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Exploring malaria parasite surface proteins to devise highly immunogenic multi-epitope subunit vaccine for *Plasmodium falciparum*

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ABSTRACT

Background: Malaria has remained a major health concern for decades among people living in tropical and sub-tropical countries. *Plasmodium falciparum* is one of the critical species that cause severe malaria and is responsible for major mortality. Moreover, the parasite has generated resistance against all WHO recommended drugs and therapies. Therefore, there is an urgent need for preventive measures in the form of reliable vaccines to achieve the target of a malaria-free world. Surface proteins are the preferable choice for subunit vaccine development because they are rapidly detected and engaged by host immune cells and vaccination-induced antibodies. Additionally, abundant surface or membrane proteins may contribute to the opsonization of pathogens by vaccine-induced antibodies.

Results: In our study, we have listed all those surface proteins from the literature that could be functionally important and essential for infection and immune evasion of the malaria parasite. Eight *Plasmodium* surface and membrane proteins from the pre-erythrocyte and erythrocyte stages were shortlisted. Thirty-seven epitopes (B-cell, CTL, and HTL epitopes) from these proteins were predicted using immune-informatic tools and joined with suitable peptide linkers to design a vaccine construct. A TLR-4 agonist peptide adjuvant was added at the N-terminus of the multi-epitope series, followed by the PADRE sequence and EAAAK linker. The TLR-4 receptor was docked with the construct's anticipated model structure. The complex of vaccine and TLR-4, with the lowest energy −1514, was found to be stable under simulated physiological settings.

Conclusion: This study has provided a novel multi-epitope construct that may be exploited further for the development of an efficient vaccine for malaria.

1. Background

Over the centuries, malaria has been one of the life-threatening diseases around the world. The most affected parts are sub-Saharan and tropical countries.[1] Around 250 million people report positive for malaria every year (247 million in 2021 as per WHO malaria report 2022) (https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022), and the *Plasmodium falciparum*, the deadliest among all the malaria species, is responsible for significant mortality (more than a lakh per year) caused by malaria.[2] It is transmitted by the female Anopheles mosquito, commonly habitat in tropical and sub-tropical regions, and poor sanitation is the major cause of the spread of these mosquitoes in such developing countries. After the discovery of *Plasmodium* as a causative agent of malaria by Alphonse Laveran and Ronald Ross, efforts are being made to understand the biology and pathogenicity of the parasite to overcome the disease.[3] Several preventive measures, including proper sanitation and vector control, play significant roles, along with drug therapies and vaccination programs for disease elimination. A massive number of drugs are being used to target the parasite in the human body. Simultaneously, the parasite has evolved with drug resistance against the drugs used to treat malaria worldwide.[4] *Plasmodium* has acquired resis-

Abbreviations: TLR, Toll-like Receptor; CSP, Circumsporozoite Protein; CTL, Cytotoxic T-Lymphocyte; HTL, Helper T-Lymphocyte; MSP, Merozoite Surface Protein; KAHRP, Knob-associated Histidine Rich Protein; ANN, Artificial Neural Network; MHC, Major Histocompatibility Complex; IEDB, Immune Epitope Database; TAP, Transporter Associated with Antigen Protein; SIB, Swiss Institute of Bioinformatics; GRAVY, Grand Average of hydropathicity index; RMSD, Root Mean Square Deviation; RMSF, Root Mean Square Fluctuation; SASA, Solvent Accessible Surface Area; Rg, Radius of Gyration; DC, Dendritic cells.

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tance to almost all anti-malarial drugs, starting from chloroquine to the most recent artemisinin.[5] Artemisinin and its derivative are currently being used in combination with other anti-malarial drugs in almost every malaria-endemic country. Therefore, it is the need of the hour to strengthen the preventive measures in the absence of curative medication.

The vaccination program has proven a reliable strategy to eradicate a disease worldwide. Recently, the RTS,S (Mosquirix) vaccine has been approved by WHO for field trials in malaria-endemic countries. The vaccine is based on virus-like particle technology for delivery of the target antigen, Circumsporozoite protein, in this case.[6] Despite multiple limitations of the RTS,S, the vaccine has managed to provide protection against malaria in children for a shorter duration. However, new and effective vaccine candidates for complete protection from malaria are yet to be identified.[7] In the whole life cycle of the parasite, *Plasmodium* goes through a variety of cells and organs in the human body. *Plasmodium* has a cassette of proteins exposed on the surface at different development stages of the parasite to make an interaction with the host cell.[8] Surface proteins are not only involved in parasite entry into the host cell but equally involved in transportation (nutrition uptake and excretion), signal transduction to neighboring parasites, sequestration, and rosetting (clumping of infected RBCs with Uninfected RBCs).[8–10] Surface proteins are sometimes highly glycosylated to perform the adhesion function to the epithelium wall of blood vessels. This huge number of glycosylated surface proteins can be targeted as vaccine candidates as they can be easily recognized by host-immune cells when an actual infection occurs. A protein present in abundance makes its repetitive exposure to host immune cells and could generate a stronger immune response than protein with low expression and exposure. A high abundance of protein not only boosts immune response but is also recognized by monocytes or phagocytic cells through the process of opsonization.[11–12] Blocking these surface-exposed proteins may interfere with the invasion as well as the cytoadherence function of the parasite, resulting in the prevention of infection and immune evasion.[13] Several proteins have been present on the surface of sporozoite, merozoite, and parasitized erythrocytes, which are reported as malaria vaccine targets. For a parasite like *Plasmodium*, which has multiple life stages, a good strategy is to opt for multiple proteins targeting all stages. The purpose behind this strategy is to block the entry of the parasite at multiple stages of development. Such as the parasite that skipped the immune clearance at the pre-erythrocyte will be blocked at the erythrocyte and later at the intra-erythrocyte development cycle. In the present study, we are targeting multiple proteins from pre-erythrocytes, erythrocytes stages, and also proteins responsible for cytoadhesion. Sequestration and rosetting are the key mechanisms evolved by the parasite to hide from the host immune system.[10,14] Generated antibody response targeting these cytoadherence proteins prevents parasite hiding as well as enhances immune clearance of infected cells. In *Plasmodium falciparum*, antigenic variations in surface protein expressed during pre-erythrocyte and erythrocyte stages are very well reported. A significant antigenic variation has been reported in Erythrocyte membrane protein (EMP), which is encoded by 60 genes known as var genes, RIFIN (rif genes), and STEVOR (stevor genes).[15] In a study, the researchers have compared PfCSP protein from non-3D7 field strains with laboratory 3D7 strain and found genetic variations in CSP proteins. However, these variations did not affect its binding with HLA and showed similar vaccine efficacy.[16] Therefore, in our study, during protein selection, we excluded the protein encoded by var genes and reported antigenic variance genes, and included the proteins involved in cytoadhesion, but no antigenic variation has been reported yet for these proteins. Finally, we applied an immunoinformatic approach to design a vaccine against *Plasmodium falciparum* targeting the multi-protein of pre-erythrocyte and erythrocyte stages. This in-silico vaccine candidate is designed by joining B-cell, CTL, and HTL epitopes using suitable linkers. Physiochemical properties, antigenicity, and allergenicity were determined, and the construct was modeled. Interaction and binding between TLR-4 and the vaccine model were checked by molecular docking and dynamics simulation. Overall, the results are encouraging and will pave the way for in-vitro and in-vivo testing of the selected vaccine candidate. A graphical summary of work flow is represented in Fig. 1.

