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Discovery of novel vaccine candidates based on the immunogenic epitopes derived from *Toxoplasma* membrane proteins

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Purpose: Due to the widespread distribution and importance of *Toxoplasma gondii* infection as a parasitic zoonosis, multi-epitope vaccine design was implemented using a set of immunodominant epitopes screened out of a wide scope of membrane proteins.
Materials and Methods: On this basis, 5 vaccine candidates were created using linkers ($[GGGGS]_2$, KK, AAY, GPGPG, GDGDG, EAAAK) and adjuvants (RS-09 peptide, *Mycobacterium tuberculosis* resuscitation-promoting factor E [RpfE] and 50S ribosomal protein, human interferon [IFN]-γ).
Results: Polytopes with RS-09 alone (Toxo-App) and with IFN-γ (Toxo-Apfn), and one with 50S ribosomal protein (Toxo-Ribos) showed the highest immunogenicity during *in silico* prediction, and their 3-dimensional structure was refined. Protein-protein docking and molecular dynamics simulation analysis was done between the Toxo-App and human toll-like receptor (TLR)-4, rendering a stable connection. Codon optimization and *in silico* cloning was done ultimately for the selected vaccine candidate.
Conclusion: In conclusion, potent multi-epitope vaccine candidates were designed against toxoplasmosis using a diverse set of *in silico* techniques, while further wet experiments are recommended.

Keywords: *Toxoplasma gondii*; Vaccine design; Vaccination; Immunoinformatics

INTRODUCTION

Toxoplasma gondii is a widely distributed coccidian parasite that is known to cause toxoplasmosis in various animal species as well as humans [1]. This protozoan, which exhibits a diverse range of genotypes, primarily opts definitive hosts from the members of the Felidae family, such as domestic cats, while other warm-blooded

animals like birds and mammals can also serve as carriers of the infection [2]. The parasite can be transmitted through various routes, including congenital transmission from infected mothers to their offspring [3], blood transfusions via the tachyzoite stage [4], organ transplants [5], and consumption of undercooked meat containing bradyzoites [6]. This parasitic infection can result in a wide range of serious clinical sequelae. In infected fetuses, it may cause chorioretinitis, hydrocephaly, microcephaly, mental retardation, and abortion [7]. In immunocompromised individuals, *T. gondii* can lead to the development of the inflammation of internal organs (e.g., brain, heart, lungs), further aggravating the severity of the infection [8]. Additionally, *T. gondii* infection has significant implications in the veterinary field, particularly for livestock such as sheep and goats, rendering abortion and stillbirth. Since current treatment approaches such as chemotherapeutics, nano-based, and herbal-based therapies are only active against tachyzoites (acute) stage [9] and may lead to unwanted side effects [9], vaccination seems to be necessary.

Toxoplasma infection usually elicits a T helper 1 (Th1)-biased immune response in susceptible hosts, including humans and sheep characterized by interferon-γ (IFN-γ) upsurge, followed by specific humoral immunity [10]. Thus far, most vaccination studies have been focused on the well-known antigenic repertoire of *T. gondii*, comprising surface antigens (SAGs), dense granule molecules (GRAs), rhoptries (ROPs), and micronemes for vaccine design and immunization purposes [11]. In this sense, less attention has been paid to the *T. gondii* membrane proteins, which play a pivotal role in host-parasite interaction and invasion. For instance, a transmembrane protein called *Toxoplasma* apical membrane antigen-1 is a critical factor in ROPs secretions and host cell penetration [12]. Moreover, a wide plethora of membrane proteins act as catalysts, solute and ion transport, immune evasion, as well as in cell signaling. Based on prediction algorithms, thousands of proteins in the *T. gondii* genome are approximately associated with the cell membrane. However, at present, major groups of membrane proteins remain fully or partially uncharacterized [13]. As membrane proteins are in close contact with immune system effectors, their immunodominant epitopes would presumably elicit robust immune responses and serve for next-generation vaccine design against *T. gondii* [14].

The only commercial vaccine against toxoplasmosis, Toxovax®, is available in the veterinary field to prevent abortion in susceptible ewes, while a human vaccine is still lacking in spite of decades of attempts in *Toxoplasma* vaccine projects [15]. The strategic development of an effective vaccine holds significant promise for stimulating both innate and adaptive immune responses, as previously indicated [16]. The utilization of cutting-edge computational techniques, specifically *in silico* approaches, to design multi-epitope vaccine candidates (MEVCs) has emerged as a potentially valuable avenue for combating a wide range of infections [17,18]. By circumventing undesirable immune reactions and fostering long-lasting immunity, these innovative approaches can significantly enhance the efficiency of vaccine development pipelines in terms of cost and time, ultimately leading to the generation of efficacious and safer candidates [18]. The present study was performed to introduce some unprecedented MEVCs derived from immunogenic peptides from membrane proteins to combat *T. gondii* infection.

MATERIAL AND METHODS

A simple schematic outline of the multi-epitope vaccine construction in the present study is illustrated in **Fig. 1**.

Prediction and screening of linear B-cell and human T-cell epitopes

The initial section of this study was completely adapted by Li et al. (2022) [19], in which about 314 membrane-bound and surface proteins of *T. gondii* were targeted in a genome-wide analysis using the UniProt database, available at https://www.uniprot.org/uniprot/. They subsequently predicted shared linear B-cell epitopes using ABCpred (threshold: 0.95) (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html) [20] and BepiPred 2.0 (threshold: 1.5) (https://services.healthtech.dtu.dk/services/BepiPred-2.0/) [21]. Also, mhc tools of the IEDB web server (https://www.iedb.org/) [22] and the human leukocyte antigen (HLA) reference sets were employed for cytotoxic T-lymphocyte (CTL) and helper T-lymphocyte (HTL) epitope prediction with further screening in terms of antigenicity (VaxiJen v2.0) (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) [23], allergenicity (AllerTOP v2.0) (https://www.ddg-pharmfac.net/AllerTOP/) [24], toxicity (ToxinPred) (https://webs.iiitd.edu.in/raghava/toxinpred/algo.php) [25], signal peptide (SignalP-5.0) (https://services.healthtech.dtu.dk/services/SignalP-5.0/) [26], and transmembrane position (TMHMM 2.0) (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) [27]. Population coverage of the discovered epitopes was done using the appropriate tool in the IEDB server, available at http://tools.iedb.org/population/.

Design and assemblage of potent MEVCs

Herein, 4 different MEVCs were engineered and designed using strictly selected linear B-cell epitopes (n=3) along

