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Exploring advanced genomic and immunoinformatics techniques for identifying drug and vaccine targets against SARS-CoV-2

Syed Luqman Ali [a,1,*], Awais Ali [a,1,*], Waseef Ullah [a], Abdulaziz Alamri [b,*], Elham Mohammed Khatrawi [c], Gulzira Sagimova [d], Aigul Almabayeva [d], Farida Rakhimzhanova [e], Gulsum Askarova [f], Fatima Suleimenova [d], Nabras Al-Mahrami [g], Prasanta Kumar Parida [h]

[a] Department of Biochemistry, Abdul Wali Khan University Mardan (AWKUM), Mardan 23200, Paksitan
[b] Department of Biochemistry, College of Science, King Saud University, Riyadh 11451, Saudi Arabia
[c] Department of Medical Microbiology and Immunology, Taibah University, College of Medicine, Madinah 42353, Saudi Arabia
[d] Department of Human Anatomy, Astana Medical University, Astana 010000, Kazakhstan
[e] Department of Microbiology, Semey Medical University, Semey, Kazakhstan
[f] Department of Clinical Disciplines, Al Farabi Kazakh National University, Almaty 050000, Kazakhstan
[g] Bioinformatician, National Genetic Center, Royal Hospital, Ministry of Health, Muscat, Oman
[h] Department of KIIT School of Rural Management, KIIT University, Bhubaneswar 751024, India

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ABSTRACT

The coronavirus that causes serious acute respiratory syndrome. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is still a major problem in public health and biomedicine. Even if there is no cure for it, the infection is still progressing naturally, and the only time that optimal treatment choices, such as doxycycline, work is at the beginning of the infection. Our project is structured into two critical parts: the first focuses on the identification of potential drug targets, and the second on vaccine design, both aimed at exploring new ways to treat the disease. Initially, cytoplasmic proteins identified through subtractive analysis underwent comprehensive evaluation for potential drug targeting, focusing on metabolic pathways, homology prediction, drugability assessment, essentiality, and protein–protein interactions. Subsequently, surface proteins underwent rigorous assessment for allergenicity, antigenicity, physiochemical attributes, conserved regions, protein interactions, and identification of B and T cell epitopes. Molecular docking and immunological simulation analyses were then employed to develop and characterize a multi-epitope vaccine, integrating findings from the aforementioned evaluations. Findings from the study point to six proteins as potential critical therapeutic targets for SARS-CoV-2, each of which is involved in a distinct metabolic process. The reverse vaccinology analysis suggested that the following proteins could be used as vaccine candidates: sp|P05106, sp|O00187, sp|Q9NYK1, sp|P05556, sp|P09958, and sp|Q9HC29. Four multi-epitope vaccine named as SARS-COV-2-, C1, C2, C3, and C4 was designed by utilizing different adjuvants and eighteen B cell overlapped epitopes which were predicted from top ranked protiens. Based on immune simulation study, the vaccine exhibited adequate immune-reactivity and favorable encounters with toll-type receptors (TLR4, TLR8, HLA, etc ACE), Among them the SARS-COV-2-C2 showed best binding affinity of which all receptors. Findings from this study could be a game-changer in the quest to develop a vaccine and medication that effectively combat SARS-CoV-2. It is necessary to do additional experimental analyses, nevertheless.

* Corresponding authors.
E-mail addresses: syedluqmanali5@gmail.com, luqmanali@awkum.edu.pk (S. Luqman Ali), awaisali@awkum.edu.pk, awaisalibio@gmail.com (A. Ali), abalamri@ksu.edu.sa (A. Alamri), ekhatrawi@taibahu.edu.sa (E. Mohammed Khatrawi), sagimova.g@amu.kz (G. Sagimova), almabaeva.a@amu.kz (A. Almabayeva), farida.rakhimzhanova@smu.edu.kz (F. Rakhimzhanova), gkaskarova@mail.ru (G. Askarova), suleymenova.f@amu.kz (F. Suleimenova).
[1] These authors contributed equally to this work.

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1. Introduction

According to S. Mahmud et al. (2021), the SARS-COV-2 coronavirus is responsible for the extremely infectious and potentially fatal sickness known as severe acute lung disease (SARS). After making its debut in late 2002 in Asia, the virus quickly moved to other regions of the globe and in 2019 caused a worldwide outbreak. Acute respiratory distress and severe respiratory symptoms are the hallmarks of severe acute respiratory syndrome, which is where the name "Severe Rapid Respiratory Syndrome" comes from.[43].

The coronavirus family, of which SARS-CoV-2 is a member, contains other respiratory disease viruses in humans, like the common cold has.[50] Infected people with SARS-CoV-2 can develop severe upper respiratory infection and pneumonia, in contrast to the mild symptoms usually seen with a common cold. The fast transmission of the virus from one person to another, most commonly in medical settings or through intimate contact with a person with the virus is one of its defining features. Respiratory droplets, released when an infected person coughs or sneezes, are the principal vector for the transmission of SARS. Additionally, indirect touch transmission is conceivable due to the potential contamination of surfaces and objects by these microscopic droplets. The virus can be transmitted more easily to those who are in close proximity to an infected person's respiratory secretions. In order to restrict the spread of the disease, strict infection control measures, such as isolation and quarantine protocols, are crucial, especially considering this mechanism of transmission.

Medical professionals and scientists around the world took action in response to the SARS-CoV-2pandemic. Crucial to successfully managing the outbreak were advancements in diagnostic testing, treatment tactics, and public health measures. Additional coronavirus outbreaks, such as the SARS-CoV-2 caused COVID-19 pandemic, have been better understood and combatted thanks to the insights obtained from the SARS epidemic.

Finding novel therapeutic options with the outdated disease-focused approaches to drug discovery is very time- and money-consuming. Some studies reported the limitations on drug target discovery time have been significantly reduced by advances in computational approaches.[29,31] Computational approaches like subtractive genomics and comparative microbial genomes have shown promise in the search for essential targets in several human diseases.[41] By discovering genes or proteins required by the pathogen but lacking counterparts in the host, subtracting and comparative genomics approaches provide potential treatment targets (M. I. Khan et al., 2020). Reverse vaccinology also improves vaccine target discovery and treatment target prediction for intracellular bacteria (like SARS-CoV-2) and other challenging-to-cultivate pathogens. Compared to conventional vaccines, which often face significant development delays and rely on in vitro culture models, epitope-based vaccine prediction allows for faster targeting of immune protein molecules across a bacterial or viral proteome.[16,42,58] Using specific features like subcellular localization, structure, as well as epitopes, bonding and antigenicity probability, and ties to major the ability complex (MHC) class I and class II molecules, the reverse vaccinology method has proven useful in prioritizing and developing vaccine targets against a wide range of pathogens.[4,9,36,39,41,45]

To identify potential therapeutic and vaccine targets inside SARS-COV-2, we used a comprehensive approach in our study that combined subtractive genomics, immuno-informatics, and reverse vaccination techniques. Finding vital and survival proteins that are unique to the pathogen and have no resemblance to the host or host microbiome was our main goal. In order to ensure non-homology, we simultaneously undertook a thorough investigation of the human microbiota and screened for B- or T-cell epitopes as well as Surface proteins. Our discovery of the top-ranking protein and epitopes could help future in vitro and in vivo investigations, which could lead to the creation of efficient medications and vaccines to prevent intracellular SARS-CoV-2 infections.

2. Methodology

2.1. Data filtration and druggability analysis

2.1.1. Whole proteome retrieval

The whole SARS-COV-2proteome, including 11,742 proteins, was obtained from UniProt of different samples from different locations which were then Submitted to CD-Hit Suite analysis (https://weizhong-lab.ucsd.edu/cdhit-web-server/cgi-bin/index.cgi).[17] Using a threshold score of 0.6 for sequence identity, duplicate sequences with 60% identical were effectively removed.. The subsequent analysis focused exclusively on non-paralogous protein sequences, ensuring a refined dataset for in-depth investigation, as illustrated in Fig. 1.

2.1.2. Identification of non-homologous human proteins

Conducting a non-homology analysis for SARS-CoV-2aims to pinpoint pathogen-specific proteins with no homology to the human genome. Through BLASTp analysis, non-paralogous proteins were scrutinized against the *Homo sapiens* reference proteome, employing a defined threshold expectation value (E value).[33] Selecting only non-homologous protein sequences post-filtering for significant hits, we meticulously screened for any potential functional resemblance with the human genome. This strategic step aims to minimize unwanted cross-reactivity of drugs, ensuring they do not bind to active sites of homologous human proteins. By prioritizing non-homologous sequences, we enhance the specificity of drug interactions and mitigate the risk of unintended physiological effects.[51]

2.1.3. Pathway analysis and prediction of hypothetical proteins

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) provides a graphically rich understanding of biological processes in living organisms by revealing complex metabolic pathways.[23] We thoroughly assembled the metabolic pathways of the pathogen (SARS-CoV-2) and the host (H. sapiens) using the three-letter KEGG organism codes. Using a manual comparison, the KEGG pathway database revealed distinct pathways that are specific to SARS-CoV-2. By identifying specific pathogenic pathways, our targeted analysis improves our understanding of SARS-CoV-2metabolism.[13] Some pathways were classified as unique, while others were categorised as common. Using BLASTp via the KEGG Automatically Annotation Server (KAAS), probable therapeutic and vaccination targets among the anticipated human non-homologous, essential amino acids of SARS-CoV-2were found. This procedure uses BLAST analyses against the entire KEGG GENES database to annotate the functional roles of genes, emphasising metabolic proteins with KEGG Orthology (KO) designations. KEGG circuits showcasing specific metabolic proteins, our research moves on to the next phase of subcellular localization estimation. Finding proteins involved in these particular pathogen metabolic pathways, as well as those assigned to KO but not taking part in specified pathways, eliminates proteins involved in common pathways between humans and pathogens.