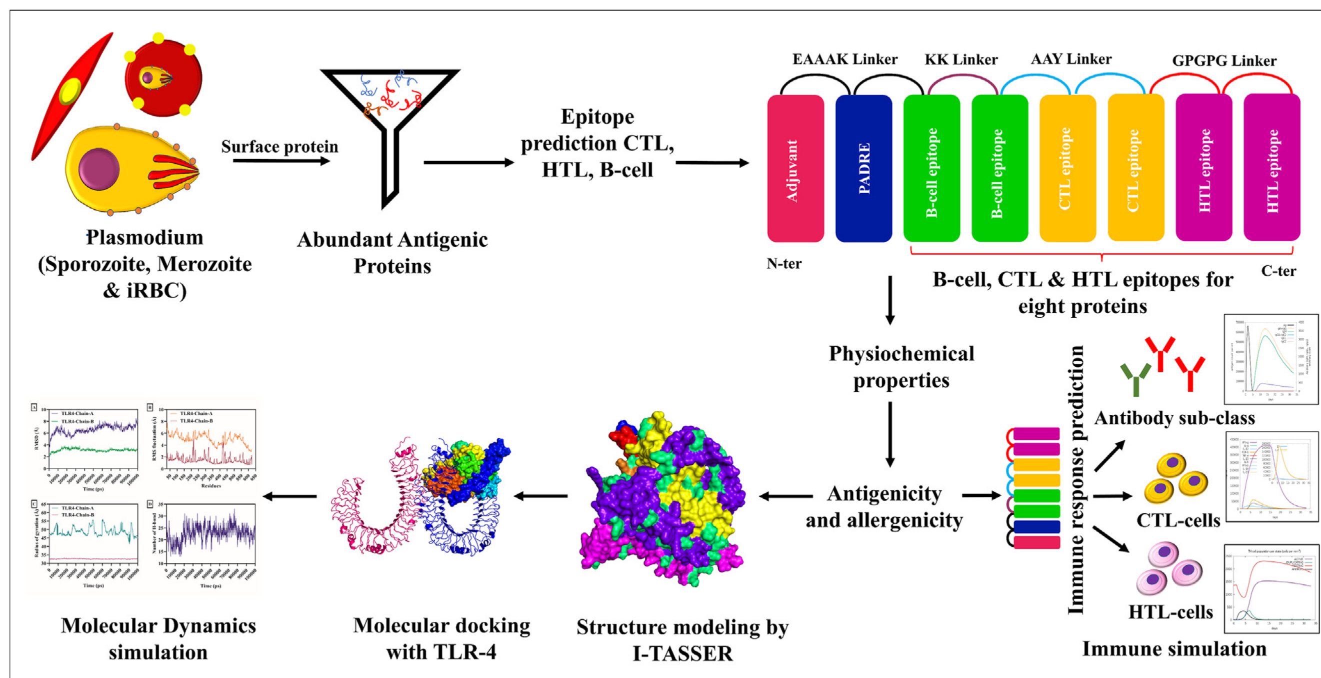

Fig. 1. Schematic representation of methodology used for designing immunogenic multi-epitope subunit vaccine for malaria.

2. Methods

2.1. Protein selection

For the protein selection, a literature survey was done to collect the surface protein of *Plasmodium falciparum*.[17–25] A list of surface proteins (Supplementary Table 1) was made, including pre-erythrocyte and erythrocyte stage surface proteins. Gametocytes (sexual stage) surface proteins were excluded from this study. Protein sequences (FASTA format) were retrieved from PlasmoDB (https://plasmodb.org/plasmo/app/-fasta-tool/gene).[26] For a potent vaccine candidate, the proteins should be antigenic enough to induce an immunogenic response and abundantly present in the pathogen to hit maximum immune memory cells when encountered in the host body. For this purpose, the immunogenicity of the proteins was checked using VaxiJen *v*2.0 ((https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html).[27] Further proteins were sorted on the basis of immunogenicity and abundance. The PAXdb[4.] [2] (https://pax-db.org/dataset/5833/943661154) database was used to check the abundance of each protein in the pathogen. PAXdb is the database for protein abundance generated from experimental as well as computational data.[28] Abundance is given in part per million (ppm) for each protein of an organism.

2.2. B-cell epitope prediction

B-cell epitopes are essential to generate a humoral immune response against malaria. ABCpred (https://crdd.osdd.net/raghava/abcpred/)[29] webserver was used to predict B-cell epitopes from target proteins. This server predicts linear and continuous B-cell epitopes, which employ Artificial Neural Network (ANN) to predict the immunogenic epitopes. The sequence of selected proteins was subjected to predict 10 amino acid-long B-cell epitopes. Top-scored and immunogenic epitopes were chosen for further study.

2.3. Cytotoxic T-cell epitope prediction

Activation of cytotoxic T-cell lineage is crucial for parasite or pathogen clearance. To activate cytotoxic T-cells, an antigen or epitope must be presented by MHC class I molecules present on all nucleated cells. For this, CTL epitopes were predicted using NetCTL 1.2 webserver (https://www.cbs.dtu.dk/services/NetCTL/).[30] An updated version of Net CTL 1.2 was used, which is trained over known 886 MHC I ligands. This server uses a method that is an integration of three individual approaches, including MHC I bind affinity prediction, proteasomal cleavage by NetChop neural network, and TAP transport efficiency based on weight matrix. The server can predict 12 supertypes of MHC class I molecules.[30] We have predicted A2, A3, and B7 supertypes in this study, which cover more than 86 % of the world's population and are relevant to African countries.[31] All predicted epitopes were subjected to an IEDB server (http://tools.iedb.org/immunogenicity/[32] to check the immunogenicity of each epitope. Top-scored immunogenic epitopes were taken for the next step.

2.4. Helper T-cell epitope prediction

Helper T-cell epitopes were predicted using the IEDB server for all eight proteins (https://tools.iedb.org/mhcii/).[33] The epitope size was kept to 15 amino acids as recommended. The immunogenicity of the predicted epitopes was calculated by the IEDB immunogenicity tool (https://tools.iedb.org/CD4episcore/)[34] by keeping the threshold value at 80. The consensus method was used, and the percentile rank threshold was kept at 1. The epitopes with a percentile rank up to 1 and IC_{50} less than 50 nM were subjected to check the Interferon-gamma inducing ability of epitopes using the IFNepitope online tool (https://crdd.osdd.net/raghava/ifnepitope/).[35]

2.5. Vaccine construct design

The vaccine construct was designed by joining the CTL, HTL, and B-cell epitopes using linkers. A synthetic peptide adjuvant (RS-09) has been reported as a TLR4 agonist is taken to improve the immunogenicity of the vaccine construct.[36] A five amino acid long linker EAAAK keeps the adjuvant at the N-terminal of the vaccine construct and a universal Pan HLA-DR binding epitope (PADRE) next to it to induce helper T-cell response.[37] Apart from the EAAAK linker, B-cell, HTL, and CTL epitopes were joined in six different orders using KK, GPGPG, and AAY linkers, respectively (Supplementary data).[38–40] The linkers allow these epitopes to function as independent epitopes more efficiently than adjacent epitopes without linkers.

2.6. Physiochemical properties analysis of vaccine constructs

The physiochemical properties of the designed vaccine construct were computed using the Expasy ProtParam server (https://web.expasy.org/protparam/).[41] The Swiss Institute of Bioinformatics (SIB) collaborated with Expasy to run this server. The physiochemical study included the analysis of molecular weight, theoretical pI, in-vivo half-life, instability index, Aliphatic Index, and grand average of hydropathicity (GRAVY) index. Vaccine candidates with greater half-life can persist long in the host body to activate maximum immune cells and can generate an elevated immune response. In the ProtParam server, the half-life of a protein is estimated by the N-end rule, which determines half-life based on the amino acid composition at the N-terminal of the protein. In contrast, the *in-vitro* stability of the protein is calculated by the instability index. A protein with an instability index of less than 40 is considered a stable protein in solution. The thermostability of the protein is determined by an aliphatic index, which calculates the total relative volume of aliphatic side chains in the protein.

2.7. Antigenicity and allergenicity prediction

Antigenicity and allergenicity of the designed vaccine construct were analyzed using VaxiJen v2.0 (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html)[27] and AllerTop (https://www.ddg-pharmfac.net/AllerTOP/)[42] servers, respectively. Both these servers are based on an alignment-independent algorithm and work on the basis of physiochemical properties and chemical composition of amino acid sequences.[43] .

2.8. Tertiary structure prediction, refinement, and validation

The I-TASSER webserver (https://zhanggroup.org/I-TASSER)[44] was used to predict the tertiary structure of the designed vaccine construct. The server uses the LOMETS threading program, which makes use of the PDB library of known protein structures to generate templates for alignment with the query sequence. The accuracy of the predicted model is determined by the Confidence score (C-score), estimated TM score, and estimated RMSD.

