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Designing and characterization of a SARS-CoV-2 immunogen with receptor binding motif grafted on a protein scaffold: An epitope-focused vaccine approach

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ABSTRACT
The COVID-19 pandemic caused by SARS-CoV-2 has a significant burden on the economy and healthcare around the world. Vaccines are the most effective tools to fight infectious diseases by containing the spread of the disease. The current vaccines against SARS-CoV-2 are mostly based on the spike protein of SARS-CoV-2, which is large and has many immune-dominant non-neutralizing epitopes that may effectively skew the antibody response towards non-neutralizing antibodies. Here, we have explored the possibility of immune-focusing the receptor binding motif (RBM) of the spike protein of SARS-CoV-2 that induces mostly neutralizing antibodies in natural infection or in vaccinees. The result shows that the scaffolded RBM can bind to Angiotensin Converting Enzyme 2 (ACE2) although with low affinity and induces a strong antibody response in mice. The immunized sera can bind both, the receptor binding domain (RBD) and the spike protein, which holds the RBM in its natural context. Sera from the immunized mice showed robust interferon γ response but poor neutralization of SARS-CoV-2 suggesting presence of a predominant T cell epitope on scaffolded RBM. Together, we provide a strategy for inducing strong antigenic T cell response which could be exploited further for future vaccine designing and development against SARS-CoV-2 infection.

1. Introduction
Coronavirus disease 2019 (COVID-19), caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has caused a global health concern since its first emergence in Wuhan, China in December 2019. It spread rapidly across countries causing the pandemic of the millennium due to its prolific inter human transmission capacity [1,2]. Till the end of March 2022, in about 2 years since its first identification, there have been over 450 million cases worldwide and over 6 million deaths. The novel betacoronavirus, SARS-CoV-2 is a positive-sense single-stranded RNA virus (+ssRNA) and is closely related to SARS-CoV and MERS-CoV (Middle East Respiratory Syndrome coronavirus), the pathogens responsible for the SARS epidemic of 2002, and the MERS epidemic in 2012 respectively [3,4]. Also, being a RNA virus it undergoes mutations that is driven by the low fidelity RNA-dependent RNA polymerase (RdRp) leading to the recent emergence of SARS-CoV-2 variants [5,6]. SARS-CoV-2 infection causes lower respiratory tract infection and elicits range of clinical presentations, from asymptomatic infection to severe viral pneumonia that may lead to multiple organ failure and death [7]. In the absence of standard medical intervention for COVID-19, current clinical management comprises mainly of supportive care [8,9]. The surface glycoprotein of SARS-CoV-2, the spike (S) protein is a homotrimeric glycoprotein on the virion surface and is a type I viral membrane fusion protein. Each monomer is composed of two

Abbreviations: SEC, Size Exclusion Chromatography; MRE, Mean Residual Ellipticity; CD, Circular Dichroism; RBD, Receptor Binding Motif; RBM, Receptor Binding Domain; ACE2, Angiotensin Converting Enzyme 2; BLI, Bio-layer Interferometry.

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subunits, the S1 subunit is responsible for binding to the host receptor and the S2 subunit facilitates membrane fusion [10]. The receptor binding domain (RBD) is located in the S1 subunit and binds with the Angiotensin Converting Enzyme 2 (ACE2) present on the host cell surface [4]. In addition to ACE2 recognition, the RBD is also responsible for eliciting neutralizing antibodies and has become a highly-investigated target for vaccine and drug development [3,11,12].