2.1.4. Prediction of subcellular localization

SARS-COV-2proteins can be recognized in two key subcellular locations within host cells, the cytoplasm during viral replication and the extracellular space when the virus is released from the host cell. CELLO v.2.5 (https://cello.life.nctu.edu.tw/) was used to estimate the locations of non-homologous essential pathogen proteins. This server calculated and generated the best score for each location.[64] The proteins' final locations underwent a three-step assessment: firstly, if consensus existed among all three servers regarding a protein's location, that determination was chosen as the last result; secondly, two servers concurred on a location, It was chosen as the ultimate outcome; and thirdly, if three different locations were predicted, the PSLpred server was consulted (https://webs.iiitd.edu.in/raghava/pslpred/index.html), and the predicted location was cross-referenced with CELLO v.2.5′s prior results. This systematic approach ensured a robust and reliable determination of the

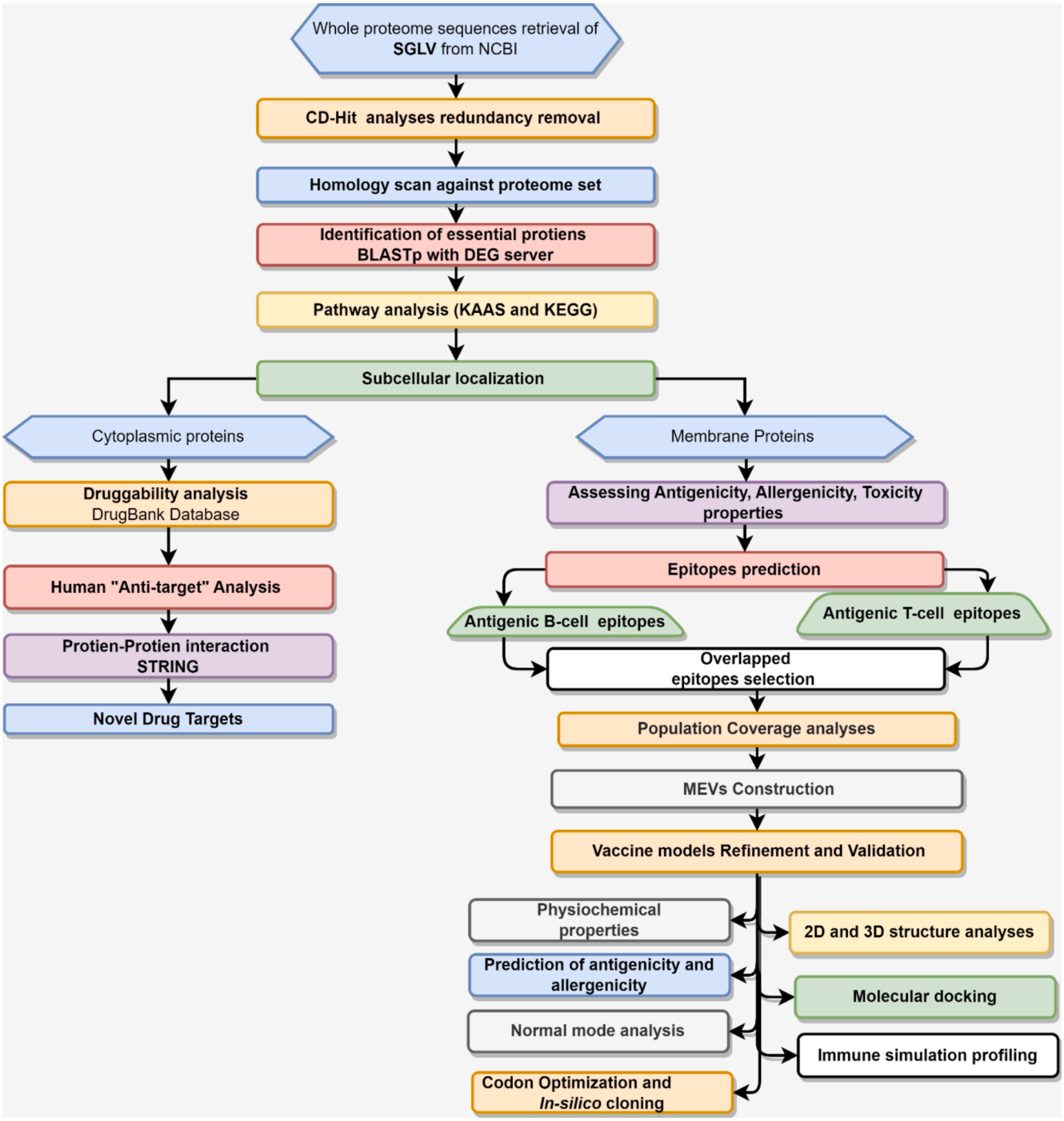

Fig. 1. Methodological frame work of druggability analysis and vaccine designing.

proteins' ultimate locations.[5]

2.1.5. Draggability analysis

Conducting draggability analysis, we assessed non-homologous cytoplasmic proteins against the entire spectrum of current FDA-approved drug targets available in the DrugBank 5.0 database. (https://go.drugbank.com/).[60] Conducting a BLASTp analysis aligned selected protein sequences with designated drug targets, identifying those with similarity in the DrugBank database as druggable targets. Conversely, proteins not meeting the threshold value were deemed novel drug targets. This approach efficiently differentiates potential drug targets, enhancing the strategic identification of novel candidates for drug targets.

2.1.6. Druggability screening and antigenicity analysis

An important aspect of finding "druggable" targets is testing how well they bind to drugs and compounds that look like medications. According to the criteria in practice, membrane and cytoplasmic proteins were shortlisted. They then were filtered by screening in DrugBank 5.1.0 database using these same default criteria therein (it is not clear what they might have been). As seen in the DrugBank database where both intracellular and extracellular proteins are listed as drug targets, this poses no obstacle to current therapeutic targets being consistent with established druggable features of shortlisted proteins. But their non-existence defines them as novel therapeutic targets, demonstrating that they are indeed novel. After the DrugBank research, proteins with no homology were designated as new therapeutic targets and potential

immunization candidates.Doytchinova & Flower,[14] report that VaxiJen v2.0 was also used to predict candidate protective antigens for sub-unit vaccines. Only those protein sequences of newly found SARS-COV2 therapeutic targets were used that are membrane proteins. The VaxiJen v 2.0 was run with a Cut off (threshold) value set at 0.4. Those that exceeded the antigenicity prediction criterion set by VaxiJen v2.0 proceeded into the next stage and were marked as potential vaccines.[14]

2.1.7. Prediction of Non–homologous essential proteins and shortlisted proteins

Utilising the Online Database of Essential Genes (DEG 15) (https://origin.tubic.org/deg/public/index.php/index) with identity=0.5, analysis was able to identify novel therapeutic targets, which is significant (Luo, Zeng, et al., 2021). Important gene products found in the DEG, which are based on experimental data, are necessary to the maintenance of cellular viability. Proteins that are believed to be possible new therapeutic drug targets and that are clinically valid in DrugBank were shortlisted. Lastly, the state-of-the-art STRING V1.1 (https://string-db.org/) web tool was expertly used to evaluate protein–protein interactions for potential therapeutic targets.[25,52]

2.2. Vaccine candidate designing

2.2.1. Selecting ideal vaccine Targets

Selecting surface proteins that elicit robust immune responses was made possible by analysing subcellular localization data from PSORTb v.3.0.2.[65] Proteins with a threshold of ≥0.4 were identified using creative antigen predicting tool VaxiJen,[14] while allerTOP 2.0 assessed the allergenicity of antigenic proteins (threshold ≥0.5). Our systematic approach successfully identifies intriguing antigen and non-allergen proteins with the aim of potentially creating subunit vaccines.[12]

2.2.2. T- and B-cell prediction

Using the FASTA-formatted transcripts and the ANN 4.0 technique, the IEDB MHC-I and MHCII Binding Predict Tool was utilized to forecast T-cell epitopes[58]. With humans designated as the host species, the output was in the form of XHTML tables. The original values were maintained for all other parameters. In order to locate MHC-II epitopes, the IEDB web tool for MHC II contact predictions was utilized in conjunction with the FASTA sequences submitted at the same time.[66] The ABCPred web tool used to predict B-cell epitopes. We looked at the molecular weight, calculated half-life, hypothesized the isoelectric points (pI), aliphatic index, and unstable index as physiochemical features of specific epitopes.[34] To learn about the molecular features they need for biological activities, this evaluation made use on the Expasy ProtParam database (https:), which supplied a rich set of data.[46,67]

2.2.3. Population coverage analysis

According to Vita et al.,[58] the chosen epitopes' population coverage was examined using the IEDB's population covering analysis webtool (http: //tools.iedb.org/population). We aimed to incorporate a wide range of populations by meticulously selecting MHC-I and the MHC- II epitopes that have been confirmed to be involved with B cell epitopes. An effective global vaccination can be designed with this strategic strategy, which provides a holistic picture.

2.2.4. Construction of the Multi-Epitope Vaccine, physicochemical and structural properties

Our study used strict criteria in designing a multiepitope vaccine against SARS-CoV-2. Adding linkers–EAAAK, GPGPG and GGGGGS–between the listed priority epitopes to raise expression levels, improve solubility as well as proper folding of the multiepitope protein structure. These linkers were all used either in isolation or combined, and went through comprehensive tests for antigenicity by AllerTOP[12]), toxicity by ToxinPred[47] and allergenicity by VaxiJen v 2.0.[14] This approach will help develop a strong, safe vaccine against the virus. In the end, a combination of linker was chosen and adjuvant applied to N-terminus EAAAK linker. As for these four vaccine candidates C1,C2,C3,C4, (Table S3, each was built using a different adjuvant–HBHA protein; β-defensin, and HBHA conserved peptide sequences.

First, the MEV was screened for homology with the human proteome but produced no matches. Antigenicity, allergenicity and toxicity of the MEV were thereafter carefully reviewed in accordance with Table 2. Moreover, our vaccine model turned out to be highly non-allergenic and nontoxic as well. Most importantly, its antigenicity was high. ProtParam was then used to explore the physicochemical aspects of vaccine construction, and its favorable characteristics were confirmed.

2.2.5. 2D and 3D structure prediction

With PSIPRED, the MEV secondary structure was designed[38]. The software also evaluated structural attributes of the vaccine such as alpha helices and beta twists. The tool uses an ANN machine learning algorithm to calculate the secondary structure of a vaccine construct from its primary sequence. In addition, the RNAfold tool in ViennaRNA Package 2.0 gives accurate prediction of the mR NA vaccine construct 's secondary structure[21], A more advanced version uses the McCaskill algorithm to find the centroid secondary structure. The construct is then analyzed in terms of its minimal free energy (MFE) as well[31]. This tool's predictive capabilities are vital to determine vaccine stability and folding patterns necessary for efficacy. Predict, refine and valid An Alphafold AI-based web-server we used with a simplified method to produce the best 3D tertiary structure for the vaccine. With this intricately designed structure that combines optimum stability and minimum energy, the protective accuracy is boosted, as is the precision of function prediction for vaccine constructed[56,68,69]. Here the homology modeling is used to predict 3D structural structure of protein from amino acid sequence. In order to refine and improve the constructs 3D structure of our vaccine, we used GalaxyRefine server. Next, its validity was tested by utilizing the results from RAMPAGE server; this ensures that our model is strong and accurate.

2.2.6. Molecular docking and molecular dynamic simulation

Molecular docking was used to evaluate the interactions between the multi-epitope vaccination and immunological receptors. TLRs are crucial for recognizing viral PAMPS. TLR4 identifies lipopolysaccharide (LPS) in Gram-negative bacteria, while TLR8 is known to recognize ssRNA as well as viral capsid proteins, enabling it to respond to diverse viral structures, including those used in the proposed protein-based vaccine[24]. HLA molecules on antigen-presenting cells (APCs) play a significant role in adaptive immune responses by presenting antigens to T cells, which then produce antibodies and other immune molecules. Additionally, SARS-CoV-2 uses ACE2R to enter host cells and trigger inflammatory cytokines. Our results show that the constructs bound well to all tested immune receptors, suggesting they could be effective adjuvants for vaccines against various diseases and pathogens[22]. The crystal structures of Toll-like receptors TLR4 (PDB ID: 4G8A), HLA (PDB ID: 5WJL), and ACE2R were retrieved. Thus, our work used HawkdocK to dock ligands with receptors[52,56], and it was confirmed using the HdocK docking webtool (Saleem Naz[49]; Y.[63], 2020).