The modeled structure was further refined using the GalaxyRefine webserver (https://galaxy.seoklab.org/refine).[45] The server employs the *ab-initio* method to model the missing loops and terminal ends. After refinement, the refined model was subjected to SWISS MODEL's structure assessment tool (https://swissmodel.expasy.org/assess)[46] to assure the quality of the predicted model. The Ramachandran plot, MolProbity, QMEAN, and QMEANDisCo values were utilized to assess the modeled structure's quality.[47] .

2.9. Continuous and discontinuous antibody epitope prediction

Continuous (linear) and discontinuous (confirmational) epitopes were predicted next after the 3D model of the vaccine construct. A

Refined model of the vaccine construct was given as input in IEDB ElliPro https://tools.iedb.org/ellipro/ online tool, keeping all parameters as default. The threshold for epitope score was 0.5.[48] .

2.10. Molecular docking with Toll-Like receptor

Molecular interaction between the designed vaccine candidate and human TLR4 was determined by the ClusPro 2.0 web server (https://cluspro.bu.edu/login.php).[49] Thirty model complexes were generated, out of which the model complex with the lowest energy and maximum binding was selected for further study.

2.11. Molecular dynamics simulation of immune receptor and vaccine construct

Molecular dynamics simulation was used to study the structure and function of the immune receptor and the vaccine construct. To understand the stability as well as the interaction between the vaccine construct and immune receptor (TLR4), molecular dynamics simulation was performed using Desmond, system builder panel (Schrödinger Inc.). The OPLS3 force field, which has the best potential for liquid simulations, was utilized for protein–protein simulation. OPLS3 was initially designed to simulate proteins and small molecules with drug-like properties.[50] In the molecular dynamics panel, the system was opened. Using the single point charge (SPC) water model, the original structure of vaccine-TLR4 was solvated, and counter ions (Na + and Cl-) were introduced to neutralize the system. To enable gentle relaxation, the system was gradually heated in NVT conditions from 0 to 300 K.[51] To examine the conformational dynamics of the vaccine construct and the TLR4, the whole system MD production run was started in NPT conditions (T = 300 K and P = 1 atm). By calculating the radius of gyration (Rg), root mean square deviation (RMSD), root mean square fluctuation (RMSF), solvent accessible surface area (SASA), and hydrogen bonding, the structural convergence properties were examined.[52–54] .

2.12. Immune simulation and antibody class prediction

Finally, we have carried out immunological simulation studies to determine whether or not the developed vaccine candidate can elicit a protective immune response (acquired immunity after immunization that significantly prevents infection through antibody production and cytotoxic killing of the pathogen) in the mammalian host following injection. The sequence of the vaccine construct was used as an input for the immune dynamics simulation that was carried out on the online webserver C-ImmSim (https://kraken.iac.rm.cnr.it/C-IMMSIM/).[55] The improved C-ImmSim utility maintains compatibility with the prior version of the program by substituting amino acid strings for Bit strings as the input data. There are very few vaccines that require a gap of 2, 3, or 6 months between the initial immunization and the booster dosage. As per the CDC, the next dose of vaccine is typically given after a month to ensure its effectiveness and is crucial for optimum immune response. Hence, we considered the time period of one month for this study, and after that, we evaluated the dose-dependent immunological response of our tailored vaccine.[56–57] Immunological response refers to the immune system's reaction when exposed to foreign particles such as vaccines. The vaccine contains antigen, and when administered, it mimics a specific pathogen. These antigens are recognized as foreign by the immune system, prompting an immunological response to neutralize them. The probability of eliciting an immunological response was further investigated through repeated administration of our vaccine candidate. In each scenario, one dosage of the vaccine was administered, and a simulation was executed. Finally, the immunological response produced by the potential vaccine candidate was determined in the form of the Simpson Index (D).[58–59] .

Antibody class prediction was also made by using the IgPred online tool https://webs.iiitd.edu.in/raghava/igpred/help.html by the Raghava group.[60] This server predicts antibody class-specific B-cell epitopes that help estimate the class of antibody and immunity response that is going to be generated by an epitope or vaccine. The whole vaccine construct was subjected to predict the antibody-specific B-cell epitope as an input. The server will predict epitopes for three classes of immunoglobulin: IgG, IgA, and IgE. The threshold was kept at 0.9 for all classes of antibody.

3. Results

In invasive pathogens like parasites (*Plasmodium*, *Leishmania*, and *Toxoplasma*), bacteria (Meningococci and Pneumococci),[61] and viruses, surface proteins are involved in a variety of processes and functions. One of the main functions of the surface proteins of the pathogen is host-parasite contact or invasion. These surface proteins are often glycosylated, performing adhesion and invasion activity. Surface polysaccharides and glycan moiety are T-cell-independent antigens that induce a humoral antibody response, which is a prior requirement of a malaria vaccine.[62] For T-cell mediated immune response (to help activate B-cell also), a protein carrier (T cell-dependent antigen) is usually conjugated in a protein-polysaccharide conjugate vaccine. Surface proteins expressed in eukaryotic cells are constitutively glycosylated through post-translation modification.[61–62] *Plasmodium* expresses a different set of surface proteins at its multiple development stages, which might go through this kind of modification. Glycosylation of surface protein for vaccine development can be achieved by chemically linking to glycan moiety (Synthetics GPI linker specific to *P. falciparum*) or utilizing a mammalian expression system for target protein expression.[63] Vaccines targeting the parasite's surface proteins interfere not only with the host-parasite surface interaction and invasion but also with the parasite's immune evasion mechanism and prevent severe malaria.

3.1. Retrieval of Plasmodium falciparum surface protein

In this study, we have screened proteins on the basis of their surface localization in sporozoite form as well as merozoite and parasitized erythrocytes. Once we listed surface proteins from the literature survey, we sorted proteins on the basis of antigenicity and part per million abundances checked by VaxiJen v2.0 and PAXdb, respectively. We excluded the surface protein belonging to the multigene family that shows antigenic variations as reported in the literature. In this way, we have selected eight proteins with antigenicity of more than 0.8 (Table 1). The first three proteins (Circumsporozoite protein (CSP), CSP-related protein/exported protein, and conserved *Plasmodium* protein) are expressed on the sporozoite surface during the pre-erythrocyte stage. Circumsporozoite protein, already reported as a potential vaccine candidate and key antigen in RTS,S malaria vaccine is found immunogenic and most abundant in our screening process. The immunogenicity of the other two proteins was found to be comparable to that of the CSP protein.

The next three proteins are localized on the merozoite surface and are functionally important to make an entry into the erythrocyte (RBC) to start a new development cycle. Antibodies generated against all the six proteins mentioned above work for the prevention of sporozoite and merozoite invasion to hepatocytes and erythrocytes, respectively. While remaining two proteins have their role in the cytoadhesion (Knob formation on RBC membrane) of infected RBC to the epithelial cells present on the wall of blood vessels, kidney, brain, and other visceral organs.[64] The proteins getting expressed during the blood stage and exported to the RBC membrane and cytoplasm are key players in knob formation and are involved in the cytoadhesion of iRBCs through receptors like I-CAM present on the epithelial cells.[65] This allows parasites to hide from macrophages and prevents splenic

Table 1
List of selected proteins for vaccine construct:

S. No.	Protein name	Accession ID	Stage	Immunogenicity	Abundance
1	Circumsporozoite protein (CSP)	PFC0210c	Sporozoite stage	0.965	19170 ppm
2	CSP-related protein/exported protein 1	PF11_0224	Pre-Erythrocyte and Erythrocyte stage	0.8018	888 ppm
3	Conserved protein	PF10_0112	Sporozoite stage	0.9127	4104
4	Merozoite surface protein 8 (MSP8)	PF3D7_0502400	Erythrocyte stage	0.8421	39.5
5	Early transcribed membrane protein 5	PF3D7_0532100	Erythrocyte stage	0.9113	276
6	Merozoite Surface Protein 2 (MSP2)	PF3D7_0206800	Erythrocyte stage	1.1807	32.4
7	Mature parasite-infected Erythrocyte Surface Antigen (MESA)	PF3D7_0500800	Erythrocyte stage	1.0116	321 ppm
8	Knob-associated Histidine Rich Protein (KAHRP)	PF3D7_0202000	Erythrocyte stage	0.9255	5540

clearance, causing malaria to worsen. Using these proteins to generate antibodies will prevent cytoadhesion and promote the outflow of parasitized RBCs to the spleen.