Vaccines are the most effective counter measures to protect and halt the spread of infectious diseases. Several vaccines and antibody therapeutics with good reported efficacy are now available for use while others are in the different stages of development and clinical trials [13]. However, current method and techniques of SARS-CoV-2 vaccine production is expensive. At present, there is a massive disparity in the global distribution of SARS-CoV-2 vaccines [14]. To control the COVID-19 pandemic in low- and middle-income countries (LMICs) where most of the global population resides, a great increase in sustainable supply of affordable vaccines is urgently needed [15,16]. Different approach of vaccine development against SARS-CoV-2 is predominantly focused on presenting the spike glycoprotein as antigen to the host. The protein subunit based vaccine approach is the development of vaccine candidate using purified viral protein(s) as antigen that is capable of inducing neutralizing antibodies. For enveloped viruses the protein subunit based vaccine development mostly targets the viral structural protein(s) produced and purified as soluble recombinant protein which is highly immunogenic and provides protection [17–21]. However, designing and development of soluble protein subunit based vaccine candidate for the viral pathogen is challenging. The viral proteins are large, complex and have posttranslational modifications such as glycosylation as the viral pathogens use complex protein synthesis system of their eukaryotic host such as mammal [22–25]. The recombinant protein production in mammalian system incurs high cost due to low yield, costly reagents and complex downstream processing [7,26]. Contrary, the prokaryotic system is cost effective because of high yield and cheap upstream and downstream stages. However, enveloped subunit protein based vaccine production in prokaryotic expression system is not successful so far as the viral protein produced in prokaryotic system forms non native-like structure and often form large protein aggregates or expressed in low quantity or found in inclusion bodies [27,28]. Secretory recombinant proteins produced in yeast results in high mannose hyperglycosylation, a major issue as it potentially alter functional properties of the proteins [29]. Ongoing efforts are being made to produce the RBD protein of SARS-CoV-2 in Pichia pastoris and E. coli. [30,31].

Here, a scaffold based approach was adapted for the designing of a SARS-CoV-2 immunoconjugate that could be expressed in the bacterial system [32]. The most immunogenic epitope of the spike protein that induces neutralizing antibodies, the receptor binding motif (RBM) present within the RBD, was placed in the middle of two CH3 domains of mouse IgG1 flanking at the either end. The idea here is that the CH3 domains at the either end will fold on itself to dimerize and exposing the RBM loop, possibly in a flexible native-like conformation, as present in the context of RBM in the viral protein produced in prokaryotic system [28]. The CH3 gene, (AA 362–463) cloned in pFUSEh-Chg-mg1M18 (Addgene Cat no. #82357) and cloned in pET28b(+) vector between the NcoI and NotI restriction sites incorporating two other restriction sites, BsWI and NotI at the C-terminal end. Another gene fragment of CH3 was amplified with 6× HisTag at the C-terminal end and cloned between NotI and Xhol. Thus, two copies of CH3 gene were cloned in the pET28b(+) vector with a 6× HisTag at the C-terminal end and cloned between NotI and XhoI. The RBM loop of RBD (AA 416–525) was PCR amplified from the RBD plasmid obtained from BEI resource (Catalog No. NR-52422; BEI Resources; NIH) and cloned in the middle of the two CH3 domain using the BsWI and NotI restriction sites. The integrity and correct frame of cloning of the whole construct was confirmed by DNA sequencing.

2. Materials and methods

2.1. Molecular modeling and structural refinement

The homology approach was used to construct the three-dimensional (3D) structure model of the primary amino acid sequence of RBD region of RBD (AA 416–525) sandwiched between two arms of CH3 domain of IgG1 antibody. The I-TASSER server [33] was used to find the best acceptable template structure. Based on I-TASSER rank, top 10 templates (pdb-ids: 6hyyA, 1o0VA, 6wibA, 6hyg, 1hz, 10ovB, 6hyg, 6wibA and 10ovB) were chosen to model the structure using a multi-template based algorithm of modeller 10.0 [34]. A total of 300 models were generated. Finally, all the model structures were aligned with the template structure and the best model with the lowest RMSD was selected. The structural validation of the selected structure was performed by the Procheck server [35].

The selected model structure was energy minimized for structural refinement using 3Drefine server [36]. The server iteratively optimizes the hydrogen bond network and steric clash and minimizes the overall energy of the model. The 3D refine server generated five refined models and all were subjected to the Procheck server [35] for further structural validation.

2.2. Construction of expression plasmid

The CH3 gene, (AA 362–463) was amplified from the mouse IgG1 heavy chain gene cloned in pFUSEh-Chg-mg1M18 (Addgene Cat no. #82357) and cloned in pET28b(+) vector between the NcoI and Xhol restriction sites incorporating two other restriction sites, BsWI and NotI at the C-terminal end. Another gene fragment of CH3 was amplified with 6× HisTag at the C-terminal end and cloned between NotI and XhoI. Thus, two copies of CH3 gene were cloned in the pET28b(+) vector with a 6× HisTag at the C-terminal end and two restriction sites, BsWI and NotI, between the two CH3 genes. The RBM loop of RBD (AA 416–525) was PCR amplified from the RBD plasmid obtained from BEI resource (Catalog No. NR-52422; BEI Resources; NIH) and cloned in the middle of the two CH3 domain using the BsWI and NotI restriction sites. The integrity and correct frame of cloning of the whole construct was confirmed by DNA sequencing.