The use of molecular dynamics into in-silico investigations to probe the stability of protein–protein complexes is essential. According to López-Blanco et al.,[30], a typical protein mobility exam can be conducted using NMA quantified through eigenvalues, covariance, B-factors, and deformability utilising the online server iMODS (NMA). What matters most is how deformable its main chain is, which determines the likelihood of deformation at each residue. Motion stiffness is indicated by an eigenvalue for the normal mode value; structures with smaller values are more prone to bending. The energy needed for deformation is directly proportional to these, but in the opposite direction. The result is a more comprehensive evaluation of stability for in-silico studies of proteins and their complex interactions.

2.2.7. Immune simulation

We used the C-ImmSim 10.1 computer, an advanced in silico immunity simulation method, to evaluate the developed MEV's immunological reactions[7,70]. A multi-protein a FASTA text file, PDB main IDs, or UniProt accession numbers are all acceptable ways for the user to supply the antigen. By utilising the C-ImmSim computer, we were able to replicate the thymus, bone marrow, and lymph node, three important mammalian systems, and model the immunological reactions that would occur in response to the intended vaccine formulation. The immunological simulations' parameters were selected with care to provide a realistic portrayal, yielding useful insights into the MEV's prospective efficacy.

2.2.8. Reverse translation, codon adaptation and in-silico cloning

For successful manufacturing of subunit vaccines, it is vital to maximise protein outputs within the E. coli production system. The JCat server and the Sequence Manipulation Suite's reverse translate tool were used for this purpose, together with codon optimisation[20]. The JCat service evaluates the codon adaptability index (CAI) & GC content, which are essential for host-based high-throughput expression. We optimised the pET28a(+) vector to increase the production of proteins in the E. coli K12 strain. The 5′ & 3′–OH of the codon-adapted vaccination sequence were carefully supplemented with start/stop codes and restriction positions for BlpI and EcoR1 enzymes to make cloning easier. This was done after evaluating probable slicing sites using the NEBcutter 2.0 server. The SnapGene v6.2 software was used to accomplish this in-silico cloning technique without any issues[6].

3. Results

3.1. Sequence retrieval and filtration

Employing genome-wide proteome exploration, this study utilized subtractive genomics and reverse vaccinology to identify novel drug and vaccine targets for SARS-CoV-2. The subtractive genomics approach, involving crosschecking with a list of human "anti-target" proteins, was complemented by screening against the human microbiome to avoid unintended physiological consequences. The successful sequences were then subjected to reverse vaccinology for potential vaccine design. Throughout the process, state-of-the-art bioinformatics approaches were employed, as illustrated in Fig. 1, with a comprehensive summary of methodologies and screened proteins in each step provided in Table 1. This streamlined workflow represents an innovative approach to discovering therapeutics against SARS-CoV-2.

Table 1
Subtractive genomic analysis scheme toward the identification of novel therapeutic targets.

Serial no.	Steps/description	SARS-CoV-2
01	Total no. of proteins	11,742
02	No. of non-paralogous proteins (.60 % identical) in CD-Hit	841
03	No. of proteins non-homologous to H. sapiens using BLASTp (E = 1023)	668
04	No. of proteins non-homologous to microbiome using BLASTp	616
05	Essential proteins in DEG 15.2 server (E # 102100, bit score 0.100)	349
06	Essential proteins involved only in unique metabolic pathways (KAAS at KEGG)	21
07	Proteins assigned KO (KEGG orthology)	250
08	Essential proteins found to be novel in DrugBank 5.1.0	10
09	Membrane proteins showing antigenicity using VaxiJen v2.0 (threshold value 0.0.4)	5
10	Proteins selected for epitope design	5
11	Novel drug targets	10

Table 2
Identified novel drug targets against SARS-COV-2.

Serial no.	Accession no.	Protein name
01	sp|P68104|EF1A1_HUMAN	Elongation factor 1-alpha
02	sp|P08865|RSSA_HUMAN	small ribosomal subunit protein uS2
03	sp|P29597|TYK2_HUMAN	Non-receptor tyrosine-protein kinase TYK2
04	sp|P31751|AKT2_HUMAN	RAC-beta serine/threonine-protein kinase
05	sp|P35232|PHB1_HUMAN	Prohibitin 1
06	sp|P49327|FAS_HUMAN	Fatty acid synthase

3.2. Proteome retrieval and finding paralogous sequence and identification of human non-homologous proteins

Utilizing the entire UniProt proteome (Proteome ID: UP000000609), consisting of 11,742 proteins, the CD-Hit tool effectively eliminated paralogous and duplicate sequences with a threshold value of 0.6, resulting in 841 non-paralogous protein sequences. BLASTp analysis against the human genome identified 868 non-homologous proteins, with only 349 displaying sequence similarity to essential proteins in the Database of Essential Genes (DEG). Remarkably, a mere 10 proteins among this subset were recognized as essential for the survival of SARS-CoV-2, offering valuable insights for targeted research and potential therapeutic strategies.

3.3. Metabolic pathway identification, druggability screening and antigenicity analysis

From the 150 identified SARS-CoV-2target pathways on the KEGG server, KAAS analysis revealed 150 proteins with assigned KEGG Orthology (KO) identifiers out of 350. Among the 134 proteins, only 5 participated in unique metabolic pathways (Table 2), while the rest had no involvement in either unique or common pathways (refer to Table S1). Notably, only 6 proteins from the entire set emerged as novel and experimental drug targets in the DrugBank 5.1.0 database (Table 2), exhibiting no hits and thus considered novel drug targets for SARS-CoV-2. Following druggability and antigenicity analysis, these proteins underwent screening for human "anti-target" and microbiome non-homologous characteristics. This multifaceted approach aims to uncover promising avenues for drug development against SARS-CoV-2.

3.4. "Anti-target" and human microbiome non-homology analysis

Minimizing cross-reaction and toxicity requires the identification of proteins called "anti-targets," which are important for human homeostasis but can cause negative medication reactions. The six new therapeutic proteins chosen for this investigation are completely unlike any known human "anti-targets." (Table 4) shows that out of 25 proteins analyzed using BLASTp, only five have a similarity level below 47% among the microbial strains included in the Human Microbiome Project. Because of their singularity, these proteins are attractive therapeutic development targets because they do not share any characteristics with human "anti-targets" or take part in typical host-pathogen pathways.

3.5. Protein-protein interaction studies

Thorough protein–protein interaction (PPI) analysis that identified interaction of six protiens (Table 2) as therapeutic targets for SARS-CoV-2. Protein production relies on GTP-dependent aminoacyl-tRNA attachment to ribosomes, and the "The elongation factor 1-the alpha" protein was a major component in this process, according to the research in STRING v11.5. This protein is an essential component of Th1 cytokine production and, when combined with PARP1 and TXK, functions as a transcription factor specific to Th1 cells. It binds to the IFN-gamma locus

Table 3
Identified druggable targets with drug names.

Serial no.	Accession no.	Protein name	Drug bank ID	Generic Name	**Groups**
01	sp|P08865| RSSA_HUMAN	Small ribosomal subunit protein uS2	DB04985	Tigapotide	Investigational
02	sp|P29597| TYK2_HUMAN	Non-receptor tyrosine-protein kinase TYK2	DB04716	2-*tert*-butyl-9-fluoro-1,6-dihydrobenzo[h]imidazo[4,5-f]isoquinolin-7-one	Experimental
03	sp|P31751| AKT2_HUMAN	RAC-beta serine/threonine-protein kinase	DB08073	(2S)-1-(1H-INDOL-3-YL)-3-{[5-(3-METHYL-1H-INDAZOL-5-YL) PYRIDIN-3-YL]OXY}PROPAN-2-AMINE	Experimental
04	sp|P31751| AKT2_HUMAN	RAC-beta serine/threonine-protein kinase	DB07859	4-(4-CHLOROPHENYL)-4-[4-(1H-PYRAZOL-4-YL)PHENYL]PIPERIDINE	Experimental
05	sp|P31751| AKT2_HUMAN	RAC-beta serine/threonine-protein kinase	DB07947	ISOQUINOLINE-5-SULFONIC ACID (2-(2-(4-CHLOROBENZYLOXY) ETHYLAMINO)ETHYL)AMIDE	Experimental
06	sp|P31751| AKT2_HUMAN	RAC-beta serine/threonine-protein kinase	DB07812	N-[(1S)-2-amino-1-phenylethyl]-5-(1H-pyrrolo[2,3-b]pyridin-4-yl) thiophene-2-carboxamide	Experimental
07	sp|P31751| AKT2_HUMAN	RAC-beta serine/threonine-protein kinase	DB15496	Didesmethylrocaglamide	Experimental
08	sp|P35232| PHB1_HUMAN	Prohibitin	DB15495	Rocaglamide	Experimental
09	sp|P49327| FAS_HUMAN	Fatty acid synthase	DB01034	Cerulenin	Experimental
10	sp|P68104| EF1A1_HUMAN	Elongation factor	DB04315	Guanosine-5′-Diphosphate	Experimental

TABLE 4
Proteins involved in pathogen specific pathways.

Serial no.		Pathway	No. of enriched protein
01	ko05171	Coronavirus disease − COVID-19	109 (Fig ko 5171)
02	ko05203	Viral carcinogenesis	12
03	ko03250	Viral life cycle	7
04	ko05416	Viral myocarditis	4
05	ko03262	Virion − Coronavirus	1

to control transcription. This discovery, which falls under the subfamily of translation factors GTPases known as the TRAFAC class, opens potentially exciting new possibilities for attacking critical pathways involved in SARS-CoV-2infections (Fig. 2 A,F). In Fig. 2B, we can see that the second protein, uS2, interacts with proteins that are involved in cell binding to the basement membrane and the activation of signalling transduction pathways. In Fig. 2C, we can see that the non-receptor tyrosine-protein enzyme TYK2 is likely to play a crucial role in inside cells signal transduction, especially in starting kind I interferon (IFN) signalling pathways. Furthermore, the protein called "RAC-beta serine/threonine-protein an enzyme" interacts with regulators that play a key role in controlling various processes like digestion, cell survival, proliferation, angiogenesis, and growth (Fig. 2D). One such regulator is Prohibitin 1, also known as PHB, which plays a role in DNA synthesis and ultimately affects cellular proliferation. Whether this suppression is due to a protein or the mRNA, the exact mechanism is still a mystery. Furthermore, PHB may have a role in ageing through its involvement in modulating mitochondrial respiration activity. Table 6 also highlights crucial therapeutic targets, such as the intracellular protein's "Fatty acid synthase" that takes acetyl-CoA, malonyl Co, and NADPH and uses them to catalyse the formation of long-chain fatty acids. This versatile protein serves as an acyl carrier and has seven catalytic activities. As shown in Fig. 2E. Using a reverse vaccinology strategy, more research was conducted to develop in-silico vaccines targeting membrane antigenic proteins.

