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Research Article

Designing of Multi Epitope-based Vaccine against SARS-CoV-2 using Reverse Vaccinology Approach

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ABSTRACT

SARS Coronavirus-2 (SARS-CoV-2) pandemic has shown a devastating effect worldwide. More than 1.62 million people have lost their life due to this dangerous virus, which has become a major concern worldwide. It has become an emerging global issue concerning different scientific communities. Here an attempt has been made to develop a vaccine against the SARS-CoV-2 virus using epitope-based subunit vaccines and reverse vaccinology and immunoinformatics methods. With the help of computational experimentations and multi-epitope-based drug designing studies, we have successfully designed five different vaccine structures, which were further analyzed for *In-silico* toxicity as well as molecular interaction studies. Significant interacting candidates were further subjected to *In-silico* immuno simulation for the data point of about a year time. This work could provide new hope for the development of effective vaccine in the face of global threats arising from the emerging virus.

Introduction

One of the many deadly viruses that took over half a million European population annually in the 18th century was smallpox.[1] And even until the 20th century, it still claimed around 500 million lives. Some patients who got mildly cased of the disease have survived and got immune to the disease in china the first method of vaccination developed using the dried scabs from those patients having mild symptoms of the disease and treating it well by drying it under sunlight, grinding the dried scab into a powder and release of the moisture from it using cotton and then inhaling the small quantity of the fully dried powder by inhaling it with the help of a wooden-straw.[2] This proces was later termed as insufflation and was one of the first processes in the history of vaccination.[3]In the 18th century, this practice was improvised in Europe in the name of variolation in England. The term variolation was derived from the scientific name of smallpox causing Variola virus.[4]Edward Jenner was a physician and scientist who lived in the eighteenth century in England who came across a similar case of cowpox disease a disease similar to smallpox that occurs in the cow.[5] According to his folkloristic practice, he notices that the milkmaids who work in close proximity sometimes attain milder symptoms of smallpox but was not lethal, simultaneously, it makes them even immune to human smallpox.[6] In 1798, He inoculates a small group of neighbors with cowpox and then variolates them with smallpox, discovering that none of them show cowpox; hence the scientific name for cowpox is variola vaccina because vaccina means "cow," and his procedure he calls vaccination. Finally, in 1980 World Health Organisation (WHO) declared smallpox irradiated. Since then, there are several different vaccines that are present for many diseases. If a major part of the healthy population is immune by the process of vaccination the remaining population which includes

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immune compromised, infants and old population are also protected through Herd immunity.[7]

A vaccine is a substance manufactured from the causal agent of a disease,[8] its derivatives, or a synthetic equivalent that has been processed to behave as an antigen without causing the disease, that stimulates antibody formation and gives protection against one or more diseases.[9] An adjuvant is a pharmacological or immunological agent that improves the immune response of a vaccine. Adjuvants are substances that are added to vaccines to increase the immune response, resulting in more antibodies and longer-lasting immunity. There are several different types of vaccines such as live attenuated vaccine, inactivated vaccine, subunit vaccine (purified antigen), toxoid vaccine, deoxyribonucleic acid (DNA) vaccine, recombinant subunit vaccine, and synthetic peptide vaccine.[10]