2.3. Protein expression and purification

The pET28b(+) plasmid carrying the gene for RBM-CH3(scaffold) was transformed into the E. coli host Rosetta(DE3) for protein expression. The transformed cells were grown overnight and a 1% secondary inoculum was added to an appropriate volume of LB medium. Cells were induced with 1 mM IPTG at an OD of 0.4–0.6 and grown overnight at 18 °C after induction. The cells were harvested and re-suspended in lysis buffer containing urea (4 M urea; 50 mM Tris-Cl, pH 8.0), for purification under denaturing conditions. The lysate was centrifuged at 18,000g for 30 mins and the supernatant obtained was loaded onto a Ni-NTA column followed by washing with 30 mM imidazole in presence of urea (4 M urea; 50 mM Tris-Cl, pH 8.0). Elution was done with 500 mM imidazole in presence of 4 M urea; 50 mM Tris-Cl, pH 8.0. The eluted protein was dialysed against PBS to remove urea and imidazole. After dialysis, protein was concentrated through a centrifugal concentrator (Millipore; with a 10 kDa membrane molecular weight cut-off) up to 0.5–1.0 mg/ml, with no precipitation. SDS-PAGE analysis of the purified protein showed a pure protein band with the anticipated mobility of 37 kDa.
2.4. Circular dichroism (CD) spectroscopy

Far-UV circular dichroism spectra were acquired on a Jasco-815 spectropolarimeter. The concentration of the protein used was 5 μM. Cuvette of path length of 0.2 cm was used and spectra were collected from 260 to 200 nm at a rate of 100 nm/min and data pitch of 1 nm, with averaging of 10 scans for noise reduction. Contribution of the buffer to the spectra was electronically subtracted and mean residual ellipticity (MRE) was calculated and plotted. Deconvolution of CD spectra data and prediction of the secondary structure of RBM-CH3(scaffold) was performed by the BeStSel method (https://bestsel.elte.hu/index.php) [37].

2.5. Biolayer interferometry (BLI)

For binding kinetics anti-human IgG Fc capture (AHC) sensor (ForteBio Inc.) was used to capture the ACE2-Fc on the sensor and the RBM-CH3(scaffold) protein was used as analyte in concentrations ranging from 5400 nM to 200 nM with 1/3 serial dilution along with a no analyte as background control. The PBS buffer background was supplemented with 0.01% Tween 20 and 0.1% BSA. The experiment was performed at room temperature (RT) with agitation at 1000 rpm. To capture the ACE2-Fc, the AHC biosensors were immersed in wells containing ACE2-Fc at a concentration of 10 μg/ml for 120 s. Association was recorded for 120 s followed by dissociation for 200 s. Data were analyzed using the ForteBio Data Analysis software, 10.0 (Forte-Bio Inc). The kinetic parameters were calculated using a global fit 1:1 model as applicable [38].

2.6. Mice immunization

For immunization study, 7–8 weeks old female BALB/c mice weighing 18–25 g and inbred in institute's (THSTI) small animal facility (SAF) were used. Ten mice were randomly divided into two groups. One group of six mice was immunized with RBM-CH3(scaffold) + AddaVax as adjuvant mixed in 1:1 ratio containing 25 μg of RBM-CH3(scaffold). The other group of four mice (control group) was treated with PBS + AddaVax mixed in 1:1 ratio. The animal study was conducted as per the institutional animal ethical regulations and ethical approval. Immunization was performed via intramuscular route (cranial thigh muscles) thrice (prime, first boost and second boost) at interval of 3 weeks. The mice were observed daily and weighed twice a week over the course of the immunization period. Blood sample from each mouse was collected at day 0 (pre immune sera), 14 (sera after priming), 35 (sera after the first boost), and at 56 (sera after the second boost). Serum was separated from the blood, heat inactivated at 56 °C for 1 h, and stored at –20 °C for future use. The splenocytes were collected 63 days after first immunization.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed to characterize the binding of RBM-CH3 (scaffold) to ACE2. Maxisorp plates (Nunc) were coated with 100 μl of ACE2-Fc protein (2 μg/ml) or BSA as control in 1× carbonate/bicarbonate buffer, pH 9.6 for overnight at 4 °C. Next day, the plate was blocked by 250 μl of PBS containing 5% skimmed milk. Different concentration of RBM-CH3(scaffold) protein ranging from 16 μg/ml to 0.25 μg/ml in 1/2 fold serial dilution was added to the wells (100 μl/well) and incubated for 2 h at RT. The wells were then washed and HRP-conjugated anti-His antibody (Abcam) in 1:2000 dilution were used for developing ELISA.

