D model of the multi-epitope vaccine construct and improve the structure quality. In this study, the quality of the refined vaccine construct has been assessed by the ProSA-web server (<https://prosa.services.came.sbg.ac.at/prosa.php>). It predicts the overall quality of the protein model in the form of a Z-score (Wiederstein et al. 2007). Also, the Ramachandran plot was generated using the PROCHECK (<https://services.mbi.ucla.edu/>) server to illustrate the Ramachandran plot with allowed and disallowed regions of amino acids.

### The Prediction of B-cell Epitopes and Interferon $\gamma$ Inducing Epitopes

The epitopes of the humoral immune response, particularly B-cell epitopes, are antigenic determinants and considerably affect vaccine designing. In the present study, these linear B-cell epitopes (20-mer peptides) of the final vaccine construct were predicted using the BCPREDS server at default parameters. This server utilizes a support vector machine (SVM) based on kernel methods for predicting linear B-cell epitopes (El-Manzalawy et al. 2008). Also, the Ellipro server (<https://tools.iedb.org/ellipro>) has been employed to identify discontinuous B-cell epitopes of the refined final vaccine construct. This server implements three algorithms based on their protrusion index (PI) values and allocates a PI value to the predicted epitopes (Ponomarenko et al. 2008). Besides, interferon-gamma (IFN- $\gamma$ ) plays a vital role in innate and adaptive immunity. In this case, the IFNepitope (<https://webs.iitd.edu.in/raghava/ifnepitope/index.php>) server has been considered for predicting the IFN- $\gamma$  epitope of the final vaccine. This server predicts epitopes via the hybrid algorithms of motif and SVM (Dhanda et al. 2013).

### Molecular Docking of the Designed Vaccine with the TLR-4 Receptor

The Computed Atlas of Surface Topography of proteins (CASTp) server V3.0 has been employed to predict the protein binding site and hydrophobic interaction on the protein surface (Dundas et al. 2006). This situation initiates an interaction between the final vaccine construct and the TLR4 immune receptor. Then, the PatchDock web server (<http://bioinfo3d.cs.tau.ac.il/PatchDock/>) has been considered for the molecular docking of the final vaccine construct with TLR4 (PDB ID: 4G8A) receptor. This procedure has been carried out to demonstrate an immune response development and analyze the interaction between the ligand and receptor (Schneidman-Duhovny et al. 2005). Afterward, the Fast Interaction Refinement in Molecular Docking (FireDock) server has been utilized to refine and re-score the rigid body molecular docking complexes (Andrusier et al. 2007). This

situation has been led to achieving the best structure. This server provides the ten best solutions for the procedure. Besides, the refined models are configured based on several factors such as atomic contact energy, partial electrostatics, Van Der Waals interaction (vdW), and binding energy estimations. Also, the TLR4 receptor- vaccine interaction was visualized using the UCSF Chimera (Pettersen et al. 2004) and LigPlot+ (Laskowski et al. 2011) tools.

### Molecular Dynamics Simulation

In addition, the stability of the interaction between TLR-4 receptor and the vaccine construct was determined by GROMACS 5.1.1 package (Van Der Spoel et al. 2005). This process has been performed using the GROMOS96 43a1 force field parameter set to implement molecular dynamics simulations. Before simulation, residues 980 to 1200 of TLR4 receptor that were located considerably far from the vaccine-TLR4 complex (based on docking analysis) were manually removed to reduce the time of simulation and further improve MD analysis. In this case, the structure was initially solvated with transferable intermolecular potential with 3 points (TIP3P) water model. Sodium and chloride ions were also added to neutralize the system. Besides, the energy has been minimized to provide a proper molecular arrangement in space. It was conducted by eliminating high-energy configurations. Also, the simulation process has been run under canonical (NVT) and isothermal–isobaric (NPT) conditions at 300 K and 1 bar pressure with the duration of 50 ps. Then, the stabilized structure has been subjected to the MD simulation of 50 ns. Finally, the root mean square deviation (RMSD), the root mean square fluctuation (RMSF), the radius of gyration (Rg) (Lobanov et al. 2008), and hydrogen bonds (Joshi et al. 2021) were calculated for all the systems. The MD complex analysis were determined by using graphs generated by Xmgrace software.

### Immune Response Simulation

The immunological responses of the designed vaccine construct have been evaluated by *in silico* method C-ImmSim (<https://www.iac.cnr.it/~filippo/c-immsim/index.html>) online dynamic immune simulation server (Rapin et al. 2010).

### Codon Adaptation and *In Silico* Cloning of the Final Vaccine

Reverse translation and codon optimization of the final vaccine construct were conducted by the Java Codon Adaptation Tool (JCat) server (<http://www.jcat.de/>) to attain an improved nucleotide sequence and make it compatible with widely used prokaryotic expression

system; *E. coli* K12 (Grote et al. 2005). Besides, three options of avoiding rho-independent transcription terminators, prokaryote ribosome binding sites, and cleavage sites restriction enzymes were selected. The JCat output includes the codon adaptation index (CAI) value and the percentage of GC content. The desired CAI value is ranged from 0.8 to 1.0, and the optimal percentage of GC content ranges from 30 to 70%. Also, SnapGene 5.1.5 software cloned the optimized codon sequence into *E. coli* pET-28a (+) vector. Afterward, the restriction sites of XhoI and NdeI were incorporated in the N- and C-terminal sequences, respectively.