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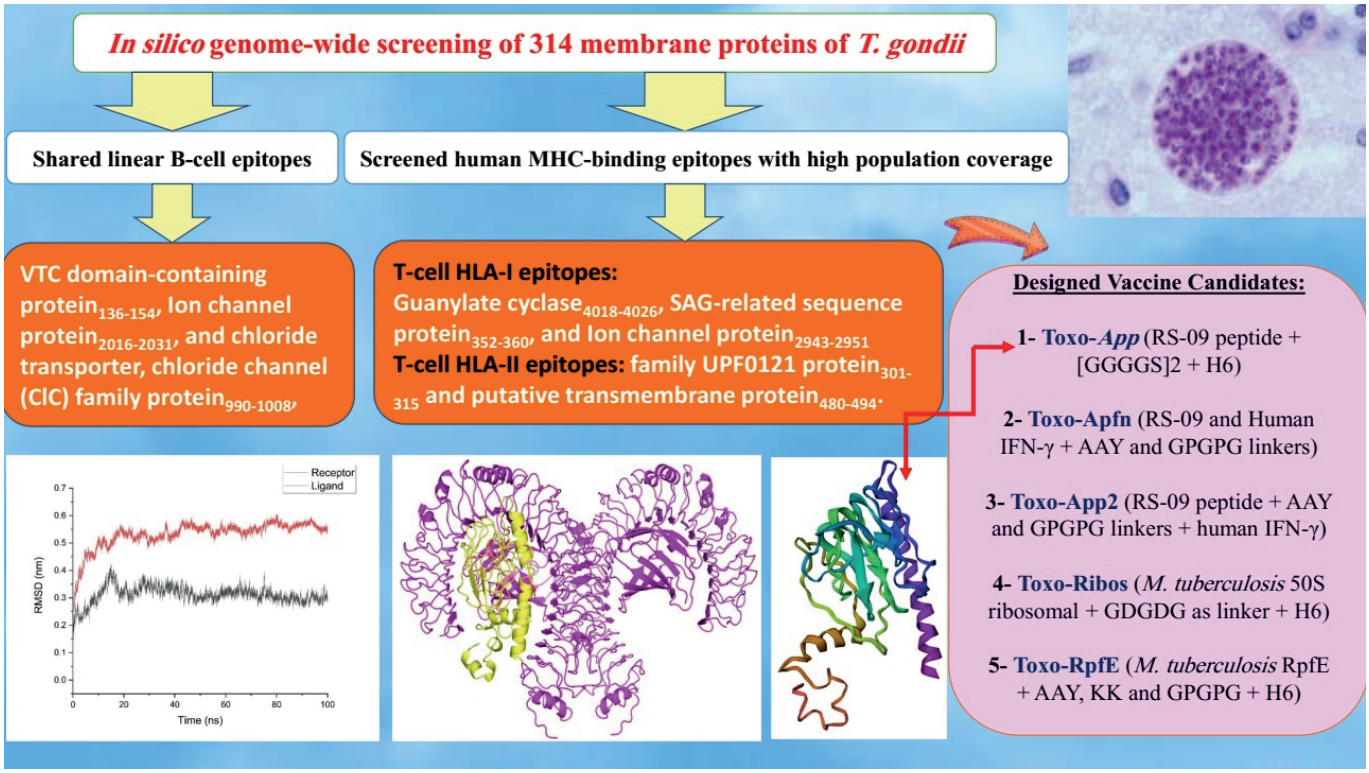

Fig. 1. Illustration of multi-epitope vaccine candidate generation using selected epitopes from *Toxoplasma gondii* membrane proteins.
ClC, chloride channel; HLA, human leukocyte antigen; SAG, surface antigen.

with CTL (n=3) and HTL (n=2) epitopes of human origin, in addition to the one assembled by Li et al. (2022) [19]. Four different adjuvants were employed in the present study, encompassing a synthetic peptide (RS-09: APPHALS) as toll-like receptor 4 (TLR-4) agonist, *Mycobacterium tuberculosis* immunogenic components (e.g., resuscitation-promoting factor E [RpfE], 50S ribosomal protein), as well as human IFN-γ. Additionally, various linkers such as "EAAAK," "$(GGGGS)_2$," "GPGPG," "AAY," "KK," and "GDGDG" were utilized to link different vaccine fragments, shaping the final sequences of MEVCs. Notably, a histidine tag was embedded C-terminally to all MEVCs, except of one with double adjuvants (RS-09 and human IFN-γ).

Basic properties of the designed MEVCs

To assess allergenicity, AllergenFP v1.0 and AllerTOP v2.0 were employed. The latter achieves a 85.3% prediction, while AllergenFP v1.0 (https://ddg-pharmfac.net/AllergenFP/) predicts with 88% accuracy based on physico-chemical and structural characteristics [28]. The antigenicity of the designed MEVCs was assessed in the following using the VaxiJen v2.0 server, which makes predictions with an accuracy of 70%–89% based on the chemical composition of the proteins. Protein solubility was predicted using the Protein-Sol server, accessible at https://protein-sol.manchester.ac.uk/; proteins with solubility values greater than 0.45 are regarded as soluble. Using the ExPASy ProtParam online tool (https://web.expasy.org/protparam/), the basic physico-chemical characteristics were predicted in the next stage. Molecular weight (MW), theoretical isoelectric point, grand average of hydropathicity (GRAVY) score, aliphatic and instability index, are among the metrics that the server forecasts [29].

Secondary and tertiary structures of different MEVCs

In this study, the NetSurfP-3.0 (https://services.healthtech.dtu.dk/services/NetSurfP-3.0/) was used that can predict solvent accessibility, secondary structure, structural disorder, and backbone dihedral angles for every residue in an amino acid sequence [30]. The Robetta web server (https://robetta.bakerlab.org/) was utilized in conjunction with RoseTTAFold, a comparatively quick and accurate deep learning-based prediction approach, to forecast 3-dimensional (3D) structures of designed MEVCs. The server provides five 3D models with defined confidence score having different error estimates. The one with the lowest error estimates and higher confidence score would be selected as appropriate for further analysis [31].

Immune profile simulation

Using the C-ImmSim online server (https://150.146.2.1/C-IMMSIM/index.php), the virtual immune simulation process elicited by 5 designed MEVCs was predicted. PSSM for machine learning techniques serves as the foundation for these forecasts. Default parameters including a random seed of 12,345, a simulation volume of 10, and 100 simulation steps, the computer-aided simulation was completed. Three extremely immunogenic MEVCs were selected for additional *in silico* evaluations [32].

Refining and validating the tertiary models of selected candidates

Regarding structural quality improvement, the GalaxyRefine web server (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) was used to improve the 3D model of the top-3 immunogenic MEVCs. The output includes a number of parameters, including the Clash score, root mean square deviation (RMSD), global distance test-high accuracy (GDT-HA), Poor rotamers, MolProbity, and Rama favored [33]. The quality of the rehashing process was assessed in the following using Ramachandran plot analysis and compared to crude models; the SAVES v6.0 server's PROCHECK tool (https://saves.mbi.ucla.edu/) was utilized to achieve this. The server analyzes the overall and regional stereochemical quality of a protein structure by generating several PostScript plots [34].

Molecular docking between the best MEVC and human TLR-4

In order to accomplish this, the 3D structure of the human TLR-4/MD2 molecule (Accession No. 3FXI) was retrieved by RCSB server. Using default settings, the likely amino acid interactions between the highly immunoreactive candidate in the current study (ligand) and TLR-4 (receptor) were predicted using the HDOCK protein–protein docking web server, accessible at http://hdock.phys.hust.edu.cn/, which forecasts the conformation and binding affinity of the ligand to its receptor. Likely binding conformations were forecasted by a rapid Fourier transform-based algorithm. Subsequently, all sampled binding conformations are assessed using the iterative scoring function based on ITScorePP knowledge. Ultimately, the macromolecular binding conformations are evaluated and ranked based on their binding energies. The structural model with the lowest binding energy score and RMSD of the ligand is used to evaluate protein-protein interactions using the Dimplot program [35].

