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Generation and utility of a single-chain fragment variable monoclonal antibody platform against a baculovirus expressed recombinant receptor binding domain of SARS-CoV-2 spike protein

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ABSTRACT

As the second wave of COVID-19 launched, various variants of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) have emerged with a dramatic global spread amongst millions of people causing unprecedented case fatalities and economic shut-downs. That initiated a necessity for developing specific diagnostics and therapeutics along with vaccines to control such a pandemic. This endeavor describes generation of murine derived recombinant single-chain fragment variable (scFv) as a monoclonal antibody (MAb) platform targeting the receptor binding domain (RBD) of Spike protein of SARS-CoV-2. A specific synthesized RBD coding sequence was cloned and expressed in Baculovirus expression system. The recombinant RBD (rRBD) was ascertained to be at the proper encoding size of ~ 600bp and expressed protein of the molecular weight of ~ 21KDa. Purified rRBD was proved genuinely antigenic and immunogenic, exhibiting specific reactivity to anti-SARS-CoV-2 antibody in an indirect enzyme-linked immunosorbent assay (ELISA), and inducing strong seroconversion in immunized mice. The scFv phage display library against rRBD was successfully constructed, revealing ~ 90 % recombination frequency, and great enriching factor reaching 88 % and 25 % in polyclonal Ab-based and MAb-based ELISAs, respectively. Typically, three unique scFvs were generated, selected, purified and molecularly identified. That was manifested by their: accurate structure, close relation to the mouse immunoglobulin (Ig) superfamily, right anchored six complementarily-determining regions (CDRs) as three within variable heavy (vH) and variable light (vL) regions each, and proper configuration of the three-dimensional (3D) structure. Besides, their expression downstream in a non-suppressive amber codon of \textit{E. coli} strain SS32 created a distinct protein band at an apparent molecular weight of ~ 27KDa. Moreover, the purified scFvs showed authentic immunoreactivity and specificity to both rRBD and SARS-CoV-2 in western blot and ELISA. Accordingly, these developed scFvs platform might be a functional candidate for research, inexpensive diagnostics and therapeutics, mitigating spread of COVID-19.

1. Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the causal agent of an acute respiratory disease termed COVID-19. It has been dramatically spread over the world due to its easy way of transmission between persons, resulting in millions of infected and dead cases. This makes a necessity for developing a fast detection test to assist in controlling of outbreaks (Nardell and Nathavitharana, 2020).

SARS-CoV-2 is a positive-sense single-stranded RNA enveloped virus with a genome size of ~ 30Kb. It was recognized as a related clade to the prototype human and bat coronaviruses of the species SARS-related coronavirus, genus \textit{Betacoronavirus}, subfamily \textit{Orthocoronavirinae}, within the family \textit{Coronaviridae} (Zhou et al., 2020). One of the SARS-CoV-2 structural components is the spike (S) protein, which is found on the surface and performs an important role in viral entrance into the host cells (Li, 2012, 2016). Particularly, the receptor binding domain (RBD) of this protein is influences in infectivity (Wang et al., 2020), its amino acid sequences are frequently divergent that determine the viral host specificity, and facilitating attachment to the targeted host receptor (Li et al., 2005). Moreover, antibodies against this RBD can potentially neutralize the virus, as it encloses the major neutralizing epitopes (Hoffmann et al., 2020). Thus, RBD is a core determinant for...
tissue tropism, host range and immunogenicity of coronaviruses (He et al., 2006; Du et al., 2009).

The main diagnostic procedure for detecting SARS-CoV-2 genomic RNA is the reverse transcription polymerase chain reaction (RT-PCR) (Wang et al., 2020; Corman et al., 2020). Because of the obstacles of RT-PCR, such as the need for a well-equipped laboratory and high reagent costs (Li et al., 2020), serological detection of viral proteins is a potential alternative. However, availability of specific antibodies against SARS-CoV-2 proteins is a key facet to carry out such serological assays (Lee et al., 2008, 2010; Dutta et al., 2008). Monoclonal antibodies (mAbs) have been accepted as the foremost element in virus detection, plus they are quickly gaining popularity as a supplemental treatment to vaccinations (Jin et al., 2017; Renn et al., 2020; Zhou and Zhao, 2020; Jiang et al., 2020).