## Results

### Sequence Retrieval of *Leishmania donovani* Protein

In this case, the amino acid sequences of three secretory proteins of *L. donovani* (LDBPK\_120390, LDBPK\_300860, and LDBPK\_312610) were retrieved from the NCBI protein database to design an immunogenic subunit vaccine. The CELLO2GO (Yu et al. 2014) and PSORTb v3.0 (Yu et al. 2010) servers predicted that these secreted proteins were localized in extracellular location. These HPs were found with an antigenic probability of greater than 0.5. This value was in agreement with VaxiJen 2.0 (Doytchinova et al. 2007).

### T-cell Epitopes Prediction

A total of five CTL epitopes with high-ranked binding affinity scores were predicted for the three nominated proteins (Table 1). This process has been completed by the NetCTL 1.2 server (Larsen et al. 2007). Likewise, nine HTL epitopes based on their lowest percentile rank with MHC-II were predicted from the IEDB server (Vita et al. 2019) for mouse alleles (H2-1Ad, H2-1Ed, and H2-1Ab) to design the vaccine (Table 2).

**Table 1** Predicted cytotoxic T-lymphocyte (CTL) epitopes of the three hypothetical secretory proteins in *Leishmania donovani*

Serial No.	Protein name	Epitopes	Score
1	LDBPK_300860	LMALVAGWY	2.0187
2	LDBPK_120390	WTLAAHVY	2.3469
		STDDGQLSV	2.1619
3	LDBPK_312610	HTENVRFPY	3.5561
		FSDNTAVVY	3.2386



**Table 2** Predicted helper T-lymphocyte (HTL) epitopes of the three hypothetical secretory proteins in *Leishmania donovani*

Serial No.	Protein name	Allele	Start	End	Epitopes	Percentile rank
1	LDBPK_300860	H2-IAb	20	34	LLYCVLAASSTPAPT	0.84
		H2-IAd	148	162	GASAVLALMALVAGW	1.27
		H2-IEd	157	171	ALVAGWYSRHKIVSV	0.78
2	LDBPK_120390	H2-IAb	435	449	AQLQLLRyatPLPLE	0.17
		H2-IAd	508	522	HRQYARAAMTVHANH	0.04
		H2-IEd	633	647	APESRYPRtarHFAA	3.3
3	LDBPK_312610	H2-IAb	303	317	CPRGQWtTPASSTAA	0.38
		H2-IAd	20	34	ILVITATAAAAASIVD	2
		H2-IEd	87	101	VRFPYYAWNstSWYA	3.75

## Developing a Multi-epitope Vaccine Protein

The vaccine sequence has been constructed by combining five CTL epitopes and nine HTL epitopes with the AAY and GPGPG linkers, respectively. Also, the 50 S ribosomal L7/L12 (P9WHE3) was added to the N-terminal of the construct using the EAAAK linker, which is ideal for achieving maximum immune response (Fig. 1).

## The Prediction of Antigenicity, Allergenicity, and Toxicity of Subunit Vaccine

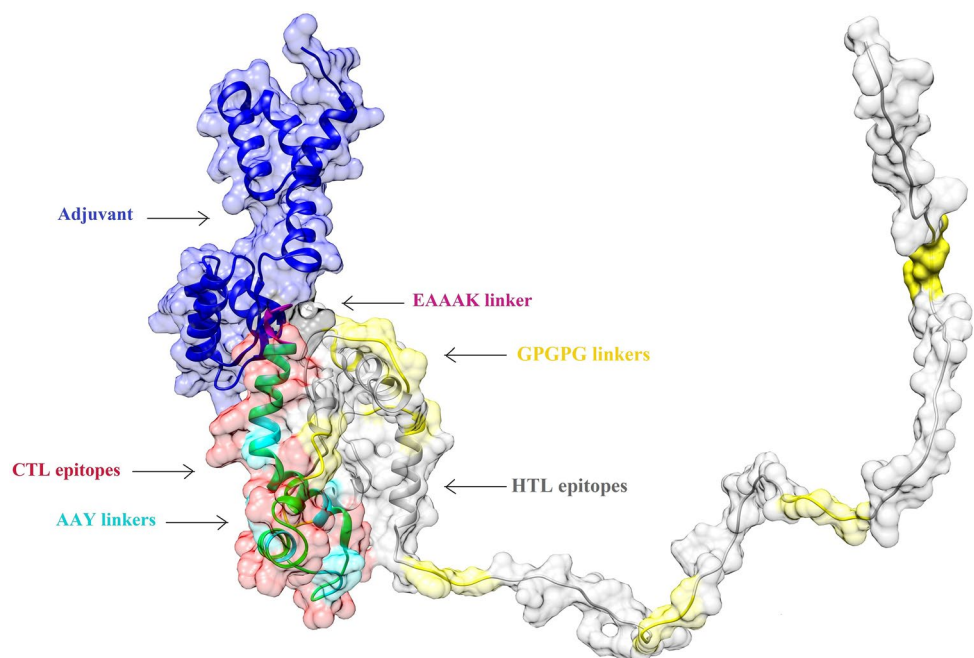
In the designed vaccine construct, the immunological properties such as antigenicity, allergenicity, and toxicity were evaluated by the three servers of ANTIGENpro (Magnan et al. 2010), AllerTOP 2.0 (Dimitrov et al. 2014), and ToxinPred (Gupta et al. 2013), respectively. The results

demonstrated that the designed vaccine protein was antigenic, non-allergenic, and non-toxic.

## Physicochemical Characterization and Solubility Prediction of Subunit Vaccine

In this case, the ProtParam (Gasteiger et al. 2005) tool has been considered to evaluate the physicochemical properties of the designed subunit vaccine sequence containing 372 amino acids. The molecular weight, theoretical isoelectric point (pI), and instability index values were 38078.35, 5.84, and 25.4, respectively. The estimated half-life was assessed 30 h in mammalian reticulocytes, > 20 h in yeast, and > 10 h in *E. coli*, in vivo. The aliphatic index (II) and GRAVY were 84.17 and 0.108, respectively. The results indicated that the designed vaccine was significantly acidic, stable, thermostable, and hydrophobic.