Molecular dynamics (MD) simulations

The interactions between the Toxo-App and the receptor complex were simulated using the GROMCAS 2021.2 software. With the use of the pdb2gmx module, data associated with the complex was provided. Subsequently, upon selection of OPLS-AA/L all-atom force field, the complex was dissolved in water. To counteract long-range electrostatic interactions, the Particle-Mesh-Ewald method was applied to the system. For this aim, KCl salt ions were also considered for electrical neutralization, at a physiological concentration (0.15 M). The ion accessible volume (V) and total system charge (Qsys) detected the quantity of Cl^- and K^+ ions automatically. Energy minimization was used in the following to enable the system to achieve the lowest energy and the most stable state prior to initiating dynamics. The proton exchange membrane decreasing slope method was used to achieve this goal, and it was kept going until the maximum force was less than 1,000 kJ/mol/nm. To keep the system from collapsing, equilibration was then applied between the solvent and the ions surrounding the protein. Usually, there are 2 steps involved in balancing. The system's temperature must first plateau at the intended value while operating under an NVT (constant number of particles, volume, and temperature) set. Following temperature stabilization, pressure equilibration was carried out using an NPT set with the Parrinello-Rahman barostat, where temperature, pressure, and particle count were all constant. The system containing the docked complex was then subjected to a 100 ns simulation step, and the simulation quality was assessed using the gmx check tool. The gmx energy tool was used to perform the thermodynamic equilibrium analysis [36].

Safety prediction, codon adaptation and *in silico* cloning

The BLAST tool (https://www.uniprot.org/blast) was used to distinguish similar regions among organisms. Identity over 35% mean is defined as homologous proteins with the human proteome [37]. A critical stage in the production of subunit vaccines is the efficient expression of proteins in *Escherichia coli*. Consequently, the Sequence Manipulation Suite's reverse translate tool was used to translate the chosen MEVCs backwards, and the JCat server was used to optimize the codons (https://www.jcat.de/). The JCat server evaluates a number of crucial DNA sequence characteristics, including codon adaptation index (CAI) and GC content, which are strongly linked to the expression of chimeric proteins in the corresponding hosts. As a result, codon optimization was done in the current study using the *E. coli* K12 strain [38]. Next, codon-adapted vaccine sequences were examined for the presence of cutting sites of various commercially available restriction enzymes using the NEBcutter 3 server (https://nc3.neb.com/NEBcutter/). In the end, the 5′ - and 3′ -OH of the codon optimized vaccine sequence were supplemented with the cutting sites of 2 restriction enzymes,

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Eco53KI and *EcoRV* to be embedded in appropriate plasmid vectors such as pET28a(+). To increase the yield of expression, the Shine-Dalgarno sequence (AGGAGG) was inserted prior to the start codon.

RESULTS

Designing MEVCs using linkers and adjuvants

Based on the *in silico* screening of a wide variety of *T. gondii* membrane proteins, the following epitopes were found eligible for vaccine design: i) B-cell epitopes: VTC domain-containing protein$_{136\text{-}154}$, Ion channel protein$_{2016\text{-}2031}$, and chloride transporter, chloride channel (ClC) family protein$_{990\text{-}1008}$, ii) T-cell HLA-I epitopes: Guanylate cyclase$_{4018\text{-}4026}$, SAG-related sequence protein$_{352\text{-}360}$, and Ion channel protein$_{2943\text{-}2951}$, iii) T-cell HLA-II epitopes: family UPF0121 protein$_{301\text{-}315}$ and putative transmembrane protein$_{480\text{-}494}$ (**Table 1**). Herein, 5 MEVCs were designed based on the arrangement of different adjuvants and linkers, as follows: Seq1 or Toxo-App containing RS-09 peptide as adjuvant and a his-tag adjoined by $(GGGGS)_2$ linkers; Seq2 or Toxo-Apfn with double adjuvants (RS-09 and human IFN-γ) connected with $(GGGGS)_2$ linkers; Seq3 or Toxo-App2 again with RS-09 adjuvant, a his-tag, along with GPGPG and AAY linkers; Seq4 or Toxo-Ribos having *M. tuberculosis* 50S ribosomal protein as adjuvant, a his-tag, and GDGDG linkers; and Seq5 or Toxo-RpfE including *M. tuberculosis* RpfE as immune enhancer, a his-tag, with KK, GPGPG and AAY linkers. All designed MEVCs have been illustrated in full details in **Fig. 2**.

Physico-chemical properties, antigenicity and allergenicity profiles

The highest and lowest lengths of the MEVCs belonged to Seq2 and Seq3 with 364 and 175 aa, respectively. The MW of different sequences ranged between 18266.38 dalton (Seq3) to 38080.22 dalton (Seq2). The highest thermotolerance (aliphatic index) and hydrophilicity (GRAVY) was predicted in Seq4 (74.31) and Seq5 (–0.286), respectively. Also, all of the vaccine sequences, except of Seq2 and Seq5 had instability index below 40, so assigned as stable polypeptides (**Table 2**). Based on the VaxiJen server, the best antigenic vaccine sequence belonged to Toxo-App (1.8151), followed by Toxo-Apfn (1.1937), Toxo-App2 (1.0660), Toxo-RpfE (0.9016) and Toxo-Ribos (0.8785). All candidates were shown to be non-allergenic using the AllerTOP v2.0 and AllergenFP v1.0 servers. The solubility scores of the vaccine candidates were as follows, respectively: 0.532, 0.489, 0.454, 0.889, and 0.392.

Structural analyses

Secondary structure prediction revealed that random coils were the most frequent structures in almost all designed

Table 1. Selected B-cell and T-cell epitopes during genome-wide analysis of *Toxoplasma* membrane protein by Li et al. (2022) [19]

Epitopes	Antigenicity	Allergenicity	Toxicity	Immunogenicity	Water solubility
B-cell epitopes					
VGSQESGQARERDDREATE	1.2285	No	No	-	Good
WESWGVPTDPESRANE	1.5416	No	No	-	Good
AEGEGRGDSRDSRDVRLCT	2.0674	No	No	-	Good
HLA-I epitopes					
YLHENVTFL	0.5471	No	No	0.24497	-
VVFTPFVSF	1.0384	No	No	0.11464	-
SPHLRLTAF	1.5632	No	No	0.09492	-
HLA-II epitopes					
IPIFLYFHLFRIRYS	1.2381	No	No	-	-
EDRLRLSAAALAAAR	0.8410	No	No	-	-

HLA, human leukocyte antigen.

Table 2. Comparable physico-chemical characteristics of 5 designed MEVCs against *Toxoplasma gondii* infection, predicted using ExPASy ProtParam web server

MEVCs	No. of residues	MW	Theoretical pI	+ charged residues	– charged residues	Half-life (hr)[a]	Instability index	Aliphatic index	GRAVY
Seq1	221	20,568.01	5.55	15	19	4.4	36.04	43.85	–0.277
Seq2	364	38,080.22	9.00	45	39	4.4	45.83	60.08	–0.510
Seq3	175	18,266.38	5.55	15	19	4.4	37.89	58.8	–0.217
Seq4	304	31,482.8	4.37	31	60	30	28.42	74.31	–0.215
Seq5	331	34,703.72	5.27	31	37	30	42.60	65.41	–0.286

MEVC, multi-epitope vaccine candidate; MW, molecular weight (Dalton); pI, isoelectric point; GRAVY, grand average of hydropathicity.
[a]It means estimated half-life in mammalian reticulocytes, *in vitro*.