Immunogenic cells follow a complex mechanism called the antigen recognition system. Antigens are the foreign molecule for the cells containing an epitope. An epitope, also known as an antigenic determinant, is a portion of an antigen that the immune system recognizes. The counterpart of an epitope is called a paratope, [11] which is a part of an antibody that recognizes and binds to an antigen immunogenic cell in our body generally encounters two types of cells self-alter cells which can also be considered as the virally infected cells and antigen-presenting cells (APC) such as macrophages, dendritic cells, neutrophil cells and many others. [12] The virally infected cells express Major histocompatibility complex-I (MHC-I) molecules on their surface which present the antigenic part of the virus to cytotoxic T cells (TC-cell) containing cluster of differentiation 8 (CD-8) receptors on their surface. [13,14] On the other hand the antigen-presenting cells such as macrophage and dendritic contain MHC-I and MHC-II on their surface with interacts with T- helper cells (TH-cells) with CD-4 molecules present on the cell surface.[15] When an antigen containing an adjuvant enters the body it creates an immunological response where the antigen is presented to the TH-cells with the assistance of APC creates a cascade of molecular cross-talk and eventually activates naïve T cells with then activates T-cells, macrophages,[16,17] plasma cells & effector T-cells which help activate the active immune response which includes effector CD-4+ T-cells, memory CD-4+ T-cells, effector CD-8+ T-cells, and memory CD-8+ T-cells.[18,19] Similarly, when the B-cells encounter the same antigenic molecule, it presents it to TH-cells and leads to the production of memory B-cells and plasma B-cells. Simultaneously, the memory B-cells help in the immunogenic memory for future interaction with the similar antigenic molecule, and plasma B-cells help in the secretion of a large number of antibodies helps in the antigen clearance.[20] An ideal immunogenic response starts with a lag phase after the antigen is introduced to the body for some time with a gradual interval of time the IgG produces as the first class of antibody and then IgM comes into the picture with other antibody classes. [21] IgG and IgM gradually Increase in the serum, which leads to a log phase with a small plateau, and then a decline this is the time if a second booster dosage gets administered, it leads to a secondary response which has a shorter lag phase and longer plateau phase which leads to immunologic memory development. [22]

An effective vaccine should have the properties of different major epitopes or the specific signature of the virus so that it can elicit a proper immune response at different stages of the viral infection cycle. [23] The goal of this study is to develop an effective vaccination against the Severe acute respiratory syndrome coronavirus 2 (SARS COV-2) virus using epitope-based subunit vaccines and reverse vaccinology and immunoinformatics approaches.[24] *In-silico* epitope mapping and immune simulation, it was not only based on spike protein but several other important protein markers of the novel coronavirus species to select promising T-cell and B-cell epitopes. [25] All the prioritized epitopes were later incorporated in a different multiepitope vaccine and then they were further studied for there immune simulation response for a time scale of about 350 days. More than one vaccine candidate had shown promising results. [26] This study can help the research community in the development and have a better idea about the probable vaccine models that can be studied further in wet bench experimentations.

Materials and Methods

Protein Sequence Retrieval

The protein sequences for different target proteins for SARS COV-2 (such as papain like protease, 3CL protease, NSP, RNA dependent RNA polymerase (NSP), helicase, spike protein, ORF3a, Ion channel, ORF8), SARS-COV (Spike Protein) and MERS-COV (Spike Protein) in FASTA format were retrieved from National Center for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov) database.

B-cell, MHC-I, MHC-II Specific Epitope Prediction

Immune Epitope Database (IEDB) (www.iedb.org) sever was used to predict and select the best probable epitopes specific to B-cell, MHC-I, and MHC-II based on the top scores.

In-silico Screening of the Selected Epitopes

All the selected epitopes were screened for the potency of generating an immunogenic response or antigenicity using the VaxiJen server (www.ddg-pharmfac.net/vaxijen),[27] Checked for anti-allergenic using AllerTop (http://www.ddg-pharmfac.net/allertop/), [28] antitoxic using Toxipred server (crdd.osdd.net/oscadd/toxipred) and are checked that these epitopes are not conserved to any related peptide present in human or conserved using Protein basic local alignment search tool (BLASTp) from NCBI. Further screened for the inducers of IL-4 (using IL-4 PRED server), IL-10 (IL-10 PRED server), and IFN- γ (IFN epitope server).