**Fig. 1** Schematic diagram of the multi-epitope vaccine construct. The 372- amino acid multi-epitope vaccine containing an adjuvant (blue) at N-terminal end linked followed by CTL and HTL epitopes. EAAAK linker (pink) has joined adjuvant and CTL epitope. Whereas, AAY (Cyan) and GPGPG (yellow) linkers were used to join the CTL (red) and HTL (grey) epitopes, respectively (Color figure online)

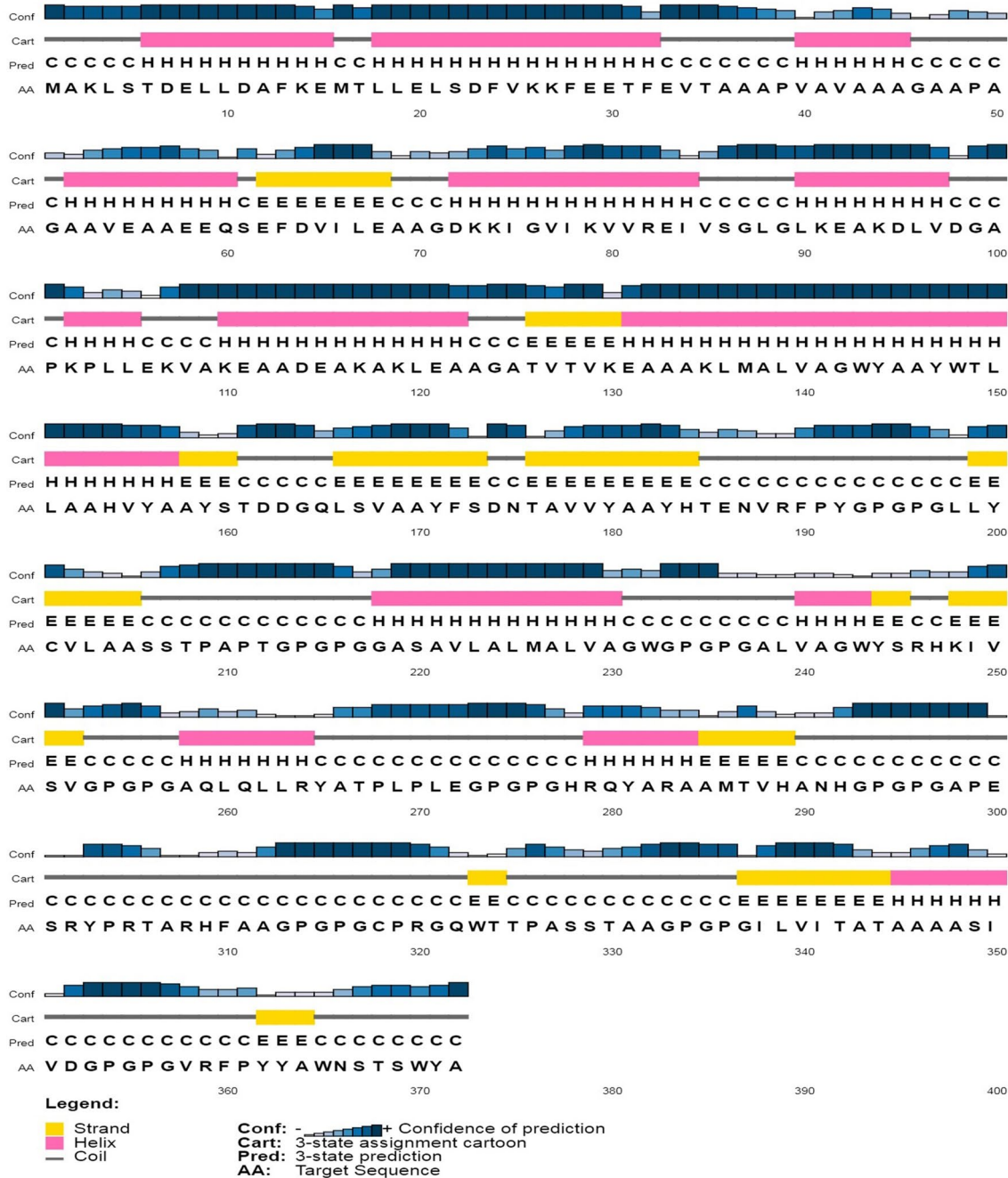


Also, the scaled solubility value of the vaccine construct was 0.496. This value was higher than the average soluble *E. coli* protein derived from the experimental solubility dataset (Niwa et al. 2009).

Besides, the NCBI BlastP has been utilized to search for similarities between the multi-epitope vaccine and the *Homo sapiens* proteome. The results showed no similarities between them. This situation reveals that the designed vaccine was non-homologous with the host proteome.