3.6. Potential protein vaccine selection and T-cell and B-cell epitopes prediction

Following a rigorous curation process, we retained 12 protein sequences that were unique to the virus and lacked significant homology to human proteins. This approach minimized the risk of autoimmune reactions by ensuring that the selected antigens were distinct from human proteins (Table S2). Subsequently, these proteins underwent a comprehensive evaluation, including assessments of antigenicity, allergenicity, toxicity, and virulence. Among them, three membrane proteins emerged as promising vaccine candidates: sp|P05106|ITB3, sp|O00187|MASP2, and sp|P05556|ITB1. (Table 5). These proteins exhibited impressive antigenicity scores, as predicted by VaxiJen[14], surpassing the critical threshold of 0.4. Furthermore, they were found to be non-allergenic, non-toxic, and involved in viral virulence. These characteristics suggest that the elicited immune responses would specifically target the virus without causing adverse reactions in the host.

By focusing on these antigens, we aim to develop a vaccine that is both effective and safe, targeting key viral proteins that are essential for the pathogen's life cycle and less likely to elicit adverse reactions in the host[4].

In order to design chimeric vaccine structures against SARS-COV-2, these three proteins were used for epitope prediction and to select lead epitopes. The epitopes of T cells (both MHC-I and MHC-II) were predicted by employing the IEDB server and a strict IC_{50} threshold of less than 100 nM. At the same time, B-cell epitopes that overlap were identified using an ABCpred server with a specificity of 75% and a mI > 0.8. In order to build the vaccine, we first found six overlapping leading epitopes that for each protein that was prioritized. The selection of epitopes was based on their high antigenicity, IFN-positivity, low toxicity, and lack of allergenicity (Table 6). The end goal was to find lead epitopes that could activate host interferons, stimulate humoral and immune responses caused by cells, or all three.

Verifying that these chosen epitopes are conserved on various SARS-COV-2 strains was critical for improving the vaccine's effectiveness and expanding its protective spectrum. Remarkably, the prioritized epitopes showed full coverage in every group on the planet. Positively, according to Oyarzun and Kobe (2015), the projected epitopes had a very high population coverage in the areas hit the worst by SARS-COV-2, which included Europe, East Africa, and Southeast Asia (Figure S4).

3.7. Chimeric vaccine Constructs: Pioneering the future of immunization

Four chimeric vaccine constructs based on their high immunogenicity and effective epitope coverage (Table S3). Each construct combined lead epitopes with GGGS and HEYGAEALERAG linkers to ensure proper presentation and immune response. The number of constructs was chosen to balance feasibility with thorough evaluation, aiming to maximize effectiveness while allowing for detailed assessment and

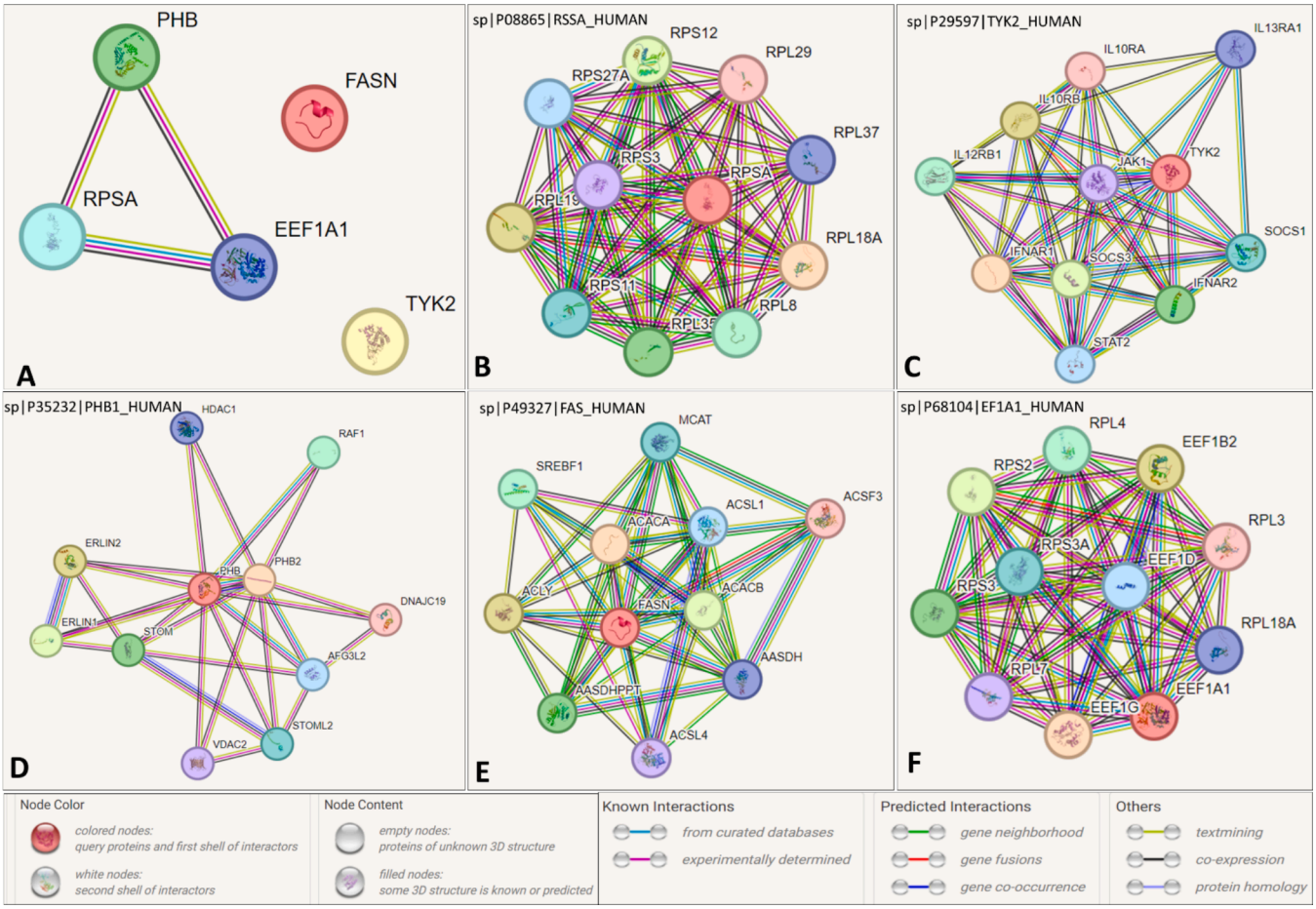

Fig. 2. Analysis of PPIs network using the STRING v11.5 webservers. (A) Elongation factor 1-alpha EF1α, (B) Small Ribosomal Subunit Protein uS2 (C) Non-Receptor Tyrosine-Protein Kinase TYK2, (D) DRAC-beta Serine/Threonine-Protein Kinase,(E) Prohibitin 1 (F) Fatty acid synthase.

TABLE 5
Probable antigenic proteins for vaccine targets.

Serial no.	Accession no.	Protein name	antigenicity	allergenicity
01	sp|P05106| ITB3_HUMAN	Integrin beta-3	0.5738 (Probable ANTIGEN)	NON-ALLERGEN
02	sp|O00187| MASP2_HUMAN	Mannan-binding lectin serine protease 2	0.5060 (Probable ANTIGEN)	NON-ALLERGEN
03	sp|P05556| ITB1_HUMAN	Integrin beta-1	0.4955 (Probable ANTIGEN	NON-ALLERGEN

optimization, these linkers make sure that each epitope works on its own once it's in the human host. The epitopes have been coupled with four separate adjuvants—HBHA protein, beta-defensin, 50S ribosome protein L7/L12 adjuvants, and HBHA repetitive peptides sequences to increase the immunogenic potential vaccine constructs. There is great potential for these adjuvants, when coupled with EAAAK linkers, to dramatically increase immunogenic responses. These vaccine designs integrate PADRE peptide chains (Table S3) to address the difficulty of HLA-DR differences across worldwide populations. A strong cytotoxic T lymphocyte (a CTL) response and enhanced immune protection can be achieved with this inclusion, according to prior studies.

ANTIGENpro scores, exceeding the 0.9 threshold, reaffirm their substantial antigenic properties. VaxiJen 2.0 scores, falling within the range, align with established viral standards. SOLpred scores, indicating exceptional solubility upon expression. The ProtParam server has further illuminated the constructs' physiochemical characteristics. Molecular weights ranging from ~48868.61 Da to ~61594.29 kDa promise versatility. Impressively, GRAVY scores, indicating hydrophilicity, range from −0.221 to −0.381. Theoretical pI values exhibit a range between 5.37 and 8.72. Aliphatic index scores reflect remarkable thermostability, ranging from 53.35 to 63.88. Instability index scores predict stability under varying temperatures, falling within the 30 to 40 range (Table 7). All four vaccine constructs were non-allergenic, Antigenic, immunogenic and non-toxic nature.

3.8. 2D and 3D structure prediction, Refinement and validation

To accomplish molecular connections with human immunological receptor proteins, the vaccines' 2D and 3D structures are crucial. depicting the constructed secondary structure with its many attributes, such as alpha helices, beta twists, coils, and the extended chain (Fig. 3A). Using the RNAFold website, we were able to predict the second structure of mRNA. With overall MFE of −544.09 kcal/mol for SARS-COV-2-C2, the predicted mRNA structural models were determined to be stable in a thermodynamic ensemble. In dot-bracket notation, the centroid secondary components have a minimum free energy of −364.89 kcal/mol (Fig. 3B-D). The vaccination models' 3D structures were created using the Aplhafold server (Fig. 4A, B) (see Figure S1-S3). Prior to conducting additional structural validation analyses, these models were fine-tuned using the DeepRefiner web-server. The Ramachandran plots revealing all of construct's residues fall within the favored region of the plots (Fig. 4C) (Table S4). Using ERRAT, our refined vaccine constructs

Table 6
Antigenicity pattern of the overlapped B-cell, MHCI and MHCII epitopes.