Shuffling of the Selected Screened Multiepitopes in the Vaccine Construction

The selected epitopes having specificity for B-cell, MHC-I and MHC-II were joined together in five different combinations (SV-1 to SV-5) so as to maintain a specific

peptide length (<500 amino acids) starting from an N-terminal EAAAK linker (Kar *et al.*, 2020, Naz *et al.,* 2020: Samad *et al.,* 2020) followed by the β-defensing-3 sequence as a selected adjuvant followed by the AAY linker specific for MHC-I specific epitopes than the epitopes itself and then the MHC-II epitopes followed by GPGPG linker specifically to connect MHC-II specific epitopes, B-cell epitopes followed by a di-lysine linker (KK).

Homology Modeling Prediction using Template-based Method (TBM) and 3D Structure Refinement

Homology modeling, also known as comparative modeling of protein, refers to constructing an atomic-resolution model of the "target" protein from its amino acid sequence and an experimental three-dimensional structure of a related homologous protein (the "template"). GalaxyWeb server (http://galaxy.seoklab.org/) [29] was used for the protein 3D structure prediction and its refinement. Protein structure prediction by Galaxy TBM is performed in two stages. In the first stage, more reliable core structures are built by selecting multiple templates (up to 20) after rescoring HHsearch results, aligning core sequence of query and templates by PROMALS3D, and model-building by MODELLERCSA. In the second stage, less reliable loop or terminus regions (called ULR, unreliable local regions) are detected and re-modeled using an optimization-based refinement method. ULRs are detected by a model-consensus method. Galaxy Refine performs repeated structure perturbation and subsequent overall structural relaxation by molecular dynamics simulation. For model 1, structure perturbation is applied only to clusters of side-chains, and for model 2~5, more aggressive perturbations to secondary structure elements and loops are also applied. The triaxial loop closure method is employed to avoid breaks in model structures caused by perturbation.

Validation of the Homology Modeled Refined Structures of the Vaccines

Ramachandran plot depicts the energetically allowed and disallowed region based on the backbone dihedral angles psi (ψ) against phi (φ) of amino acid residues in protein structure. Procheck (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) was used to generate the Ramachandran plot for the refined 3D- structures for the designed vaccines SV-1 to SV-5. [30]

Physiochemical Properties of the Designed Vaccine

Antigenicity, allergenic properties, toxicity, solubility and the stability of the vaccine was checked.

Molecular Interaction Studies

All Toll-like receptor (TLR) are present in the cell membrane hence they are the first line of contact with the viral pathogen. TLR's plays a major role in the activation of the intracellular signaling cascade which leads to the induction of interferon regulatory factor 3 (IRF-3) and nuclear factor kappa B (NF-κB) which helps elicit the cellular immume responses. Molecular interaction studies of the respective TLR's with the constructed vaccines were performed using HDOCK server. [31]

In-silico codon adaptation and vector designing

Jcat server was user for the *in-silico* codon adaptation of the protein coding gene sequence in accordance to the bacterial strain of *Escherichia coli* K-12 strain and the plasmid vector designing was performed using snapgene tool. [32]

Immune Simulation

The immunogenic effect of the designed vaccine with the best interacting scores were simulated using the C-ImmSim server for 350 days, giving 3 consecutive booster dosages within a time gap of 4 months each. [33]

Results and Discussion

Epitope Prediction and Screening of the Peptide Fragments

Top 20 results were selected from the IEDB server with a fragment length of 9 amino acid each for the peptide fragments having specificity for MHC-I AND MHC-II and 15 amino acid fragments for B-cell epitopes. Peptides were screened further for antigenicity, anti-allergen, anti-toxic, and not conserved with humans (Table 1). MHC-II epitopes were further screened for the probable inducers for IL-4, IL-10 and interferon gamma (IFN-γ). Selected epitopes were used for vaccine construction (Fig 1).

Vaccine Designing

Selected epitopes were shuffled into different combinations and lined by specific linkers to form the sequences of the designed vaccine. β-defensins 3 adjuvant sequence were attached for an improved antigenicity of the designed vaccine sequence.