For characterizing the binding of immunized sera with the RBM-CH3 (scaffold), the RBD and the prefusion spike protein, Maxisorp plates (Nunc) were coated with 100 μl of the proteins at concentration of 2 μg/ml in 1× coating buffer for overnight at 4 °C. Next day, the plates were blocked using 250 μl of PBS containing 5% skimmed milk. The immunized sera were diluted serially 1:3 times in the dilution buffer with a starting concentration of 1:300 dilution and incubated for 2 h followed by washing and blocking. HRP-conjugated anti mouse secondary antibody (Jackson Immuno Research, USA) in 1:2000 dilution was used for developing ELISA.

2.8. Western blot analysis

For Western blot analysis, 2 μg of each of RBM-CH3(scaffold), RBD and the soluble spike proteins were loaded on 12% SDS-PAGE followed by transformation to PVDF membrane. The membrane was blocked with 5% skimmed milk, incubated with pooled serum from the immunized mice or the control group in 1:2000 dilutions for overnight at 4 °C. The membrane was developed with HRP-conjugated anti-mouse secondary antibody (Jackson Immuno Research, USA).

2.9. Immuno-fluorescence analysis

HEK293T cells (ATCC, CRL-3216) at 0.25 million in each well were seeded in Dulbecco’s Modified Eagle Medium (DMEM from Gibco) containing 10% FBS and 100 U/ml Penicillin-Streptomycin (P/S from Gibco) in a 12-well plate. Next day, 1 μg of Spike or RBD plasmid was transfected using FuGENE HD transfecting reagent (Promega) at 1:3 ratio of plasmid DNA and FuGENE HD reagent. After 24 h of transfection, cells were fixed in 4% paraformaldehyde (PFA) for 15 min. Non-specific binding was blocked using 3% goat serum in PBS for 1 h at RT. Cells were then incubated at RT for 1 h with heat inactivated pooled serum sample from terminal bleed of immunized mice or the control group in 1:200 dilution. After incubation, cells were washed and incubated for 1 h at RT with the Alexa Fluor 488 labeled rabbit anti-mouse IgG secondary antibody (1:1000 dilutions). Cells were washed three times with PBS and their nuclei were counterstained with DAPI (D9542, Sigma-Aldrich, United States) for another 10 min at RT. After three washes with PBS, images were acquired on an Olympus IX-71 fluorescence microscope.

2.10. In vitro stimulation of splenocytes and intracellular cytokine staining

The spleens from the immunized mice were isolated and single-cell suspension was prepared. About 0.5 million splenocytes were then cultured in 96-well plates in Iscove’s Modified Dulbecco’s Medium (IMDM) complete media and stimulated with (a) PMA(50 ng/ml) and ionomycin (1 μg/ml) for 4 h or (b) with 50 μg/ml of RBD for 72 h in 5% CO2 incubator at 37 °C. Thereafter, cells were washed and stained for intracellular cytokines. For intracellular cytokine staining in vitro-stimulated splenocytes were first surface stained for CD4, and CD8 markers at RT for 20 min in dark and thereafter fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, CA, USA) according to manufacturer protocol. The permeabilized cells were then stained for intracellular cytokines, such as IFNγ, interleukin (IL)-4, and IL-17A at RT for 20 min in dark. The cells were then washed and data acquired on BD FACSCanto II (BD Biosciences, CA, USA) and the data were then analyzed on FlowJo software (Tree Star, Inc., OR, USA) and plotted on GraphPad prism software (59,40).

2.11. Plaque reduction neutralization test (pRNT)

SARS-CoV-2, isolate USA-WA1/2020 virus was used to perform pRNT at institute’s (THSTI) Infectious Disease Research Facility (Biosafety level 3 facility). In a 12-well plate, 0.4 million of Vero E6 cells in 1 ml of DMEM (Gibco) containing 10% FBS (Gibco) and 100 U/ml P/S (Gibco) were seeded. Next day, heat inactivated pooled serum sample from terminal bleed of individual mice were diluted 5-fold in 100 μl of DMEM supplemented with 2% FBS and incubated with 100 μl of 50 PFU of SARS-CoV-2 for 1 h at 37 °C. Following incubation, confluent Vero E6 cells in 12 well plates were infected with the sera-virus mixture for 1 h at 37 °C. After 1 h of adsorption, the wells were washed one time with PBS.
One ml of DMEM containing 2% FBS, 1 x P/S and 0.5% carboxy methyl cellulose (CMC) was added to each well and the plate was incubated at 37°C with 5% CO2. After 48 h media from each well were removed and the cells were fixed with 4% PFA for 1 h. Cells were then stained with 0.5% crystal violet for half an hour and washed with water. The plates were allowed to air dry, and the numbers of plaques in respective wells were counted.