Constructing an antibody library through phage display technology provides a functional approach for obtaining recombinant mAbs (rMabs), as the traditional technique for mAbs production is costly and time-consuming (Schofield et al., 2005; Dai et al., 2003; Hu et al., 2003). This method utilizes recombinant DNA technology to facilitate selection of rMabs and their coding genes (Witrup, 1999). Furthermore, it could be used to create a therapeutic antibody cocktail of Mabs against a variety of epitopes and might endorse resistance to variant mutations, where SARS-CoV-2 can quickly evolve versions with mutations that circumvent immune responses (Wang et al., 2018; Noy-Port et al., 2020; Baum et al., 2020; Zost et al., 2020).

Single-chain fragment variable (scFv) is one of the most beneficial rMabs; it can be easily selected through displaying on a filamentous phage (Moghaddam et al., 2003). A number of rMabs have already been generated against various antigens (Little et al., 1999; Knappik et al., 2000; Salem et al., 2019a).

Baculovirus expression system is an effective approach for expression of foreign genes, because the yields of expressed proteins are very high and their biological properties are sustained. The baculovirus expressing system has been previously used for the synthesis of different recombinant proteins (El-Gaied et al., 2017; Elmenofy et al., 2020), and vaccine manufacturing (Cox, 2012; Van Oers, 2006).

As waves of COVID-19 infections have been continuing worldwide, there was increased demand for development of detection assays targeting RBD for sensitive and accurate diagnosis of COVID-19. In this paper we described the using of phage bio-panning technique to generate murine recombinant scFv antibodies targeting the recombinant SARS-CoV-2 RBD protein. We believe that these antibodies will be valuable in laboratory research, and inexpensive diagnostics and therapeutics, mitigating spread of COVID-19. Further study for field detection and therapy applications for generated scFv antibodies against the pathogenic SARS-CoV-2 may be developed.

2. Materials and methods

2.1. Constructing and expression of the recombinant RBD of SARS-CoV-2

The nucleotide sequence fragment encoding for SARS-CoV-2-RBD was correspondingly designed based on the reference isolate Wuhan-Hu-1(GenBank: MN908947.3). The selected nucleotide sequence was synthesized (Synbio Technologies Company, USA) after being subjected for codon usage optimization to best counterpart the machinery condition of the insect cells Spodoptera frugiperda expression system (Sf9, Invitrogen) using the GenSmart codon optimization, version Beta 1.0 (https://www.genec rept.com/tools/gensmart-codon-optimization). Antigenicity along with the hydrophobic and hydrophilic regions were assessed through the three-dimension (3-D) structure of optimized RBD coding sequence using different softwares (I-Tasser, Pymol, and DNA-Laser). Briefly, the synthesized RBD coding fragment was directly cloned into the baculovirus cloning vector pFast- Bac1 of the Bac-to-Bac baculovirus Expression System kit (ThermoFisher Scientific). The recombinant pFast-Bac1-RBD plasmid was then transformed into E. coli competent cells of DH10Bac strain to generate the recombinant bacmid, following the manufacturer’s instructions (ThermoFisher Scientific). Subsequently, log-phase insect cells Sf9 were transfected with the recombinant bacmid DNA purified from the positively selected DH10Bac transformants. The budded recombinant baculovirus was harvested, clarified, screened for proper recombination, titrated and utilized to transfect log-phase Sf9 cells to obtain the recombinant baculovirus (rBac/SARS-CoV-2-RBD). This step was repeated until a high-titer purified viral stock was obtained according to standard procedure provided by the manufacturer (ThermoFisher Scientific). The recombinant RBD protein of SARS-CoV-2 (rRBD/SARS) was expressed in Sf9 insect cells (log-phase 2.5 × 10^6 cells/mL, in 75 cm^2 tissue culture flasks; infected at m.o.i. of 5 and incubated at 27 °C) using the generated rBac/SARS-CoV-2-RBD, as supernatants from infected cells were harvested 5 days post infection and analyzed by SDS-PAGE. Consequently, rRBD/SARS fused with N-terminal 6xHis-tag was purified from the infected cell culture using Ni-NTA agarose resin (Qiagen, Germany) and its concentration was determined using Bradford protocol (Salem et al., 2020).

2.2. Antigenicity and immunogenicity of the purified rRBD/SARS2 protein

Two female 21 days old BALB/c mice, obtained from Theodor Bilharz Research Institute, Egypt, were treated in compliance with the principles and policies of the National Institute of Health (NIH) animal care; and upon the approval of the Animal Care and Use Committee in Veterinary Medicine, Cairo University (Vet CU16072020181), they were utilized in the experiments. The primary immune response was initiated in mice by intraperitoneal injection with a 0.2 mL dose containing 50 μg of the rRBD/SARS2 protein emulsified in complete Freund’s adjuvant, followed by 3 subsequent intravenous boosters (once/weekly), each with a 100 μg per dose emulsified in incomplete Freund’s adjuvant that was excluded from the last booster. Seven days post the 5th injection, sera were collected and spleens were gathered and kept at −80 °C after mice being euthanized.