3.2. B-cell epitope prediction

Humoral immune response is crucial to preventing malaria by blocking the invading parasite from infecting a host cell. Antibodies not only block the infectious sporozoite or merozoite from infecting hepatocytes or erythrocytes but also target infected cells to antibody-dependent cell-mediated cytotoxicity, resulting in advanced prevention from causing disease.[66] Hence, B-cell epitopes were predicted for eight selected proteins using the ABCpred server. The immunogenicity of predicted epitopes was determined by the VaxiJen v2.0 server. Ten amino acid long epitopes from each protein with top-score and immunogenicity were selected (Table 2).

3.3. Cytotoxic T-lymphocytes epitope prediction

Alongside the Humoral immune response, Cytotoxic T-lymphocytes is responsible for parasite killing and clearance by releasing certain chemokines, cytokines, and enzymes like granzymes.[67] For this purpose, it is essential to activate and induce proliferation of Cytotoxic T-lymphocytes. Cytotoxic T-lymphocytes are restricted to antigen presented by MHC class-I molecules. This way, binding of epitopes to MHC class I molecules is a must to generate a cell-mediated immune response. Epitopes specific to MHC class-I are then predicted by NetCTL 1.2 webserver for three supertypes, i.e., A2, A3, and B7. A total of 24 epitopes were sorted for eight proteins based on their immunogenicity obtained by the IEDB immunogenicity tool (Table 3).

3.4. Helper t-lymphocyte epitope prediction

Helper T-lymphocyte plays an important role in cell-mediated as well as humoral immune response by releasing specific cytokines to activate and proliferate T-cells and inducing antibody production. To generate a helper T-cell response, an antigen should be presented by an MHC class II molecule. MHC II binding epitopes were predicted by the IEDB web server, keeping the 15-mer standard size for epitopes. All epitopes with percentile rank less than 1 and IC_{50} less than 50 nM were subjected to the IEDB immunogenicity tool. Epitopes having immunogenicity of more than 80 were taken to check Interferon-γ

Table 2
List of B-cell epitopes:

S. No.	Protein	B-cell epitope	Score	Immunogenicity (VaxiJen 2.0)
1	Circumsporozoite protein (CSP)	NDDGNNEDNE	0.84	2.03
2	CSP-related protein/exported protein 1	KSKYKLATSV	0.83	1.11
3	Mature parasite-infected Erythrocyte Surface Antigen (MESA)	VKEEIEKQVE	0.83	0.94
4	Knob-associated Histidine Rich Protein (KAHRP)	KKSKKHKDNE	0.79	1.4289
5	Conserved Protein	IIFVFLVIAL	0.78	1.79
6	Merozoite Surface Protein 2 (MSP2)	AEASTSTSSE	0.85	1.49
7	Merozoite Surface Protein 8 (MSP8)	VTSNVGDTNN	0.78	0.82
8	Early Transcribed membrane protein 5	DDSKNASLDK	0.79	0.72

Table 3
List of Cytotoxic T-cell epitope:

S. No	Protein	A2	Score/Immunogenicity	A3	Score/Immunogenicity	B7	Score/ Immunogenicity
1	Circumsporozoite protein (CSP)	YLNKIQNSL	1.247/-0.301	KQENWYSLK	1.206/0.092	MPNDPNRNV	0.937/ 0.043
3	CSP-related protein/ exported protein 1	ILSVFFLAL	1.062/0.225	ALFFIIFNK	1.734/0.506	EPLIDVHDL	0.064/0.231
3	Mature parasite-infected Erythrocyte Surface Antigen (MESA)	VILDPIITF	0.224/0.268	RMYEENAAR	1.061/ 0.247	RGRLNTVIL	1.288/ 0.148
4	Knob-associated Histidine Rich Protein(KAHRP)	LVSFLVWVL	0.804/0.303	VSFLVWVLK	1.604/0.304	APYGVPHGA	0.846/ 0.110
5	Conserved Protein	LGIYIIIFV	0.751/0.475	FVAFLLGIY	0.823/0.823	IIVLITASF	0.778/ 0.105
6	Merozoite Surface Protein 2 (MSP2)	SIINFFIFV	1.301/0.441	FIFVTFNIK	1.179/0.300	TPATTTTTK	0.802/ 0.185
7	Merozoite Surface Protein 8 (MSP8)	ILCLFIFIL	0.994/0.387	SMNDDFINK	1.220/0.260	LPGFNNIKI	0.999/ 0.103
8	Early Transcribed membrane protein 5	VLVGGIIGT	0.799/0.338	FFAFFIALK	1.268/0.396	SIATGLAVL	0.126/ 0.131

Table 4
List of Helper T-cell epitopes:

S. No.	Protein	Allele	Epitope	Method/ Percentile rank	SMM align IC50 (nM)	Immuno-genecity	INF inducer/ Score
	Circum-sporozoite protein (CSP)	HLA DPA1*01:03/ DPB1*02:01	SSFLFVEALFQEYQC	Consensus (comb.lib./ smm/nn) /0.86	42	97.009	Positive 0.044291
	CSP-related protein/ exported protein 1	N/A	Not Found	N/A	N/A	N/A	N/A
	Mature parasite-infected Erythrocyte Surface Antigen (MESA)	HLA-DPA1*03:01/ DPB1*04:02	EKAFRELQFIKLRDR	Consensus (comb.lib./ smm/nn) /0.85	47	92.21	Positive 0.449241
	Knob-associated Histidine Rich Protein (KAHRP)	N/A	Not Found	N/A	N/A	N/A	N/A
	Conserved Protein	HLA-DPA1*03:01/ DPB1*04:02	IVLITASFLNIYIST	IEDB / 0.25	19	87.23	Positive 0.402274
	Merozoite Surface Protein 2 (MSP2)	N/A	Not Found	N/A	N/A	N/A	N/A
	Merozoite Surface Protein 8 (MSP8)	HLA-DPA1*03:01/ DPB1*04:02	SIHNFIFFFILCLF	Consensus (comb.lib./ smm/nn) / 0.2	17	93.93	Positive 0.68617
	Early Transcribed membrane protein 5	HLA-DPA1*01:03/ DPB1*02:01	FSKVFSFFAFFIALK	Consensus (comb.lib./ smm/nn) /0.15	13	84.03	Positive 0.081576

producing capacity. A total of five epitopes were found to be interferon-γ producing and selected for further study (Table 4).

3.5. Vaccine construct design

The multiprotein, multi-epitope vaccine candidate was constructed by joining eight B-cell linear epitopes, twenty-four cytotoxic T-cell epitopes, and five Helper T-cell epitopes. KK, AAY, and GPGPG linkers were employed to join the said epitopes. A universal Helper T-cell epitope (PADRE) was added at the N-terminal following synthetic peptide RS09 TLR4 agonist as an adjuvant. Adjuvants are the non-replaceable partners of a vaccine, which is essential to induce a potent immune response through toll-like receptor (TLR) signaling. TLR4 is one of the receptors present on cell membranes, and performing signaling through both MYD88 and TIRF pathways has been reported to induce an anti-malarial immune response.[68] EAAAK linker is used to join adjuvant and PADRE sequence. Linkers help epitopes to behave as individual structures and as efficient immunogens. A sum of 512 amino acid residues with a molecular weight of around 56KDa made the final vaccine construct (Fig. 2). The amino acid sequence of the vaccine construct is given below. (Red-Adjuvant, Brown-PADRE sequence, Purple-B cell epitope, Blue-CTL epitope, Yellow-HTL epitopes, Green-Linkers).

