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ORIGINAL PAPER

In silico designing of a novel polyvalent multi-subunit peptide vaccine leveraging cross-immunity against human visceral and cutaneous leishmaniasis: an immunoinformatics-based approach

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Abstract

Context Leishmaniasis is a group of vector-borne infectious diseases caused by over 20 pathogenic *Leishmania* species that are endemic in many tropical and subtropical countries. The emergence of drug-resistant strains, the adverse side effects of anti-*Leishmania* drugs, and the absence of a preventative vaccination strategy threaten the sensitive population. Recently, many groups of researchers have exploited the field of reverse vaccinology to develop vaccines, focusing chiefly on inducing immunity against either visceral or cutaneous leishmaniasis.

Methods This present work involves retrieving twelve experimentally validated leishmanial antigenic protein sequences from the UniProt database, followed by their antigenicity profiling employing ANTIGENpro and Vaxijen 2.0 servers. MHC-binding epitopes for the same were predicted using both NetCTL 1.2 and SYFPEITHI servers, while epitopes for B cell were computed using ABCpred and BepiPred 2.0 servers. The screened epitopes with significantly higher scores were utilized for designing the vaccine construct with appropriate linkers and natural adjuvant. The secondary and tertiary structures of the synthetic peptide were determined by conditional random fields, shallow neural networks, and profile-profile threading alignment with iterative structure assembly simulations, respectively. The 3-D vaccine model was validated through CASP10-tested refinement and the MolProbity web server. Molecular docking and multi-scale normal mode analysis simulation were performed to analyze the best vaccine-TLR complex. Finally, computational immune simulation findings revealed promising cellular and humoral immune responses, suggesting that the engineered chimeric peptide is a potential broad-spectrum vaccine against visceral and cutaneous leishmaniasis.

Keywords Leishmaniasis · Reverse vaccinology · TLR · Multi-epitope vaccine · Molecular dynamic simulation · Homology modeling

Introduction

Leishmaniasis is a group of parasitic diseases caused by various members of the genus *Leishmania*, a dimorphic intracellular parasitic protozoan [1]. Leishmaniasis affects most populations from the low socio-economic status of tropical and subtropical countries worldwide [2]. It is a vector-borne disease, and the parasites are transmitted by biting the infected female phlebotomine sand flies (WHO, 2019). According to the latest reports by World Health Organization, 700, 000 to 1 million new cases and some 26,000 to 65, 000 deaths occur annually (WHO, 2019). In East Africa, outbreaks of visceral leishmaniasis occur frequently. The Eastern Mediterranean Region accounts for 70% of the cutaneous leishmaniasis cases worldwide (WHO, 2019). In 2017, 20,792 out of 22,145 (94%) new cases reported to WHO occurred in seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan (WHO, 2017). Due to the alarming scenario, measures for controlling and eradicating the disease should also be underscored in our country. Leishmaniasis has a variety of clinical manifestations, and there are three primary forms of the disease—visceral (also known as kala-azar and the most severe form of

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the disease), cutaneous (the most common), and mucocutaneous (WHO, 2019). Evidence of self-cure in a few cases of cutaneous leishmaniasis (CL) and the resultant immunity to re-infection infers the possible scope of vaccination as a feasible method to control leishmaniasis over the period [3]. However, to date, no licensed vaccine is available against human leishmaniasis. Although a few potential candidate vaccines have advanced to clinical trial, many are still in the early research and development phase [4]. The generation of peptide vaccines is a predominant and promising area of research in this field.

In contrast to whole live or attenuated parasites or their subunit-based vaccines, synthetic peptides have several advantages that include the absence of potentially harmful materials, lower antigen complexity, good stability, and low cost to scale up [1, 5]. Developing new vaccines demands deeper insights into immunology, molecular biology, and chemical engineering. Therefore, scientists across all these arenas work hand-in-hand to design and produce safe, effective vaccines [6]. An ideal vaccine against leishmaniasis should elicit long-lasting immunity and a balanced T_H-1 and T_H-2-mediated immune response [4, 7]. Immunoinformatics-based approach for designing such a novel candidate vaccine is a newer strategy that primarily employs computational tools to predict a structurally stable and immunogenic multi-subunit peptide vaccine within a short time.

Proteins that were primarily found to have requisite immunogenic features for conferring immunity against the disease in previous immunological studies involving experimental animal models or other in silico analysis were the best possible epitope candidates [1, 8–10]. Among the proteins under investigation, the LACK (*Leishmania*-activated C-kinase antigen) protein is a highly conserved protein across *Leishmania* species, and it is considered a viable vaccine candidate against human leishmaniasis [11]. It has been proven to be a key target of immune response in sensitive BAL b/c mice [12]. KMP-11 (kinetoplastid membrane protein-11) is present in all kinetoplastid protozoa and is also considered a potential vaccine candidate that is shown to increase IL-10 levels in murine models, indicating its immunogenic potential [13]. Leishmanolysin, or GP63, is a surface proteinase that has been postulated as a virulence factor involved in the direct interaction of the parasite with the host macrophage receptor [14]. The membrane proteins LCR1 and GP46 are also found to increase IFN-gamma production, and hence, are regarded as helpful in developing a general vaccine against *Leishmania* infections [5, 15]. Heat shock proteins (HSPs) are highly conserved molecules that are highly immunogenic to inducing both MHC-I and MHC-II pathways of adaptive immunity and are thought to have a pertinent role in vaccine development against infectious leishmaniasis [16]. *Leishmania* hydrophilic acylated surface protein-B (HASP-B) is expressed only in infective parasites suggesting a role in parasite virulence and, therefore, a potential vaccine candidate [17]. Kinetoplastid paraflagellar rod (PFR) proteins are also of therapeutic and prophylactic importance due to their restricted evolutionary distribution, high-order organization, and high immunogenicity [18]. Likewise, cysteine proteases and proteophosphoglycans also act as essential virulence factors for the parasites in mammalian hosts and are attractive drug targets for leishmaniasis [19–21]. Moreover, beta-tubulin protein had also previously been characterized as T cell-stimulating antigen from *Leishmania*, although its efficacy as a vaccine candidate has not been tried as yet [22]. Beta-tubulin of *L. donovani* is also considered a potential drug target against visceral leishmaniasis [23]. Lastly, the translation factor protein eIF5A from *L. braziliensis* has been shown to induce heterologous protection against leishmaniasis in animals when administered as a vaccine in combination with another recombinant protein. It increases the secretion of parasite-specific cytokines in vaccinated animals. eIF5A protein is also conserved in all eukaryotes [24].

Earlier, Khatoon et al. described an immunoinformatic-based approach to design a multi-subunit peptide vaccine against visceral leishmaniasis (VL) by exploring the secretory proteins of *L. donovani* [6]. Here, we selected various leishmanial antigens across various infectious species of the parasite. At the same time, all these proteins have already been identified (either by in vitro animal studies or by utilizing computational tools) to instigate an immune response, provoking protective immunity. The selected protein sequences belonged to any of the five pathogenic species of *Leishmania*, namely, *Leishmania major*, *Leishmania maxicana*, *Leishmania donovani*, *Leishmania chagasi*, and *Leishmania amazoensis,* among which *L. donovani* is the primary parasite causing visceral leishmaniasis (VL), while the others are primarily responsible for causing cutaneous (CL) or mucocutaneous leishmaniasis (MCL). The rationale behind the choice of such diverse types of conserved and immunogenic proteins across different species of *Leishmania* was to design a general peptide vaccine that would confer broad-spectrum cross-immunity against both the forms of leishmaniasis (VL and CL) that commonly affect humans.

Materials and methods

Selection and retrieval of Leishmania antigenic protein sequences

The complete amino acid sequences of twelve *Leishmania*-specific antigenic proteins were retrieved from Uniprot (Universal Protein Resource) database (https://www.uniprot.org/uniprot/) in FASTA format (accessed on 21.03.2022). As stated earlier, all these proteins were reported as

immunogenic, highly conserved, and potential vaccine candidates, as found in previous immunological studies involving animal models or computational in silico prediction methods. The antigenicity of the selected proteins was computed by employing the ANTIGENpro server (http://scratch.proteomics.ics.uci.edu/) that computes antigenicity of an input protein sequence by exploiting an SVM-based two-stage classifier validated by tenfold cross-validation approach [25]. The proteins were then subjected to signal peptide analysis usingSignalP 4.1 server (SignalP—4.1—Services—DTU Health Tech) in order to discriminate between classical secretory and non-secretory (transmembrane) proteins. This method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks [26]. Localization predictions for all the proteins were performed next using DeepLoc (DeepLoc—1.0—Services—DTU Health Tech), a template-free algorithm that uses deep neural networks to predict protein subcellular localization exploiting only sequence information, achieving good accuracy [27]. All functional protein sequences, as obtained from SignalP 4.1 server, were next subjected to predict the presence of antigenic determinants (epitopes) that will be recognized by B and T cell receptors. This was done to ensure that the designed vaccine construct contains only immuno-dominant T cell and B cell epitopes and, so, would be able to drive an efficient immune response involving both humoral and cell-mediated pathways of immune mechanisms.

Cytotoxic T lymphocyte (CTL) epitope prediction

Prediction of the cytotoxic T lymphocyte (CTL) epitope is an essential aspect of designing an ideal peptide [28]. A freely accessible web server, namely, NetCTL1.2 (https://www.cbs.dtu.dk/services/NetCTL), was utilized to predict the CTL epitopes for all the selected proteins. The method integrates the prediction of peptide MHC class I binding, proteasomal C terminal cleavage, and TAP transport efficiency [29], all of which are essential features that a sequence of CTL-binding epitope should possess. The server allows for predictions of CTL epitopes restricted to 12 MHC class I supertype, among which only the A1 supertype was used for the present study. MHC class I binding and proteasomal cleavage are performed using artificial neural networks. TAP transport efficiency is predicted using a weight matrix [29]. Sequences with higher binding scores are considered potent CTL epitopes.

In addition to NetCTL 1.2, another publicly available web server-based approach, namely, SYFPEITHI (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm), was also availed for the prediction of CTL epitopes from all the selected *Leishmania* proteins. The forecast is based on published motifs and considers the amino acids in the anchor and auxiliary anchor positions and other frequent amino acids [30]. From the list of MHC types provided in this server, HLA-A*01, HLA-A*02:01, and HLA-A*03 were chosen for the prediction. The epitope search was specified for nonamer sequences for uniform comparison with the results given by the NetCTL 1.2 server. Only the common epitopes from each protein (if any), which were predicted by both the NetCTL 1.2 and SYFPEITHI servers with compatible higher scores, were selected for the final vaccine construct.

Helper T lymphocyte (HTL) epitope prediction

Helper T lymphocytes are the most important immune cells as they are required in all types of adaptive immune responses. Prediction of HTL epitopes (mainly of the Th-1 type), therefore, becomes an essential element for designing an effective peptide vaccine. In the current study, MHC-II-binding analysis tool in the IEDB (Immuno Epitope Database and Analysis) server (http://tools.iedb.org/mhcii/) was employed for the prediction of 15-mer HTL epitopes for a set of three human alleles, namely, HLA-DRB1*01:01, HLA-DRB1*01:02, and HLA-DRB1*01:03. Human HLA alleles were chosen for more realistic estimation of the MHC-II-binding affinity of the epitopes as the vaccine was being designed against human leishmaniasis. The predicted output is given in units of IC_{50}nM for the combinatorial library and SMM alignment. Therefore, a lower number indicates higher affinity. IEDB recommended prediction method was used in this approach in which low adjusted rank is indicative of suitable MHC-II binders and, hence, can be defined as potent HTL epitopes.