### The Prediction of Secondary and Tertiary Structures of Subunit Vaccine Construct

Figure 2 depicts the secondary structure prediction of the subunit vaccine construct using the PSIPRED (Buchan and Jones 2019) server. It contains alpha-helix (38%), beta strands (17%), and coils (45%). Besides, the RaptorX (Kallberg et al. 2012) server has been utilized to construct a 3D structure of the subunit vaccine. The best template for



**Fig. 2** Schematic illustration of secondary structure of the subunit vaccine construct including alpha-helix, beta strands, and coils

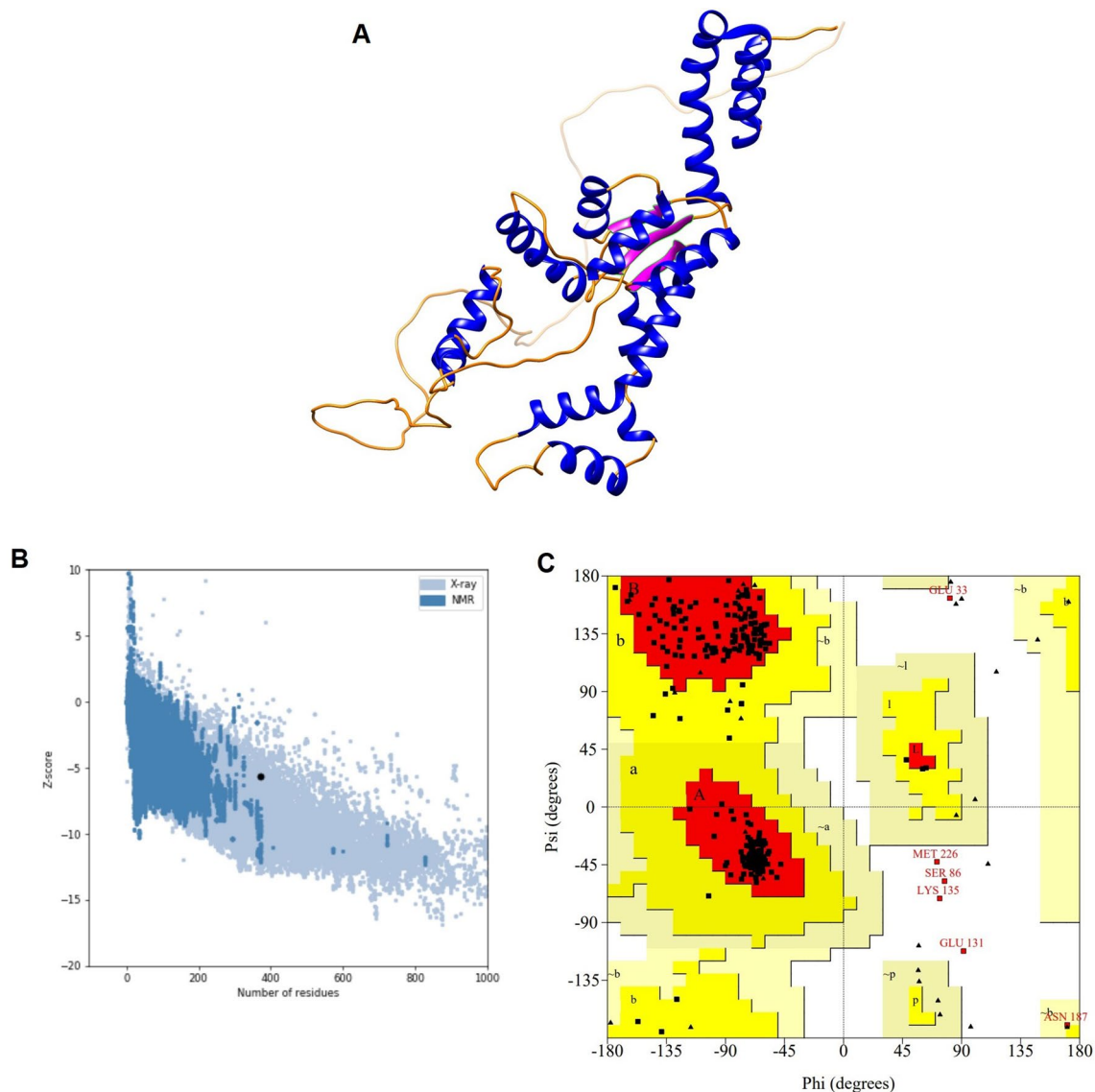
modeling the designed vaccine was the crystal structure of *Thermotoga maritima* (PDB ID: 1dd3A) with the p-value of  $6.19 \times 10^{-5}$ . Then, the GalaxyRefine (Heo et al. 2013) server refined the vaccine structure and generated five models. Among these generated models, model 1 (Fig. 3a) has been chosen due to its properties such as GDT-HA (0.9348), RMSD (0.458), MolProbity (1.734), Clash score (7.2), Poor rotamers (0.4), and Rama favored (95.1).

In this case, the final vaccine construct has been validated by the ProSA-web and Ramachandran plots after the model refinement. As shown in Fig. 3b, the ProSA-web server provided a Z-score of  $-5.67$  for the input vaccine protein. Figure 3c illustrates the Ramachandran plot of the

vaccine construct, and only 1.7% of the residues are in the disallowed regions. These issues confirm the quality of the designed vaccine.

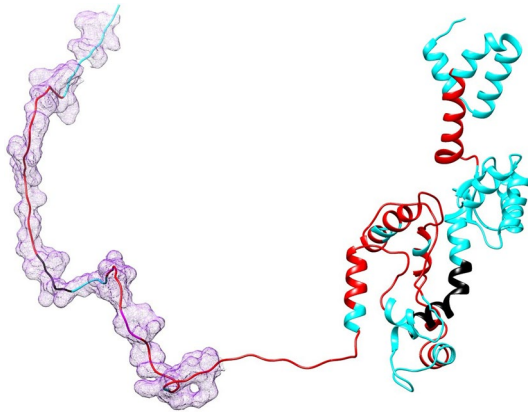
### Analysis of B-cell Epitopes and Interferon $\gamma$ Inducing Epitopes Prediction

Two servers, BCPred (El-Manzalawy et al. 2008) and ElliPro (Ponomarenko et al. 2008), have been employed to predict the linear and conformational B-cell binding epitopes among the primary input sequence of the final vaccine construct. Figure 4 depicts information about the eight epitopes. These epitopes had a BCPreds cut-off score of more than 0.9. A total of 66



**Fig. 3** The tertiary structure of the final vaccine construct and its validation. **a** The 3D model of the final vaccine construct was obtained by RaptorX. **b** ProSA-web z-score plot indicating a Z-score =  $-5.67$ .