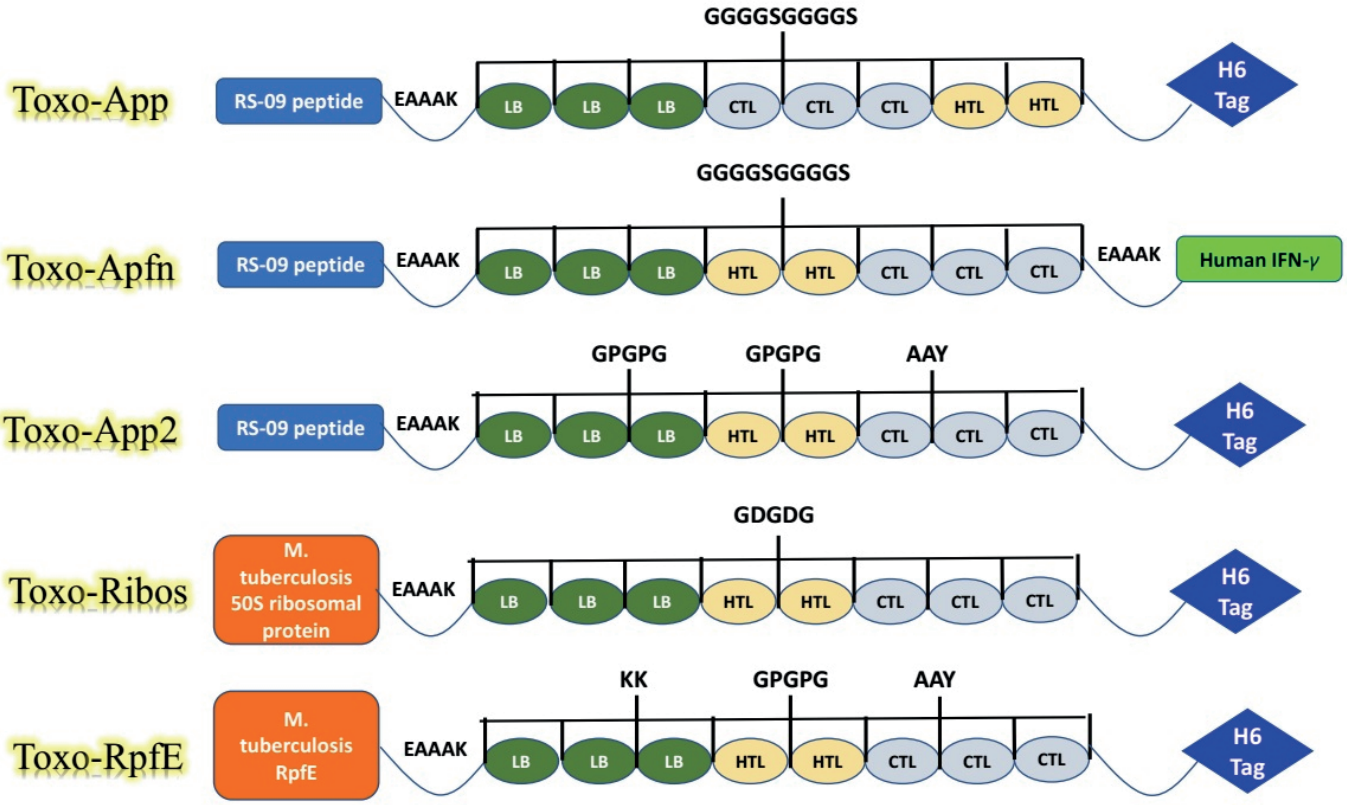

Fig. 2. Arrangement of LB, HTL and CTL epitopes to construct 5 potent multi-epitope vaccine candidates using linkers, adjuvants and His-tag sequences.
LB, linear B-cell; HTL, helper T-lymphocyte; CTL, cytotoxic T-lymphocyte; IFN, interferon; RpfE, resuscitation-promoting factor E.

polypeptides (seq1: 62%; seq2: 46.43%; seq3: 60.57%; seq5: 58.31%), except of seq4 where helix (42.76%) was predominant. Next, 3D homology modeling was performed using the powerful Robetta web server, where a higher confidence score demonstrates a more precise modeling. In this sense, Toxo-App model 1 (confidence: 0.31), Toxo-Apfn model 2 (confidence: 0.43), Toxo-App2 model 1 (confidence: 0.46), Toxo-Ribos model 1 (confidence: 0.47), and Toxo-RpfE model 1 (confidence: 0.51) were selected as the best 3D models for further analysis (**Supplementary Figs. 1-7**).

Immune response induction analysis

Major immune-related parameters that are important for immunity against *T. gondii* infection, such as cytokine induction, antibodies and B-lymphocytes, T cytotoxic cell population, and Th cell population, were assessed for each vaccine candidate using the C-ImmSim web server. Two cytokines involved in the immunity against the infection, IFN-γ (range: 41,000 ng/mL [seq5] to 44,000 ng/mL [seq1]) and IL-2 (range: 23,000 ng/mL [seq5] to 39,000 ng/mL [seq1]), were adequately induced particularly by Toxo-App vaccine candidate. Immunoglobulin M (IgM) elicited during acute infection was predominant upon seq1 administration (13,000), followed by seq2 (9,000) and seq4 (5,300). Moreover, the highest total antibody titers (IgM + IgG) were predicted for seq1 (20,000), seq2 (12,000), and seq4 (8,000). Plasma B lymphocytes and memory B-cells were mostly predominant in seq1, seq2, and seq4, respectively. Also, the highest Tc and Th cell populations (per mm^3) belonged to seq1 (Toxo-App) vaccine candidate, rendering it as the most immunogenic among all other MEVCs (data not shown). Accordingly, based on our results, Toxo-App, Toxo-Apfn and Toxo-Ribos were selected for further 3D model refinement, and the best (Toxo-App) was docked with the human TLR-4. The results of the immune simulation for the Toxo-App are provided in **Fig. 3**.

Three-dimensional model refinement and validation

Based on the GalaxyRefine output, model #4 was the best refined model for Toxo-App 3D model, having the following evaluated parameters: GDT-HA of 0.9966, RMSD of 0.260, MolProbity of 2.121, Clash score of 15.0, no poor rotamers, and Rama favored of 93.2. Regarding Toxo-Apfn model #1 was selected with GDT-HA of 0.9870, RMSD of

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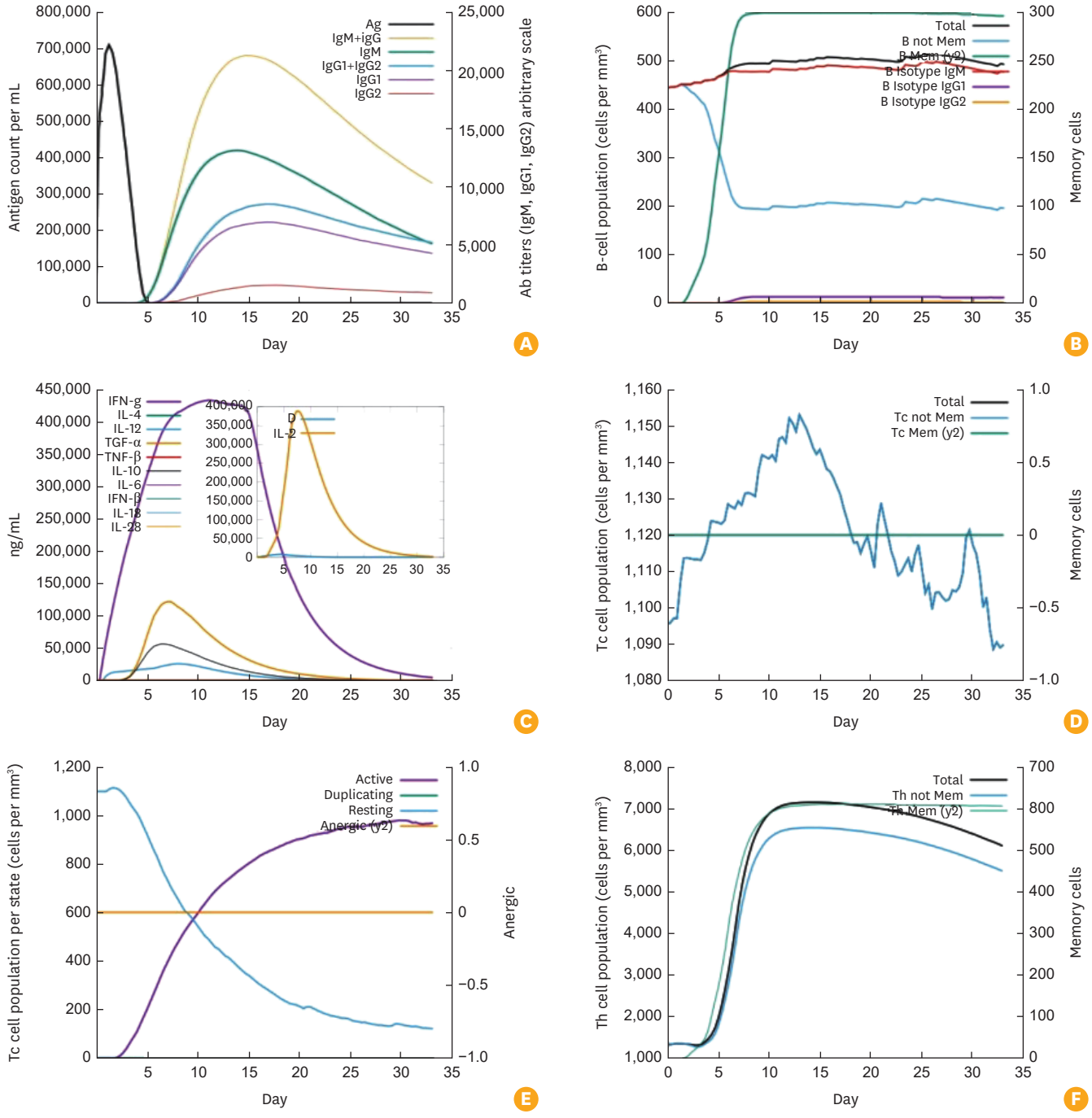