Identification and selection of cytokine-inducing HT epitopes

Cytokines are of immense importance for the proper functioning of the immune system. A battery of cytokines like IL-2, IL-4, IL-6, and interferon-γ can induce both CTL-mediated and humoral immune responses [31]. Hence, the HTL epitopes that can trigger cytokine production are preferable for designing an immune-prophylactic vaccine. With this view, IFNepitope server (http://crdd.osdd.net/raghava/ifnepitope/) was used for the identification of HTL epitopes having cytokine-inducing abilities. IFNepitope is an online prediction server that aims to predict the peptides from protein sequences that can induce IFN-gamma released from $CD4^+$ T cells. The web server has been developed on the basis of a dataset that comprises IFN-gamma-inducing and non-inducing MHC class II binders [32]. The HTL epitopes predicted by the IEDB server with higher MHC-II-binding affinities (lower adjusted ranks) were provided as input to

check for their cytokine-producing ability by the IFNepitope server. The epitopes thus identified were then subjected to the next level of analysis by the IL4pred server (IFNepitope(iiitd.edu.in) and also by the IL10Pred server (https://webs.iiitd.edu.in/raghava/il10pred/predict3.php) in order to ensure that the selected HTL epitopes would as well evoke the secretion of IL-4 and IL-10, respectively. They also consider positional conservation of amino acid residues and amino acid composition of the peptides for more accurate prediction [33, 34]. The MHC-II-binding HTL epitopes predicted to be positive by all three servers were selected for incorporation in the final vaccine construct. This strategy was adopted to improve the vaccine's efficacy since it could instigate an intense response involving a wide variety of cytokines (IFN-gamma, IL-4, and IL-10), leading to accelerated and long-lasting immunity.

Epitope conservation analysis

Epitope conservation analysis becomes essential to check whether the chosen epitope(s) is conserved across different species of *Leishmania* proteins. Designing a subunit vaccine consisting of conserved epitopes offers a promising scope for broad-spectrum protective immunity against leishmaniasis. Conservation analysis for each of the selected CTL and HTL epitopes was done by using IEDB "Epitope Conservation Analysis" tool available within the IEDB portal (http://tools.iedb.org/conservancy/). This tool calculates the degree of conservation of the target epitope (e) within a set of homologous protein sequences (P) as the fraction of {p} that matched the aligned e above the chosen identity level while considering that the target epitope is sequential [35]. Firstly, the corresponding source proteins from which the CTL and HTL epitopes were finally selected were subjected to a BLAST search, for which we used the Uniprot BLAST tool (https://www.uniprot.org/blast/). From the BLAST result, the aligned protein sequences (belonging to the genus *Leishmania*) that showed significantly high similarity with the query sequence (> 40%) were selected. Such sequences were compiled to constitute an epitope-specific dataset, and the same was provided as the input set of proteins (P) to be used for conservation analysis by the tool. Next, the sequences for selected CTL and HTL epitopes were screened individually against their respective datasets that revealed the relative conservation of the epitopes across a set of related *Leishmania* proteins. The threshold identity level was set at > 100% (default).

Designing of multi-epitope vaccine sequence

Based on the immunoinformatic analysis, a primary sequence of the subunit vaccine was constructed by incorporating the selected CTL and HTL epitope sequences. These epitopes were linked together by AAY and GPGPG linkers, respectively [28, 31]. Linkers are inserted for proper separation of the individual epitopes that are required for the efficient functioning of the vaccine construct [28, 36]. Secondly, the addition of an appropriate adjuvant is crucial in a subunit vaccine to boost the immune response [31]. The choice of a suitable adjuvant is cardinal in designing vaccines for human trials. IL-12 produced by various immune cells is essential in developing cell-mediated immunity against leishmaniasis, and the potential of IL-12 as an adjuvant in vaccines against leishmaniasis has been reported in murine models [3, 37, 38]. This IL-12 was selected as an adjuvant for designing the vaccine, and the sequence of the same was included in the N terminal of the vaccine construct using the EAAK linker [28, 31]. The sequence of the human IL-12 alpha chain was retrieved from the Uniprot database (www.uniprot.org; Accession No. P29459). Finally, the peptide vaccine construct was obtained having adjuvant, linker, CTL epitopes, and HTL epitopes (with intra-epitopic AAY and GPGPG linkers) in a sequence moving from N terminal to C terminal.

Conformational and linear B cell epitope prediction

Humoral immunity involves the synthesis and secretion of specific antibodies by activated B cells, which requires the presence of antigenic determinants that are recognized by B cell receptors present on the surface of B lymphocytes. An ideal immune-protective peptide vaccine should have such components in order to interact with and, thereby, stimulate the B cells. Therefore, two servers, namely, ABCpred and BepiPred 2.0, were applied for the precise prediction of B cell epitopes in the primary sequence of the designed vaccine. ABCpred server (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html) is an artificial neural network (ANN) based approach which predicts B cell epitopes in a given antigen sequence using fixed length parameters. The training dataset used in this method contains B cell epitopes from the B cell epitope database (BCIPEP). The server is able to predict epitopes with 65.93% accuracy using a recurrent neural network [39]. The primary sequence of the final vaccine construct was provided as the input in a plain format, and the ABCpred server default parameters, including a threshold value of 0.51 with a window length of 16 amino acid residues, were used for the prediction. BepiPred 2.0 server (BepiPred—2.0—Services—DTU Health Tech), on the other hand, predicts sequential B cell epitopes from an input sequence by a random forest algorithm trained on epitopes and non-epitope amino acids determined from crystal structures followed by a sequential prediction smoothing [40]. The primary sequence of the vaccine was provided in a FASTA format as the input. The application of two different servers that predict B cell epitopes based on two different

algorithms makes the prediction more realistic. Most of the antigenic determinants recognized by the B cell receptor and antibodies are discontinuous, meaning they contain amino acid residues located distantly in the primary structure of the immunogen but are brought adjacent to each other during the folding of the protein [41].

Prediction of such discontinuous B cell epitopes in the validated tertiary structure of the vaccine was performed by utilizing the ElliPro server (http://tools.iedb.org/ellipro/) incorporated within the IEDB portal. ElliPro is a freely accessible web tool that implements modified Thronton's method and takes into account each residue's center of mass rather than the Cα atoms. Doing so relates each of the epitopes to an assigned score defined as PI (protrusion index) averaged over epitope residues. Discontinuous epitopes are determined based on their respective PI values and clustered based on the distance between the residue's centers of mass (*R*). Larger values of *R* correlate to more significant discontinuous epitopes being predicted [42]. At the same time, linear B cell epitopes in the input protein model can also be predicted and visualized by using ElliPro. Detection of both linear and discontinuous B cell epitopes in the refined 3D model of the vaccine ensures a successful vaccine-designing strategy.

Profiling of antigenicity, allergenicity, and toxicity of the vaccine construct

Antigenicity is an essential criterion for an immune-prophylactic vaccine. To ensure that the designed vaccine construct would elicit a long-lasting immune response by interacting with the B and T cell receptors, the antigenicity of the same was evaluated by using ANTIGENpro and VaxiJen 2.0 servers. ANTIGENpro (http://scratch.proteomics.ics.uci.edu/) server correctly classifies 82% of the known protective antigens when trained using only the protein microarray datasets. The accuracy of the combined dataset is estimated at 76% by tenfold cross-validation experiments that allow significantly better recognition of antigenic peptides. It runs on a two-stage architecture, including an SVM-based second-stage classifier.

For a new input protein sequence, the probability computed by the second stage SVM predictor is the final ANTIGENpro prediction score [25]. VaxiJen2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) is another publicly accessible web server that evaluates antigenicity of a protein sequence by an alignment-free approach which is based on auto cross-covariance (ACC) transformation of protein sequences into uniform vectors of principal amino acid properties and allows antigen classification solely based on various physicochemical properties of the protein without referring to sequence alignment [43].

Secondly, in order to rule out any possibility that the vaccine construct could trigger an allergic response in an individual, AlgPred (https://webs.iiitd.edu.in/raghava/algpred/submission.html) server was used to determine its allergenicity. The server tool provides a variety of approaches, including mapping of IgE epitopes, MEME/MAST motif, SVM method based on dipeptide composition, and BLAST ARPs, all of which are exploited for an accurate prediction. The threshold was set at − 0.4 (default) for higher prediction precision. In addition, AllerTop v.2.0 (https://www.ddg-pharmfac.net/AllerTOP/index.html), a freely accessible web server-based tool, was also applied to confirm the non-allergic nature of the vaccine construct. This method is based on the auto cross-covariance (ACC) transformation of protein sequences into uniform equal-length vectors. It has been applied to quantitative structure–activity relationships (QSAR) studies of peptides with different lengths, and the proteins are classified by the *k*-nearest neighbor algorithm [44]. Toxicity, if any, was predicted by utilizing the ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/protein.php) server. This prediction tool works based on both machine learning technique and quantitative method that is trained on a dataset of various toxic and non-toxic peptides obtained from SwissProt and TrEMBL [45]. Toxicity profiling is crucial to assess the safety of the vaccine and other such immunotherapeutic peptides that are intended for a human trial. The analysis was performed by choosing SVM (TrEMBL) + motif-based approach at an *E* value threshold of 10.0 for motif-based method and an SVM threshold of 0.1.

Evaluation of physicochemical properties

Describing the physical and chemical features of a chimeric peptide becomes vital since, when administered as a vaccine, it should be able to induce a proper immune response. The ProtParam server available with the ExPASY (Expert Protein Analysis System) portal (https://web.expasy.org/protparam/) was used to compute various physicochemical parameters of the primary vaccine construct that included features like molecular weight, theoretical pI, instability index, aliphatic index, in vivo and in vitro half-life, grand average of hydrophobicity (GRAVY), and others.

Secondary structure prediction of the vaccine peptide

Secondary structural elements of the designed vaccine construct were predicted by applying the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/) and RaptorX property server (http://raptorx.uchicago.edu/StructurePropertyPred/predict/) both of which are freely accessible online protein structure analysis servers. The primary sequence of the

vaccine peptide was provided as the input. PSIPRED predicts the secondary structure of the input protein sequence by exploiting two feed-forward neural networks that function based on position-specific scoring matrices (PSSM) generated by PSI-BLAST [46]. It incorporates the identification of sequences that are homologous to our vaccine construct by PSI-BLAST, followed by the generation of PSSM. PSIPRED 3.2 and higher versions were found to achieve an average Q_3 score of 81.6% as evaluated by a stringent three-fold cross-validation method which makes this approach significantly precise and accurate. RaptorX property web server engages a very recent machine learning technique called DeepCNF (deep convolutional neural fields) for predicting various features like secondary structure, solvent accessibility, and disordered regions simultaneously. DeepCNF is an integrated approach combining both conditional random fields (CRFs) and shallow neural networks that models complex sequence-structure relationships by a deep hierarchical architecture and can obtain approximately 84% Q3 accuracy for 3-class secondary structure [47].

Tertiary structure prediction

The tertiary structure of the final vaccine peptide was predicted by using the online web server I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). I-TASSER is a hierarchical protein structure modeling approach based on profile-profile threading alignment (PPA) and iterative structure assembly simulations followed by atomic-level structure refinement. It works in three stages: structural template identification, iterative structure assembly, and structure-based function annotation. The top-ranked structure models with global and local accuracy estimations are returned with their representative *C*-scores and TM scores, which are correlated with the quality of the predicted model. The I-TASSER program represents one of the most successful methods demonstrated in CASP for automated predictions of protein structure and function [48, 49].

Refinement of vaccine tertiary structure

Conventional computational modeling of a protein structure alone does not guarantee the authenticity and accuracy of the predicted model since such modeling strategies largely depend on the degree of likeliness of the input (target) with the available template structures. Enhancement of the quality of the template-based model beyond the accuracy was, therefore, thought to be necessary, and this could be achieved by refining the whole protein structure. With this perspective, the tridimensional model output from the I-TASSER server with the best *C*-score was subjected to further refinement by the GalaxyRefine server (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE). It is a CASP10-tested refinement approach in which protein side chains are rebuilt first, and then side chain repacking, and the overall structure relaxation is performed by molecular dynamics simulation. This leads to precise improvement of both local and global tertiary structures of target proteins [50].