**c** Ramachandran plot showing 98.3% of the residues are present in the favored and allowed regions, and 1.7% of residues are in disallowed region



**Fig. 4** Schematic representation of predicted B-cell epitopes and IFN- $\gamma$  inducing epitopes. **a** Red color ribbons show the liner B-cell epitopes, **b** Purple mesh surface color sequences show the discontinuous B-cell epitopes, and **c** Black color ribbons illustrate IFN- $\gamma$  inducing epitopes in the constructed vaccine (Color figure online)

residues were predicted as the conformational B-cell epitopes with a probability score greater than 0.8 and residue numbers ranging from 303 to 370. These scores start with Tyrosine residue (score 0.85) and end with Tryptophan residue (score 0.98). The findings showed that the designed vaccine could enhance humoral immunity (Khatoun et al. 2017; Shi et al. 2015).

The IFNepitope (Dhanda et al. 2013) server has been utilized to predict 364 epitopes (15-mer) based on SVM. This case included both negative and positive predictions. In this regard, two IFN- $\gamma$  inducing epitopes were selected. These epitopes had a prediction score of one (Fig. 4).

### Molecular Docking of the Final Vaccine Construct with the Immune Receptor (TLR-4)

The CASTp server analyzed the active binding sites of the protein. It could serve as a possible site for interacting with the TLR4 receptor. Besides, this server predicted the potential binding site with 2073.8  $\text{\AA}^2$  surface area and 2980.2  $\text{\AA}^3$  molecular surface volume. Also, the PatchDock (Schneidman-DuhovnyInbar et al. 2005) server has been considered to perform the molecular docking of the vaccine construct against TLR4 at the predicted binding pocket. In this case, the top ten models were refined via their binding energy using the FireDock (Andrusier et al. 2007) server. Figure 5 displays the selected final model (model No. 2) for molecular docking based on the binding energy.

### Molecular Dynamics Simulation of Vaccine- TLR4 Complex

In order to validate structural stability of the subunit vaccine-TLR4 complex, molecular dynamics simulation was

performed using a 50 ns MD simulation study. Obtained potential energy for the complex was  $-7.72982e + 6$  KJ/mol. The complex attained equilibrium during the simulation trajectory after about 20 ns of simulation and acquired a value between 1.9 and 2.23 nm. The RMSD plot shows the complex stabilized in the last 30 ns of the simulation (Fig. 6a). The RMSF plot of the TLR4- vaccine complex indicated that most of the peaks are belong to residues, which are located in the loop regions and the terminal residues of strand (Fig. 6b). Because the loop structure is much more flexible, the loop region consisting of Val288-Ala372 residues showed larger RMSF values compared to  $\alpha$ -helix structures. The overall predicted RMSF graph suggested, the ligand have a little fluctuated (less than 2 nm) throughout the simulation time. The compactness of vaccine and TLR4 complex evaluated and the average Rg was found to be 3.34  $\text{\AA}$ . As shown in Fig. 6c, we could assume that this complex did not induce noticeable fluctuation after 50 ns of simulation and remain compact after 25 ns that is in agreement with the RMSD values. The number of hydrogen bonds formed between the vaccine and the TLR4 through 50 ns simulation period is shown in Fig. 6d. The observed bonding graph, demonstrated that hydrogen bond interaction patterns increased slightly and then remained constant during the last 30 ns of simulation time. All evidences indicate that receptor tends to bind to vaccine and remain stable as previous RMSD and Rg represented.