Screening of the designed vaccine

Screening of the designed vaccines were done based on different parameters such as antigenicity, allergenicity, toxicity, solubility characteristics. Similarly, various physiochemical properties were studied to check the overall stability of the designed vaccines.Homology modeling of the designed vaccine.

Homology Modeling of the Designed Vaccine

Homology modeling of the designed vaccine was performed using GalaxyWEB server. The server uses template-based method in defining the respective 3-D structures from the designed vaccines sequences and the structures were further minimized (Table 2).

Fig 1: Showing the selected epitopes for the proposed vaccine

Table 1: Screening parameters for physiochemical properties, antigenicity, anti-allergen, anti-toxic, solubility and protein stability of the proposed vaccine.

Physiochemical properties of the designed vaccines									
						Prot-param results			
S. no	*Vaccines*	*Antigenicity*	*Allergenicity*	*Toxicity*	*Solubility*	*GRAVY*	*Aliphatic index*	*Instability index*	
1	SV-1	yes	no	no	yes	-0.242	79.3	34.59	stable
2	SV-2	yes	no	no	yes	-0.21	84.66	31.74	stable
3	SV-3	yes	no	no	yes	-0.327	78.2	33.82	stable
4	SV-4	yes	no	no	yes	-0.233	80.51	32.52	stable
5	SV-5	yes	no	no	yes	-0.187	86.95	25.97	stable

Table 2: Showing the 3-D structures of the designed vaccine in ribbon and surface forms.

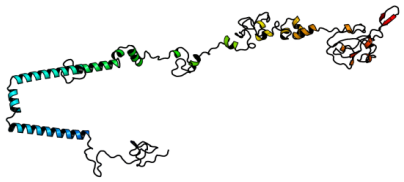
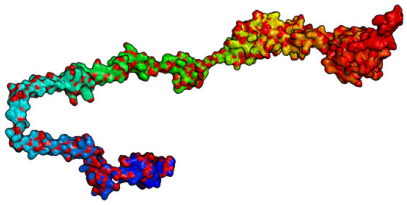
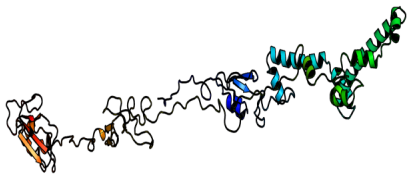
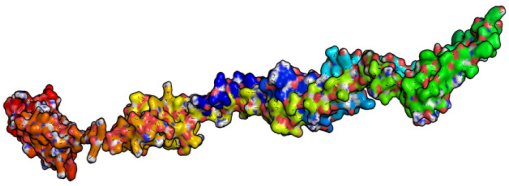
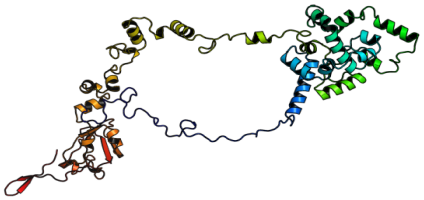
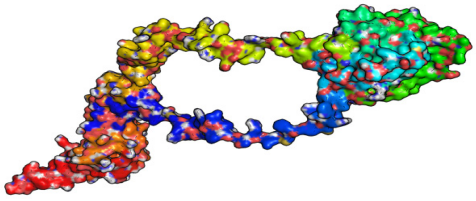
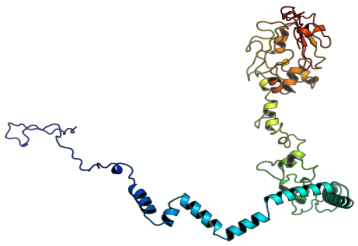
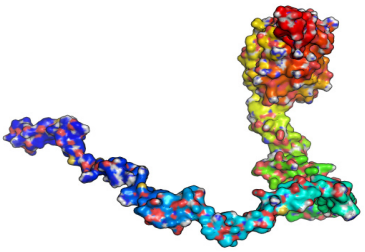
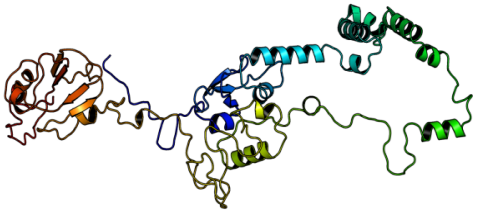
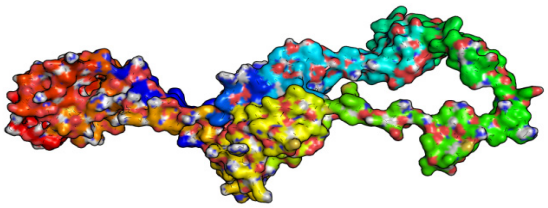
	3D Structure in cartoon form	***3D Structure in surface view***
SV-1		
SV-2		
SV-3		
SV-4		
SV-5		