3. Results

3.1. Construct design

The interaction of RBD with the host ACE2 is the critical event for the SARS-CoV-2 viral infection. The RBD within S glycoprotein is structurally defined as a region between two cysteine residues (C336 and C525) that form a disulfide bridge. The detailed structure of SARS-CoV-2 RBD bound to ACE2, highlighting the key regions of the RBD responsible for ACE2 binding has been determined and explained by Lan et al. [4]. The RBD mostly consists of extensive β-sheets structure with a short linear segment called receptor binding motif (RBM) that contains most of the amino acid residues making contact with the ACE2. Most of the neutralizing antibodies identified so far for SARS-CoV-2, irrespective of their source of origin, such as monoclonal antibodies isolated from patient sera or neutralizing antibodies (nAbs) generated in mice, or antibodies isolated through phage display techniques [41], have their binding surface on or close to the RBM region of RBD [42]. The mechanism of neutralization by these nAbs is mainly through direct competition with ACE2 for binding to the RBD. This suggests that the epitopes near the ACE2 binding site is not only immunogenic but induces nAbs. The other parts of the RBD might induces non neutralizing antibodies or neutralizing antibodies that work through allosteric mechanism (destruction of spike), such as CR3022, but not directly interfere with ACE2 binding [43]. This is important from a vaccine development standpoint, especially for designing RBD-based immunogens, because if the non neutralizing epitopes are highly immuno-dominant, they can potentially skew the dominance of non-neutralizing antibodies or interfere with the induction of nAbs.

We conceptualize a RBD based immunogen design strategy, whereby to present only the most important epitope of the spike protein, the RBM region, grafted on a protein scaffold, such that the displayed RBM mimics the structure of RBM present in the context of RBD or spike protein on the virion surface. Thus the designed immunogen will selectively display only the RBM to the host immune-system through an immune-focusing approach. Hence, it is expected that such designed construct if maintain a native like conformation of the RBM will also bind to ACE2 and induce conformation dependent neutralizing antibodies for SARS-CoV-2. As scaffold protein we choose a self antigen, the CH3 domain of IgG1 antibody of the host, to minimize the chance of inducing antibodies directed against the scaffold. The overall design strategy is shown in Fig. 1A and B. The CH3 domain is placed at the either end of the RBM such that when the two CH3 domains fold on itself and dimerize, the RBM loop is displayed as is present on the RBD. The full sequence of the construct is shown in Fig. S1. The designed construct was verified in silico for appropriate folding using structure analysis tools and energy minimization.

3.2. Homology modeling and structural refinement

The primary amino acids sequence of the designed construct of the RBM-CH3(scaffold) with the RBM region of RBD sandwiched between two CH3 domain of mouse IgG1 on either side was loaded on the I-TASSER (Iterative Threading ASSEmbly Refinement) [33] server for homology modeling to find out the best fit 3D model for the designed construct. As expected, the best 3D models obtained after molecular modeling were mostly aligned with the structure of the Fc domain of antibodies available in the structural database. To further improve the

| Secondary structure prediction | Sequence analysis (GOR IV) (%) | I-TASSER (%) | Circular dichroism (BebSel) (%) |
|-------------------------------|--------------------------------|--------------|-------------------------------|
| α-Helix                       | 6.6                            | 4.1          | 5.0                           |
| β-Sheet                       | 28.8                           | 36.6         | 32.7                          |
| Random coil                   | 64.6                           | 59.3         | 62.3                          |