Validation of vaccine tertiary structure

The refined tertiary structure of the vaccine needed to be validated to spot potential errors that might occur in the predicted 3D model. The refined model obtained from the GalaxyRefine server was provided as the input structure in a.pdb format. Besides, Ramachandran plot is another very useful approach for visualizing energetically allowed and disallowed regions of *phi* (ϕ) and *psi* (ψ) dihedral angles of the amino acid side chains that are considered crucial components for validation of a protein structure. Hence, the MolProbity server [Main page—MolProbity (duke.edu)] was employed that generate a Ramachandran plot for the given model of the vaccine based on Python-based MolProbity-style Phenix validation [51]. We further used the ERRAT server [SAVESv6.0—Structure Validation Server (ucla.edu)] that analyzes the statistics of non-bonded interactions between different atoms in the model and plots the value of error function versus a 9-residue sliding [52]. ProSA web server (https://prosa.services.came.sbg.ac.at/prosa.php), an interactive web-based platform based on the standard ProSA program, was used for this purpose [53]. This step was performed to get a more sophisticated and precise validation of the modeled structure.

Disulfide engineering of the vaccine peptide

Disulfide bonds contribute to protein stability by lowering the conformational entropy and also by increasing the free energy of the denatured state. The introduction of novel disulfide bonds has been considered a critical biotechnological tool to improve the thermostability of native, folded proteins. Disulfide by Design 2 (DbD 2) v 2.12 server was used to enhance the overall structural stability of the designed vaccine by introducing disulfide linkages. This method utilizes native geometry and calculates an energy value for each potential disulfide, thereby providing a means to rank the candidate disulfide bonds. The disulfide bonds that confer maximum stability were the candidates with high chi3 value, bond energy, and B factor [54]. The flexible regions of the peptide were selected based on the parameters mentioned earlier, and corresponding stabilizing mutations were created by forming disulfide linkages between the residues chosen pairs.

Molecular docking of vaccine construct with immune receptors (Human TLRs-2, 4, 5, 8, and mouse TLR-9)

Toll-like receptors (TLR) are major attributes of cellular immune responses that recognize pathogen-associated molecular patterns (PAMPs) and evoke innate immune responses against infections. The direct activation of TLR-2 with *Leishmania* components was subsequently reported [55]. In other related experimental studies, the lack of TLR-4 resulted in increased parasitic growth and delayed healing of cutaneous lesions caused by *L. major* infections indicating the plausible role of TLR-4 in inducing immunity against *Leishmania* [56]. On the other hand, TLR-2, 4, and 9 were found to be involved in an immunopathologic spectrum of CL caused by *L. braziliensis* and *L. amazonensis* [57]. TLR-9 was also evaluated as a key player in the activation of dendritic cells in the pathogenesis of VL in humans [58]. Some of the *Leishmania*-derived components have been shown to activate TLR-2, 4, and 9 in the majority of studies conducted in this field. These observations emphasize the fact that various TLRs are involved in triggering protective immunity against *Leishmania*. Therefore, an interaction between the TLRs and the designed vaccine construct was considered necessary for eliciting effective immunity against *Leishmania* infections. This was checked by performing molecular docking of the multi-epitopic vaccine peptide with human TLRs-2, 4, 5, 8 and mouse TLR-9 using PatchDock server [PatchDock Server (tau.ac.il)]. The PatchDock algorithm has three main stages, namely, molecular shape representation, surface patch matching, and filtering and scoring. It is a geometry-based molecular docking algorithm that principally operates on molecular shape complementarity between the receptor and the ligand. The complementary patches are matched, and candidate transformations are generated. Each of these transformations is further ranked by assigning a scoring function that considers both atomic desolvation energy and geometric fit [59, 60]. In our study, human TLR-2 (PDB id: 6NIG), TLR-4 (PDB id: 4G8A), TLR-5 (PDB id: 3J0A), and TLR-8 (PDB id: 4QC0), and mouse TLR-9 (PDB id: 3WPF) were selected as receptors, and their separate PDB files were retrieved from Protein DataBank (www.rcsb.org). The refined 3D model of the vaccine peptide was used as the ligand for all the docking simulations. The PatchDock server was set at default parameters (clustering RMSD: 4.0). We further analyzed the best docking model using the Cluspro server based on a possible bond rotation algorithm, and bonds were evaluated using PyMOL software [61].

Codon optimization, mRNA structure prediction, and in silico *cloning for expression of vaccine protein*

Optimization of the codon is required to achieve the maximum expression of foreign genes in the host organism when the usage of a codon by the host differs significantly from that of the native host from which the original sequences for the final vaccine construct had been culled. JCat (Java Codon Adaptation Tool), available publicly at (http://www.jcat.de/), was used for reverse translation and codon optimization by providing a primary sequence of the final vaccine construct as the input. The codon usage was optimized to the most sequenced prokaryotic organism, *E. coli* K12 [6]. Three additional options provided by the tool were selected to avoid rho-independent transcription terminators, prokaryotic ribosome-binding sites, and unwanted restriction cleavage sites. The codon adaptation index (CAI) and GC content, as calculated by the JCat tool, indicate how good the optimization was. Better optimization ensures higher expression of the vaccine protein. Further, restriction sites for the enzymes *XhoI and NdeI* were incorporated into the optimized cDNA sequence provided by the JCat tool to facilitate cloning. The modified sequence (with restriction sites) was then inserted into the *E. coli* pET 28-a(+) plasmid vector by using the restriction cloning module of the SnapGene tool [6, 31, 62, 63]. SnapGene software is available on the web at (https://www.snapgene.com/). Besides, we also used the mfold web server (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) for the prediction of the secondary structure of the mRNA encoded by the genes of the chimeric peptide. The reverse-translated optimized sequence obtained from the JCat tool was provided as the input. The mfold program is based on a core algorithm that predicts minimum free energy, Δ G, and minimum free energies for foldings containing any particular base pair. Various folding constraints are also applied for the accurate prediction of folding energies. The folding energy for RNA folding is fixed at 37 °C, while the ionic conditions are fixed at $[Na^{+}] = 1$ M and $[Mg^{++}] = 0$ [64].

C-immsim-based immune simulation

C-immsim (https://kraken.iac.rm.cnr.it/C-IMMSIM/index.php?page=1) is a server-based immune response prediction tool that utilizes agent-based class, and its prediction relies on neural networks. It can predict both humoral and cell-mediated immune responses. C-immsim utilizes the Celda-Seiden model, bit-string polyclonal lattice model, and Simpson index to characterize the prediction of active, resting, and memory cells and their longevity. It helps to predict the cytokine profile of a given reaction to evaluate inflammatory responses [65].

Molecular dynamic simulation

The molecular dynamic simulation was performed to evaluate the stability of our best complex, i.e., TLR, with the

proposed vaccine using iMOD (iMod Server home page (csic.es) server. This web-based server uses improved normal mode analysis (NMA) for the characterization of various dynamic properties of biomolecules, like protein flexibility and stability, by means of an intra-atomic force field. In this current study, we analyzed the main chain deformities, B factor, eigenvalues, covariance factor, and elastic network model as standard NMA parameters for the evaluation of protein–protein complexes obtained by in silico approaches [66–69].

Results

Selection and retrieval of Leishmania antigenic protein sequences

The amino acid sequences of twelve *Leishmania* proteins were retrieved from the Uniprot database in FASTA format and subjected to further analysis for the purpose of designing a multi-subunit peptide vaccine against leishmaniasis. These proteins were selected based on previous studies that reported them as potential vaccine candidates as evaluated in experimental animal models or in silico prediction tools. Antigenicity analysis by the ANTIGENpro server showed all the chosen proteins as potential antigens with varying degrees of antigenicity, keeping with the information obtained from literature mining. However, ten among the twelve proteins were presented with relatively higher prediction scores (> 0.51), reflecting their higher potency for vaccine design (Table 1). The proteins were discriminated as secretory and non-secretory based on the prediction of signal peptide cleavage sites by the SignalP 1.4 server. Two of the selected *Leishmania* proteins viz. *L. mexicana* cysteine proteinase A (Uniprot id: P25775) and *L. amazoensis* surface glycoprotein GP-46/M2 (Uniprot id: P21978) were found to contain signal peptides and, hence, predicted as secretory proteins. The rest of the ten *Leishmania* antigens were not found to have any signal peptide indicating their non-secretory nature. Localization analysis predicted that the protein cysteine proteinase A of *L. mexicana* (Uniprot id: P25775) to be localized in lysosome/vacuole, proteins leishmanolysin C1 and proteophosphoglycan of *L. mexicana* (Uniprot ids: P43150 and Q9TW13, respectively) to be membrane proteins, LACK protective antigen of *L. donovani* (Uniprot id: Q9BIJ5) to be in the nucleus and heat shock 70 related protein 1 of *L. major* (Uniprot id: P12076) to be localized in mitochondria. All the other proteins were predicted as cytoplasmic (Table 1). In addition to this, the amino acid sequence of the alpha chain of human IL-12 was also obtained from the Uniprot database (Uniprot id: P29459) to be used as an adjuvant in the final vaccine construct.

Prediction of CTL and HTL epitopes

From all the proteins being investigated, a total number of 59 common nonameric CTL epitopes were identified jointly by NetCTL 1.2 and SYFPEITHI servers. Each of the predicted CTL epitopes was assigned a particular score computed by the respective servers. Only 26 of these common 9-mer CTL motifs were selected due to their higher scores (indicating higher MHC I-binding affinity). These twenty-six CTL epitopes identified commonly by both the servers with compatible higher scores were included in the final vaccine

Table 1 Twelve potential antigenic peptides from different species of *Leishmania* have been exploited for vaccine construction and selected based on their high antigen score as predicted by the ANTIGENpro server

Serial no	Protein	Uniprot Id	Organism	ANTIGENpro Score	SignalP 1.4 server outcomes
1	Paraflagellar rod protein 2C	Q4QEM2	*Leishmania major*	0.832853	Non-secretory
2	Kinetoplast membrane protein 11 (KMP-11)	E9AF45	*Leishmania major*	0.456471	Non-secretory
3	Cysteine proteinase A	P25775	*Leishmania mexicana*	0.944768	Secretory
4	Leishmanolysin C1	P43150	*Leishmania mexicana*	0.887777	Non-secretory
5	Antigen Lcr1 (fragment)	Q25240	*Leishmania chagasi*	0.622478	Non-secretory
6	Hydrophilic acylated surface protein b	Q4QB56	*Leishmania major*	0.516465	Non-secretory
7	LACK protective antigen	Q9BIJ5	*Leishmania donovani*	0.827942	Non-secretory
8	Surface membrane glycoprotein GP46/M-2	P21978	*Leishmania amazonensis*	0.302615	Secretory
9	Proteophosphoglycan	Q9TW13	*Leishmania mexicana*	0.912010	Non-secretory
10	Heat shock 70-related protein 1	P12076	*Leishmania major*	0.871139	Non-secretory
11	Tubulin beta chain	P21148	*Leishmania mexicana*	0.736898	Non-secretory
12	Eukaryotic translation initiation factor 5A	A4GVE9	*Leishmania donovani*	0.868310	Non-secretory

All of these antigenic peptides have been retrieved from the UniProt database

construct (supplementary Table S1). Likewise, IEDB MHC-II-binding analysis tool predicted 15-mer HTL epitopes from all the selected proteins against a set of three human HLA alleles (HLA- DRB1*01:01, HLA- DRB1*01:02 and HLA-DRB1*01:03). The predicted HTL epitopes with lowest adjusted rank for each one of these alleles are documented as in supplementary Table S2. From this pool, a total number of nine sequences were chosen on the basis of their low adjusted ranks (Table 2) that make these HTL epitopes the most potent MHC-II binders for any of the three HLA alleles. The selected HTL epitopes were next subjected to further computational analysis in order to predict their cytokine-producing abilities.