**MAPPHSSIEAAAKAKFVAAWTLKAAAEAAAKNDDGNNEDNEKKKSKYKLATSVK
KVKEEIEKQVEKKKKSKKHKDNEKKIIFVFLVIALKKAEASTSTSSEKKVTSNVGDT
NNKKDDSKNASLDKAAYYLNKIQNSLAAYKQENWYSLKAAYMPNDPNRNVAAYIL
SVFFLALAAYALFFIIFNKAAYEPLIDVHDLAAYVILDPIITFAAYRMYEENAARAAY
RGRLNTVILAAYLVSFLVWVLAAYVSFLVWVLKAAYAPYGVPHGAAAYLGIYIIIF
VAAYFVAFLLGIYAAYIIVLITASFAAYSIINFFIFVAAYFIFVTFNIKAAYTPATTTTT
KAAYILCLFIFILAAYSMNDDFINKAAYLPGFNNIKIAAYVLVGGIIGTAAYFFAFFIA
LKAAYSIATGLAVLGPGPGSSFLFVEALFQEYQCGPGPGEKAFRELQFIKLRDRGP
GPGIVLITASFLNIYISTGPGPGSIHNFIFFFILCLFGPGPGFSKVFSFFAFFIALK**

3.6. Physiochemical properties analysis of vaccine constructs

To be an effective vaccine candidate, the designed vaccine construct must be stable in the host body as well as in *in-vitro* conditions. Physiochemical properties determined by the Expasy tool confirmed that the construct is stable. The molecular weight of the construct was found to be 56.3 KDa with 9.38 basic pI. The half-life of the construct was 30 hrs in mammalian reticulocytes *in-vitro* conditions, and the instability Index was 24.08, which says the construct is stable at *in-vitro* conditions. The aliphatic Index showed the thermostability of the proteins is 101.76, which is at par score for a thermostable protein Table 5.

3.7. Antigenicity and allergenicity prediction

To achieve the goal of disease eradication, a vaccine should have reliable efficacy, and at the same time, it should be safe to use in the community. Antigenicity determines the effectiveness of the vaccine, while non-allergic or non-toxic behavior determines the safe use of the vaccine. VaxiJen v2.0 online tool was used to assess the immunogenicity of the vaccine. All six combinations of vaccine construct were found antigenic with a score > 0.7 (Supplementary data). The threshold was kept at 0.5 on the VaxiJen v2.0 tool. The allergenicity of the designed construct was tested by the AllerTop web tool. All combinations of designed vaccine constructs were found to be non-allergen.

3.8. Tertiary structure prediction, refinement, and validation

Tertiary structure of the designed vaccine construct was modeled by the i-TASSER webserver. Five models were obtained in the result. A model with a more positive C-score was taken for further refinement to GalaxyRefine webserver. The refined structure was further assessed by SWISS MODEL's structure assessment tool. The Ramachandran favored region achieved after refinement was 91.2 % compared to an initial score of 76.3 %. MolProbity obtained was 2.683, and overall QMEANDisCo was 0.35 ± 0.05. All these assessments show that the

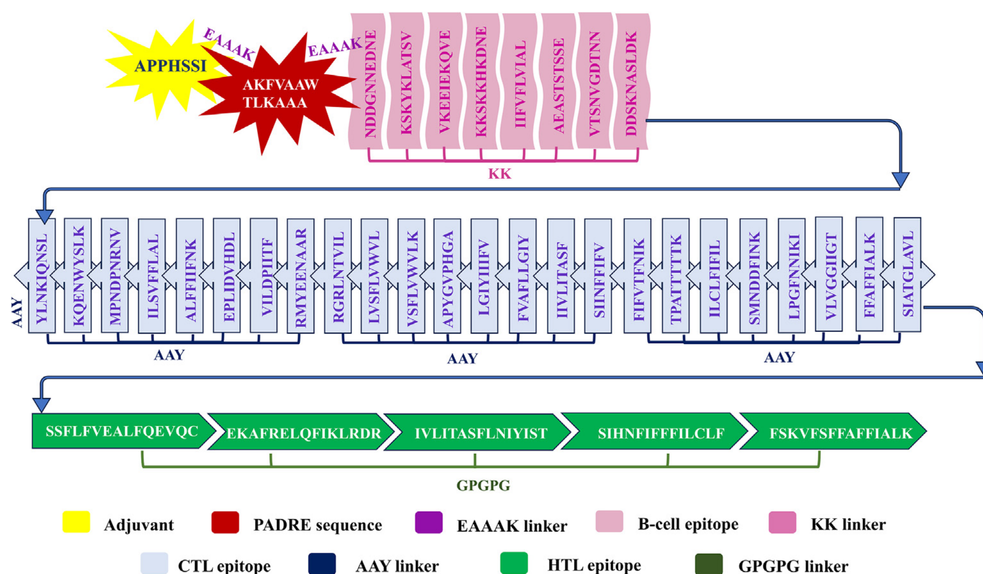

Fig. 2. Shows a cartoon representation of the designed vaccine construct.

Table 5
Physiochemical properties of designed vaccine construct:

S. No	Vaccine construct Combination	Mol. wt.	Theoretical pI	Estimated half-life	Instability Index	Aliphatic index	GRAVY
1	B-CTL-HTL	56299.80	9.38	30 h (mammalian reticulocytes, in vitro). > 20 h (yeast, in vivo). > 10 h (*E coli*, in vivo)	24.08	101.76	0.421
2	B-HTL-CTL	56299.80	9.38	30 h (mammalian reticulocytes, in vitro). > 20 h (yeast, in vivo). > 10 h (*E coli*, in vivo)	23.92	101.76	0.421
3	HTL-CTL-B	56190.76	9.43	30 h (mammalian reticulocytes, in vitro). > 20 h (yeast, in vivo). > 10 h (*E coli*, in vivo)	24.00	102.36	0.417
4	HTL-B-CTL	56190.76	9.43	30 h (mammalian reticulocytes, in vitro). > 20 h (yeast, in vivo). > 10 h (*E coli*, in vivo)	24.17	102.36	0.417
5	CTL-B-HTL	56250.82	9.44	30 h (mammalian reticulocytes, in vitro). > 20 h (yeast, in vivo). > 10 h (*E coli*, in vivo)	23.54	101.57	0.402
6	CTL-HTL-B	56250.82	9.44	30 h (mammalian reticulocytes, in vitro). > 20 h (yeast, in vivo). > 10 h (*E coli*, in vivo)	23.87	101.57	0.402

model quality has been improved after refinement, and the overall quality of the model is good for further analysis (Fig. 3).

3.9. Continuous and discontinuous antibody epitope prediction

Both linear and confirmational (discontinuous) epitopes are important to be identified for antigen–antibody or humoral immune response once the 3D model of the vaccine has been generated.[69] ElliPro server was used to identify the epitopes on the 3D model structure of the vaccine construct. Ten linear or continuous epitopes (Table 6 and Fig. 4A) and five discontinuous epitopes (Table 7 and Fig. 4B-F) of different lengths were predicted from the vaccine model.

3.10. Molecular docking with Toll-Like receptor

In order to induce and activate a range of immune cells and responses, an antigen should be recognized and shall interact with the Toll-like receptor present on antigen-presenting cells (APC). This interaction initiates signal transduction, resulting in nuclear translocation of NFκB and transcription of certain genes. A variety of cytokines and chemokines that can be pro-inflammatory and activating signals for innate and/or adaptive immune cells get expressed as a gene product. With such a critical binding, the interaction between the antigen (vaccine model) and TLR4 was determined by molecular docking. The ClusPro online tool was used for the molecular docking of the vaccine model and TLR4. A total of thirty models were obtained. Model number 15, which has the lowest energy of −1514.2 and a center value of −1413.7 (Fig. 5), was selected for the simulation study to analyze the stability of the complex in the simulated biological environment.