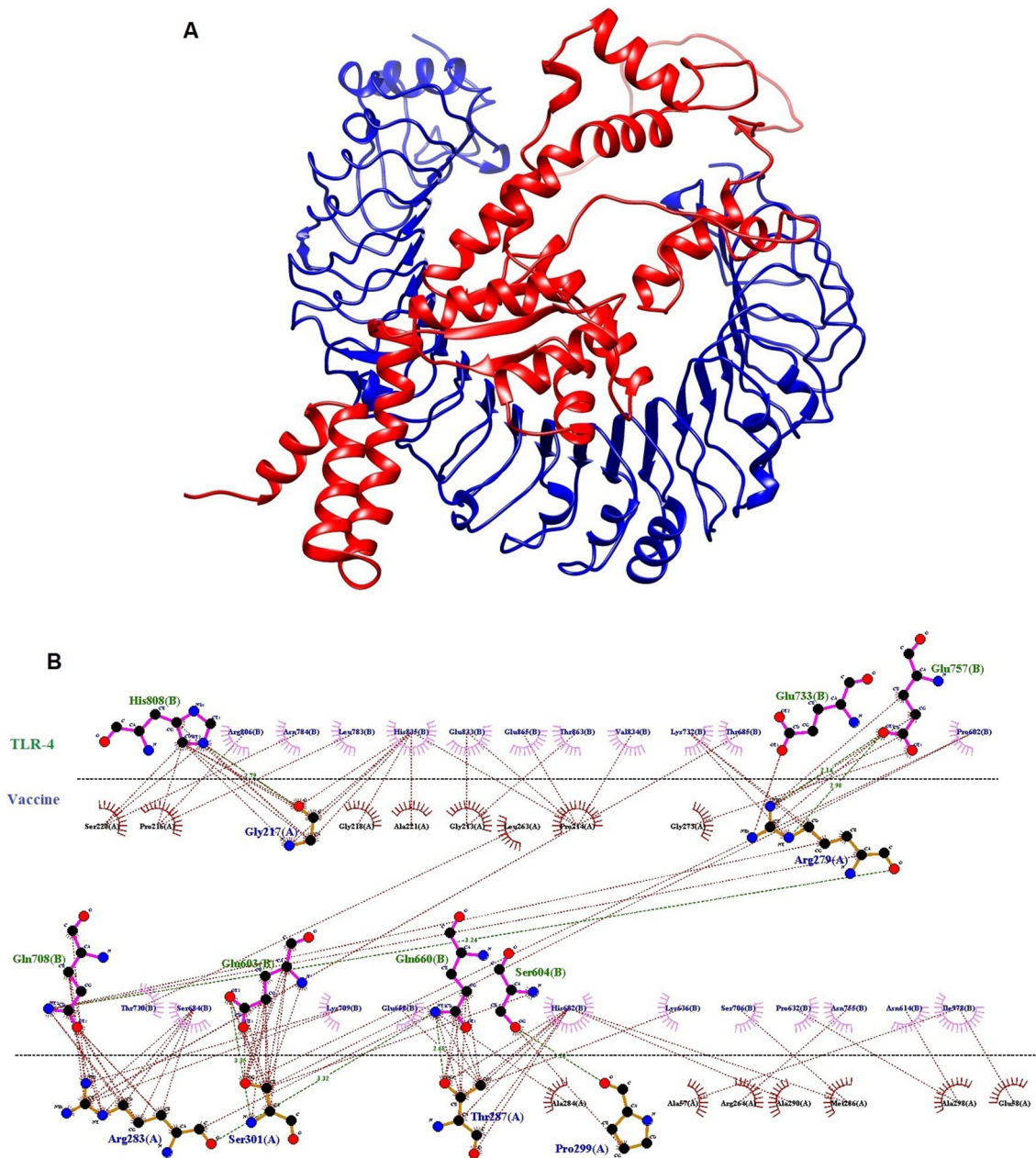
### Immune Response Simulation

The refined vaccine protein was subjected to in silico immune simulations to check the proficiency of the vaccine construct to the adaptive immune system. The simulation results showed that responses (secondary and tertiary) produced high levels of IgG and IgM antibodies (IgM + IgG and IgM), while the antigen level decreased (Fig. 7a). Besides, Fig. 7b exhibits an effective antiviral response by the vaccine in the elevated levels of IFN- $\gamma$  and interleukin-2. Also, Fig. 7c displays the estimation of the B-cell population in the elevated levels of B memory cells and B-cell isotype IgM. In addition, a high response was observed in the T cells (both  $T_h$  and  $T_c$ ) populations (Fig. 7d and e). The obtained results suggest that the proposed vaccine construct can effectively trigger the immune response and provide immunity against VL infections.

### Virtual Cloning of the Final Vaccine Construct

The Jcat (Grote et al. 2005) server has been utilized to adapt the codon for multi-epitope vaccine as per *E. coli* K12 strain. In this case, the Codon Adaptation Index (CAI) of the improved sequence was 0.968 with GC contents of 56.45%. Also, the length of the optimized codon sequence





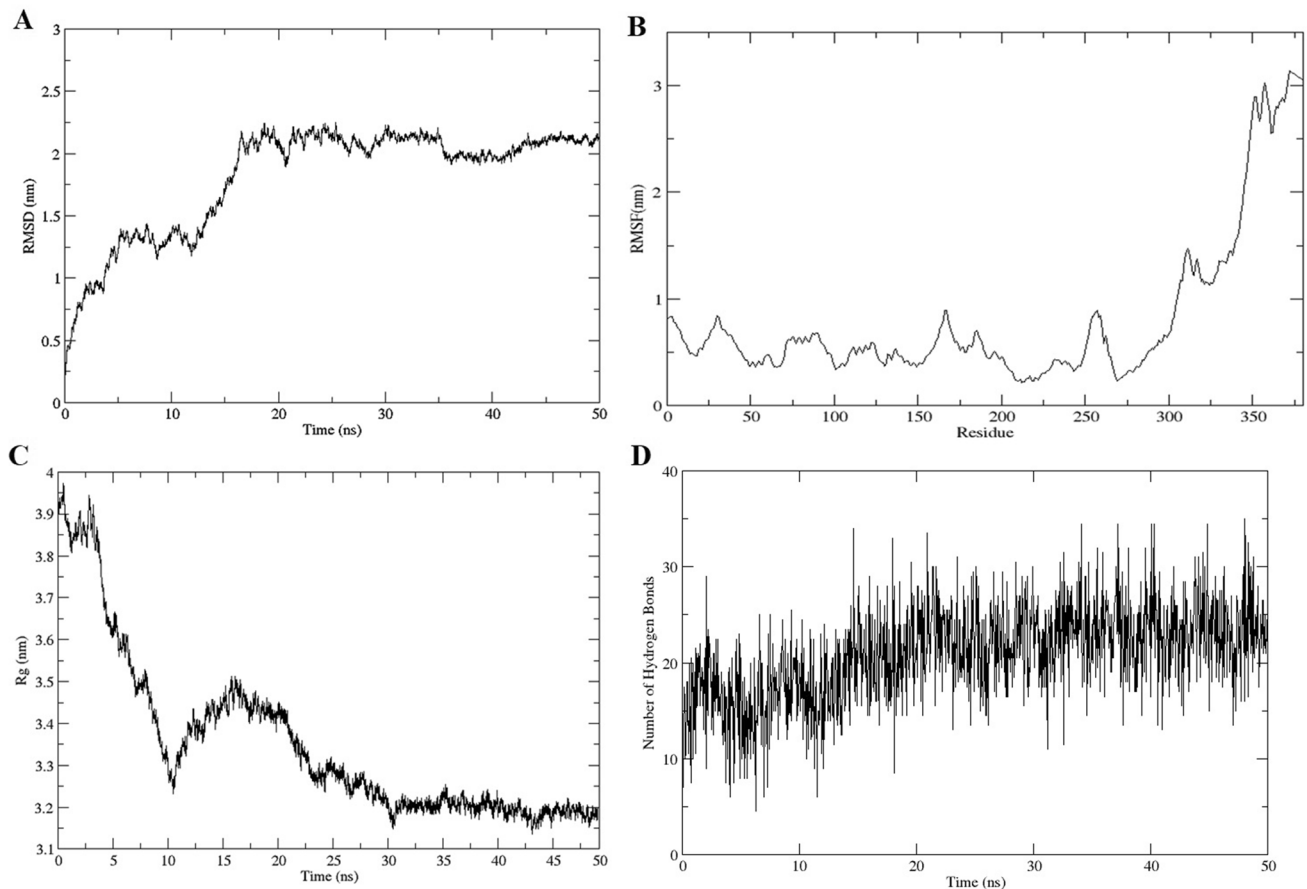
**Fig. 5** Molecular docking of the final vaccine construct with human TLR4 receptor. **a** Graphical representation Patch-docking of the vaccine construct (red color) and TLR-4 receptor (blue color). **b** The