An Indirect ELISA was carried out to assess the seroconversion against rRBD/SARS2 in sera of immunized mice. Briefly, 96-well Immunolon-1 microtitration plates (Nunc, IL, USA) were coated with rRBD/SARS2 diluted in carbonate - bicarbonate buffer, pH 9.6 (~ 50 ng/well) and incubated for overnight at 4 °C. The wells were washed with Phosphate-Buffered Saline (PBS) containing 0.01 % Tween, then blocked with 3 % bovine serum albumin (BSA) in PBS, pH 7.4, for 2 h at 37 °C. The plates were decanted and washed three times as before. A 100 μL/well of each serum sample diluted 1:10 in PBS, pH 7.4 containing 3 % BSA was added in duplicates to the wells and incubated for 2 h at 37 °C then, decanted and washed. The zero day mouse serum (prior to the first mouse immunizations) was utilized as negative control. The wells were intensively-washed with PBS-Tween 20 (PBST). The rabbit anti-mouse IgG (Sigma Aldrich) conjugated with alkaline phosphatase (ALP) was added at a dilution of 1:20000 and incubated for 2 h at RT. The reaction was developed with p-Nitrophenyl Phosphate (PNPP) substrate solution after washing with PBST. The absorbance values were determined at wavelength 405 nm using Bio-Rad 1 Mark microplate reader.

For determining the rRBD authentic immunoreactivity, its sensitivity to capture the SARS-CoV-2 specific antibodies in the serum of infected patients was assessed by indirect ELISA. Different concentrations of purified rRBD protein, ranging from 500 to 50 ng per well, were coated in ELISA microtitration plate. Sera from SARS-CoV-2 patients (all serum samples were collected after obtaining the written informed consent from the patients) were diluted in PBS containing 3 % BSA, added at 100 μL per well in duplicate (4 times), and incubated at 37 °C for 2 h. After decanting the sera and washing, anti-human IgG (ThermoFisher Scientific) labeled with ALP was added. After incubation for 1 h at 37 °C, the plates were decanted and washed, and detection was performed as aforementioned.
RNA was isolated from dissected spleens of rRBD immunized mice. The complementary DNA (cDNA) was generated from isolated RNA. The variable chains (variable heavy (vH) and variable light (vL)) amplification and their assembly were conducted through subsequent PCRs as diagrammed in Fig. 1, using different pairs of primers designed according to the V-BASE database. In the first PCR, repertoires of vH and vL coding sequences were amplified separately from cDNA. The same amounts of amplified vH and vL repertoires (~ 100 ng/each) were combined to be assembled through the second PCR, called splicing overlapping extension (SOE)-PCR (SOE-PCR). The coding sequence of SfiI restriction enzyme was added in the third PCR to the amplified scFv repertoires to be subsequently batch-ligated into pADL-22c phagemid and then transformed into E. coli TG1 competent cells. All of the clones that resulted were gathered in LB medium; and after centrifugation, pelletized cells were resuspended in 2xYT medium containing 15 % glycerol and saved as a library stock.

2.4. Phage display and bio-panning

An aliquot of saved library stock was activated and infected with M13K107 helper phage. Following our published protocol (Salem et al., 2019a), the rescued phages were purified and exposed to six rounds of panning (three against the rRBD, and three against the inactivated SARS-CoV-2 strain hCoV-19/Egypt/NRC-03/2020, kindly provided by the Center for Scientific Excellence for Influenza Virus, Environmental Research Division, National Research Centre). In each panning, an ELISA microplate was coated with the antigen either inactivated SARS-CoV-2 or the rRBD (5 μg /well) for overnight at 4 °C; after washing and blocking steps, rescued phages were added to the coated wells for 1 h. Unbound residual phages were discarded; after intensive washing, phages were eluted with the tri-ethylamine. After their neutralization, and washing steps, rescued phages were added to the coated wells for 1 h, washed and blocked using 5 % skimmed milk for 2 h at room temperature (RT). Phages from each round of panning (after and before) were added. After incubation and washing steps, anti-M13 PIII monoclonal antibody was added. Following the washing steps, anti-mouse IgG labeled with ALP was added, and then detection was developed by PNPP substrate solution.