3.11. Molecular dynamics simulation using Desmond

The molecular dynamics simulation study of vaccine candidate-TLR4 complex was performed using Desmond (Schrodinger Inc.). MD trajectory was analyzed to check the behavior of the vaccine candidate in virtual physiological conditions. The RMSD for C atoms in Chain A and Chain B as a function of time in picoseconds is presented in Fig. 6A. The complex's RMSD value was found to be in the range of 2.82 Å to 8.48 Å (chain A) and 1.68 Å to 3.85 Å (chain B), and these values might indicate the stability of the complex. The magnitude of chain flexibility in the complex was illustrated in terms of RMSF for each residue from average position vs time fluctuation between 2.49 Å and 7.76 Å (chain A) and 0.64 Å and 5.21 Å (chain B). Consid-

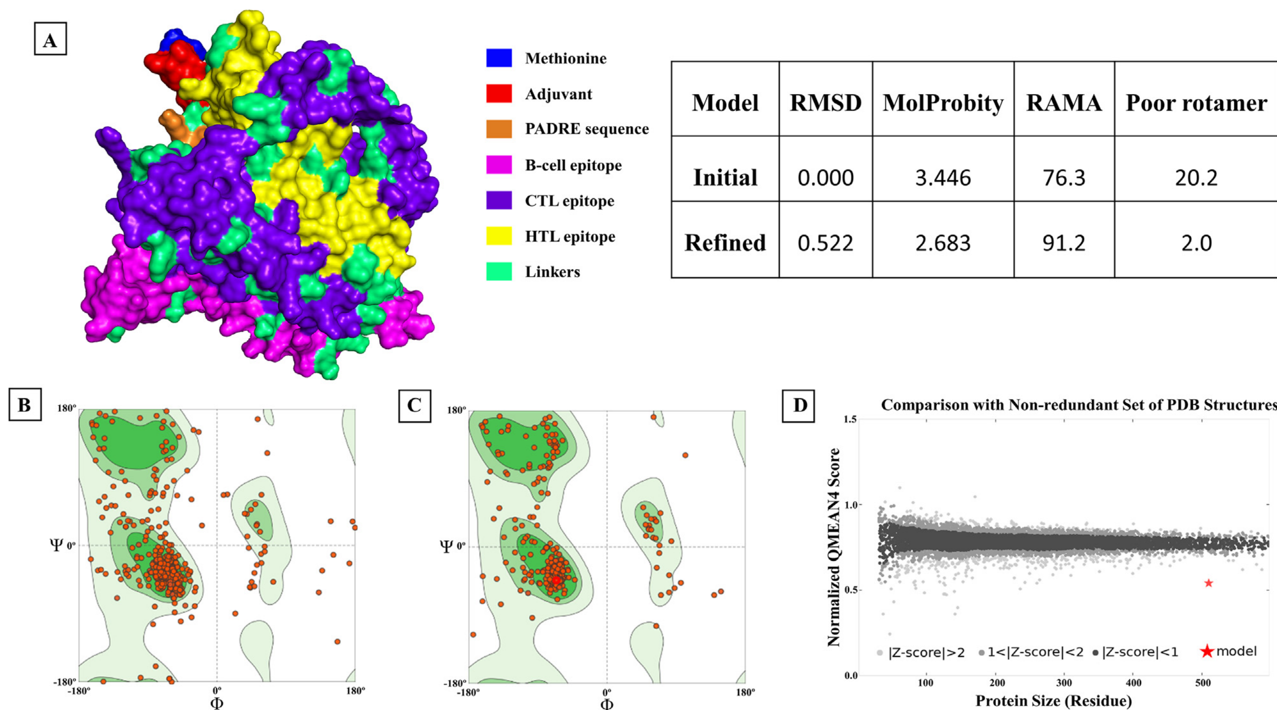

Fig. 3. [A] Modeled structure of designed multi-epitope subunit vaccine. [B] and [C] represent Ramachandran plots showing the distribution of amino acids in favored, allowed, and disallowed regions before and after refinement. [D] QMEAN4 score (Z-score) for constructed vaccine model.

Table 6
Linear or continuous antibody epitope predicted by ElliPro.

S. No.	Start	End	Peptide	Residues	Score
1	26	55	AEAAAKNDDGNNEDNEKKKSKYKLATSVKK	30	0.812
2	1	16	MAPPHSSIEAAAKAKF	16	0.79
3	65	134	EKKKKSKKHKDNEKKIIFVFLVIALKKAEASTSTSSEKKVTSNVGDTNNKKDDSKNASLDKAAYYLNKIQ	70	0.782
4	430	449	EYQCGPGPGEKAFRELQFIK	20	0.758
5	291	348	GIYAAYIIVLITASFAAYSIINFFIFVAAYFIFVTFNIKAAYTPATTTTTKAAYILCL	58	0.74
6	220	230	ARAAYRGRLNT	11	0.69
7	152	161	YMPNDPNRNV	10	0.654
8	394	400	FAFFIAL	7	0.614
9	473	480	TGPGPGSI	8	0.561
10	369	373	LPGFN	5	0.539

ering that chain B has more interactions with the vaccine candidate than chain A, the total fluctuation of chain B is lower (Fig. 6B). We also examined the gyration radius to determine whether the complex in the dynamic system was compact. The average Rg was estimated to be 48.60 Å (chain A) and 32.56 Å (chain B), indicating that chain B interacts with the vaccine candidate more strongly than chain A (Fig. 6C). Hydrogen bonding is critical for the biomolecular complex's sustainability and stability. As a result, the average number of hydrogen bond breakdowns and formations in the complex during the molecular dynamics simulation was calculated to be 22.65 (Fig. 6D). The results of the MD trajectory analysis indicate that more residues from TLR4 chain B interact with the vaccine candidate than residues from TLR4 chain A, and the vaccine-TLR4 complex was found to be stable.

3.12. Immune simulation and antibody class prediction

The final step for vaccine development is vaccine evaluation. Before coming to the clinical trials, a vaccine needs to be evaluated in laboratories (in-vitro & in-vivo) for its immunogenicity, safety, and, more importantly, its efficacy in preventing disease. With advanced immunoinformatic techniques, it is now possible to predict or investigate the immune response going to be generated by a designed vaccine. For immune simulation, C-ImmSim online tool was used to predict the immune response induced by our designed vaccine construct. An activated B-cell population was increased and maintained after immunization with the designed vaccine construct, even when antigen concentration was subsequently decreased (Fig. 7A). Similarly, active cytotoxic T-cell and helper T-cell population was found to be generated after injection of antigen (Fig. 7B and 7C). The humoral response generated by the vaccine construct was calculated by antibody titer and class analysis (Fig. 7D). The combination of IgM and IgG antibody titer was found to be the highest (600000 xx per mL) among all other classes, followed by individual IgM and IgG (IgG1 + IgG2), and it was maintained for up to 15 days and more. At the same time, antigen concentration decreased within 5 days after injection. Production of INF-gamma (400000 ng/mL) and IL2 (160000 ng/mL) represent a stable cell-mediated immune response (Fig. 7E). Parallely, the whole construct sequence was predicted to be an IgG class antibody epitope

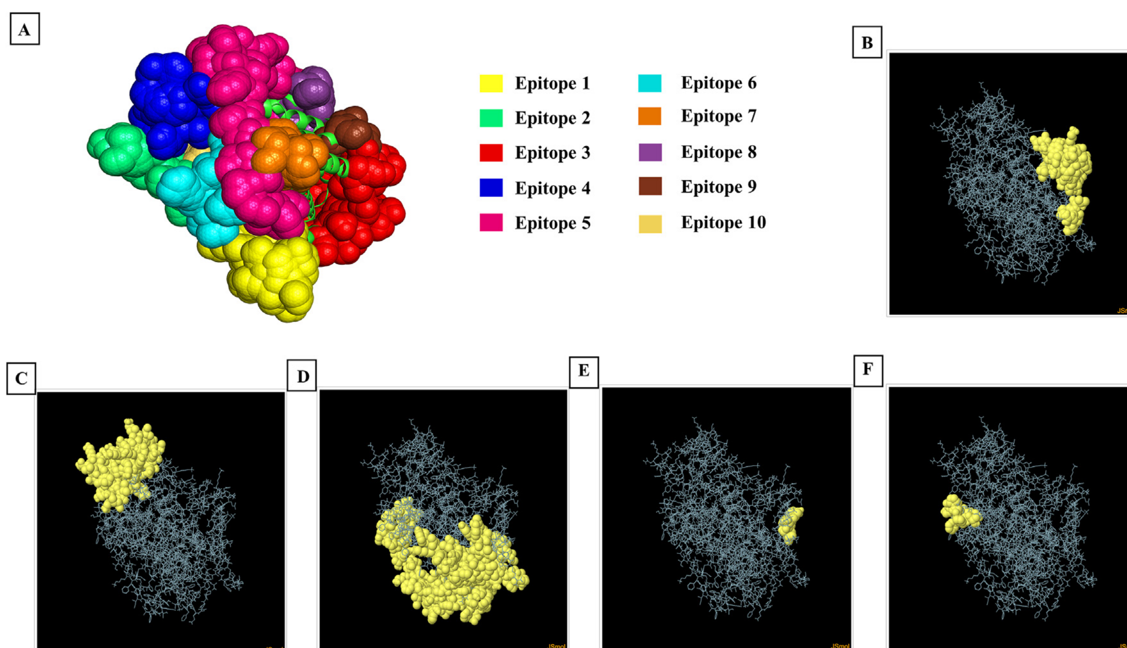

Fig. 4. [A] Linear or continuous antibody epitopes (a total of ten epitopes) present on the 3D structure of vaccine predicted by ElliPro server. [B-F] Five Confirmational or discontinuous antibody epitopes (highlighted in yellow) present on the 3D structure of the vaccine predicted by the ElliPro server. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 7
Confirmational or Discontinuous antibody epitope predicted by ElliPro.