interaction map of the TLR4-vaccine complex showing residues involved in hydrophobic (red dotted lines) and hydrogen bonding (green dotted lines) (Color figure online)

was 1,116 nucleotides. The results showed the possibility of good expression of the vaccine candidate in the *E. coli* host. Afterward, the SnapGene software generated the sequence of the recombinant plasmid to insert the optimized sequence into the pET28a (+) vector (Fig. 8). This procedure has been performed for in silico cloning.

## Discussion

The VL infection is a serious public health problem, which annually affects millions of people across the globe. Leishmaniasis treatment is confined to several anti-leishmaniasis



**Fig. 6** Molecular dynamics (MD) simulation analysis of the vaccine-TLR4 complex. **a** Root mean square deviation (RMSD) of the vaccine-receptor complex backbone, **b** root mean square fluctuations

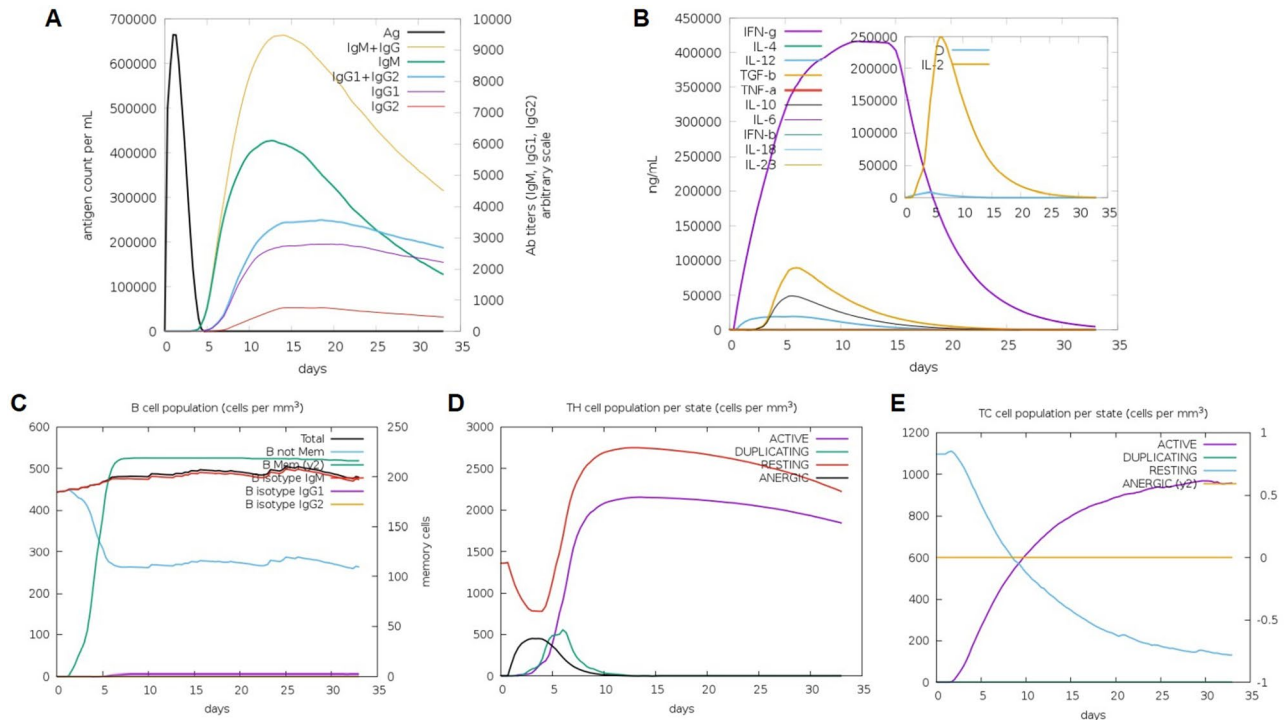
(RMSF) of the docked complex side chains, **c** radius of gyration ( $R_g$ ) of the docked complex, and **d** Number of hydrogen bonds formed during the 50 ns MD simulations

drugs, but these drugs showed increased drug resistance and toxicity. Therefore, there is no effective controlling approach for VL (Nagill et al. 2011). Despite the efforts of scientists to develop a vaccine against leishmaniasis, there is still no effective vaccine against this disease. Several antigens (e.g., cysteine protease C and kinetoplastid membrane protein-11) have been evaluated as targets for vaccines against *Leishmania* (Basu et al. 2005; Das et al. 2014). Also, recombinant vaccines (e.g., Leish111f and Leishmune) have been developed as vaccine candidates against leishmaniasis (Jain et al. 2015). Nevertheless, no vaccine has been approved against leishmaniasis for use in humans (Kedzierski et al. 2006).