Fig. 1. A) The RBM region of RBD (AA 416–525) used in the design of the vaccine candidate is shown in blue interacting with the receptor ACE2 shown in yellow. The RBM region is flexible and forms a loop like structure. B) Schematic presentation of the designed vaccine. The RBM region of RBD (AAs 416–525) shown in red, is sandwiched between two CH3 domains (yellow) of IgG1 at the either end of RBM. The final energy minimized 3D model is shown below the schematic presentation. The two CH3 domains at the either end fold to dimerize and thereby exposing the RBM loop on the CH3 scaffold. C) Superimposed image of 3D model structure is shown with the CH3 domain (PDB ID: 6HYG) in green and the RBM loop of RBD (PDB ID: 7BJW) in blue. The modeled structure is shown in red (RBM) and yellow (CH3) respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
immunogen have the potential to adopt a structure where the two CH3 domain of the IgG1 folds on itself to form a dimer and the RBM loop is displayed on the CH3 scaffold with some deviation as it present on the RBD structure. The overall RMSD of the structure is inflated as the RBM structure is trapped between two rigid CH3 terminals, however, the secondary structure contents are considerably stable. A high resolution structure would have provided a better insight of the final modeled structure particularly for the native-like conformation of the RBM loop. Indeed, in the absence of actual structure, we based our study on the modeled structure of the designed immunogen.

3.3. Expression, purification and characterization

The protein was expressed in *E. coli* Rosetta(DE3) strain and purified to homogeneity under denatured condition using Ni-NTA affinity chromatography followed by refolding. On SDS-PAGE the protein migrated with a molecular weight of about 37 kDa which is the expected molecular weight with >95% purity. It eluted as a single peak from the Superdex 200 increase 10/30 size exclusion column at elution volume of 15.2 ml, slightly less than the elution volume of ovalbumin with mol. wt. of 44 kDa. Extrapolation from the calibration curve of Superdex 200 increase 10/30 column estimates the molecular weight as 37 kDa, suggesting that the protein is monomeric in solution (Fig. 2A and S4). To assess the secondary structure signature, we performed circular dichroism spectroscopy of RBM-CH3(scaffold). Assessment of the CD curve using BeStSel method revealed 5.0% α-helix, 32.7% β-sheet and 62.3% of random coil [37]. Amino acid sequence based analysis for secondary structure prediction using GOR IV was also performed [44]. A comparative analysis of the secondary structure estimation with different methods is given in Table 1. The secondary structure estimates based on prediction methods (GOR IV and I-TASSER) and CD analysis of actual purified protein are comparable. All methods estimated a predominantly β-sheet based structure with high degree of random coil and very little α-helix. This is expected as the CH3 domains of antibodies are rich in anti parallel β-sheet and the RBM is mostly flexible loop (Fig. 2B).

To evaluate the presence of native like conformation of the displayed RBM on the CH3 scaffold and its binding with ACE2, we performed ELISA binding of the RBM-CH3(scaffold) with ACE2 protein fused to Fc domain (ACE2-Fc). The result suggests that the RBM-CH3(scaffold) binds to ACE2 but with low affinity (Fig. 2C). To measure the binding affinity and kinetics of RBM-CH3(scaffold) to ACE2 we performed bi-layer interferometry (BLI). For this purpose, ACE2-Fc protein was captured on the anti-hIgG Fc capture (AHC) biosensor and titrated with different concentration of RBM-CH3(scaffold). Based on the binding kinetics the calculated apparent affinity of RBM-CH3(scaffold) to ACE2 was 350 ± 100 nM with an on rate of 4.5 ± 0.5 × 10^3 (1/Ms) and off rate of 1.6 ± 0.3 × 10^-3 (1/s) (Fig. 2D). Suggesting that the RBM-CH3 (scaffold) binds with ACE2 with 20 fold less affinity compared to the soluble RBD protein [45,46]. This suggest that the RBM loop in the designed RBM-CH3(scaffold) construct maintains native-like conformation to some extent enabling the RBM-CH3(scaffold) to bind to ACE2. This is important from the vaccine design prospective to induce conformational antibodies that binds with the RBM in its original context and interferes with the attachment of ACE2 with the spike protein and thereby neutralizes the SARS-CoV-2 virus entry to the host cell.

3.4. RBM-CH3(scaffold) induced strong RBD and S protein directed antibody responses in mice

To determine the immunogenicity of the RBM-CH3(scaffold), 7–8 weeks old BALB/c mice were immunized as described in the “Materials and Method” section. The immunization scheme is shown in Fig. 3A. The whole IgG response of the immunized sera were measured against the RBM-CH3(scaffold), the RBD and the soluble prefusion spike protein by ELISA. In each case very good titer of the whole sera was seen for each of the antigens and after boosting the titer increased considerably as expected. Highest antibody titer was seen for the RBM-CH3(scaffold) antigen that was used for immunization with significant increase in binding titer after first boost. Second boost further improved the titer (Fig. 3B). The sera also showed significant binding titer for the RBD protein as well as the soluble prefusion spike protein (Fig. 3C and D). No ELISA binding was seen for any of the antigen for sera from the control group of mice. The result suggests that the antibodies induced in
response to the immunization of RBM-CH3(scaffold) also binds to the RBD and spike protein which holds the RBM loop in its natural context on the virus particle.