Fig. 3. The immune responses elicited by the highly immunogenic Toxo-App (Seq1) vaccine candidate. Dynamics of antibody responses (A), B-cell population (B), cytokines (C), cytotoxic T-lymphocyte count (D) and per state (E), as well as helper T-lymphocytes (F).
Ag, antigen; Ig, immunoglobulin; Th, T helper; Tc, cytotoxic T; INF, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

0.295, MolProbity of 2.151, Clash score of 17.0, poor rotamers of 0.4, and Rama favored of 93.6. Also, model #1 was the best rehashed 3D model for Toxo-Ribos vaccine candidate, with GDT-HA of 0.9910, RMSD of 0.270, MolProbity of 2.068, Clash score of 12.1, no poor rotamers, and Rama favored of 92.4. The quality improvement for the Toxo-App candidate between crude and refined models was evaluated using the PROCHECK web tool; on this basis, the amino acid distribution in specific regions between crude and refined models for the seq1 were as follows: 78.2% vs. 85.2% (the most favored regions), 19% vs. 12.7% (additionally favored regions), 2.1% vs. 0.7% (generously allowed regions), and

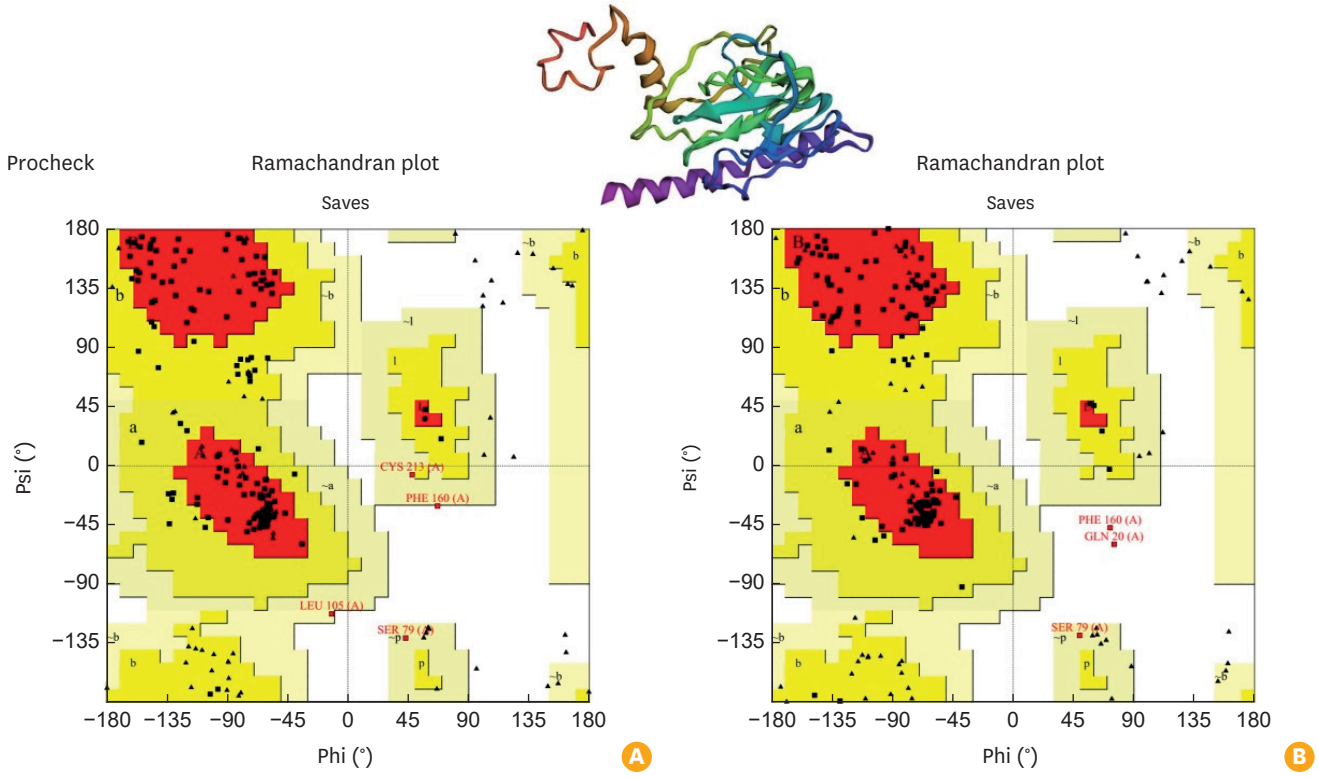

Fig. 4. Three-dimensional model validation of the Toxo-App using Ramachandran plot analysis by PROCHECK web tool. (A) The amino acid distribution in the crude model in the most favored, additionally favored regions, generously allowed regions, and disallowed regions were 78.2%, 19%, 2.1%, and 0.7%, respectively. (B) These parameters were improved in the refined model, as follows: 85.2% (the most favored regions), 12.7% (additionally favored regions), 0.7% (generously allowed regions), and 1.4% (disallowed regions).

0.7% vs. 1.4% (disallowed regions) (**Fig. 4**). Further details are tabulated in **Table 3**.

Molecular docking and MD simulation for Toxo-App candidate

Based on the HDOCK server, top-ten docked models were predicted regarding Toxo-App and human TLR-4. The docking scores of models 1 to 10 were as follows: –306.04, –282.47, –282.38, –275.07, –273.48, –271.81, –267.36, –266.86, –266.29, and –266.09. Moreover, the highest and lowest confidence scores belonged to model 1 (0.9577) and model 10 (0.9107), respectively. The model 1 was the best docking pose that had the lowest energy, hence it was selected for MD analysis. The likely protein-protein interactions in the selected pose are illustrated in **Figs. 5** and **6**.