Screening for HTL epitopes with cytokine-inducing abilities

All of the nine chosen HTL epitopes (Table 2) were evaluated for their ability to produce interferon-γ by employing an IFNepitope server. The server predicted (IFN-γ vs. non-IFNγ, using SVM and motif-based hybrid analysis) only three such peptides as FRRLYKTLGQLVYKK from paraflagellar rod protein 2C of *L. major*, DNGAYLGMEPSSVAA from proteophosphoglycan of *L. mexicana*, and RLHFFMMGFAPLTSR from tubulin beta chain of *L. mexicana* as "positive" with scores lesser than 1.0 (Table 3).

These three peptides were further checked for their IL-4 and IL-10-producing abilities using the IL4pred server and IL10pred server, respectively. Both the servers utilize an SVM classifier and consider various SVM input parameters like amino acid composition, dipeptide composition, amino acid propensity, and physicochemical properties for the prediction [32, 33]. The respective servers predicted that these three 15-mer IFN-γ-producing epitopes were inducers for IL-4 and IL-10 as well (Supplementary Table S3). Hence, these three MHC-II-binding 15-mer HTL epitopes were finally selected for the construction of the vaccine considering their ability to elicit the production of cytokines IFN- γ, IL-4, and IL-10 and thereby mediating a heightened immune response.

Epitope conservation analysis

The IEDB module "conservation across antigen" available within the IEDB server was availed to find the degree of conservation of the selected CTL and HTL epitopes that would be incorporated in the final vaccine construct. Each of the twenty-six CTL epitopes and three HTL epitopes was screened individually against a specific set of homologous protein sequences obtained by Uniprot BLAST. The BLAST results showed a high degree of conservation of the selected proteins in various species of *Leishmania* and also in other related parasitic protozoa, mostly *Trypanosoma.* All the HTL epitopes were found to be highly conserved (100%) across the set of related *Leishmania* proteins used in the analysis, while the range of conservation for selected CTL epitopes varied from 0 to 100% (Supplementary Table S4). The incorporation of such conserved CTL and HTL epitopes in the final vaccine construct would ensure broad-spectrum cross-immunity

Table 2 Details of selected HTL epitopes (15 mer) with their source proteins, corresponding binding alleles, and adjusted ranks predicted by the IEDB MHC-II-binding tool

Serial no	Uniprot sequence Id	Alleles	Start position	End position	The sequence of HTL epitope	Adjusted rank
1	Q4QEM2	DRB1*01:03	446	460	FRRLYKTLGQLVYKK	0.58
2	P25775	DRB1*01:03	258	272	SLCLAWSLNHGVLIV	0.42
3	P43150	DRB1*01:01	616	630	GMVLSLMALLVVRLL	0.10
4		DRB1*01:02	15	29	AAPLVRLAAAGAAVT	0.33
5	P21978	DRB1*01:01	18	32	LQAFARAIPALGDTW	0.14
6	Q9TW13	DRB1*01:01	401	415	DNGAYLGMEPSSVAA	0.24
7		DRB1*01:02	345	359	ALVLFFVILFIVALI	0.30
8	P21148	DRB1*01:01	263	277	RLHFFMMGFAPLTSR	0.14
9		DRB1*01:03	162	176	RIMMTFSVIPSPRVS	0.17

Table 3 IFNepitope server predicted MHC II-inducing epitopes that are capable of interferon-γ production

Serial no	UniProt Id	Sequence	Method	Result	Score
1	Q4QEM2	FRRLYKTLGQLVYKK	SVM	Positive	0.060141565
2	Q9TW13	DNGAYLGMEPSSVAA	SVM	Positive	0.003934284
3	P21148	RLHFFMMGFAPLTSR	SVM	Positive	0.028001530

against multiple species of pathogenic *Leishmania* that commonly infect humans.

Construction of multi-epitope vaccine sequence

The primary sequence of the multi-subunit peptide vaccine was constructed by merging the sequences of twenty-six CTL epitopes and three HTL epitopes selected on the basis of their antigenicity, MHC-binding affinity, and cytokine-inducing ability. The individual CTL and HTL epitopes were amalgamated by using AAY and GPGPG linkers, respectively, and the overall length of the resulting vaccine peptide was 369 amino acids. The adjuvant, 219 amino acids long alpha chain of human IL-12 (Uniprot id: P29459), was added to the N terminal of the designed peptide by EAAK linker. The addition of IL-12 as a natural adjuvant was supposed to enhance the vaccine's effectiveness by accelerating the immune response, as already validated in animal studies [3, 37]. In addition, a 6XHis tag was also added to the C terminal of the peptide to facilitate the identification and purification of the protein following its production by recombinant DNA technology. After the addition of linkers, adjuvant, and histidine tag, the length of the final vaccine peptide was found to be 598 amino acid residues (Fig. 1).

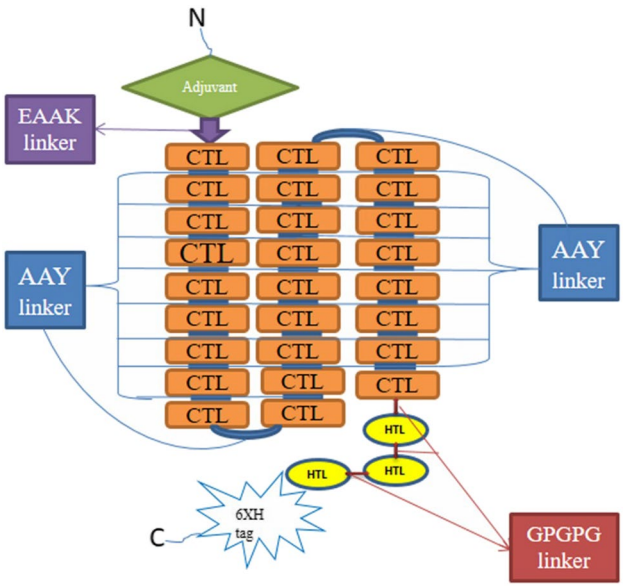


Fig. 1 Schematic depiction of designed multi-epitope peptide vaccine containing a total of 598 amino acid sequences representing possible vaccine construct with N terminal natural adjuvant (green) linked with EAAK (purple) with 26 CTL (orange) using AAY (blue) linker, and 3HTL (yellow) are connected with GPGPG (red) linker. It ends with 6-His tag at the C terminal for purification purposes

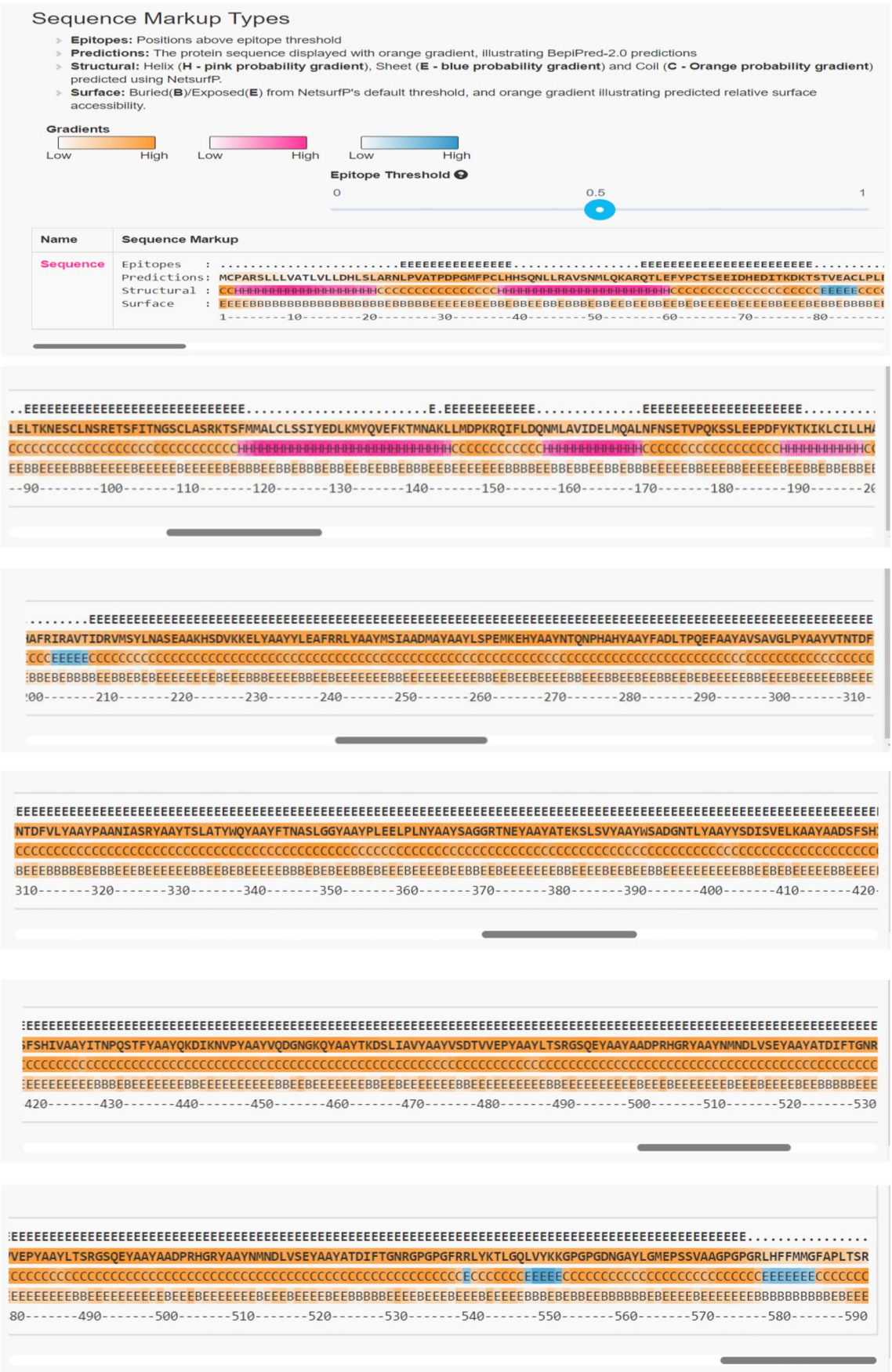
Prediction of linear, continuous, and discontinuous B cell epitope

The presence of antigenic determinants in the primary sequence of the designed vaccine was identified by employing two different servers, namely, ABCpred and BepiPred 2.0, for more effective prediction. ABCpred server predicts B cell epitopes by using a trained recurrent neural network and represents the candidate sequences as 16-mer peptides ranked according to their binding scores (Supplementary Table S5). A higher score of the peptide means a higher probability of being an epitope. The designed vaccine peptide was found to have seven non-overlapping 16-mer peptides with predicted scores of 0.90 and above at a default threshold value of 0.51, reflecting their very high potency to bind and stimulate B cells. The other server, BepiPred 2.0, predicts the probability of each amino acid residue in the input sequence to be a part of the B cell epitope and depicts the output as an illustration with an orange color gradation indicating the degree of probability. The predicted residues that belong to B cell epitopes are marked as "E." The server identified a large number of residues to be linear B cell epitopes with high probability within a residue stretch of 380–530 position of the peptide (Fig. 2) when the epitope threshold was set at 0.5 (default). Thus, the identification of antigenic determinants for B cell receptors by two independent servers established the fact that the peptide, when administered as a vaccine, would be able to stimulate the production of antibodies by B cells to protect individuals from *Leishmania* infections. Ellipro suit (available within the IEDB portal) predicted a total of 323 amino acid residues located in eight discontinuous B cell epitopes in the refined tertiary structure of the peptide vaccine, with scores ranging from 0.509 to 0.802. Among them, the largest conformational epitope contained 107 amino acid residues with a predicted score of 0.772. The default parameters of the server were used for the analysis. Ellipro also predicted twelve linear B cell epitopes with a total number of 324 residues within a score range of 0.535–0.885 (Supplementary Table S6 and supplementary image M1).