The ability of the immunized sera to bind to RBM-CH3(scaffold), the RBD and the spike protein was further confirmed by Western blot. The pooled serum of the immunized mice group detected the antigen, the RBD and the spike protein efficiently while the sera from the control group failed to detect the antigens (Fig. 4A and S5). To confirm whether the immunized sera could also bind to the cell surface expressed spike protein, HEK293T cells transiently transfected with the full-length spike protein was probed with the pooled immunized serum. The pooled serum from the control group was used as negative control. The cell surface expressed spike protein was detected in immuno-fluorescence when probed with Alexa Fluor 488 labeled rabbit anti-mouse IgG antibody for the immunized serum only (Fig. 4B and S5). These results suggests that the antibodies induced in response to the RBM-CH3(scaffold), binds with the RBD and the spike protein irrespective of expression in soluble form or in the membrane anchored form (cell surface expressed) of the spike protein confirming the induced antibodies binds to the RBM region of the RBD and the spike protein in its quaternary conformation and in natural context. Above observation is also corroborating with the ELISA data in Fig. 3.

### 3.5. T-cell specific response

To understand the T cell specific immune response by the RBM-CH3 (scaffold) protein, spleen of the immunized mice were collected 3 weeks after the second booster does and the splenocytes were stimulated under antigen specific (RBD) or non-specific (PMA + ionomycin) condition (Fig. 5, S6 and S7). Our result indicated a heightened IFNγ response in immunized mice for both antigen specific and non-specific expansion suggesting induction of Th1 response by the immunized protein. Interestingly, IFNγ secretion was increased by more than 2-fold for both CD4+ and CD8+ T-cell population. IFNγ is well documented to directly inhibits viral replication and enhances the antigen presentation [47]. Moreover, we also found elevation of IL4 (in CD8+ T-cells) and IL17A (in both CD4 and CD8+ T-cells) secretion suggesting that the designed immunogen displays potent T cell epitopes and may generate strong inflammatory response (Fig. 5). The data for T-cell response suggest that the designed immunogen has significant potential to induce T cell population which is important for strong anti viral activity and virus clearance.

### 3.6. Serum neutralization assay

To test the ability of the RBM-CH3(scaffold) immunized sera to neutralize the SARS-CoV-2 was evaluated by classical virus pRNT assay in Vero E6 cells. Sera from the terminal bleed (21 days later after the second boost) of individual mice were incubated with 50 plaque forming unit (PFU) of SARS-CoV-2 Wuhan strain (USA-WA1/2020 isolate) at 1:5 dilutions. The number of plaques formed was visualized by crystal violet staining. Neutralization of about 50% plaques were seen for sera from all the mice immunized with RBM-CH3(scaffold) at 1:5 dilution and no neutralization was seen by the sera of the control group of mice (Fig. 6). The result suggests that though RBM-CH3(scaffold) induced strong immune response, no strong neutralization response was induced. This indicates that further refinement in designing is needed to improve the neutralization potential of the induced sera by RBM-CH3(scaffold).
4. Discussion

Subunit vaccines face their own set of challenges which are unique to each pathogen and require distinctive protein design and engineering approach to suitably modify to meet the desired properties. However, given the large number of efforts to develop subunit vaccines against other pathogens including influenza, respiratory syncytial virus and HIV, a great advancement has been made in the innovation in structure based antigen design [48]. For soluble protein subunit based vaccine design, the target antigen must be reliably and consistently produced with a desired conformation and high yield that is stable throughout manufacturing, transport, and storage, right up until the moment of vaccination.