Advances in bioinformatics tools have been led to the development of subunit vaccines containing specific immunogenic components of pathogens that stimulated an immune response (Adhikari et al. 2018; Pandey et al. 2018; Rahman et al. 2020; Shey et al. 2019). Since T-cells play a crucial role in controlling VL infection, most researchers have designed the vaccine via identifying *L. donovani* antigenic epitopes and their interaction with host MHC alleles

and immune cells (Stager et al. 2012). In this regard, most recent studies have focused on the *L. donovani* genome sequences to identify specific immunogenic components for designing novel vaccines using immunoinformatics methods (Atapour et al. 2021; Khan et al. 2020; Singh et al. 2020; Vakili et al. 2018). Study on the pathogenic secretory proteins revealed that these proteins were antigenic in nature and had diverse functions during infection (Khatoun et al. 2017). Identification of these proteins and understanding of their antigenic epitopes with host MHC alleles can be used to produce an effective vaccine against leishmaniasis (Kaye et al. 2011). For example, the literature survey showed that amastigote-associated potential secretory proteins were an effective vaccine design against VL (Khan et al. 2020). Also, the KMP-11 protein sequence (as a target antigen) and B-type flagellin (as adjuvant) have been used for designing vaccine construct (Atapour et al. 2021).

This study emphasized the computational approaches for predicting the binding of peptides to MHC molecules. This procedure is performed to select the three hypothetical secretory proteins in *L. donovani*. These are used as



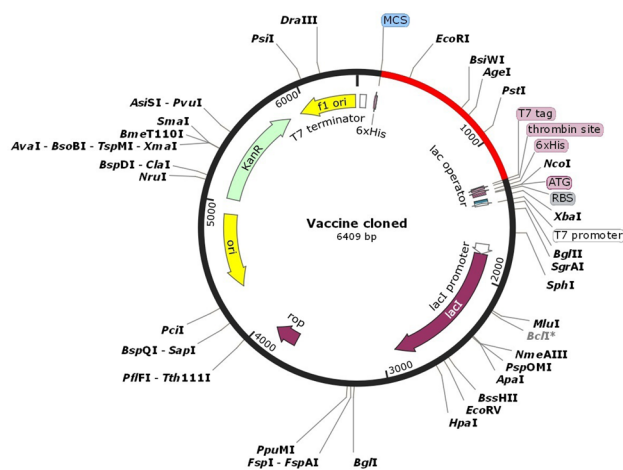
**Fig. 7** In silico immune simulation of the final vaccine construct. **a** Immunoglobulins levels upon antigen exposure, **b** The level of cytokines and interleukins, **c** Population of B -cells, **d** and **e** Population of T lymphocytes (T-helper and T-cytotoxic)

effective multi-epitope vaccine candidates. In the present study, three *L. donovani* hypothetical secretory proteins have been scanned to predict the presence of a signal peptide, subcellular localization, and antigenicity. The potential epitopes of these proteins were selected against T-cell (Cytotoxic and Helper T-lymphocyte) epitopes using the available online tools. In this case, five CTL and nine HTL epitopes were chosen as part of the developed multi-epitope vaccine

construct. This procedure was conducted by joining suitable linkers and an adjuvant together. None of these predicted epitopes have been used earlier that shows the novelty of our study.

The designed vaccine protein has been subjected to BLASTP search against human proteins to prevent the undesired pathogen cross-reactivity with the host. The analysis results demonstrated that the designed vaccine did not have any homology with the host proteome. Besides, the secondary structure of the vaccine construct including alpha-helices, beta-sheets, and coils was predicted. Also, the RaptorX and GalaxyRefine servers have been utilized to generate the 3D structure and model refinement, respectively. The Ramachandran plot showed that the refined tertiary structure of the vaccine improved to the desired level. Meanwhile, the final vaccine construct had physicochemical and antigenic properties with no probable allergenicity or toxicity.

Besides, molecular docking and MD simulation were performed to understand the binding affinity and stability of the TLR4- vaccine complex. The vaccine- TLR4 complex showed stability and compactness during MD simulation. Also, an immune simulation was carried out for the designed vaccine. This procedure has been accomplished to characterize its ability to produce immunological responses against the VL. The simulation results revealed that the final multi-epitope vaccine construct elicited unique immune responses. In addition, the in silico optimization of codon



**Fig. 8** The in silico cloning of the optimized vaccine (red lines) into pET28a (+) expression vector (black lines) (Color figure online)

has been implemented for the final vaccine construct. The values of CAI and GC contents demonstrated that the vaccine construct could be expressed in the *E. coli* host. The in silico cloning of the optimized vaccine showed its better integration for the expression in a bacterial system. This study focuses on the design of a multi-epitope subunit vaccine using immunoinformatics tools, which suggests that the final vaccine construct may have a good protective effect against VL infection.

## Conclusions

One of the major causes of mortality in tropical and subtropical regions across the world is VL. In this study, we utilized immunoinformatics tools to design a novel multi-epitope vaccine against VL. Based on the results, our multi-epitope subunit vaccine is immunogenic and non-allergic, which has the potential to induce cellular and humoral immune responses. Although in silico results showed the effectiveness of this vaccine, immunogenic potency of the proposed vaccine needs to be experimentally validated.

## Declarations

**Conflict of interest** The author(s) declare no competing interests in this manuscript.

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