B-cell	MHCI	IC_{50}	MHCII	IC-50	antigenicity
YGKIRSKVELEVRDLP	DAYGKIRSK	2.99647	LQLIVDAYGKIRSKV	240	1.4073 (Probable ANTIGEN).
NFSIQVRQVEDYPVDI	KNFSIQVRQ	17.38641	ALRLRPDDSKNFSIQ	35	1.4144 (Probable ANTIGEN).
TGKYCECDDFSCVRYK	TGKYCECDD	7.947124	HSSDFGKITGKYCEC	498	0.8877 (Probable ANTIGEN).
GGRIYGGQKAKPGDFP	TGGRIYGGQ	27.81058	PGGRIYGGQNLTLTI	446	0.5751 (Probable ANTIGEN).
FGRLASPGFPGEYAND	PVFGRLASP	14.46139	PGQPVLBGRLASPQL	22	0.4979 (Probable ANTIGEN).
CSCRAGYVLHRNKRTC	GFYCSCRAG	5.452805	CSCRAGHFLPSLIQL	6	0.8906 (Probable ANTIGEN).
CPRCYNAPFPCAPCKN	CPRCYNAPF	19.62863	LCPYDFLKRCYNAPF	115	0.7486 (Probable ANTIGEN).
EVTIPYLATDVTCVGP	TEVTIPYLA	19.73377	LLGLVTIPYLAPLGP	26	0.9190 (Probable ANTIGEN).
SFMSVNESCYKYGQTL	EASFMSVNE	8.843211	PICLPRKESFMSVNE	355	0.7135 (Probable ANTIGEN).
GECIQAGPNCGWCTNS	CGECIQAGP	27.30298	WCGECIQAQEGMPTS	264	0.6138 (Probable ANTIGEN).
TVMPYISTTPAKLRNP	TVMPYISTT	25.65724	EKTVMPYISTTPAKL	16	0.6871 (Probable ANTIGEN).
CVQCRAFNKGEKKDTC	EHKECVQCR	7.983806	CVQCRAFNKGEKKDT	471	1.1517 (Probable ANTIGEN).
PPEVEAGQRLRAGLLP	LPPEVEAGQ	21.09648	LPPEVEAGQRLRAGL	0.0269	1.1334 (Probable ANTIGEN)
HIHIYSASWGPEDDGK	GPEDDGKTV	15.14293	NHIHIYSASWGPEDD	0.0772	0.5823 (Probable ANTIGEN
AGNYDPGASFDVNDQD	LAGNYDPGA	7.298103	AGNYDPGASFDVNDQ	0.0464	0.8913 (Probable ANTIGEN).
TMCIQASEGKDSSVAA	PTMCIQASE	21.8882	DIYTMCIQEVWADVG	1471	0.9070 (Probable ANTIGEN).
VPGSTAPLEFLHITFQ	GVVPGSTAP	12.43398	AKGVVPGSTAPLEFL	226	1.2413 (Probable ANTIGEN).
ADVGMAGPPQKSPATL	WADVGMAGP	3.106061	TENVLEVWADVGMAG	1864	0.7522 (Probable ANTIGEN)

Red color* Shows the overlapped region in B-cell, MHCI and MHCII epitopes.

TABLE 7
Physiochemical properties of designed vaccine constructs via JCAT server and ProtParam server.

Vaccine constructs	Adjuvants in construct	No of Amino Acids	GC content	Instability index	Aliphatic index	Molecular weight (Da)	Theoretical PI	Grand average of hydropathicity (GRAVY)	CAI (0.85–1.0)
SARS-COV-2-C1	HBHA adjuvant	600	52.22	38.32 protein as stable	61.80	61594.29	5.50	−0.282	1.0
SARS-COV-2-C2	Beta definsin adjuvant	482	55.43	45.40 protein as stable	53.15	48868.61	8.72	−0.381	1.0
SARS-COV-2-C3	HBHA conserved adjuvant	586	43.23	47.93 protein as unstable	63.26	60118.70	5.37	−0.377	1.0
SARS-COV-2-C4	Ribosomal protein adjuvant	565	62.73	39.92 protein as stable	63.88	56975.75	5.65	−0.221	1.0

achieved an overall quality factor ranging from an impressive 85% to a staggering 100%. Likewise, the ProSA-web server weighed in, assigning Z scores to our vaccine constructs, which ranged from a remarkable −4.52 (Fig. 4D) and also indicting 3D structure with their energy levels (Fig. 4E). These findings collectively culminated in a resounding verdict on the exceptional quality of designed vaccine 3D structures (Fig. 4).

3.9. Molecular docking with immune receptors

In order to uncover the most effective binding interactions between our vaccine designs and their receptor counterparts. The Hawkdock webserver and the Hdock web server, revealed that the docking scores of our vaccine-receptor unions yielded fascinating results, with all complexes displaying similar affinities. But the SARS-COV-2-C2 (Beta defensin adjuvant), shows the lowest binding energy with the receptors (Fig. 5). This revelation illuminated the remarkable binding affinities between our designed vaccine constructs with receptors (Table 8).“.

3.10. NMA analysis

Using normal mode analysis (NMA), we explored the stability and mobility of the top vaccine model. The iMODS server calculated internal coordinates, revealing the complex’s deformability linked to each residue’s unique deformation. Our analysis resulted in a precise value of 2.394982e-05. We converted variance to eigenvalues for each normal mode, leading to a B-factor proportional to RMS. The covariance matrix highlighted residue pairs, displaying related motions in red, anti-related motions in blue, and unrelated motions white. Additionally, our Elastic map revealed spring-connected atom pairs, with gray areas indicating stiffness intensity. This visually captivating analysis can be seen in Fig. 6A–E.

3.11. Immune simulation

In our study, we utilized the C-ImmSim server to conduct an immune simulation of a vaccine. Our findings demonstrate that the secondary and tertiary immune responses (specifically IgG1, IgG2, IgG + IgM) are more robust than the primary immune response (IgM) following vaccine administration. Post-vaccination immunoglobulin concentration (IgM, I IgG + IgM, gG1+ IgG2) rises as antigen concentration drops. Referring to Fig 9(A), the initial and subsequent vaccine injections elicited elevated immunoglobulin titers. The surge in IgM concentration during the primary response is a typical early antibody response. Throughout the simulated period, antibody levels (IgM + IgG, IgM + IgG1, IgG1+IgG2) rose during secondary and tertiary responses, coinciding with declining antigen levels (Fig. 7A). Post-vaccination, A significant increase in B cell population, including memory B cells, suggests the possibility of isotype switching and memory development (Fig. 7B). Cell proliferation in B cells and antigen presentation post-vaccine injection (Fig. 7C). Fig. 7D-F, TH (helper) and TC (cytotoxic) cell populations have increased significantly, indicating memory development. (Fig. 7G), During vaccine administration, there is a notable increase in macrophage activity and antigen presentation. Post the third vaccine injection, Fig. 7(H) displays a substantial increase in interferon-gamma (IFN-γ)

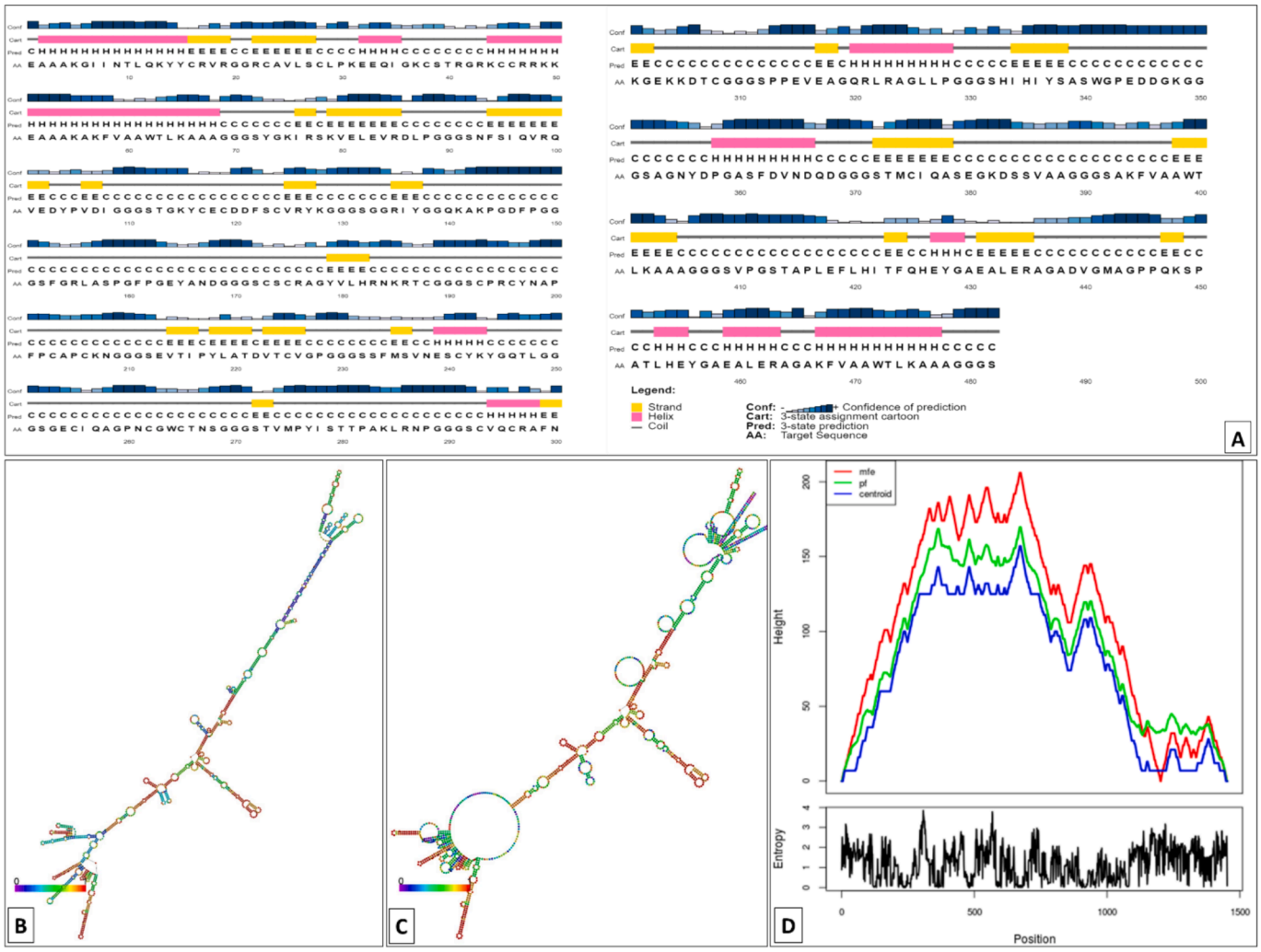

Fig. 3. Presenting the secondary structure prediction of SARS-COV-2-C2 and mRNA construct, (A) 2D structure of vaccine constructs presenting alpha helices, beta turns, coils, and extended chain. we unveil a captivating model. (B) The model eloquently displays the minimum free energy of the SARS-COV-2-C2 mRNA structure, offering a visual journey into its intricate details. (C) the minimum free energy of the secondary centroid SARS-COV-2-C2 mRNA structure, we delve into a nuanced understanding. (D) mountain plot highlights the key features of the SARS-COV-2-C2 mRNA structure, alongside the positional entropy plot enhancing our understanding of its unique characteristic.

titer and a moderate elevation in interleukin-2 (IL-2). Lastly, Fig. 7I, demonstrates a significant increase in IFN-g. These findings highlight the potential of our candidate SARS-COV-2-C2 to elicit a strong immune response capable of delivering disease protection.

3.12. Codon optimization and In-Silico Cloning

To certify efficient protein expression in the Escherichia coli system, codon optimization is crucial. To optimize the vaccine constructs the Java Codon Adaptation Tool (JCat) was employed, resulting in a 1446-nucleotide cDNA sequence. The Codon Adaptation Index (CAI) exceeded 0.8, reaching 0.96 for the vaccine, indicating high gene expression potential. With an average GC content of 53.98%, our adapted sequence falls within the optimal range of 30–70% for expression. We achieved in silico recombinant plasmid design by integrating the optimized codon sequences into the pET-28a (+) vector utilizing SnapGene software (Fig. 8). This research establishes an effective strategy for creating a multi-epitope vaccine construct, promising enhanced vaccine production.