Table 3: Showing the Ramachandran plot statistics.

S. No.	*Designed vaccine*	*Most favoured region*	*Additional allowed regions*	*Generously allowed regions*
1	SV-1	93.50%	4.90%	0.80%
2	SV-2	94.50%	4.50%	0.30%
3	SV-3	94.20%	4.60%	0.80%
4	SV-4	93.30%	5.10%	0.30%
5	SV-5	92.20%	6.70%	0

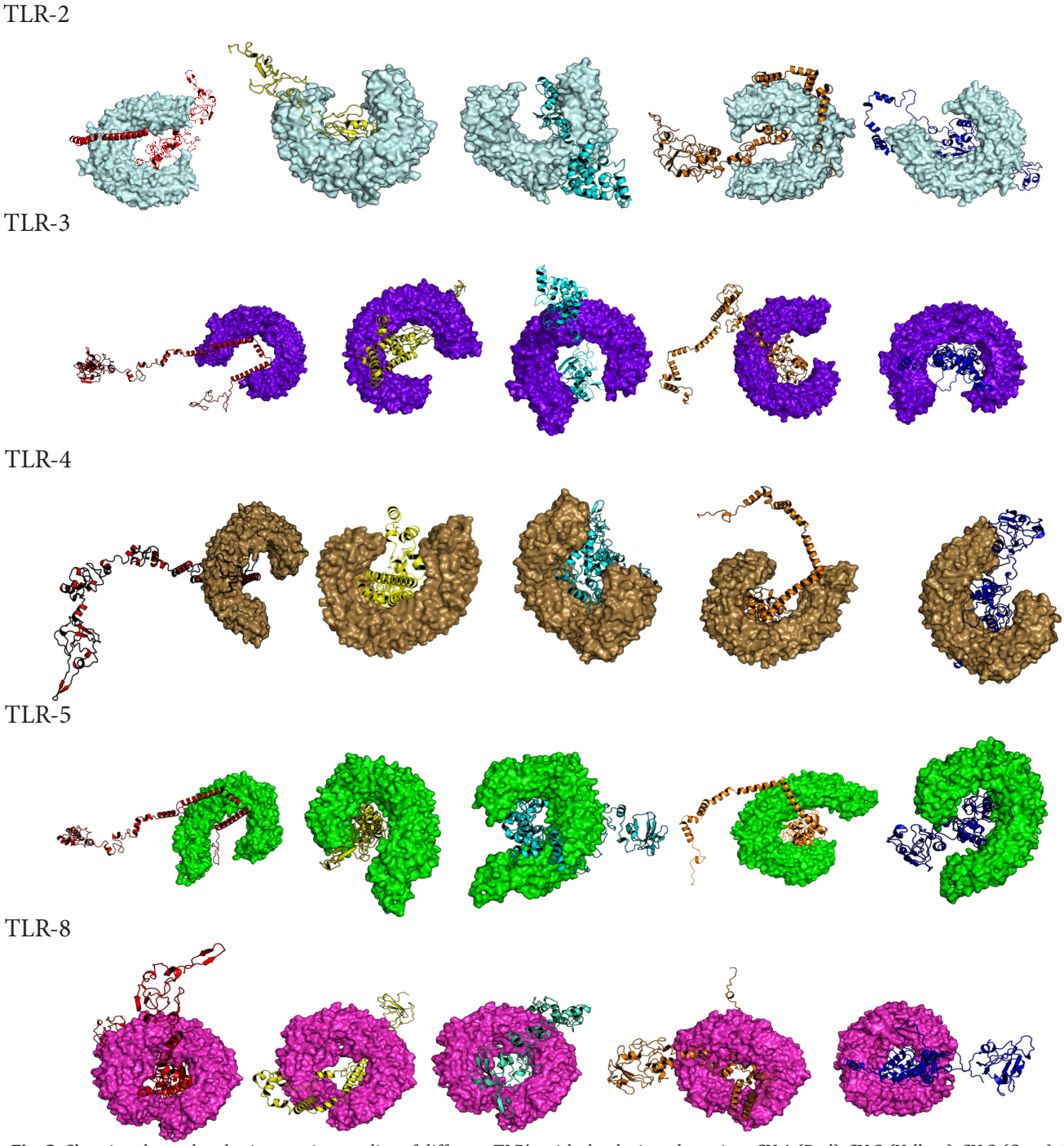
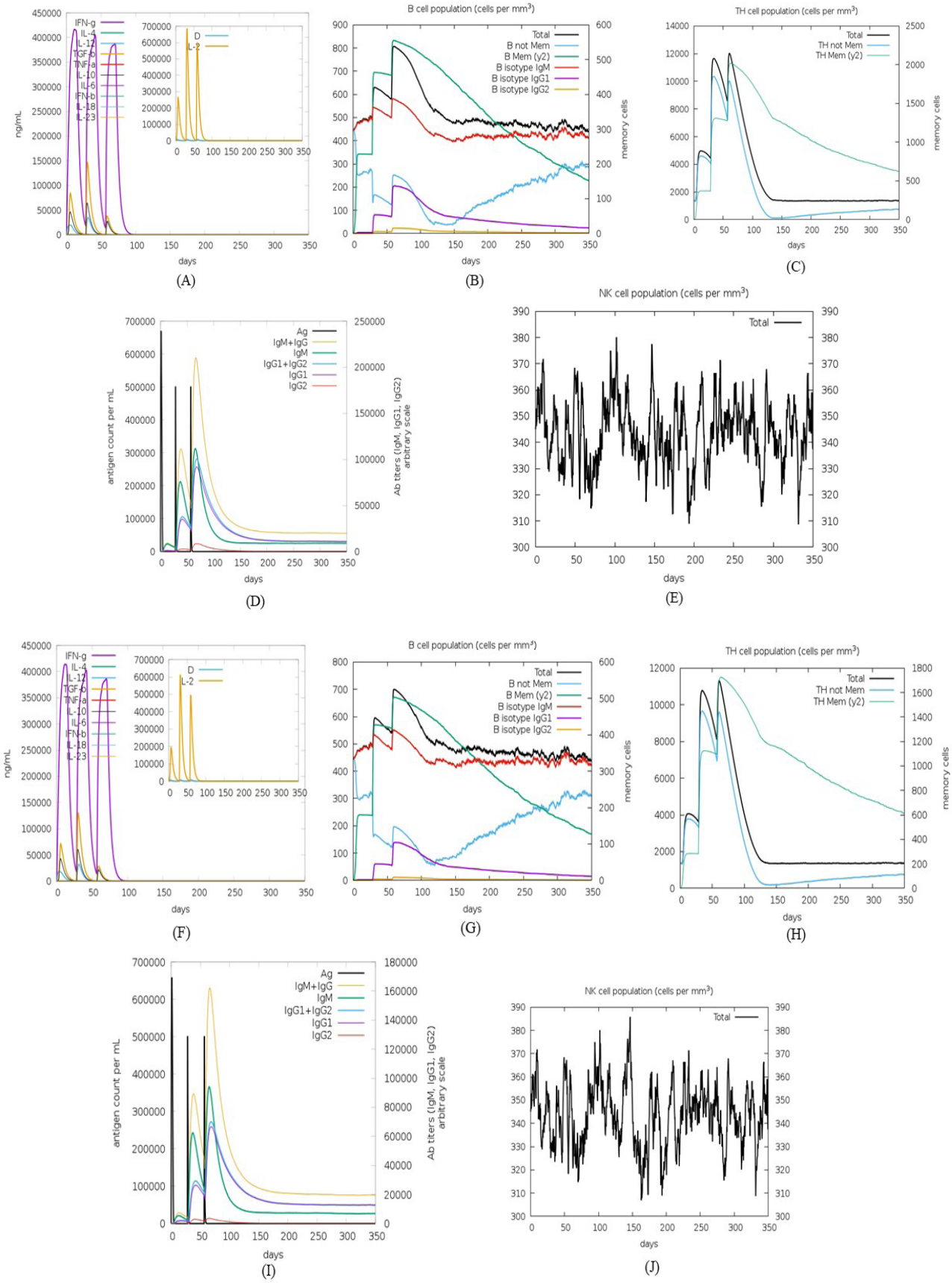