This study describes the design and development of a unique protein subunit based vaccine candidate for SARS-CoV-2. The objective here was to immune-focus an epitope of SARS-CoV-2 spike protein that is not

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**Fig. 5.** Assessment of antigen-specific cellular immune responses. In vitro antigen (RBD) stimulated splenocytes were used for intracellular cytokine staining viz. IFNγ, IL-4, and IL-17A response. A) Representative fluorescence-activated cell sorting (FACS) contour plots showing expression of cytokines in CD4 or CD8 gated cells in immunized/unimmunized group B) Representative bar graph of un-induced (UI) and induced (I) splenocytes were plotted with percent of positive±standard errors of the mean for each group. Statistical significance was determined using the one-way ANOVA test where *p < 0.05, ***p < 0.001, and ****p < 0.0001.

**Fig. 6.** A) Wells of pRNT assay using SARS-CoV-2 virus and sera from mice immunized with RBM-CH3(scaffold) (T1-T5) and control sera (C1–C2). Commercial neutralizing antibody was used as positive control (+VE) and PBS background was used in the virus control (VC). B) Bar graph presentation of the average of control group sera (C1–C2) and immunized group sera (T1-T5) showing 50% neutralization at 1:5 dilutions of sera. C) Table of individual plaque count.
only immuno-dominant but induces neutralizing antibodies. The RBM region of RBD is such an epitope [42]. The neutralizing antibodies against SARS-CoV-2 so far identified from different sources including natural infection, vaccinees and animal immunization induces antibodies against the RBM and are conformational in nature [42]. The mechanism of neutralization by these antibodies is mainly by direct competition with ACE2 for binding to the RBD. No neutralizing antibodies are so far reported for SARS-CoV-2 that binds to a linear peptide chain of spike glycoprotein and hence a linear peptide based approach of immunogen design may not be successful [49,50]. Moreover, peptides alone are not immunogenic enough to induce sufficient titer of antibodies [51,52]. The challenge here was to present the selected epitope on a protein scaffold such that it adopts a conformation which mimics the conformation of RBM present on the spike protein on the surface of SARS-CoV-2 and remove rest of the redundant part of the protein to minimize off target immune response.

One way to reduce the off-target immune responses is to alter the immunogen surface to remove, reduce or hide the non-neutralizing epitopes using protein engineering approach [53–56]. To more effectively focus the antibody immune response to the RBM loop, we hypothesized that the RBM loop being flexible and an independent domain with no long distance interaction from other structural element of the spike protein will have the propensity to adopt a native like structure by itself when scaffolded to another appropriate protein structure. The selection of the scaffold was also important to minimize the induction of antibodies against the scaffold itself and avoid off target induction of antibodies. The CH3 domain of antibody forms dimer and is a self-antigen and envisaged as an appropriate protein structure for grafting the RBM loop. We selected the CH3 domain of the host IgG1 antibody (mice in this case) as the self antigen to the host and expected not to induce off target antibodies.

Here we have demonstrated that by grafting the RBM loop in the middle of two small, independent and conformationally stable protein scaffold that forms dimer, the CH3 domain of IgG1, presented the RBM loop in native like conformation that mimic the structure of the RBM loop present on the spike protein in context with the full length spike ectodomain. The designed immunogen induced high titer of binding antibodies that not only binds to the designed immunogen used for immunization but also binds with the soluble RBD and the full length pre-fusion soluble spike protein. The antibodies also bind to the spike protein expressed on the cell surface of transiently transfected HEK293T cells. Our immunogenicity data showed that the mice immunized with scaffolded RBM protein produced a robust T-cell mediated IFNγ, IL-4, and IL-17A cytokines response which may be helpful in mounting immunity against SARS-CoV-2. When tested for the neutralization potential of the immunized sera, the sera showed 50% neutralization of live SARS-CoV-2 virus of Wuhan strain in pRNT based assay at 1:5 dilution. However, induction of weak neutralization is a concern and requires further optimization of the design to elicit predominantly neutralizing antibodies.

In summary our results demonstrate the feasibility of epitope-focused and scaffold-based vaccine design and support the application of such strategies for designing a variety of vaccine targets for different diseases. These studies demonstrate how structure-guided antigen design can be used to rapidly develop vaccine candidates for pandemic threats and support the use of prototype pathogens as an approach to pandemic preparedness and response.

CRedit authorship contribution statement

R.K., H. A. P., S. S., and S. A. methodology and investigation; S. S., and S.A. supervision; A.K.A., and Sh.A., performed and evaluated the modeling experiments; Z.A.R and A.A., design, performed and evaluated the T-cell assays; R.K., H. A. P., S.R, R. Kaul and Sh. Ma design and performed experiments; and S.A., S.S., and A.A., conceptualization, project administration, writing, reviewing and editing of the manuscript.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.04.148.

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