The estimated average temperature for the selected complex was 303.15 K (range: 296–310 K), average potential energy of –2,923,024.156 KJ/mol, average total energy of –2,347,317.461 KJ/mol, and average pressure of 0.82 bar (range: –400 to +400 bar) during 100 ns. The RMSD plot of the multi-epitope vaccine demonstrated a sharply increasing trend from 0.15 to 0.56 nm, followed by a relative plateau until 100 ns, because of receptor's flexibility. Also, the human receptor's RMSD plot indicated an upward trend from

Table 3. Comparative amino acid distribution of the crude and refined 3D models of the most immunogenic designed MEVCs using the PROCHECK tool of the SAVES 6.0 server

Immunogenic MEVCs	Amino acid distribution in the crude 3D model				Amino acid distribution in the refined 3D model			
	MA	AA	GA	DA	MA	AA	GA	DA
Toxo-App (Seq1)	111 (78.2)	27 (19)	3 (2.1)	1 (0.7)	121 (85.2)	18 (12.7)	1 (0.7)	2 (1.4)
Toxo-Apfn (Seq2)	249 (87.7)	26 (9.2)	6 (2.1)	3 (1.1)	256 (90.1)	20 (7)	3 (1.1)	5 (1.8)
Toxo-Ribos (Seq4)	201 (78.8)	48 (18.8)	3 (1.2)	3 (1.2)	210 (82.4)	39 (15.3)	3 (1.2)	3 (1.2)

Values are presented as number (%).
MEVC, multi-epitope vaccine candidate; 3D, three-dimensional; MA, mostly allowed; AA, additionally allowed; GA, generously allowed; DA, disallowed.

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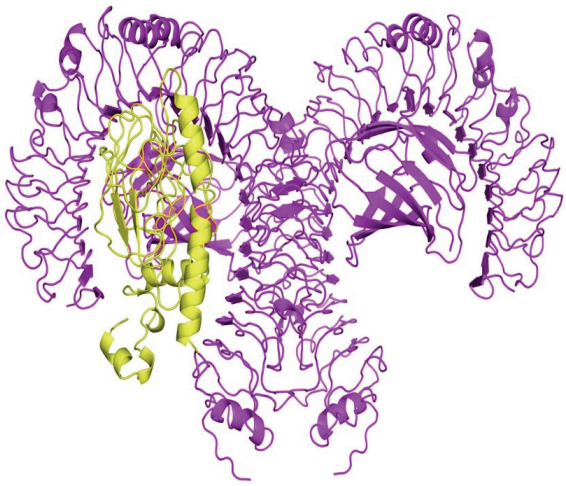

Fig. 5. The best docking pose for Toxo-App (yellow) and human TLR-4 (violet) complex using PyMol, representing a docking score of –306.04 and confidence score of 0.9577.

about 0.2 nm to 0.42 nm. In total, The RMSD of the receptor showed lower values than the ligand protein. The amount of RMSD for both structures gradually increases over time and finally reaches relative equilibrium from about 50 ns to the end of the simulation with low fluctuations. The average RMSD value was 0.31 for the protein and 0.53 for the ligand. In the root mean square fluctuation (RMSF) graph, the peaks demonstrate the regions with the most fluctuation in the entire simulation. Most fluctuations are observed in the N-terminal, C-terminal regions, as well as loops and coils of the protein, because these regions are highly flexible in terms of structure. The average RMSF of the protein during the simulation was 0.113 nm for the receptor and 0.145 nm for the ligand. The average total radius of gyration (Rg) value for the protein during the simulation was calculated to be 4.15 nm and was associated with a gentle decreasing slope during the simulation. Also, the average total Rg value for the ligand protein during the simulation was calculated to be 1.72 nm. A higher Rg is associated with increased instability of the system, while a lower Rg indicates the compact nature of the complex. In the following, hydrogen bonds were analyzed to show the dynamic equilibrium of the complex. Hydrogen bonds were formed within 0.35 nm. It was found that there is a hydrogen bond network between the components, which varies between 10 and 28 bonds. These links were formed and broken due to angle and distance fluctuations during the simulation. A schematic picture of the hydrophobic interactions between the protein and the ligand was simulated by LigPlot+ program, during which hydrogen and hydrophobic bonds are shown in green and red, respectively. The chain E is associated with the ligand protein and chains B and D are related to the receptor protein (**Fig. 7**, **Supplementary Figs. 1-7**).

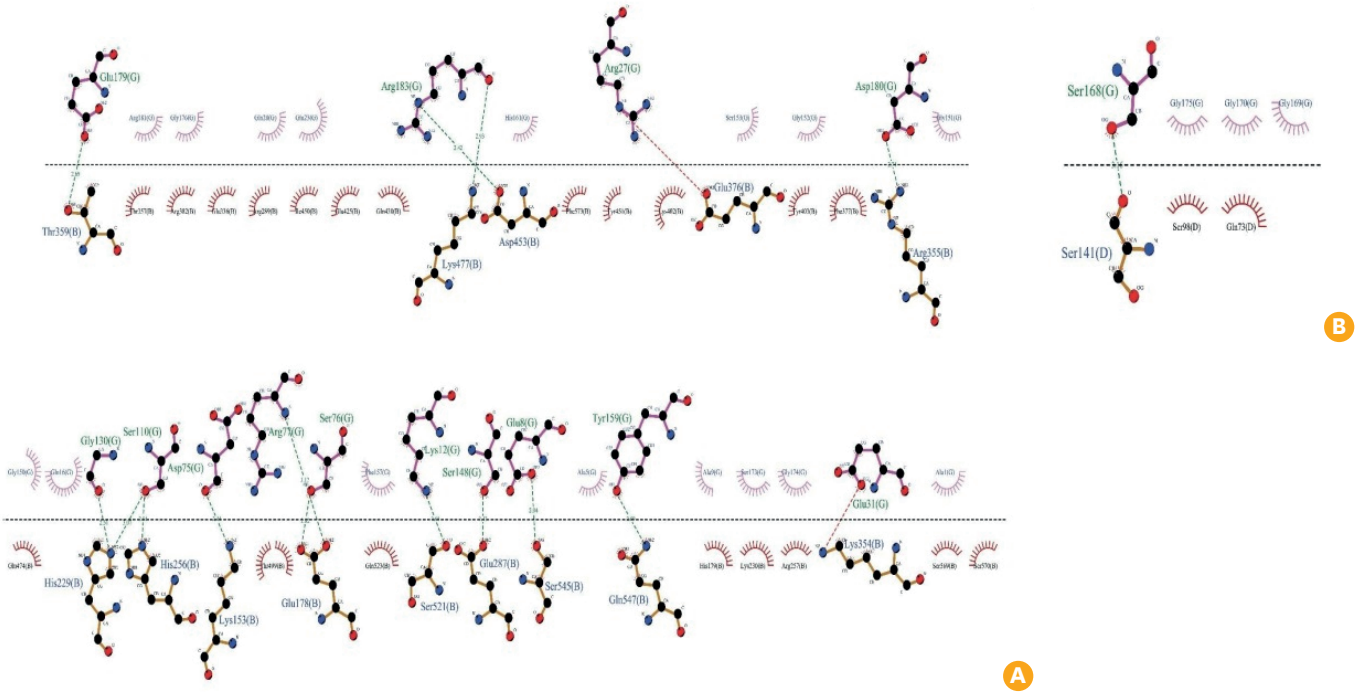

Fig. 6. Interactions between the docked ligand-receptor visualized using DimPlot software. (A) Interaction of the ligand with receptor chain B. (B) Interaction of the ligand with receptor chain D.