Fig. 2: Showing the molecular interaction studies of different TLR's with the designed vaccines SV-1 (Red), SV-2 (Yellow), SV-3 (Cyan), SV-4 (Orange), SV-5 (Blue).


IFN-g
IL-4
IL-12
TGF-b
TNF-a
IL-10
IL-6
IFN-b
IL-18
IL-23
ng/mL
days
D
L-2
(A)
B cell population (cells per mm3)
Total
B not Mem
B Mem (y2)
B isotype IgM
B isotype IgG1
B isotype IgG2
memory cells
(B)
TH cell population (cells per mm3)
Total
TH not Mem
TH Mem (y2)
(C)
Ag
IgM+IgG
IgM
IgG1+IgG2
IgG1
IgG2
antigen count per mL
Ab titers (IgM, IgG1, IgG2) arbitrary scale
(D)
NK cell population (cells per mm3)
Total
(E)
(F)
(G)
(H)
(I)
(J)

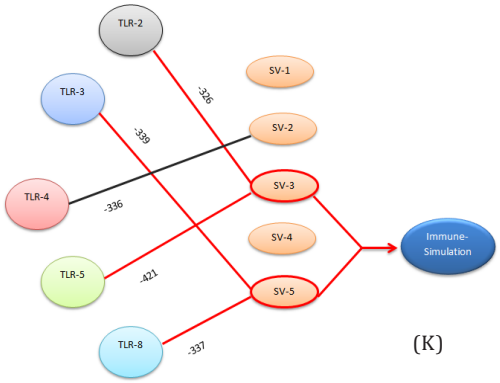


Fig. 3: Showing (A) the effect of different interleukins with respect to time in the process of immune buildup for SV3 vaccine model (B) the changes in the B-cell population with respect to time for SV3 vaccine model (C) the effect of t-helper cell population with respect to time for SV3 vaccine model (D) the observed immunological changes over a year timescale for three different booster dosage within 3 weeks of time of SV3 vaccine model (E) the changes in the population of natural killer cells with respect to time for SV3 vaccine model (F) the effect of different interleukins with respect to time in the process of immune buildup for SV5 vaccine model (G) the changes in the B-cell population with respect to time for SV5 vaccine model (H) the effect of t-helper cell population with respect to time for SV5 vaccine model (I) the observed immunological changes over a year timescale for three different booster dosage within 3 weeks of time of SV5 vaccine model (J) the changes in the population of natural killer cells with respect to time for SV5 vaccine model (K) selection of SV3 and SV5 over other vaccine model based on selection scores.