Antigenicity, allergenicity, and toxicity profiling of the vaccine construct

The antigenicity of the final vaccine sequence (with adjuvant) was evaluated by ANTIGENpro and VaxiJen 2.0 servers. ANTIGENpro server predicted the same to be a probable antigen with an antigenicity score of 0.745396. VaxiJen

Fig. 2 B cell epitope predicted by Bepipred 2.0 server. Stretches at ▶ different points from 1 to 590 amino acids sequence were predicted with potential epitope above the default threshold level. The first line marked as **E** shows the predicted epitope location


Sequence Markup Types
Epitopes: Positions above epitope threshold
Predictions: The protein sequence displayed with orange gradient, illustrating BepiPred-2.0 predictions
Structural: Helix (H - pink probability gradient), Sheet (E - blue probability gradient) and Coil (C - Orange probability gradient) predicted using NetsurfP.
Surface: Buried(B)/Exposed(E) from NetsurfP's default threshold, and orange gradient illustrating predicted relative surface accessibility.
Gradients
Low High
Low High
Low High
Epitope Threshold
0
0.5
1
Name
Sequence Markup
Sequence
Epitopes :
Predictions:
Structural :
Surface :

2.0 server evaluated the vaccine peptide as a probable antigen with a score of 0.5325 at a threshold of 0.5 when the target organism was selected to be a parasite (Table 4).

However, when the original vaccine sequence without the adjuvant was analyzed by VaxiJen 2.0, it too was predicted as a probable antigen with a score of 0.5768 in parasite models. The ANTIGENpro prediction score for the original sequence was 0.714582. Hence, both the constructed vaccine sequences (with and without adjuvant) were found to be antigenic in nature, supporting the fact that the peptide components of the vaccine were antigenic by themselves, even in the absence of the added adjuvant (Table 4).

As far as allergenicity is concerned, the final vaccine sequence was found to be non-allergen by AlgPred with a predicted score of 0.4018 at a threshold of 0.4. In addition, the protein sequence was not found to contain any experimentally proven IgE epitope, and no hits were found for BLAST results for allergen-representative peptides (ARPs), indicating the non-allergen nature of the vaccine. AllerTOP v.2.0 server also defined the protein as a probable non-allergen. Toxicity analysis by the ToxinPred server predicted the peptide to be non-toxic when the SVM (TrEMBL) + motif-based approach was adopted at an SVM threshold of 0.1, and the *E* value cut-off for motif based was set at 10.0. Hence, the generated vaccine sequence was predicted as a probable antigen, non-allergen, and non-toxic, all of which are important criteria for an ideal multi-subunit peptide vaccine.

Evaluation of physicochemical parameters

The physicochemical parameters of the final vaccine construct were computed by the Protparam server on the basis of the amino acid residues present in the given sequence. The molecular weight (MW) of the designed peptide was calculated to be 65.68 kDa which makes it suitable for inducing an immunogenic response. The theoretical pI (isoelectric point) was computed as 5.98, which designates the peptide as slightly acidic in nature. The total numbers of positively charged and negatively charged residues in the peptide were 48 and 55, respectively. The half-life was calculated as 30 h in mammalian reticulocytes in vitro, more than 20 h in yeast, and more than 10 h in *E. coli* in vivo. The persistence of the protein for 30 h in mammalian cells (including humans) was sufficient for eliciting a desirable immune response since the mechanism requires processing and presentation of the protein by the immune cells. The aliphatic index (relative volume occupied by the aliphatic side chains) was predicted to be 78.34, indicating the protein is thermostable in nature as the aliphatic index may be considered as a positive factor contributing to the increase in thermostability of globular proteins. The instability index was computed to 47.10. The predicted grand average of hydropathy (GRAVY) value of the vaccine construct was predicted to be − 0.140; the negative value defines it as hydrophilic in nature and will interact with water molecules [28, 70–72]. To conclude, the immunoinformatic analysis predicted the designed peptide construct as antigenic, thermostable, persistent in mammalian cells, and hydrophilic. All of these factors impact the effectiveness of the construct as a probable vaccine candidate.

Prediction of secondary structure

Prediction of the secondary structure for the 592 residues long chimeric peptide by PSIPRED server reveals the presence of 45.77% alpha helix, 4.89% beta-sheet, and 49.32% coils, as depicted in the following Fig. 3. In addition, the RaptorX structure prediction server analyzed the relevant solvent accessibility for the peptide in which 36% of residues were predicted to be exposed, 28% were predicted as medium buried, and 35% of residues were predicted as buried, indicating that the protein will have an overall fair contact with water (solvent). Only 32 residues (5% of the peptide) were predicted to be in the disordered domain of the protein by the RaptorX server.

Tertiary structure prediction

I-TASSER web server was utilized for the prediction of the 3-D structure of the designed vaccine. The server predicted five models for the said peptide on the basis of multiple threading alignments using ten different templates, among which the template with PDB id 7e2cl showed the best alignment with a TM score of 0.931 and an RMSD value of 2.75. The top-ranked model exhibiting the highest confidence score (*C*-score) of − 1.11 and an estimated RMSD

Table 4 Antigenicity results computed from ANTIGENpro server and Vaxijen2.0 server with and without antigen

Serial no	Selection type	Antigenicity score	Predicted result
1	ANTIGENpro server	0.745396 (with adjuvant)	Probable antigen (at threshold value 0.5)
2	ANTIGENpro server	0.714582 (without adjuvant)	Probable antigen (at threshold value 0.5)
3	Vaxijen2.0 server	0.5325 (with adjuvant)	Probable antigen as a parasite (at threshold value 0.5)
4	Vaxijen2.0 server	0.5768 (without adjuvant)	Probable antigen as a parasite (at threshold value 0.5)

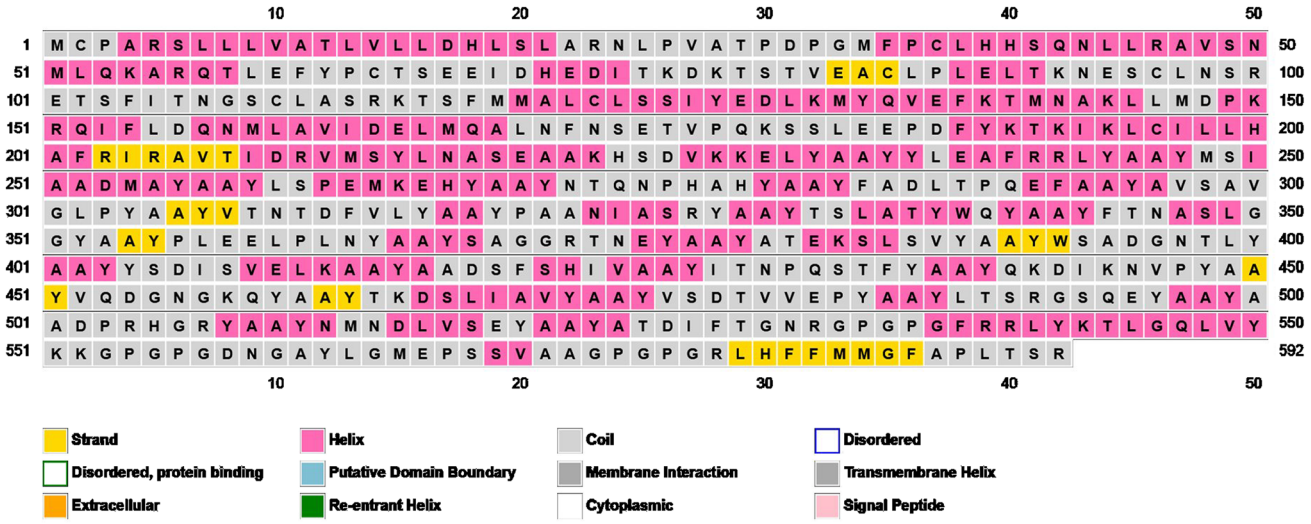


Fig. 3 Secondary structure prediction to linear vaccine sequence by PSIPRED server

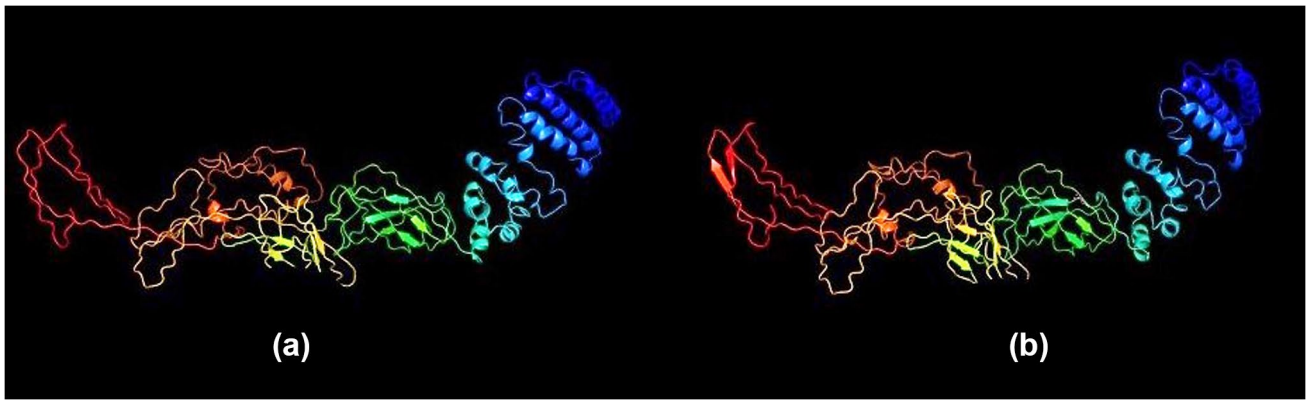


Fig. 4 Tertiary structure prediction and refinement **a** 3-D modeling of vaccine structure as predicted by I-TASSER server. **b** Refinement of the crude 3-D model by GalaxyRefine server

of 10.3 ± 4.6 Å (Fig. 4a) was selected for further refinement. A higher *C*-score indicates a higher level of confidence with which the server predicts the tertiary structure of the target protein based on the predictions obtained from modeling simulations [46, 66]. The *C*-score has a strong correlation with the overall quality of the tertiary structure, and it has been used widely for quantitative estimation of the RMSD and TM scores of predicted models in comparison with the native models; further, the estimated TM score of the chosen model was found to be 0.58 ± 0.14 which is another strong indicator of good modeling strategy since a TM score of > 0.5 indicates a model with the correct fold. TM score is a sequence-length independent parameter for measuring the structural similarity of the predicted model with other identical proteins in the same SCOP/CATH fold family [47].

Refinement and validation of vaccine 3-D model

The "crude" tertiary model for the peptide vaccine was subsequently refined by processing it through the GalaxyRefine server. GalaxyRefine yielded five models, out of which model 1 was found to be the best after the evaluation and comparison of the associated parameters such as GDT-HA (0.8826); RMSD (0.608); MolProbity (2.398); clash score (21.1); poor rotamers (0.6); and Rama-favored region (88.8) with the rest of the models (Fig. 4b).

The Ramachandran plot generated by the MolProbity server showed that the refined 3-D model has 88.8% residues in the favored region, 98.6% residues in the allowed region, and only 0.013% outliers. In contrast, the initial crude model was found to have 77.1%, 94.2%, and 0.057% residues in the

favored, allowed, and outlier regions, respectively. These results clearly conclude that the quality of the refined tertiary model was significantly improved. ERRAT server was also exploited for additional validation of the refined model. ERRAT server analyzed the model with an overall quality factor of 43.542, which actually represents the percentage of the input protein model which falls below the 95% rejection limit. Thus, we successfully obtained an appreciably refined and validated tertiary structure for the vaccine construct. The refined tertiary structure of the vaccine, along with the plots obtained from the MolProbity server and ERRAT server, is represented in Fig. 5. ProSA web server had shown a Z score of − 1.08 (supplementary image M2), in which the negative value indicates that the model falls outside the scores' range of similar proteins with comparable sizes whose structures had been determined by X-ray diffraction or by NMR [51].