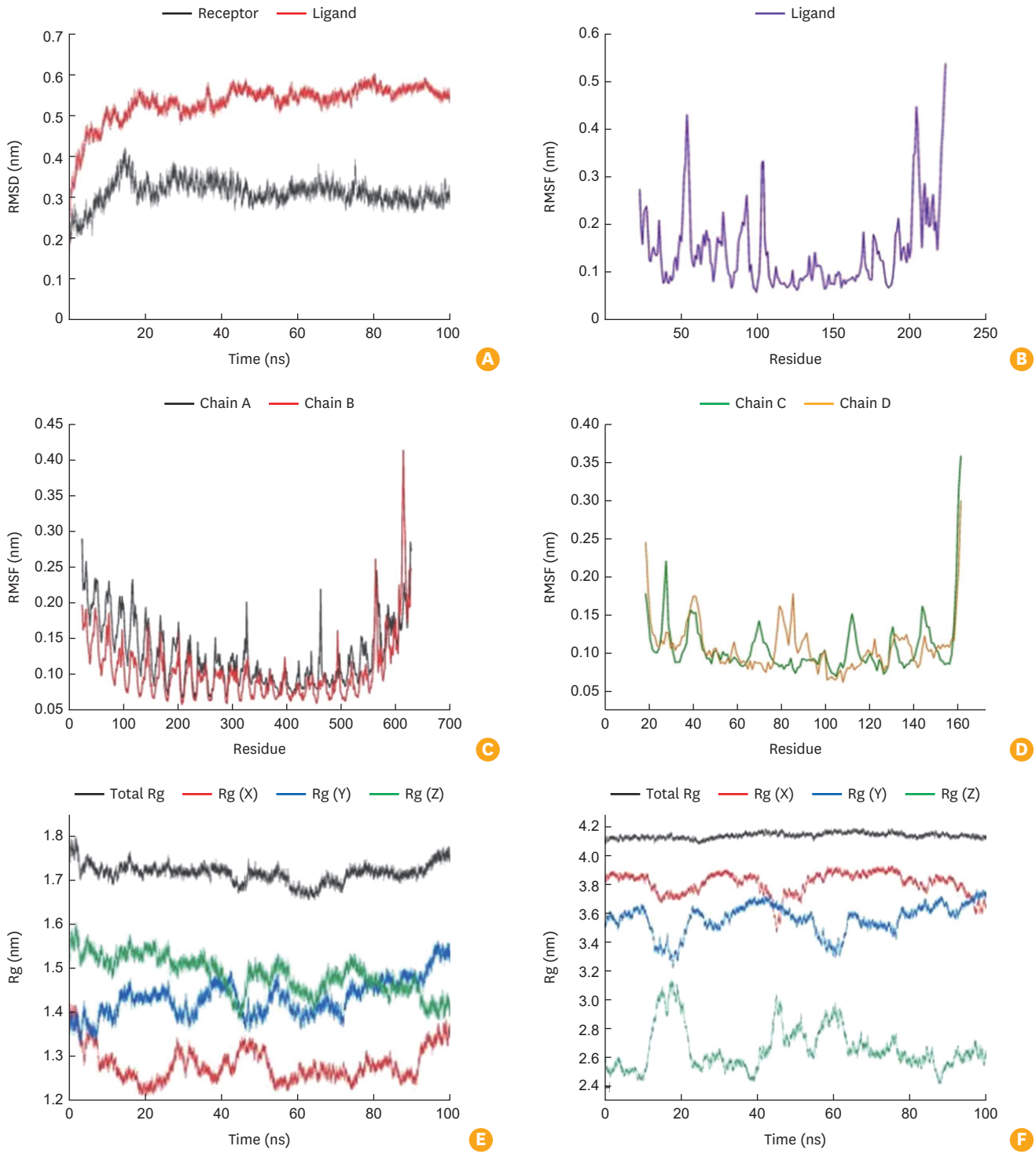

Fig. 7. The molecular dynamics simulation study for the docked complex (Toxo-App and toll-like receptor-4), representing RMSD (A), RMSF of the ligand (B) and those in association with the receptor (C, D), Rg of the ligand (E) and the receptor (F).
RMSD, root mean square deviation; RMSF, root mean square fluctuation; Rg, radius of gyration.

Vaccine safety and *in silico* cloning

The Toxo-App vaccine candidate which showed the highest immunogenicity among all candidates and was shown to be safe for human use, as evidenced by no homology with the human proteome using the BLASTp online tool. The reverse-translated "Toxo-App" vaccine model was codon

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optimized for enhanced expression in *E. coli* K12 strain using the JCat web tool. The CAI-value and GC% of the crude DNA sequence of Toxo-App were 0.57 and 75.41%, which were improved to 1 and 58.06% in the codon-adapted vaccine model.

DISCUSSION

The development of vaccines against toxoplasmosis has been the subject of extensive research over the past 30 years, employing a variety of vaccination techniques, including live-attenuated or inactivated vaccines, subunit vaccines, and vector-based ones. Toxoplasmosis-related public health concerns still include the prevention of congenital transmission and the total eradication of tissue cysts [14]. Toxovax®, the only vaccine currently in use, provides exceptional immunity in ewes through the use of live, attenuated tachyzoites in prevention of congenital infection; however, safety concerns preclude its application in humans [39]. Therefore, when designing a vaccine against *T. gondii* infection in humans, a subunit or multi-epitope-based vaccine candidates are more preferred. Reverse vaccinology and immunomics are 2 recent developments that have improved the identification of probable antigenic targets and immunogenic epitopes, resulting in a more logical approach towards vaccine design with higher efficacy, stability and safety [40]. Since *T. gondii* is a ubiquitous parasite invading different tissues and nucleated cells, both arms of the acquired immunity are involved in limiting the infection. Different membrane proteins of *Toxoplasma* are in close contact with the immune compartments, hence they may be eligible to be considered as potent vaccine candidates [41], including SAGs, transmembrane proteins and surface lipoproteins, ion channels, vesicle-associated protein, aquaporins, nucleoside triphosphatase, Streptococcal SAG repeat-containing protein, N-Methyl-D-aspartate receptor-associated protein, GOLD domain-containing protein, copper transporter, and many other proteins [42]. The hypothesis behind the current study was originated from a previous study by Li et al. (2022) [19], who deeply explored 314 membranous proteins of *T. gondii* during an *in silico* genome-wide analysis and proposed a crude vaccine construct based on the best B-cell and HLA-associated epitopes out of 8 screened proteins. In the present study, this proposed construct was further complemented using appropriate adjuvant and his-tag. In the meanwhile, we proposed 4 other MEVCs using a set of adjuvants and spacers and analyzed using a various of immunoinformatics web tools.