S.No.	Discontinuous Epitope No.	Number of residues	Score
1	Epitope 1	37	0.786
2	Epitope 2	71	0.772
3	Epitope 3	131	0.709
4	Epitope 4	5	0.64
5	Epitope 5	11	0.52

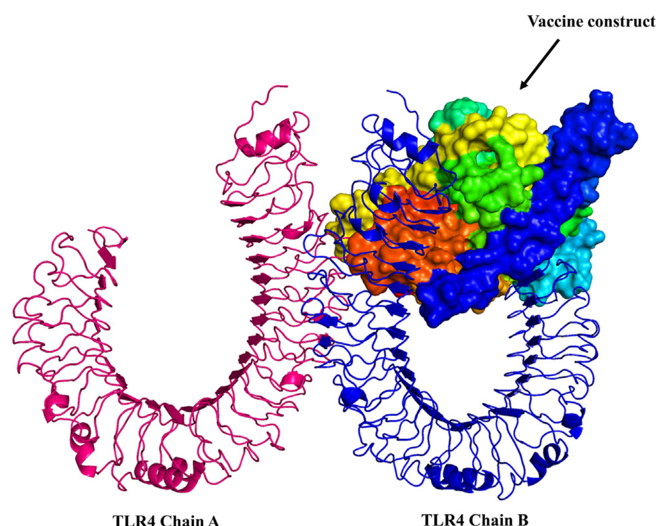

Fig. 5. Docked complex of vaccine model with Toll-like receptor 4 (TLR4).

by using the IgPred online tool. This tool predicts antibody class (IgG, IgA, and IgE) going to be generated for an epitope. IgG is one of the major class of antibodies responsible for opsonization, complement fixation, and antibody-dependent cellular cytotoxicity (ADCC). Our vaccine will be able to induce IgG class antibody with a score of 1.472, which, in response, is capable of generating humoral and cellular immunity.

4. Discussion

The advancement in the field of bioinformatics and immunoinformatics enables researchers to make advanced predictions of the effectiveness and efficiency of a vaccine. Before commercialization or reaching to the immunization state, a vaccine goes through different phases of development. This includes target selection, designing, evaluation, laboratory trials to clinical trials, which takes a huge amount of time, costs, and ethical concern (animal and human). Bioinformatics not only helps in designing a vaccine, starting from screening of immunogenic antigen (target) to modeling the vaccine structure, but it also allows us to predict the immune response and efficacy of the designed vaccine. This method will not only provide the best results but also save time and money. One can have all the safety and effectiveness information before its actual production.[70] Currently, reverse vaccinology and immune informatics are commonly used to design a multi-epitope subunit vaccine. With the availability of resources like the pathogen's genome database[71] (even for emerging species or strains)[72] and techniques like next-generation sequencing, it becomes easy to identify the suitable targets and receptors (TLRs, B and T-cell receptors). Structural vaccinology is another approach researchers have used in recent years to identify immunogenic epitopes based on the native structure of the target protein.[73] P27A, or a coiled-coil domain from *P. falciparum* blood stage protein, was identified by scanning the parasite's proteome. All these approaches enable the researchers to design a vaccine that is primarily immune-focused in contrast to traditional parasite/pathogen-focused.[73] There are several queries, such as i) how does a host immune system responds to a natural infection, ii) how it will respond upon immunization, and iii) which type of immune response is actually required.[69] An accurate design of vaccines

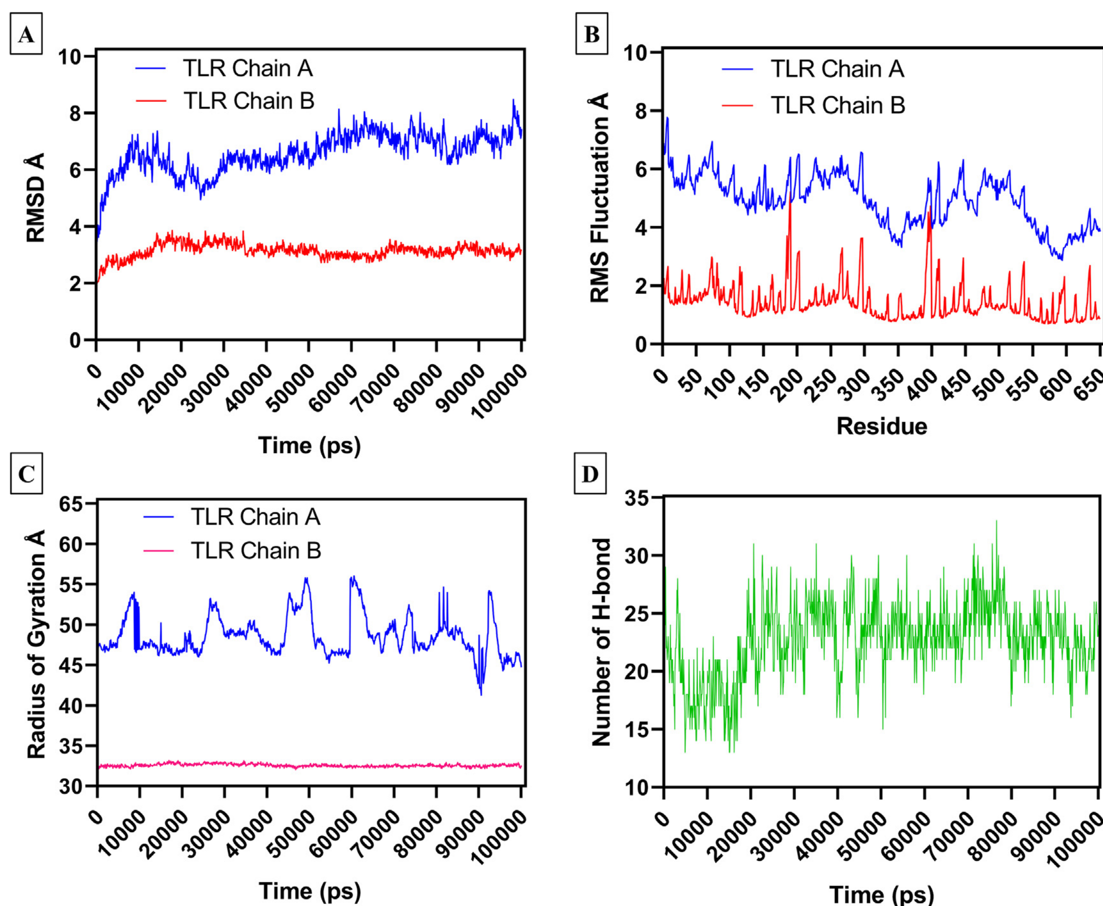

Fig. 6. Molecular dynamics simulation of docked complex (TLR4-Vaccine model) using Desmond. (A) Shows Root mean square deviation (RMSD) plot, (B) Shows residual fluctuation of chain A and chain B of TLR4 within the docked complex, (C) Radius of Gyration (Rg), (D) Hydrogen bond numbers between TLR4 and vaccine model.

by finding the answer to the above questions will lead us to a successful vaccine against parasites like *Plasmodium,* for which several vaccine candidates with different adjuvants have already been investigated without much success.[74] While for the Leishmania vaccine, Cytotoxic CD8 + T-cells and T_H1 response are the prior requirement that kills the infected cells[75] but, a malaria vaccine may require to generate a balanced immune response where T_H2 mediated activation and proliferation of antigen-specific B-cell and memory B-cells is also essential. Expression of highly specific invasive-blocking antibodies and antibodies that help in opsonization and complement fixation might play a role in blocking malaria invasion.[76–78]

Therefore, in this study, we have focused on designing a balanced vaccine construct with an emphasis on humoral and T_H2 responses. Our results show that the vaccine construct is producing a good amount of IgG class antibodies (Fig. 7), and the whole construct is considered as an IgG epitope (IgPred results). Antibody or humoral response is required in a malaria vaccine to block the parasite's multiple entry. Many researchers believes that parasite need to block at multiple stage by targeting multiple antigens from parasite's different stage. Single antigen from a single stage will not be sufficient to solve the purpose.[79] The most advance vaccine RTS,S and its upcoming generation R21 for instance, have used single antigen from pre-erythrocyte stage which may not be able to block the parasite at erythrocyte stage if by any chance skipped or evade from immune response. To overcome this limitation of existing vaccine candidates, we have used protein epitopes from the parasite's pre-erythrocytes and erythrocytes, as well as infected RBC (iRBC) surface protein, which make our vaccine candidate potent to provide multi-stage protection. Also, for the first time, we have selected majorly the surface and membrane proteins to design our malaria vaccine, which directly helps to opsonize the parasite and prevent the invasion into host cells (Hepatocytes and Erythrocytes). Surface protein will also provide a platform for glycosylation, which again promotes B-cell or humoral response.