Disulfide engineering of the vaccine peptide

Disulfide by Design 2.12 server returned 45 pairs of amino acid residues that are probable sites for disulfide bond formation. We have identified the four most crucial candidate residue pairs that might be located in the flexible loop region of the vaccine protein based on their high bond energy and Chi3 values (Supplementary Table S7). These residues, THR58-PHE61 (chi3: + 126.18), LEU216-ALA234 (chi3: + 125.92), THR323-GLU359 (chi3: + 121.54), and TYR472-ARG504 (chi3: + 125.75), were replaced with cysteine in the refined tertiary model of the final vaccine protein, thereby improving the thermal stability of peptide (Fig. 6). However the accuracy of B factor is limited to the attenuation of neutron scattering or X-ray by the thermal motion of molecules in protein crystallography. Thus, we stringently selected high bond energy and Chi3 values over the B factor for this computer-assisted refine vaccine model [73, 74].

Analysis of docking interactions of the vaccine peptide with the TLRs

The interactions of the chimeric peptide with the immune receptors (human TLRs-2, 4, 5, 8 and mouse TLR-9) were studied by performing docking analysis using PatchDock server in which the binding affinities of the vaccine (ligand)

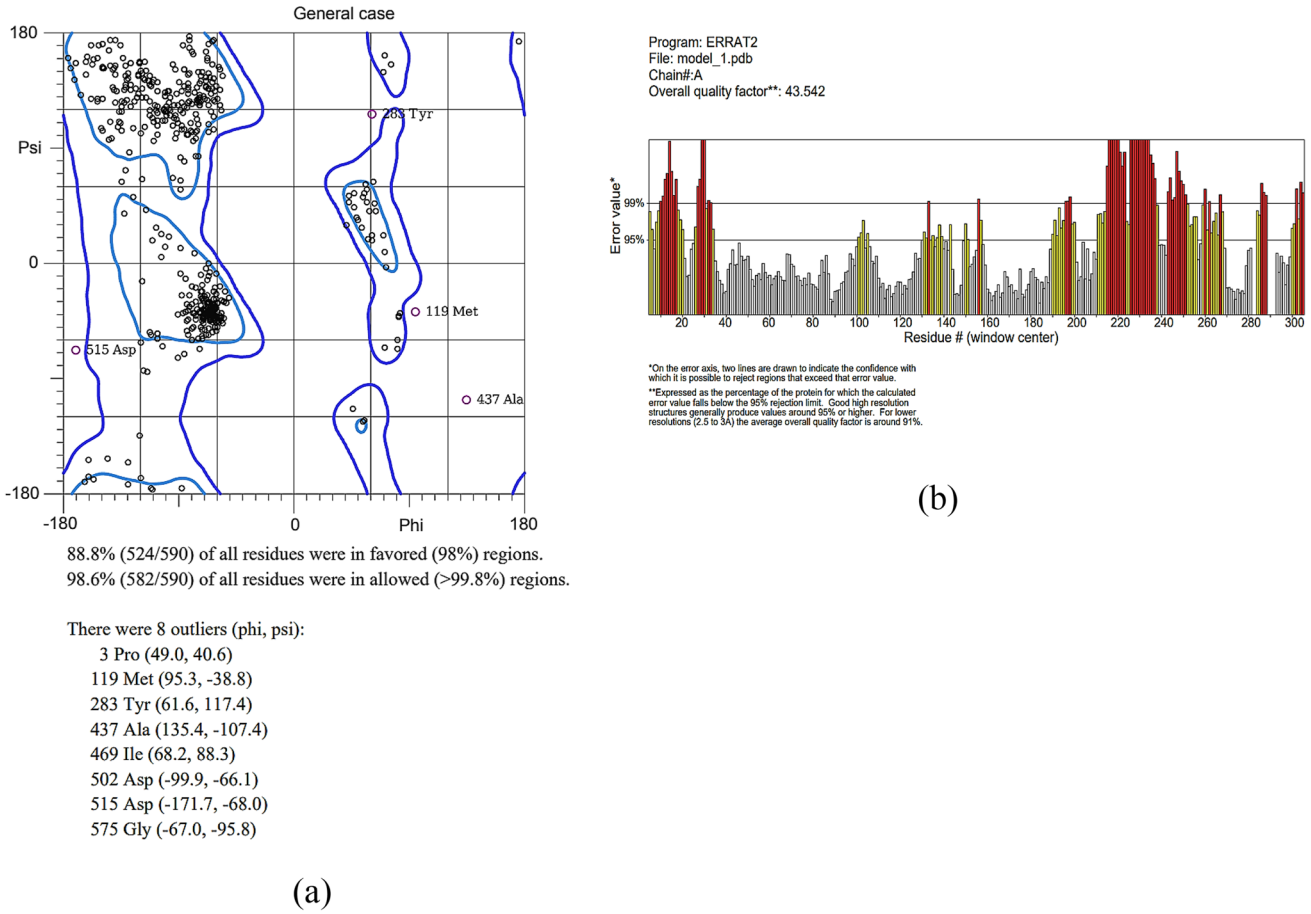


Fig. 5 **a** Ramachandran plot for GalaxyRefine model. **b** ERRAT server predicted quality assessment of protein structure

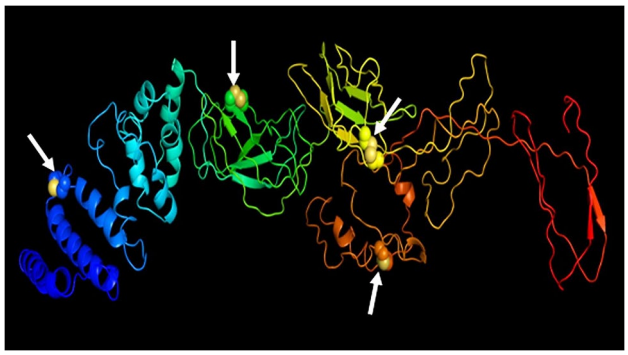


Fig. 6 Disulfide by design 2.0 predicted four (denoted by arrow) potential disulfide bond formations to improve the thermal stability of the final vaccine construct

with various TLRs (receptors) were evaluated. The top ten models generated by PatchDock for each docking were considered, among which the best-ranking models with the highest docking scores were selected for each case. The scores represented the shape and electrostatic complementarities between the receptors and ligands. The best complementarity was found in the case of human TLR-2 (PDB id: 6NIG) with a score of 18,714, surface area 3963.10, and atomic contact energy (ACE) 217.99 (Fig. 7a). The vaccine also exhibited efficient binding with TLR-5 (PDB id: 3J0A) and TLR-9 (PDB id: 3WPF) with a docking score of 18,258; surface area 2879.80 and ACE of − 666.94 and docking score 18,164; and surface area 4090.70 and ACE of 495.74, respectively. Docking with other TLRs showed weaker binding affinities as reflected by lower docking scores generated by PatchDock, 16,708 for TLR-4 (PDB id: 4G8A), and 17,800 for TLR-8 (PDB id: 4QC0). Thus, our vaccine construct was able to bind with different immune receptors with varying binding affinities. This would be highly desirable for an effective vaccine in terms of inducing a proper and heightened immunity to prevent *Leishmania* infections. Further, the FireDock web tool was employed for refinement and rescoring of the best complex (TLR-2 vaccine complex), which refined the same with a minimum global energy of − 19.01, Van Der Waals associations (− 25.74), atomic contact energy (− 0.03), and binding free energy (− 24.26). These results corroborate the fact that the vaccine peptide interacts most efficiently with human TLR-2 and, thereby, forms a reasonably stable complex that might eventuate a marked immune response against *Leishmania* parasites. In addition to this, we further investigate docking between TLR-2 and vaccine; using the Cluspro web server, top model (Fig. 7b) is selected based on 5D rotational FFT and CAPRI (critical assessment of predicted interactions) with the balanced weighted score − 1192.5 having largest cluster size of 42 members. Table 5 contains a list of 10 interacting amino acid residues from Fig. 7b that form a stable receptor-ligand complex.

Codon optimization and in silico *cloning for expression of the vaccine protein*

In an attempt to overcome the problem of codon bias, the JCat tool was utilized for codon optimization that returned the input amino acid sequence of the vaccine peptide in the form of a reverse translated cDNA sequence with an optimized CAI value of 1. This value lies within the accepted CAI value range of 0.8–1.0 [28], ensuring a high rate of expression of the gene in the host organism, *E. coli* K12. The higher the value of CAI, the better will be the expression rate of the foreign gene. In addition, the GC content of the improved sequence was found to be 51.85%, indicating a good codon optimization. For better gene expression, 35–70% GC content is required [63]. The restriction sites for the enzymes *XhoI* and *NdeI* were created at the 5′ and 3′ ends of the sequence, followed by the insertion of the modified sequence into the pET28 a(+) vector. The cloned insert, represented in red color, lies in between the specified restriction sites in the vector. A stretch of six histidine residues is also located at both ends of the cloned sequence to facilitate the purification process after the production of the recombinant vaccine protein [6, 31]. The size of the final plasmid vector construct with the inserted gene fragment was about 7 kb (Fig. 8). The mfold web server predicted a total number

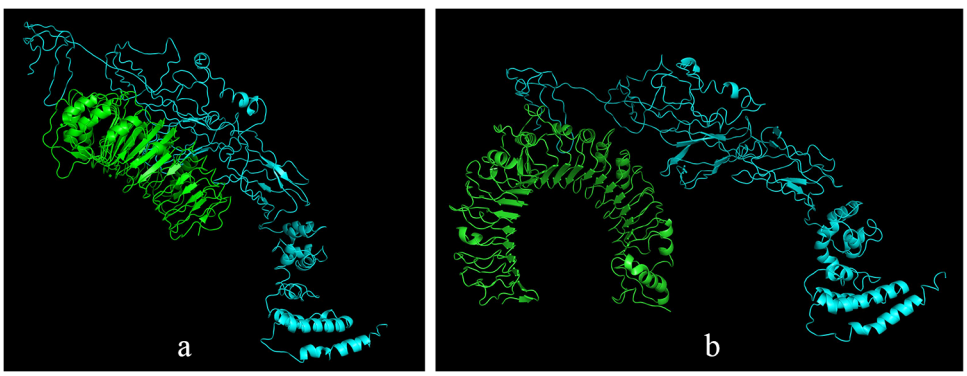


Fig. 7 Molecular docking interaction analysis. **a** PatchDock predicted model of proposed vaccine(cyan)-TLR-2 (green). **b** ClusPro generated TLR-2 (green)-proposed vaccine (cyan) complex

Table 5 PyMOL generated ten interacting amino acid residues of receptor and ligand from Fig. 7b, and the number in the () indicates the respective position of amino acids in receptor and ligand

Serial no	Interacting amino acid residue in TLR-2 (receptor)	Interacting amino acid residue in the proposed vaccine (ligand)
1	Glutamate (383)	Tyrosine (436)
2	Lysine (457)	Glutamate (258)
3	Lysine (413)	Tyrosine (436)
4	Serine (496)	Tyrosine (337)
5	Asparagine (231)	Proline (431)
6	Aspartic acid (235)	Glutamine (432)
7	Aspartic acid (223)	Threonine (398)
8	Histidine (580)	Threonine (330)
9	Arginine (302)	Lysine (551)
10	Lysine (260)	Tyrosine (400)

of fifty folding conformations of the mRNA, among which the top structure was chosen due to its lowest energy score ($\Delta G = -492.50$). This particular folded conformation was predicted to contain a single external loop and four stacks with minimum free energies indicating the stability of the conformation. The loop was formed with 21 single-stranded bases and two closing helices. The energy dot plot calculated the optimal energy (δG) to be -494.4 kcal/mol. The different colored dots in the field refer to the different energy grades of a particular base pair present in the mRNA considering the δG calculated by the server [64]. According to the plot, found the free energies of most of the base pairs are within the $-487.5 < \delta G <= -485.8$ kcal/mol range (supplementary image M3). Conclusively, a thermodynamically favored and energetically stable secondary structure of the mRNA was predicted (supplementary image M4). All the folding constraints were set at their default values, and the RNA was considered to be linear in nature.