2.5. Evaluation of scFv clones by ELISA

Phages obtained after each round of panning was tested by ELISA to see whether they have been enriched for inactivated SARS-CoV-2-specific phages. An ELISA microplate was coated with 100 ng/well of inactivated SARS-CoV-2. ELISA plate was incubated at 4 °C for overnight, washed and blocked using 5 % skimmed milk for 2 h at room temperature (RT). Phages from each round of panning (after and before) were added. After incubation and washing steps, anti-M13 PIII monoclonal antibody was added. Following the washing steps, anti-mouse IgG labeled with ALP was added, and then detection was developed by PNPP substrate solution.

Furthermore, after each panning, random individual colonies were chosen and cultivated in deep 96-well microplates on 2xYT medium. Phages were recovered by inoculating the bacterial cultures with 10^{11} pfu of M13KO7, shaking at 150 rpm for 2 h at 37 °C, and centrifuging for 15 min at 1000 xg. After dissolving the pellet in LB medium and culturing for overnight, the phage-containing supernatant was screened by ELISA.

2.6. Molecular characterization of scFv sequences

The recombinant PADL-22c-scFv phagemid was recovered from positive clones detected by ELISA as abovementioned, and subjected for sequencing and molecular analysis. ScFv sequences were analyzed using Immunoglobulin BLAST (IgBlast, https://www.ncbi.nlm.nih.gov/igblast/), the GenBank database, and an integrative database of germline variable genes (VBASE2, http://www.vbase2.org/) to determine their relatedness to published scFv sequences from mice. Furthermore, using MegaAlign lasergene software 4.0, scFv-generated sequences were aligned together, and their complementary-determining regions (CDRs) were identified using the Kabat numbering system (Kabat, 1991).

2.7. ScFv expression and purification

E. coli strain SS320, possessing a non-suppressive amber codon, was used to express scFv to devoid the fusion with the coat protein of M13 phage. The PADL-22c phagemids with the unique scFv coding sequences were individually transformed into competent E. coli. After induction, total periplasmic proteins were recovered and separated on a SDS-PAGE and examined by western blot. As 6x His-tag was located at the C-terminal of scFv peptide, the latest was purified using Ni-NiTA agarose resin (Qiagen, Germany) according to the manufacturer’s instructions, and dialyzed against 1x PBS.

2.8. Determining the binding affinity of scFv to SARS-CoV-2 by ELISA

Indirect ELISA was used to confirm the scFv binding affinity for inactivated SARS-CoV-2. Briefly, a 96-well ELISA plates was coated with inactivated SARS-CoV-2 (0.04 g /well) for overnight at 4 °C. BSA was used to block uncoated spaces in the wells. After washing, scFv diluted in PBS with 3 % BSA was added for 2 h at room temperature. After washing, ALP-labeled anti-His (ThermoFisher Scientific) was applied for 1 h at RT, followed by washing. The reaction was started with adding the PNPP substrate solution. Microplate reader (iMark, Bio-Rad) was used to determine absorbance at 405 nm.

3. Results

3.1. Determining the antigenicity and immunogenicity properties, and expression of recombinant RBD

As we selected the Baculovirus Expression Vector System (BEVS) for expression, the RBD coding sequence of SARS-CoV-2 isolate Wuhan-Hu-1 was modified to better fit S. frugiperda’s preferred codon profile (the source for Sf9 cell line). Before initiating the expression steps, the three-dimensional (3D) structure, prediction of antigenic determinant regions, examination of hydrophobicity and hydrophobicity properties, and surface probabilities, were all scrutinized for the optimized RBD sequence. Analysis results (Fig. 2) revealed the proper and normal properties of optimized RBD coding sequence, where sufficient epitopic regions were clearly noticed either through epitope prediction or visualization of the 3D-structure, along with the normal hydrophilic and hydrophobicity properties, and surface probabilities.

For expression, the RBD optimized coding sequence was synthesized and cloned in the pFast-Bac1 vector, fused with a His-tag sequence to monitor its expression and purification. PCR was used to confirm the
successful cloning into the pFast-Bac1 vector (not shown). To integrate the RBD optimized sequence into the bacmid through site-specific transposition, the recombinant pFast-Bac1-RBD plasmid was transformed into E. coli strain DH10Bac (where the bacmid is located). Consequently, the successful generation of recombinant bacmid was confirmed by specific RBD-derived PCR analysis of its genomic DNA, exhibiting a

Fig. 2. *In silico* analysis of optimized RBD’s codon sequence. (A), the epitopic regions were predicted; (B), 3D structure in cartoon shape showed the epitopic regions (1-5) in surface shape visualized in different colors; (C), showed the normality and quality of other protein properties.