It was reported that synthetic mannose moiety tethered with TLR7 agonist activates the Dendritic cells (DCs) through mannose-binding receptor and TLR pathway. In response, both CD8 + and CD4 + T cells were found to be proliferated.[80] However, it is difficult to track the subsets of activated T-cells (CD8+, CD4+, or Treg) and their cytokines profiles in both vaccine and naturally induced immunity during malaria infection.[67] Besides these limitations, researchers are continuously working on a wide range of adjuvants or TLR agonists to get a more specific immune response. A pathogen-derived antigen, either in native or modified form, results in the activation and maturation of different sets of Dendritic cells upon binding with TLRs. These DCs can be differentiated based on their co-stimulatory molecules and cytokines production.[81] TLR4, which signals through both MyD88 and TRIF pathways, allows maximum DC activation and production of pro-inflammatory cytokines and type-I interferons.[81] Our modeled vaccine showed stable interaction with the lowest energy of −1514 when docked with TLR4 (Fig. 5), and the complex was stable with 1.68 Å to 3.85 Å root mean square deviation (RMSD) (Fig. 6A) when examined under a simulated biological environment. Finally, our designed vaccine is predicted to produce a good amount of Interferon-γ (IFN-γ) and interleukin-2 (IL-2) along with a stable active

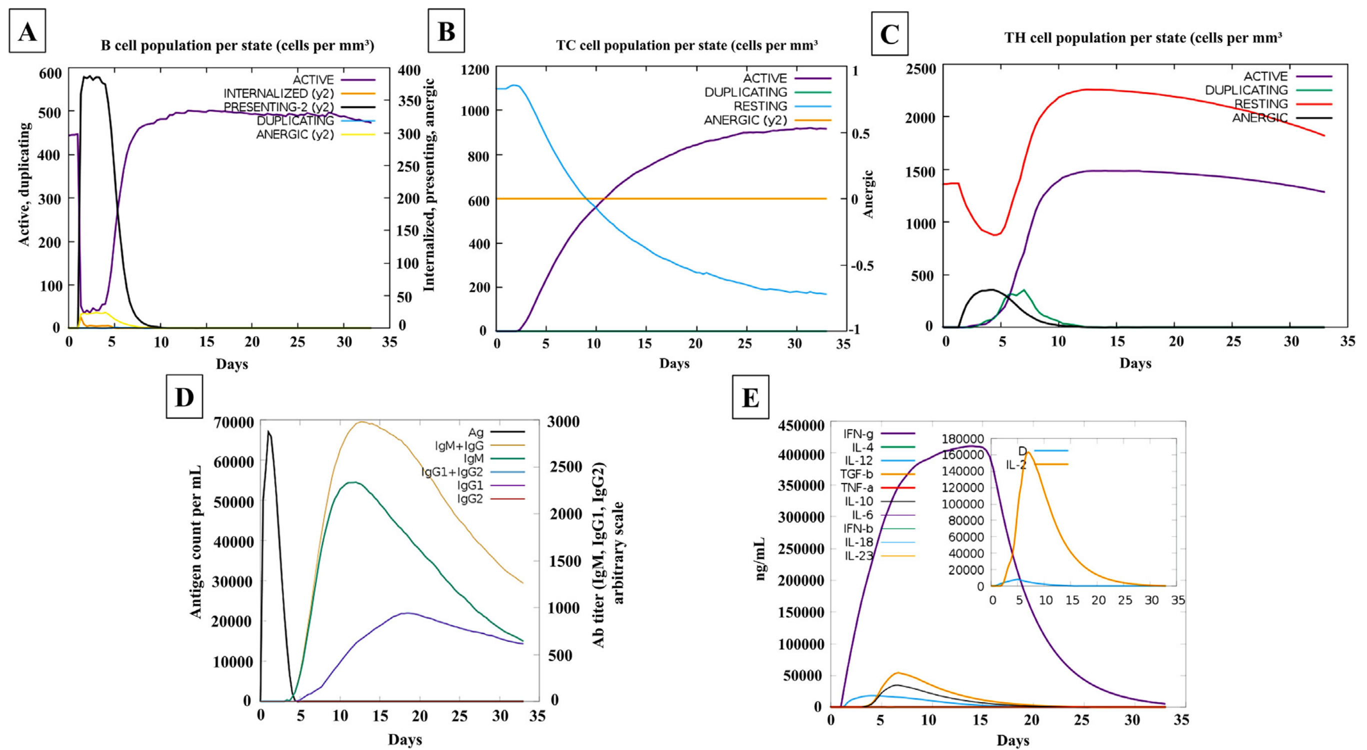

Fig. 7. Immune simulation of vaccine construct. (A) Active B-cell, (B) Cytotoxic T-cell, (C) Helper T-cell population generated in response to the designed vaccine construct, (D) shows antibody subclass profile induced by the designed vaccine, (E) graphical representation of concentration and duration of induced cytokines.

T-cell population (Cytotoxic T-cells and Helper T-cells). Overall, the proposed vaccine construct is able to generate a balanced immune response, which might be suitable for the malaria parasite's growth inhibition.

5. Conclusions

Malaria is the most prominent parasitic killer disease, accounting for significant deaths in tropical and subtropical regions. The parasite is developing resistance to the currently available treatments. As a result, there is an urgent need for an effective vaccination against this deadliest disease. We investigated the *Plasmodium falciparum* surface protein for the development of an effective vaccine due to several factors, including easy accessibility to the immune system, recognition by immune cells, strong antibody generation, role in opsonization and pathogen clearance, specific immune memory, and reduced risk of pathogenicity. The study begins with selecting eight highly antigenic proteins from pre-erythrocyte and erythrocyte stages. ABCpred, NetCTL 1.2, and the IEDB web server were used to predict B-cell, CTL, and HTL epitopes, and top-scoring antigenic and non-allergenic epitopes were selected. Linkers KK (B-cell), AAY (CTL), and GPGPG (HTL) were used for joining epitopes. An EAAAK linker was used to link the TLR-4 agonist adjuvant and pan-HLA DR sequence at the N-terminus of the construct. The proposed vaccine construct was docked with TLR4, resulting in the lowest binding energy of −1514 kcal/mol. Finally, molecular dynamics and immune dynamics simulations were performed, and findings indicated that the developed vaccine candidate is highly stable under virtual physiological conditions and can elicit secondary and tertiary immune responses through the production of IgG1, IgG2, and IgM antibodies. The intended vaccine candidate produces B-cell, HTL, and CTL populations, as well as an IFN-γ response, indicating that the vaccine candidate developed in this study may elicit neutralizing antibodies against the *Pf along with the activated T cell.*

6. Consent of publication

Not applicable.

Ethics approval

This is an observational study; no ethical approval is required.

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9. License or permission/Guidelines for plant studies

Not applicable.

CRediT authorship contribution statement

Preshita Bhalerao: Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Satyendra Singh:** Writing – original draft, Software, Formal analysis. **Vijay Kumar Prajapati:** Writing – review & editing. **Tarun Kumar Bhatt:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgeb.2024.100377.

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