Immune simulation

In silico immune simulation using C-immsim web server predicted antigenic count reaching up to 6.9×10^5/ml within 2 days of an initial dose of injection, and antigen level dropped to 0 on the fourth day onwards. This increment of antigen count is correlated with a remarkable increase in the concentration of both IgM + IgG level and IgM level alone during the early infection period and reaching peak level approximately within 12 days of the infection cycle on an arbitrary scale. Other subtypes of IgG1 + IgG2 level and

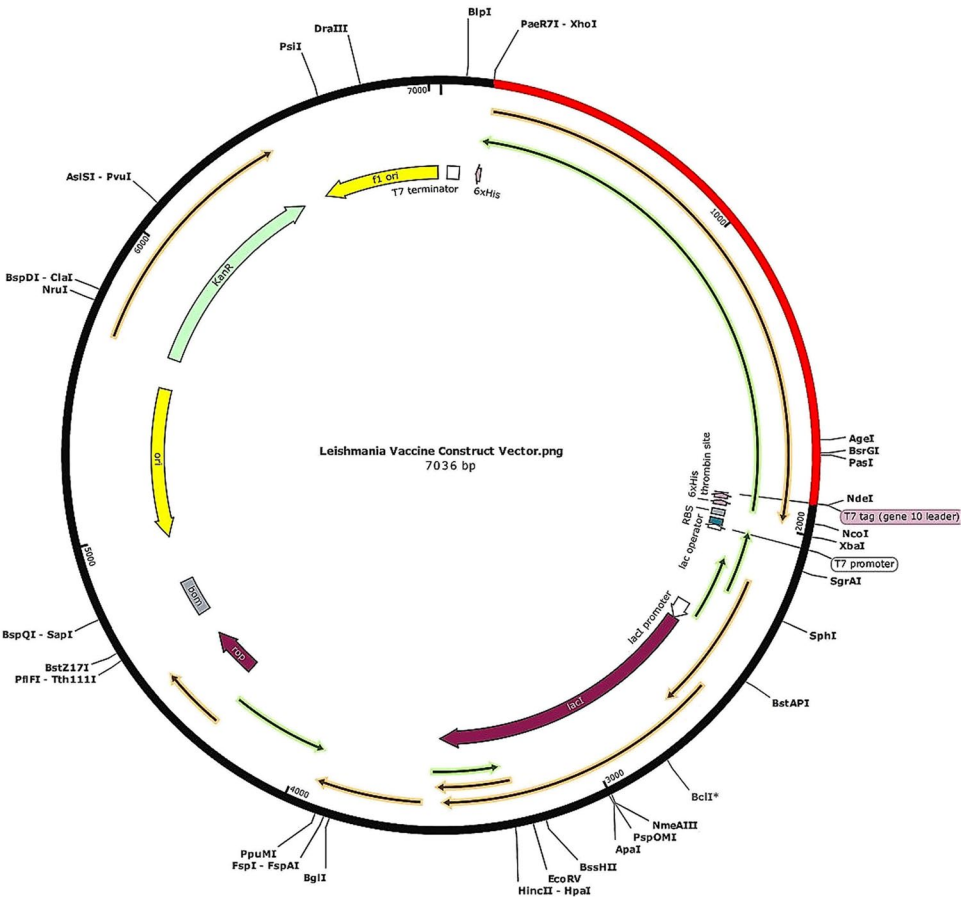


Fig. 8 *Leishmania* vaccine expression system using pET28 a(+) vector, red region indicates inserted nucleic acid sequence of vaccine flanked by NdeI and XhoI restriction sites

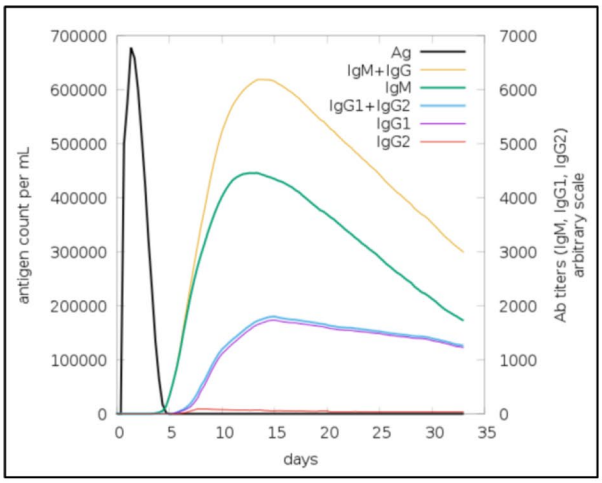
IgG1 increase markedly as a result of secondary immune response (Fig. 9a). Cytokine profiles show inflammatory mediators like INF-γ reaching level up to 4×10^6 ng/ml on the 12th day, while other cytokine levels remain quite low (Fig. 9b). Cell-mediated immunological responses suggested by the presence of active and resting cytotoxic T lymphocytes increase after 2–3 days after initial injection, and the presence of proliferative memory helper T cell was also prominent (Fig. 9c and d). The total B cell population also increases within 2–3 days, along with the generation of memory B cell after 1 week of administration of the primary dose (Fig. 9e).

Interpretation of molecular dynamic simulation

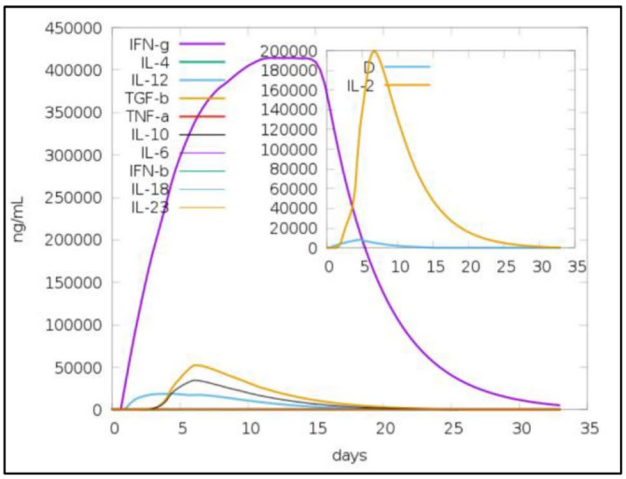
The best docking score achieved by the TLR2-vaccine complex was further analyzed by NMA-assisted molecular dynamic simulation. The deformability of the main chain is represented by high hinges (Fig. 10a), which means high deformability. B factor (Fig. 10b) was calculated by NMA, which signifies protein flexibility based on atomic displacement parameters [73]. The eigenvalue (Fig. 10c) delineates about required energy to deform the structure. TLR-2 vaccine complex shows an eigenvalue of about 7.778×10^{-7}. Variance (Fig. 10d) is inversely related to the eigenvalue. The covariance matrix (Fig. 10e) illustrates whether pairs of residues are correlated (red), uncorrelated (white), or anti-correlated (blue). The elastic network model (Fig. 10f) computes springs between corresponding atom pairs, dots in the graph represented by color gradient indicating rigidity or flexibility of spring, and the darker color reflects stiffer spring. Thus, results of molecular dynamic simulation suggested that our proposed peptide vaccine is stable.

Discussion

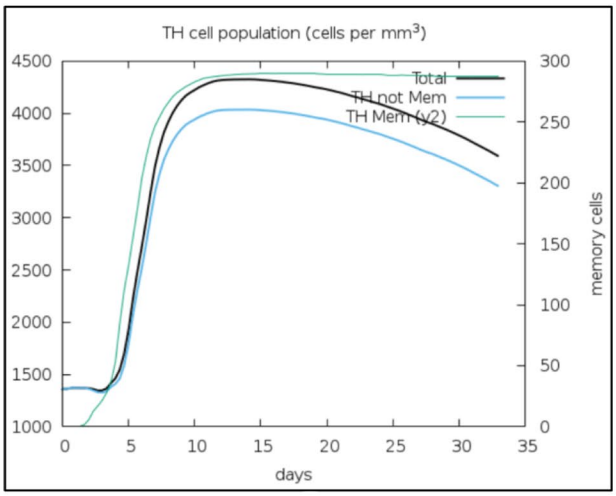
Leishmaniasis is considered one of the most neglected tropical diseases that primarily affect people living in poverty-stricken areas of developing countries, including India [2]. According to WHO reports, India is endemic for both forms of the disease, CL and VL. CL caused by *L. tropica* and *L. major* occurs in the northwestern states of India, Punjab, and Rajasthan being the foci (https://www.who.int/leishmaniasis/burden/Leishmaniasis_India/en/). Furthermore, with the increasing occurrence of the human immunodeficiency virus (HIV) epidemic across the globe, leishmaniasis has emerged as a resurfacing opportunistic pathogen in AIDS patients making the situation even more dreadful [22, 38]. In the current study, we aimed to design a novel polyvalent general vaccine for leishmaniasis that might provide heterologous protection from common pathogenic parasite species. We selected twelve *Leishmania*-specific proteins encompassing five pathogenic strains of the parasite that cause VL and CL infections in humans. These proteins had already been appraised for their role in virulence and immunogenicity and their potential as vaccine candidates in various immunological studies[1, 8–10, 75]. Therefore, a vaccine constructs designed in silico by incorporating such well-characterized and highly conserved immunogenic proteins from different pathogenic species of *Leishmania* might provide cross-immunity against all forms of human leishmaniasis (mainly VL and CL) and, hence, could be used for a general vaccination program to prevent *Leishmania* infections in humans. The selected proteins were primarily screened for the presence of potential CTL and HTL epitopes. Detection of T cell epitopes is important since T cells can only recognize antigens when processed either by endogenous or exogenous pathway, complexed with MHC class I or MHC class II molecules and are subsequently presented to the T cell receptors by the antigen-presenting cells (APCs). MHC-I is specific to CD8 + CTLs, while MHC-II binds specifically to CD4 + HTL receptors. Identification for CTL epitopes was performed stringently by employing two independent servers simultaneously. Only those epitopes predicted by both servers with significantly higher MHC-binding affinities were finally included in the vaccine peptide. Identified HTL epitopes were further evaluated for their cytokine-inducing abilities by exploiting three web servers. The ones that could stimulate the production of a wide variety of cytokines, namely, IFN-γ, IL-4, and IL-10, were selected for designing the chimeric peptide vaccine to ensure that it would instigate the production of all of these parasite-specific cytokines in the host. This is of utmost importance since Kedzierski and colleagues had proposed that the secretion of IL-10 is as important as the secretion of IFN-γ to determine if a vaccine can elicit a protective immune response and that proper immunity is not induced if IL-10 levels are not proportionately elevated [8, 75]. The exclusive CTL and HTL epitopes were next fused by using AAY and GPGPG linkers to construct the chimeric peptide. AAY and GPGPG linkers have previously been reported to be useful for multi-epitope vaccine design as they allow minimal junctional immunogenicity and facilitate the processing and presentation of selected epitopes by MHC-II [6, 28, 71]. The adjuvant alpha chain of human IL-12 was linked to the N terminal of the resultant peptide via the EAAK linker. IL-12 was chosen as an adjuvant since it is one of the most effective adjuvants for the *Leishmania* vaccine in animal models, as documented in previous immunogenic studies [3, 37, 71]. Since the vaccine is meant for human administration, incorporating human IL-12 as a natural adjuvant instead of any synthetic TLR agonist is expected to boost immunity without causing adverse side effects. The role of IL-12 as a general adjuvant for vaccines is also underscored due to its potency in enhancing both protein and polysaccharide vaccine antibody responses and inducing adequate local


Ag
IgM+IgG
IgM
IgG1+IgG2
IgG1
IgG2
antigen count per mL
Ab titers (IgM, IgG1, IgG2) arbitrary scale
days