4. Discussion

SARS-CoV-2, a formidable viral pathogen responsible for pneumonia and respiratory tract infections, stands as a prime culprit displaying resistance to a myriad of antiviral drugs[53]. Despite sporadic clinical investigations into antiviral treatments for Legionnaires' disease, their numbers remain inadequate, and our reservoir of treatment recommendations is stunted, owing to the lack of validating evidence.[28,54]

Nonetheless, study revealing the robust resistance exhibited by SARS-COV-2 strains against a cadre of antiviral, including amantadine, zanamivir, rimantadine, oseltamivir, ribavirin, ganciclovir, foscarnet, acyclovir, and rimantadine.[11] Faced with the urgent challenge posed by SARS-CoV-2, our research focuses on two primary objectives: employing subtractive genomics and a reverse vaccinology approach. Through subtractive genomics, we aim to identify unique genomic features of SARS-CoV-2 that distinguish it from human genomic sequences, facilitating the pinpointing of potential drug targets critical for viral replication and infection mechanisms. Simultaneously, utilizing a reverse vaccinology approach enables the prediction and prioritization of novel vaccine candidates by analyzing viral proteins that elicit immune responses, thereby advancing the development of effective vaccines against SARS-CoV-2. Previous studies have used bioinformatics to

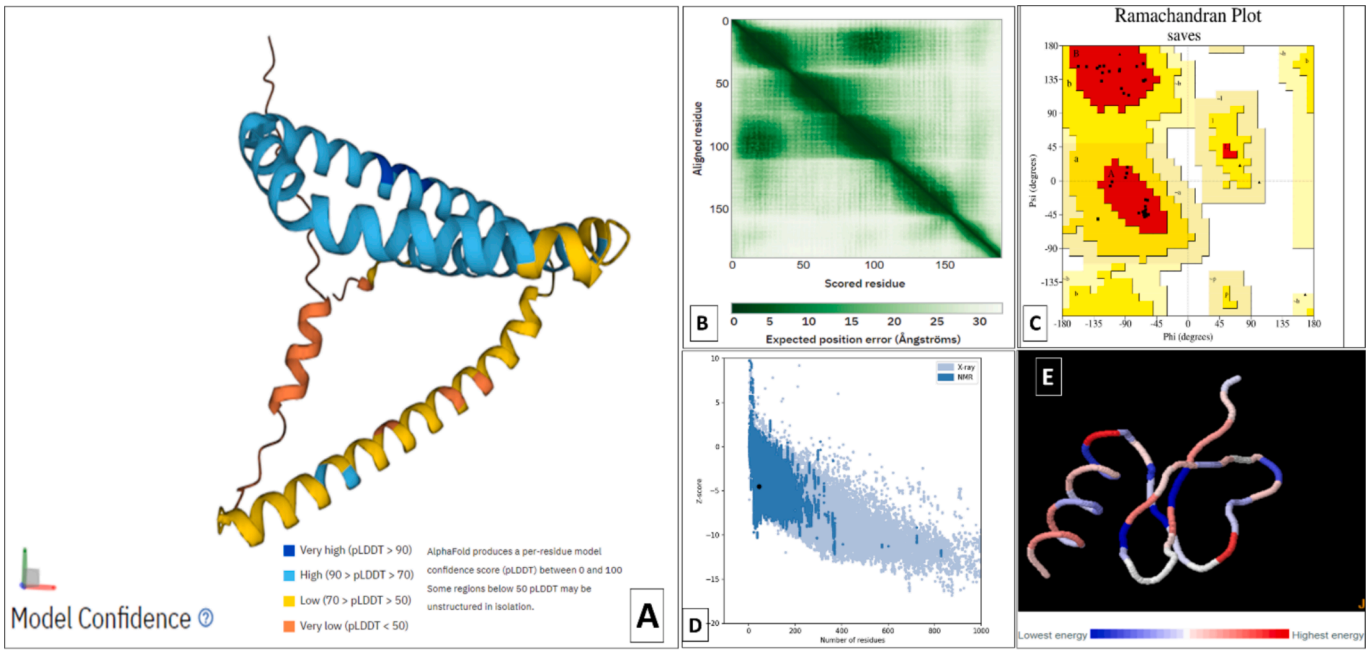

Fig. 4. Structural validation of vaccine constructs. (A) 3D structure of SARS-COV-2-C2 construct showing 96.4% sequence identify with AFDB: Q7WUL2. (B) PAE showing inter-domain accuracy. (C) Ramachandran plot showing Rama favored region of constructs. (D) Z-score of vaccine constructs (E) 3D structural validation showing energy of Vaccine construct.

design peptide-based SARS-CoV-2 vaccines, cutting costs and saving time. However, these methods often overlook the variability in mutation-prone sequences, limiting their effectiveness against new strains. To address this, we developed a multi-epitope mRNA vaccine targeting antigenic epitopes from the surface proteins of new COVID-19 strains, which shows promise for both diagnosis and prevention.

Our research employed computational predictions to identify crucial proteins essential for SARS-CoV-2 survival. We analyzed a comprehensive reference proteome of 2,930 proteins retrieved from the NCBI protein database. Evolutionary biology highlights proteins with shared functions between humans and viruses[19,34].These proteins, with significant homology to human proteins, were excluded from further analysis. Essential proteins, the lifeblood of the virus, present promising targets for novel antiviral drugs. Antiviral drugs often target these crucial gene products, making them ideal candidates for pathogen-specific drug discovery[54]. Through our analysis, we identified an impressive roster of 6 unique, essential proteins intrinsic to SARS-CoV-2 – a promising group of potential drug targets (Table 3).

Subcellular localization analysis is critical, as protein function often hinges on their cellular location. Understanding subcellular localization is crucial for unraveling disease mechanisms and developing vaccines and drug targets. While both membrane and cytoplasmic proteins can be potential therapeutic targets[40],historically, membrane proteins have been favored for vaccine design[3].We have prioritized membrane proteins for potential vaccine development, while cytoplasmic proteins remain promising candidates for future drug design.

The effectiveness of antiviral drugs has been significantly compromised due to gene mutations, leading to the rapid emergence of multidrug-resistant viruses (MDRVs)[1,32]. We currently face a crisis of viral resistance, largely stemming from the misuse and overuse of these drugs, coupled with a dearth of new drug development efforts within the pharmaceutical industry[15,33]. The indiscriminate use of broad-spectrum antiviral drugs against specific viruses or groups of viruses can trigger mutational changes and promote the transfer of genes among viruses. This, in turn, fosters drug resistance and the proliferation of resistant viruses[36].

It is crucial to identify novel drug targets and vaccine candidates to overcome existing challenges in therapeutic development. To this end, we shortlisted 10 proteins and subjected them to further analysis using the DrugBank 5.1.0 database with default parameters (Table 1)[60]. For vaccine design, the selection of membrane proteins is critical. We identified 12 membrane proteins (Table S1) that passed antigenicity, allergenicity, and toxicity tests. Among these, one protein was antigenically negative, while the remaining 11 exceeded the antigenicity threshold. After additional toxicity and allergenicity evaluations, five membrane proteins were selected as the best immunogenic candidates. These proteins were subsequently used for B-cell and T-cell epitope prediction, focusing on epitopes capable of activating cytotoxic T lymphocytes (CTLs) and helper T lymphocytes (HTLs).

In fact, targeting proteins within the human microbiome non-homology domain is particularly promising. These are completely independent of common host-pathogen pathways and have no homology with human “anti-targets,” so drugs or vaccines targeted against them could well produce little effect on commensal microbial strains at various body sites in humans generally.

This study discovered that the large majority of cytoplasmic proteins are intimately involved in outer membrane biosynthesis pathways and necessary for cell division. But six proteins (Table 2) elongation factor 1-alpha, small ribosomal subunit protein uS2, non-receptor tyrosine-protein kinase TYK2 (DRACbeta the serine/threonine −protein phosphorylating enzyme), Prohibitin 1 and Fatty acid synthase all impact In consequence (Fig. 2) these proteins actually become compelling potential targets for future drugs.

Using whole proteins as vaccine candidates exposes the danger of triggering virulence properties to emerge. To address this problem, subunit vaccines focus only on the antigenic portion of a pathogen. They promise to induce protective immunity in the host. With the aid of various bioinformatics tools, we constructed several antigenic epitopes. Comprehensive evaluation of these putative epitopes was given with respect to their toxicity profile, immunogenicity and allergen-type distribution as well as population analysis. Furthermore, the presence of PADRE in fact reduced polymorphisms at HLA cross populations[2,17]. In vivo experiments in the past also showed that incorporation of linkers increased vaccine immunogenicity[48]; X.[62]. Our prioritized epitopes are first made into four vaccine candidates (C1, C2, C3 & C4) (Table S3), with appropriate Linkers and Adjuvants and PADRE sequences.