Structure Validation

The 3D structures of the designed vaccines were validated using Ramachandran plot. All the designed vaccines showed a good score of above 90% in the most favored region (Table 3).

Molecular Interaction Studies

All TLR's are present in the cell membrane hence they are the first line of contact with the viral pathogen. TLR's plays a major role in the activation of the intracellular signaling cascade which leads to the induction of IRF-3 and NF-κB which helps elicit the cellular immune responses. Molecular interaction studies of the respective TLR's with the constructed vaccines have the potential to do the same. All the five designed vaccines have shown really good interaction scores with TLR-2, TLR-3, TLR-4, TLR-5 and TLR-8.

Codon Adaptation and Vector Construction

Codon adaptation was performed for all five vaccines and all of them showed a codon adaptation index (CAI) value of above 90% and a guanine-cytosine (GC) content of more than 50%. So all the five vaccines were subjected to vector contraction within the size limit of 7 kb.

Immune Simulation

Based on the molecular interaction between different TLR's and the five designed vaccines, SV-3 and SV-5 have shown the best scores with multiple TLR's. Immune simulations were carried out for the designed vaccines SV-3 and SV-5. Three consecutive booster dosages were injected on the 1st day, 28th day, and 56th day, respectively with the help of the C-ImmSim server. Similar immunogenic patterns were observed where the IgM at a very low level was observed for SV-3 whereas for SV-5 low level of IgM and IgG were also present. After the subsequent 2nd and 3rd dosage, there was a significant peak in the levels of IgG, IgM and there was a stable concentration of Ig's for 350 days' time. A stable B cell, Helper T cells (TH) , and Neutral killer cell population were observed.

Discussion

The current study was an attempt to design multi-epitopes based vaccine against SARS-COV-2 . To design the vaccine for SARS-COV-2 , 12 different target proteins were used specifically to SARS-COV-2 as well as SARS-COV and MERS-COV. More than five hundred epitopes were individually selected based on its specificity towards MHC-I, MHC-II and B cells, which were further screened for several different such as antigenicity, non-toxic to the cells, non-allergen, and doesn't have a conserved sequence similar to any human peptide. The selected epitopes were further used in the vaccine construction, where the screened epitopes were selected arbitrarily attached by specific linkers and an adjuvant sequence. Adjuvants help in the heightening of the immunogenic property of a vaccine. Five vaccine molecules were designed and individually checked for the anti-toxicity, anti-allergenic, antigenicity and conceivability with other human peptides. Other physiochemical properties such as solubility, extinction coefficient and GRAVY values were found to be stable for a vaccine candidate for all the five (SV-1 to SV-5) designed vaccines. 3D structures were generated, minimized and then validated. Validation of the structures has shown extremely good results for carrying out further studies. Molecular interaction studies between selected TLR's with the designed vaccines showed good interaction scores. As vaccines are polypeptide molecules with the basic building block of connecting amino acids which are translated from a codon during translation. Codon adaptation was performed using *Escherichia coli* K-12 strain as the host organism. The plasmid vector construct was designed using pET-28a(+) as the plasmid vector containing

the insert improved sequenced retrieved after codon adaptation. The insert sequence was deliberately flanked by two restriction enzyme sites containing EcoRI and BamHI for site-directed insertion. The designed vaccines that showed the best molecular interaction scores with more than one TLR, such as SV-3 and SV-5 were further subjected to immune-simulation studies.

Conclusion

An effective vaccine should have a different important viral signature and be smaller in size. In this study modeled SV3 and SV5 have shown promising results in immune simulation over a time frame time-frame of about 350 days. This work can help the research community in the development of an effective multiepitope based vaccine and this work can be taken further for wet-lab experiments and trials in the future. Till date no viable multiepitope vaccine have been reported. Multiepitope vaccine covers a diverse range of immunological targets at different stages of viral infection as compared to single epitope subunit vaccine. Hence, this vaccine could be extremely useful at a time when the virus has tendency to emerge with newer strains.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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