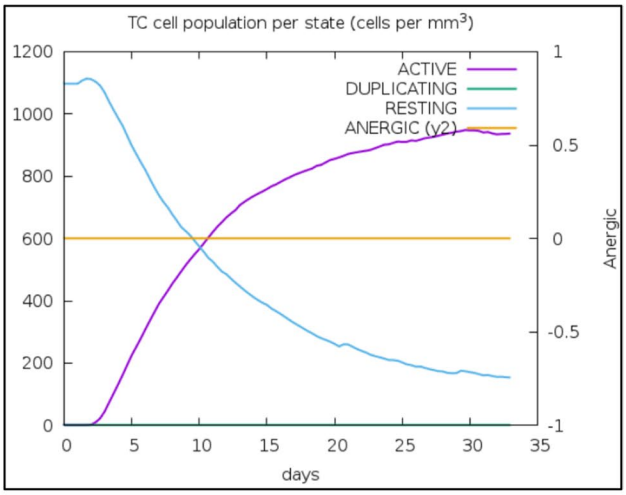
(a)


IFN-g
IL-4
IL-12
TGF-b
TNF-a
IL-10
IL-6
IFN-b
IL-18
IL-23
D
IL-2
ng/mL
days

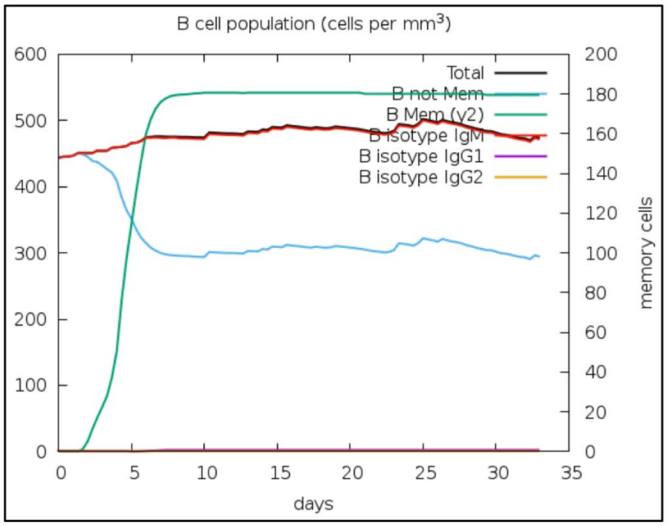
(b)


TH cell population (cells per mm3)
Total
TH not Mem
TH Mem (y2)
memory cells
days

(c)


TC cell population per state (cells per mm3)
ACTIVE
DUPLICATING
RESTING
ANERGIC (y2)
Anergic
days

(d)


B cell population (cells per mm3)
Total
B not Mem
B Mem (y2)
B isotype IgM
B isotype IgG1
B isotype IgG2
memory cells
days

(e)

◂**Fig. 9** C-immsim server-based immune simulation. **a** Total antigen and respective antibody generation, **b** cytokine production profile, **c** and **d** generation of helper T cell and total (active and resting) cytotoxic T lymphocyte count, **e** B cell response after vaccine administration

inflammation that permits IgG exudation leading to a heightened immune response [72]. EAAK linker was added to achieve a higher rate of expression and to promote the biological function of the fusion peptide by restricting the interaction among the vaccine domains [76, 77]. Immunoinformatic analysis showed that our vaccine construct has many linear B cell epitopes that are highly desirable for a vaccine made for immunoprophylactic use. Again, we used two different tools for more accurate predictions. Both servers predicted sufficient B cell epitopes in the vaccine sequence. The designed vaccine was predicted to be non-allergen, non-toxic, and sufficiently immunogenic to trigger a proper immune reaction. Subsequent physicochemical analysis revealed the vaccine peptide to be slightly acidic (pI 5.98) with a considerably higher aliphatic index (78.34), suggesting its thermostable nature. A negative GRAVY value (−0.14) indicates the peptide is hydrophilic, which further contributes to its potency as a vaccine. The secondary structural elements of the vaccine were determined by the PSIPRED v 4.0 server, which is one of the most accurate and widely used computational methods for protein secondary structure prediction. Moreover, the 3D structure for the designed peptide was also predicted using the I-TASSER server that provided detailed information about the coordinates of the important residues of the protein, which is of paramount importance in order to study the dynamics, bioactivity, and interaction of the vaccine with other ligands. The initial tertiary structure was refined and validated by utilizing protein structure validation tools to locate and rectify potential errors in the side chain or backbone of the predicted model. Ramachandran plot analysis showed that more than 90% of residues of the refined

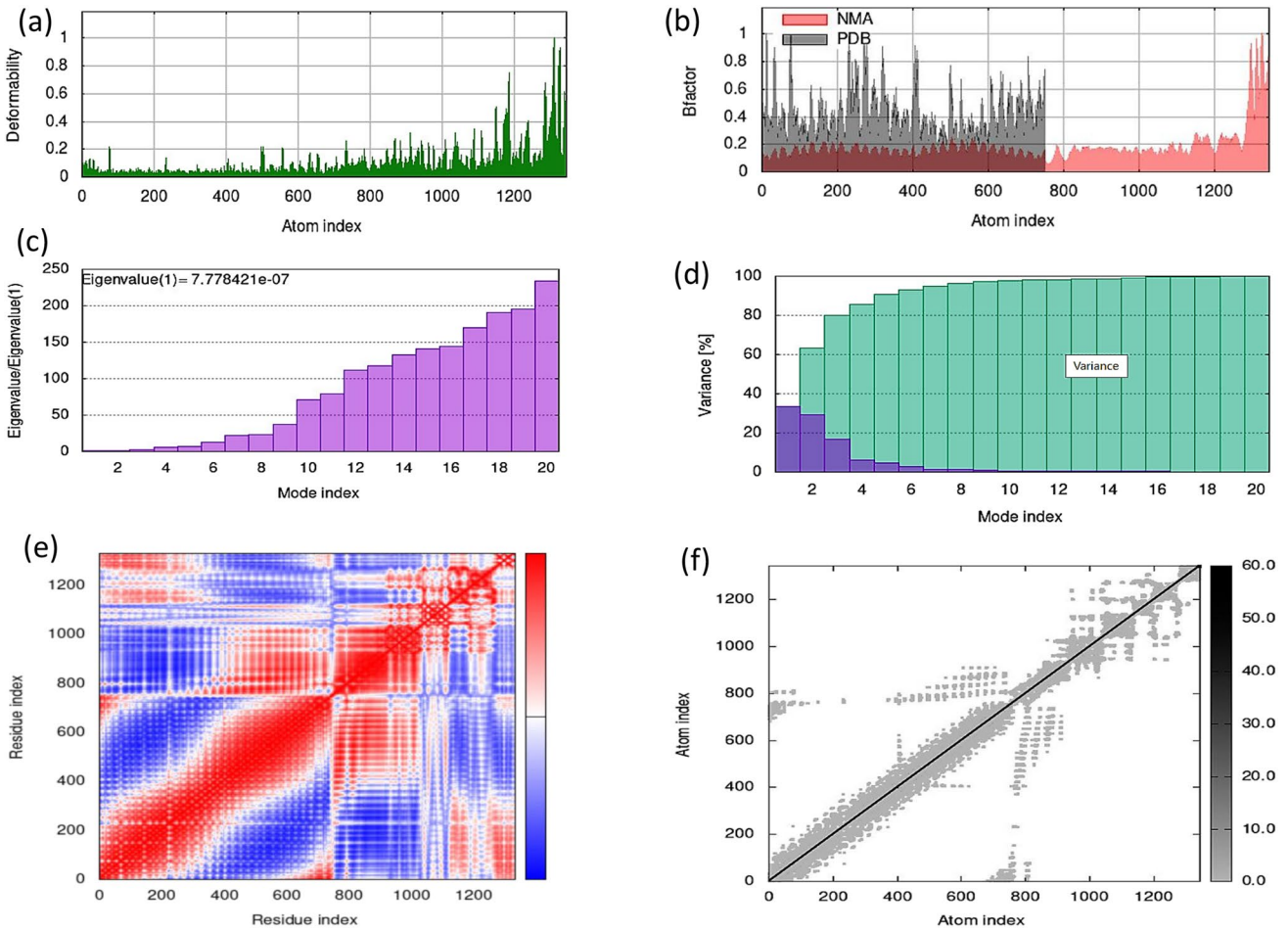


Fig. 10 Molecular dynamic simulation. **a** The main chain deformability indicated by hinges, **b** NMA generated B factor showing protein stability, **c** eigenvalue to deform the structure of docking complex, **d** individual (purple) % of the variance and cumulative (green) of the structure, **e** covariance matrix correlated (red), uncorrelated (white) or anti-correlated (blue) between pairs of atoms, **f** elastic network model showing connected springs between pairs of the atom, darker grey indicated rigid springs and vice versa

model fall within the allowed region with a small percentage of outliers, while the ERRAT server showed a quality factor of 43.542 which are all implicative of satisfactory model quality. The validated 3D model was found to have an adequate number of conformational B cell epitopes by the ElliPro server, implicating that it might generate a desirable B cell response and stimulate the production of specific antibodies in the host. B cell activation is also crucial in immunologic memory and responsible for long-lasting immunity in vaccinated individuals. The interaction of the vaccine with the immune receptors (TLRs) was also evaluated by performing docking analysis. The vaccine peptide was found to have the highest affinity for TLR-2, followed by TLR-5, although it was able to bind with other TLRs to a lesser extent. The interaction with TLRs is essential for eliciting protective immunity since previous studies involving animal models documented that different species of *Leishmania* or their antigenic components interact with various TLRs leading to an intense immune response in challenged hosts. To meet the requirements for satisfactory expression of the recombinant vaccine protein in the *E. coli* (K12) host system, codon optimization was performed by applying the JCat tool that adjusted the CAI and the GC contents of the sequence, making it suitable for expression in *E. coli*. In silico cloning was also performed using the plasmid-based expression vector pET28a(+) to show the feasibility of the sequence for cloning and overexpression to achieve enhanced production of recombinant vaccine protein. Prediction of the secondary structure of the mRNA encoded by the chimeric gene sequence was made to show the stability of the folded conformation of the mRNA that will influence the efficient translation process in the bacterial host. Stabilizing mutations were created by adding novel disulfide linkages in the vaccine protein since it would contribute to the strength of the native protein, making it amenable for additional biochemical, immunological, and biotechnological explorations.

Conclusion

In the present work, we have exploited a reverse vaccinology approach for in silico designing of a polyvalent multi-subunit peptide vaccine against human leishmaniasis (both visceral and cutaneous) that might be able to confer heterologous protection from common *Leishmania* parasites infecting humans and, thus, can be considered to be broad-spectrum in nature. The same could be used for a general vaccination program to control and eradicate the disease as a complementary strategy along with the currently available chemotherapeutic agents. In addition, we have used IL-12 as a natural adjuvant instead of any non-humanized synthetic peptide. Using IL-12 as the adjuvant will increase the immunogenicity of the vaccine without causing any adverse side effects in the host body. Provided that the vaccine peptide is composed of a variety of highly conserved and potentially immunogenic peptides obtained from five species of pathogenic *Leishmania*, it is helpful to elicit a cross-immunity in vaccinated individuals, thus also having therapeutic and prophylactic advantages. Various stringent immunoinformatic analyses utilizing several computational tools have found the proposed vaccine immunogenic, stable, and reproducible as a recombinant protein and safe for human trials. Nonetheless, experimental validation remains to be performed to guarantee proper elicitation of protective and long-lasting immunity and rule out toxicity.

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Author contribution Mainak Bhattacharjee and Monojit Banerjee conceptualized and designed the study and also made specific methodology, performing software-related analysis, and writing the original draft. Validation and data curating were performed by Monojit Banerjee. Arun Mukherjee performed the interpretation and validation of data, writing, and editing of the final manuscript, as well as supervision of work.

Data availability Data can be available on request.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval Not applicable to this research work.

Competing interest The authors declare no competing interests.

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