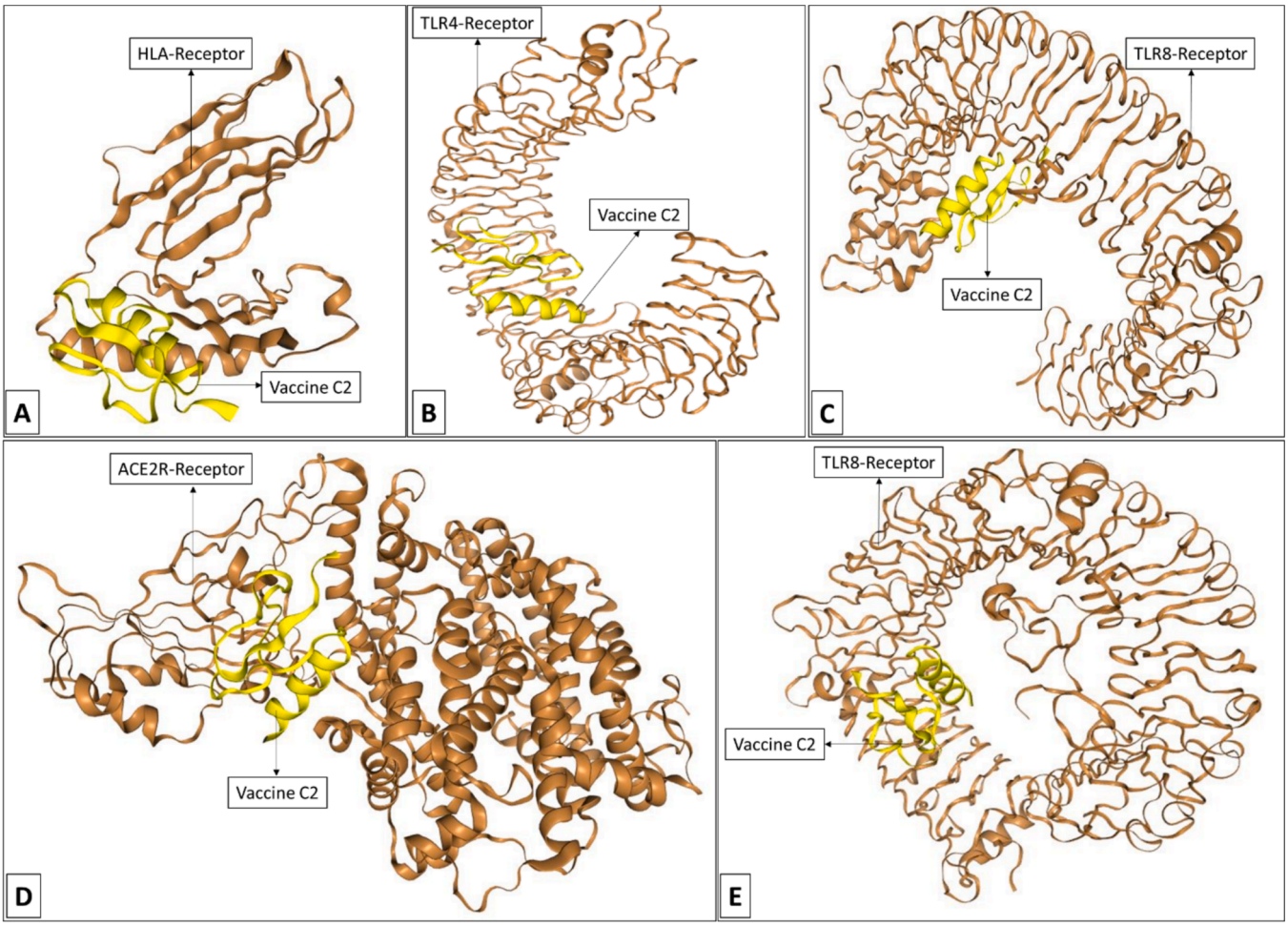

Fig. 5. Docking complexes of immune receptors and SARS-COV-2-C2. i.e (A) Docked complex of SARS-COV-2-C2 in HLA receptor, (B) Docked complex of SARS-COV-2-C2 in TLR3 receptor, (C) SARS-COV-2-C2 docked complex in ACE2R receptor, (D) SARS-COV-2-C2 docked complex in ACE2R receptor, (E) TLR8 receptor docked complex of SARS-COV-2-C2.

Table 8
Docking score of receptors with vaccine constructs.

S.No	Vaccine construct	Receptors	Binding energy (kcal/mol)	Ligand rmsd (Å)	Confidence Score	Docking score
1	SARS-COV-2-C1	TLR4	−24.49	15.6	0.9247	−336.4
2	SARS-COV-2-C2	TLR4	−50.92	25.0	0.9227	−266.5
3	SARS-COV-2-C3	TLR4	2.44	18.8	0.9228	−332.9
4	SARS-COV-2-C4	TLR4	11.35	96.3	0.9223	−200.2
5	SARS-COV-2-C1	HLA	−35.77	13.7	0.9209	−298.6
6	SARS-COV-2-C2	HLA	−72.46	48.0	0.921	−229.7
7	SARS-COV-2-C3	HLA	−67.72	64.88	0.9222	−259.1
8	SARS-COV-2-C4	HLA	−23.6	99.39	0.9267	−226.1
9	SARS-COV-2-C1	ACE2R	−6.31	43.0	0.9218	−327.3
10	SARS-COV-2-C2	ACE2R	−55.72	48.0	0.9203	−266.9
11	SARS-COV-2-C3	ACE2R	−47.17	64.88	0.9227	−323.2
12	SARS-COV-2-C4	ACE2R	34.15	99.39	0.9238	−207.3
13	SARS-COV-2-C1	TLR8	**−6.48**	56.33	0.9288	−278.42
14	SARS-COV-2-C2	TLR8	**−27.73**	63.99	0.9227	−274.59
15	SARS-COV-2-C3	TLR8	**−23.6**	12.8	0.9267	−253.1
16	SARS-COV-2-C4	TLR8	**−16.91**	56.3	0.9256	−222.6
17	SARS-COV-2-C1	TLR3	**−35.95**	43.0	0.984	−357.0
18	SARS-COV-2-C2	TLR3	**−35.78**	48.0	0.944	−302.0
19	SARS-COV-2-C3	TLR3	**−29.8**	64.88	0.958	−306.84
20	SARS-COV-2-C4	TLR3	**−2.49**	99.39	0.920	−272.1

The final vaccine models are docked with certain immune receptors (HLA, TLRs and ACE2R). The best outcome is SARS-COV-2-C2 with the least binding affinity for receptors as mentioned (−70.46 to − 27.73 kcal/mol) (Fig. 5). Besides, we also looked at the submolecular interactions of these constructions with immune receptors such as Toll-like receptors (TLRs), Human Leukocyte Antigens (HLA) and ACE2R to measure how effective they were in adjuvanting. The TLRs (trans-membrane protein family) are involved in innate immunity. They can

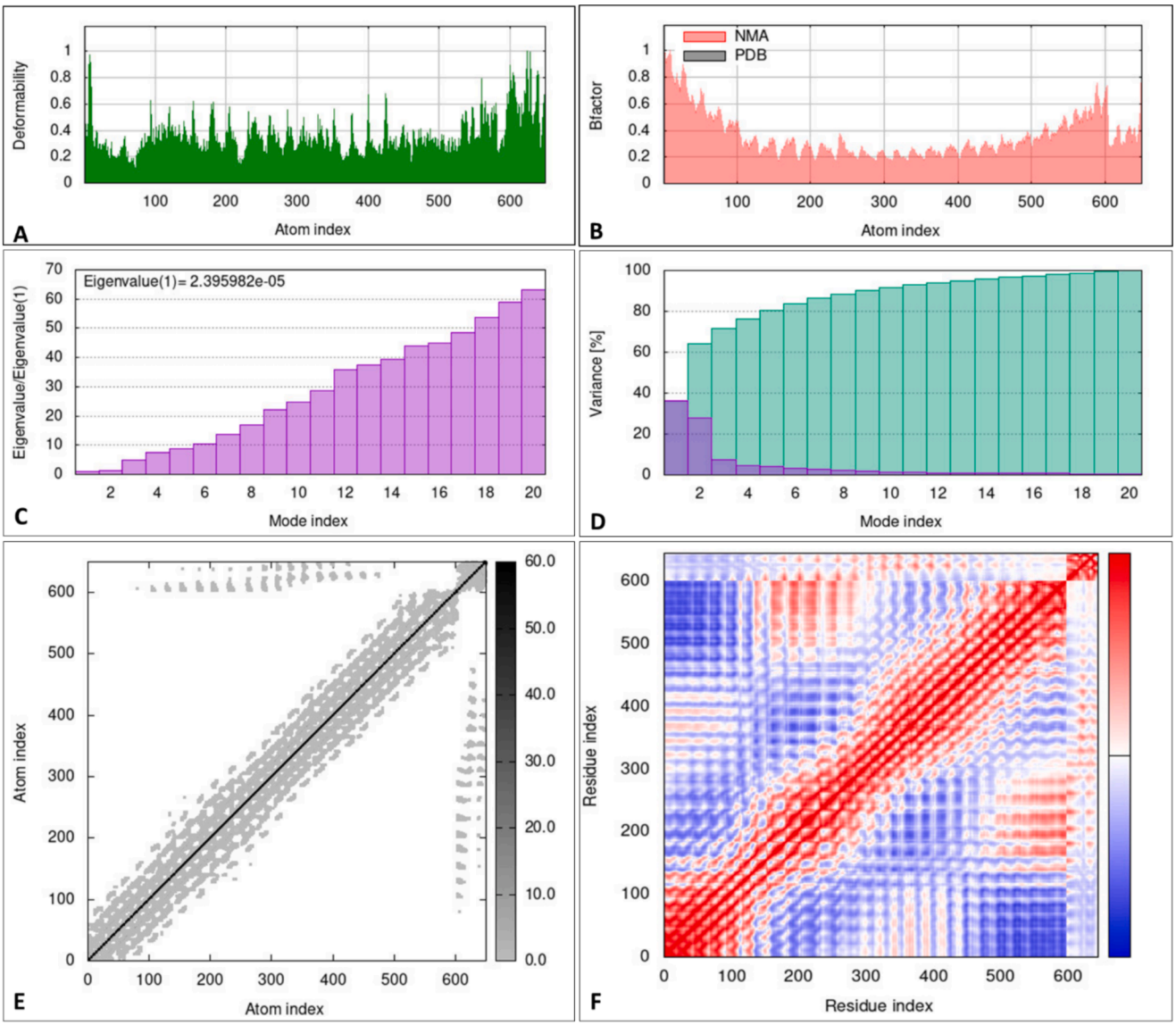

Fig. 6. (A) Simulation of main-chain deformability, identifying highly deformable regions. (B) Computation of B-factor values through normal mode analysis, indicating atom-level uncertainty. (C) Determination of the eigenvalue for the docked complex, representing structural deformation energy. (D) Variance (%). (E) elastic network model, illustrating atom-spring connections, with darker shades indicating greater rigidity. (F) Assessment of covariance relationships among residue pairs (red shows correlated, white illustrate uncorrelated, blue displays anti-correlated).

actively recognize various pathogen-associated molecular patterns (PAMPs). After the interaction between TLRs and PAMPs, inflammatory chemokines and cytokines are released to attract immune cells to the infection site.

All TLRs are involved in the recognition of viral PAMPS. TLR3: Recognizes double-stranded RNA (dsRNA), which is a common component of viruses. The reason: TLR4, which recognizes lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria[9]. TLR8 recognizes both ssRNA and viral capsid proteins, allowing it to detect viral components beyond RNA alone. This recognition of viral proteins can trigger immune responses, which supports the proposed protein-based vaccine's ability to activate TLR8[8].

HLA molecules are on the surface of antigen-presenting cells (APCs) and play an important role in triggering adaptive immune responses. APCs combine antigens with HLA molecules, for presentation to T cells. T cells then detect the antigens and become stimulated, making antibodies as well as other immune effector molecules.

SARS-CoV-2 uses ACE2R as a receptor to enter host cells. ACE2R is also found on immune cells, and it can interact with SARS-CoV-2to produce inflammatory cytokines[22,26]. The results show that the constructs bound very well to all of the tested immune receptors, so they are likely to be good adjuvants for both vaccines against many different diseases and pathogens.