As mentioned, 5 MEVCs were engineered and designed in the present study using appropriate linkers and adjuvants. The main structure of all candidates relied on 3 B-cell epitopes (VTC domain-containing protein$_{136\text{-}154}$, Ion channel protein$_{2016\text{-}2031}$, and chloride transporter, ClC family protein$_{990\text{-}1008}$), 3 T-cell HLA-I epitopes (Guanylate cyclase$_{4018\text{-}4026}$, SAG-related sequence protein$_{352\text{-}360}$, and Ion channel protein$_{2943\text{-}2951}$), and 2 T-cell HLA-II epitopes (family UPF0121 protein$_{301\text{-}315}$ and putative transmembrane protein$_{480\text{-}494}$), being connected with linkers (spacers). Suitable linkers are an essential component of the multi-epitope vaccine design. The flexibility of the polyprotein, correct folding, and separation of functional domains—all of which contribute to a more stable protein structure—are all made possible by linkers or spacers [43]. The "AAY" linker improves epitope presentation and are considered as the proteasome cleavage site in mammalian cells [44]. Furthermore, a glycine-rich linker called "GPGPG" offers flexibility, free activity, and high accessibility for neighboring domains in addition to improving the construct's solubility [45]. Due to its polar amino acid composition (Gly and Ser) and small size, the "GGGGS" linker is a great option for fusion protein domains requiring particular movements or interactions, offering good flexibility and solubility. The "KK" spacer is a cathepsin B target, a critical protease enzyme during processing the antigen [46]. The "GDGDG" has been known as a flexible linker used to join both B-cell and T-cell epitopes [47]. Four candidates possessed an adjuvant to enhance their immunogenicity upon injection. Of note, Seq2 (Toxo-Apfn) had a double-adjuvant structure using RS-09 peptide at the N-termini and the human IFN-γ at the C-termini. The adjuvant sequence was connected to the vaccine sequence using a rigid linker, "EAAAK" [48]. Numerous genetic adjuvants are present as innate immune enhancers and they are selected based on the demanded type of immune responses. Herein, 4 adjuvant sequences, including a synthetic peptide (RS-09), *M. tuberculosis* RpfE, *M. tuberculosis* 50S ribosomal protein, and human IFN-γ, were used. The first 3 are known as TLR-4 agonists, capable to bridge towards a Th1-biased immune response. The RS-09 peptide, used in many studies [49,50], acts as a co-stimulator of CTL epitopes and provides a robust immune response [51]. Also, specific *M. tuberculosis* components are involved in dendritic cell maturation [52]. Moreover, the IFN-γ as an adjuvant has been used in previous studies [53-55].

It was discovered that the final length of the proposed MEVCs in the present study was 221 (Toxo-App), 364 (Toxo-Apfn), 175 (Toxo-App2), 304 (Toxo-Ribos), and 331 (Toxo-RpfE) residues. Physico-chemical properties and well-established immune induction are essential for a

promising vaccine candidate during the production phase. Based on this, physico-chemical assessment was done using the ProtParam web server. The lowest MW belonged to Toxo-App2 with 18.26 kDa, while the highest MW was in relation with Toxo-Apfn (about 38 kDa). All candidates were shown to be hydrophilic (negative GRAVY) and moderately thermophilic in nature based on their aliphatic index. Also, 2 candidates (Seq2 and Seq5) were recognized as unstable in the laboratory settings, based on >40 instability index. All candidates were shown to be non-allergenic using the AllerTOP v2.0 and AllergenFP v1.0 servers. The highest and lowest antigenicity was associated with Toxo-App (VaxiJen score: 1.8151) and Toxo-Ribos (VaxiJen score: 0.889). The latter candidate had the highest solubility (0.889), followed by Toxo-App (0.532). Based on the secondary structure analysis, random coils were highly abundant in almost all candidates (except of Toxo-Ribos).

In the current study, 3 out of 5 MEVCs were selected for further tertiary model refinement, based on their immune profile upon injection using C-ImmSim virtual immune simulation. Actually, as compared with other constructs, Toxo-App, Toxo-Apfn and Toxo-Ribos demonstrated high B-cell induction with humoral (IgG + IgM) responses, upregulation of Th1-biased cytokines (IFN-γ and IL-2), and memory Th and Tc cells. The immunogenic properties of the adjuvants—the synthetic peptide RS-09, human IFN-γ, and the *M. tuberculosis* RpfE—as strong immune enhancers, may contribute to the higher immunity elicited by these 3 vaccine candidates. Using the GalaxyRefine server, the 3D structures of 3 chosen MEVCs were rehashed regarding total and regional structural relaxations. The Ramachandran plot analysis performed using the PROCHECK tool and resulted in proper results, showing improvements in the refined models comparable to the crude models.

Since Toxo-Apfn was shown to be unstable in nature and Toxo-Ribos had the lowest immune induction among 2 other selected candidates, Toxo-App (Seq1) was chosen for protein-protein docking using HDOCK powerful docking server. It is a widely used online server for end-to-end protein-protein docking that uses a hybrid approach that combines both template-based and template-free methods to generate high-quality docking predictions, and is particularly useful for predicting protein interactions involving flexible regions. Several studies have shown that HDOCK has comparable or even better performance than other advanced docking servers such as ZDOCK, PatchDock, and HADDOCK. Furthermore, HDOCK has been successfully used to predict the structure of protein complexes involved in various biological processes [35]. The server provided top-ten docked models for the Toxo-App/human TLR-4 complex, among which model 1 possessed the lowest energy –306.04 and the highest confidence of prediction (0.9577). Thus, it was selected for a 100ns MD analysis. The polypeptide chain deviations after docking and during simulation were displayed as RMSD plots. Based on the output of RMSD plots, the stability of the vaccine-TLR4 complex was substantiated during the MD simulation. In order to identify the most flexible residues, the RMSF analysis was also performed; in general, the majority of the regions within analyzed complex were fairly stiff and stable, especially the internal residues. An average of 19 hydrogen bonds (range: 10–28) between both proteins showed a highly stable complex. It was significant that the final vaccine candidate (Toxo-App) displayed no homology to the human proteome, hence deemed safe for use in humans. In order to achieve an optimized expression yield for the recombinant proteins, codon adaptation was done with improvements in transcriptional and translational efficiency. Accordingly, using the JCat server, the total GC content and CAI value of Toxo-App candidate were evaluated in relation to the *E. coli* (strain K12). The final step involved the addition of the *Eco53KI* and *EcoRV* restriction nucleases for a successful ligation of the proposed MEVC into the appropriate plasmid vectors, pET28a(+) vector for instance, paving the way for the production of the vaccine in the future.

The development of vaccines against *T. gondii* is challenging due to its diverse strains, resulting in different virulence factors, antigenic profiles, and immune evasion mechanisms. To ensure broad protection and effectiveness, vaccines must use different strains, identify conserved antigens, tailor vaccines to specific geographic locations or demographics, and avoid antigens that may induce immune evasion or exacerbate disease symptoms. Computational vaccinology can assist us in prediction of such parameters. In the current *in silico* study, 5 MEVCs were designed based on the most immunodominant B- and T-cell epitopes of *T. gondii* membrane proteins. Critical items such as safety, antigenicity, allergenicity, vaccine adjuvanticity, immune response profile, and codon usage were checked using web-based tools. Molecular docking and dynamic simulations provided insights into the vaccine candidate's interactions with human TLR4 as ligand and receptor. However, experimental validation is needed to provide a more realistic understanding of vaccine interactions with the immune system and cell types *in vivo*.

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

SUPPLEMENTARY MATERIALS

Supplementary Fig. 1
Secondary structure analysis of 5 multi-epitope vaccine candidates against *Toxoplasma gondii* infection using B- and T-cell epitopes derived from membrane proteins. (A) Toxo-App; (B) Toxo-Apfn; (C) Toxo-App2; (D) Toxo-Ribos; (E) Toxo-RpfE.

Supplementary Fig. 2
Three-dimensional models predicted for Seq2 (Toxo-Apfn) and Seq4 (Toxo-RpfE) vaccine candidates.

Supplementary Fig. 3
The trend of temperature during molecular dynamics simulation.

Supplementary Fig. 4
The trend of pressure during molecular dynamics simulation.

Supplementary Fig. 5
The trend of potential energy during molecular dynamics simulation.

Supplementary Fig. 6
The trend of total energy during molecular dynamics simulation.

Supplementary Fig. 7
The number of hydrogen bonds per 100 ns of molecular dynamics simulation.

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