In-silico immune simulation showed that the vaccine induced cellular immune reactions similar to those seen in natural infections. With repeat antigen exposure, the immune system responded strongly. It showed that adaptive immunity had been induced. A: For example, the long-term memory formed in B and T cells was still there several months later. After the first immunization, levels of T cytotoxic cells, T helper cells and Ig were all considerably up. Levels of IFN-gamma and interleukins also rose markedly. The continued rise in these immune markers after repeated antigen stimulation implied the establishment of humoral immunity. Moreover, the Simpson diversity index (D) for research on clonal specificity pointed to possible different immune responses. Immunoinformatics provides a cost-effective method for developing novel polypeptide vaccines against coronaviruses[26]. Our vaccine has shown promising efficacy in immune simulations (Fig. 7), and now we must validate it experimentally. We will use solid-phase

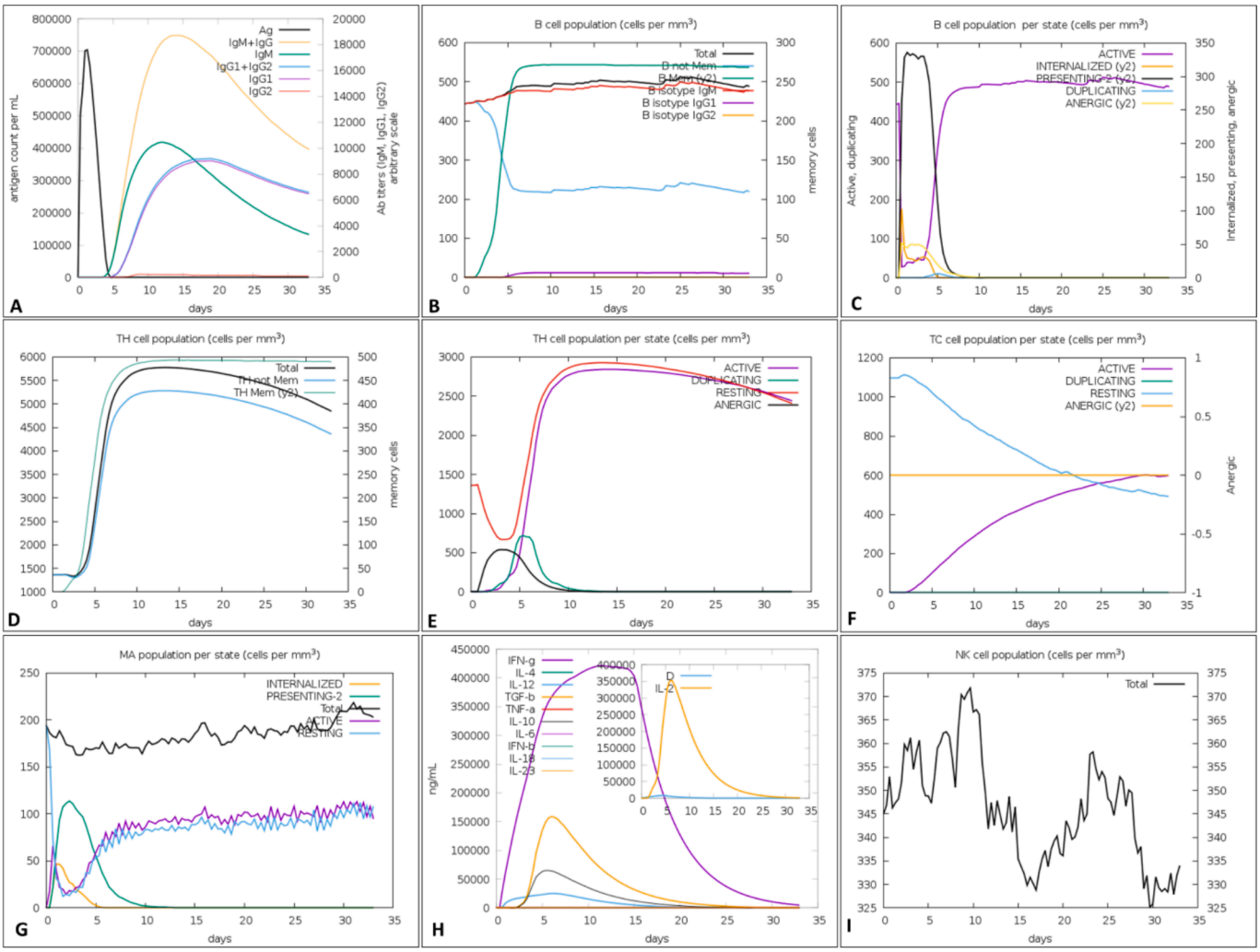

Fig. 7. Immune simulation results. (A) Generation of immunoglobulins/antibodies following antigen administration. Different subtypes of immunoglobulins are depicted as colored peaks. (B) Presentation of B-cell population post three rounds of injections. (C) Distribution of B-cell counts across states. (D) Expansion of T-helper cell population (demonstrating a notable rise in TH memory cells). (E) Distribution of T-helper cell counts across states. (F) Induction of cytotoxic T cells after vaccine inoculation. (G) Distribution of macrophage counts across states. (H) Enhanced production of cytokines and interleukins (elevated synthesis of IFN-γ and IL-2) following vaccination. (I) Deviations in natural killer (NK) cell population after three vaccine injections.

synthesis to produce the multi-epitope antigen, formulating the vaccine with adjuvants like aluminum or nano-liposome to enhance the immune response. Immunization studies in mice will evaluate the immune response by analyzing specific antibody and cytokine levels in blood and spleen, as well as T cell differentiation. Clinical trials will then assess the vaccine's ability to prevent SARS-CoV-2 infection and its complications in humans, providing real-world data on its effectiveness and safety. This approach aims to develop a robust multi-epitope vaccine against COVID-19.

5. Conclusion

Our study employed two basic and advance approaches subtractive genomics, immunoinformatics, and reverse vaccinology to identify potential drug and vaccine targets against SARS-CoV-2. We focused on essential, non-human homologous proteins and identified 6 promising candidates with potential for further drug development. Additionally, through in-silico analysis of surface proteins, we predicted a multi-epitope vaccine candidate mRNA targeting B and T cell epitopes from three prioritized surface proteins. These findings provide a valuable starting point for future research, but *in-vitro* and *in-vivo* studies are necessary to validate our predictions.

Author contributions statement.

Syed Luqman Ali and Awais Ali: Conceptualized the study, conducted the research, and co-wrote the manuscript. **Abdulaziz Alamri:** Provided critical insights, supervised the research, and contributed to the manuscript's intellectual content. **Elham Mohammed Khatrawi, Saqer S. Alotaibi, Gulzira Sagimova, Aigul Almabayeva, Farida Rakhimzhanova, Gulsum Askarova, and Fatima Suleimenova:** Participated in the research, data analysis, and manuscript preparation. All authors: Reviewed and approved the final manuscript.

The contributions of Syed Luqman Ali and Awais Ali are considered equal, and both are designated as joint first authors.

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CRediT authorship contribution statement

Syed Luqman Ali: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Awais Ali:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Investigation, Formal analysis, Data curation,

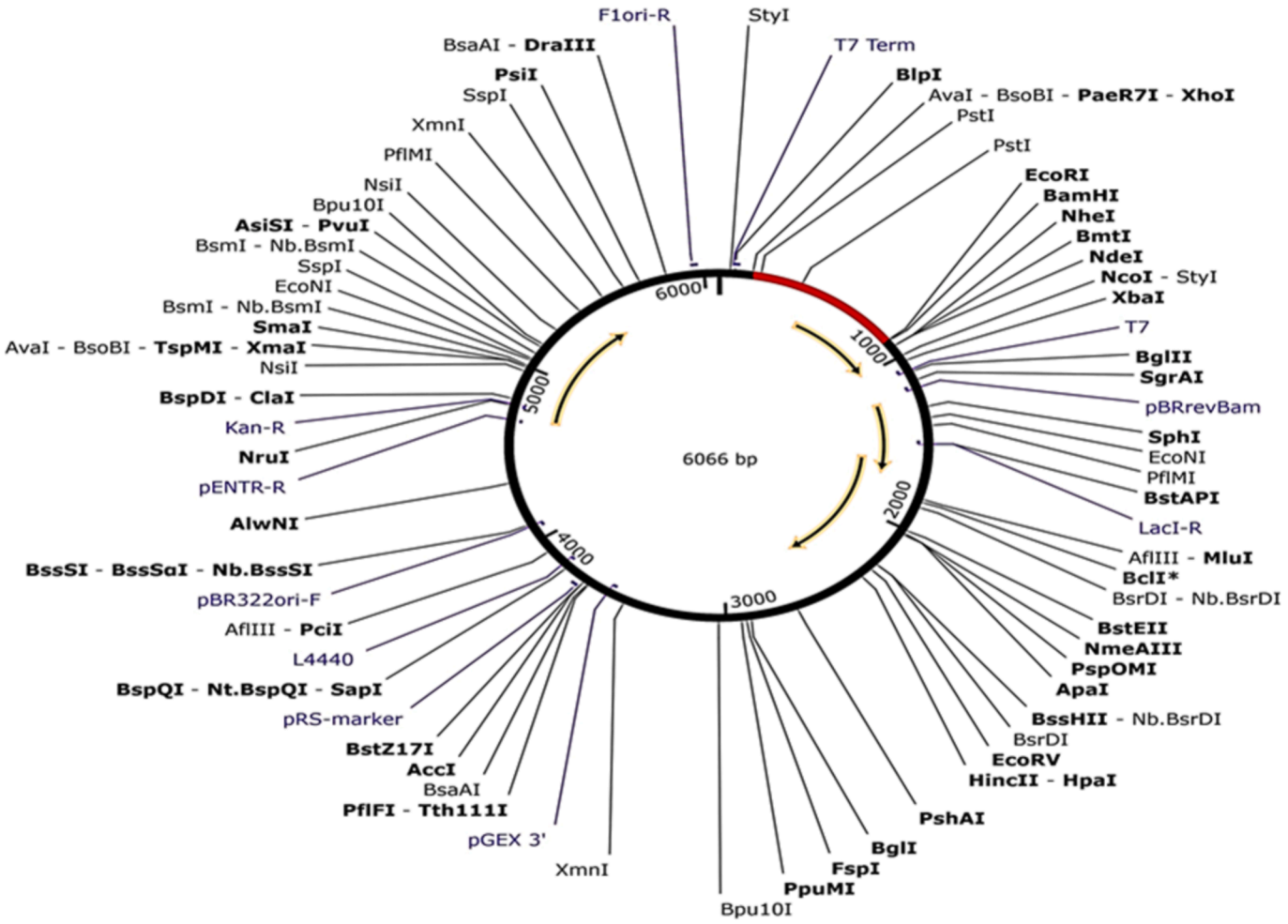

Fig. 8. Cloning the ultimate vaccine structure into the pET28a (+) vector involves in silico insertion between the BlpI and EcoRI restriction enzyme recognition sites.

Conceptualization. **Waseef Ullah:** Project administration, Methodology, Investigation, Formal analysis, Data curation. **Abdulaziz Alamri:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Conceptualization. **Elham Mohammed Khatrawi:** Software, Formal analysis, Data curation, Conceptualization. **Gulzira Sagimova:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Aigul Almabayeva:** Validation, Project administration, Methodology, Formal analysis. **Farida Rakhimzhanova:** Resources, Project administration, Conceptualization. **Gulsum Askarova:** Software, Resources, Methodology, Funding acquisition. **Fatima Suleimenova:** Writing – review & editing, Resources, Investigation, Conceptualization. **Nabras Al-Mahrami:** Methodology, Data curation. **Prasanta Kumar Parida:** Project administration, Investigation, Formal analysis, Data curation.

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 material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgeb.2024.100439.

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