Fig. 3. Verification the expression of rRBD in Sf9 cells infected with recombinant baculoviruses at ~ 21kDa using SDS-PAGE (A) and western blotting (B). Lanes, 1: Protein ladder, 2: non-infected Sf9 cell lysate, and 3: Sf9 cells infected with rRBD recombinant baculoviruses.
discrete amplicon at the proper size of ~ 600bp (data not shown). Furthermore, proper expression of the rRBD was verified using SDS-PAGE and western blotting revealing the expressed protein at the expected molecular weight of ~ 21KDa (Fig. 3).

Thus, the rRBD protein fused to His-tag was purified using Ni-NTA affinity chromatography. To assess the immunoreactivity of purified rRBD against SARS-CoV-2 antibodies, an indirect ELISA was exploited to test serum samples from infected and vaccinated human sera. As a positive control, inactivated SARS-CoV-2 was used as a coating antigen, and unrelated recombinant purified protein (FMDV 1B, Salem et al., 2019b) was used as a control negative coating antigen. Clear signals indicating the immunoreactivity of purified rRBD against SARS-CoV-2 antibodies, were appeared with serum samples either from convalescent or vaccinated sera (Fig. 4A). Furthermore, the immunogenicity of purified rRBD was demonstrated by its ability to raise antibodies in mouse serum, where ELISA was used to test the precise seroconversion of mice against the purified rRBD. ELISA detected a strong seroconversion in sera of immunized mice with a considerable rise in reactivity to the coating antigen (rRBD) with the mice sera collected after each booster immunization (Fig. 4B).

### 3.2. Isolation and cloning of scFv coding sequences

The recombinant scFv monoclonal antibodies developed against rRBD, were created utilizing the cDNA template generated from total RNA extracted from immunized mice’s spleen. Electrophoresis was used to verify the integrity of the isolated RNA (data not shown). PCR correctly amplified the vL and vH domains at the expected molecular sizes of ~ 360bp and ~ 420bp, respectively. Using the SOE-PCR, the two domains’ repertoires were connected using a 45-mer polynucleotide linker (GGGGSG), resulting in combinatorial scFv repertoires with a molecular size of ~ 825bp (Fig. 5A). ScFv coding sequences were batch cloned into the P<sup>ADL-22c</sup> phagemid in the middle between gene III of the M13 phage and the leader sequence (PeB peptide) for the consequent phage displaying and secretion in the bacterial periplasmic space, respectively. The latest two amino acids (MA) were re-presented by PCR for right frame proofreading and cleavage process (because the PeB sequence was initially interrupted by a stop codon). Between the scFv and g3 sequences, His and HA tags were located and followed by an amber codon. The integration of scFv into P<sup>ADL-22c</sup> vector (Fig. 5B) was validated by SfiI digestion, which resulted in the release of a scFv corresponding band of ~ 825bp from P<sup>ADL-22c</sup> of ~ 4000bp (not shown).

### 3.3. Construction the scFv phage library against rRBD

The scFv phage library against rRBD protein was created by transforming recombinant phagemids (P<sup>ADL-22c-scFv</sup>) into E. coli strain TG1 that suppressing the amber codon located between the scFv and g3 coding sequences to allow scFv antibodies to be displayed on the N-terminus of phage M13’s g3 protein (P<sup>g3</sup>). After library infection with the M13K07 helper phage, a significant enrichment factor was observed (Fig. 6A); the library size was computed based on the number of growing colonies (the dilution factor was taken into account). PCR analysis for the presence of scFv in 100 randomly selected clones revealed a 90 percent library recombination frequency, with individual scFv DNA fragments amplified with unique sizes of ~ 900bp from all examined clones (not shown). Phage recovery was multiplied severally following the fifth panning related to the first round of panning (Fig. 6B), revealing a greatly enriching factor of RBD antigen-specific scFv.

Phage polyclonal ELISA was used to determine the specificity of scFv, and it revealed that 88 percent of the chosen clones reacted positively with the rRBD, whereas none reacted with the BSA control antigen. Furthermore, the Phage monoclonal ELISA revealed that 50 of 200 (25%) individual clones were clearly reactive to rRBD, as seen by high ELISA OD values.

### 3.4. Selection and characterization the unique scFv sequences

The variable domain coding sequences of chosen scFv antibodies were identified and compared, revealing that three of seventeen scFv coding sequences are unique. The whole length and accurate vH-linker-vL structure of these scfv sequences were analyzed. When their amino acid sequences were compared to the Protein Data Bank (PDB), it was discovered that their vH- and vL-domains are 90 percent identical to the variable parts of mouse Ig. The findings of blasting the scFv deduced amino acids sequence against the protein data bank revealed a close link and specific hits with the mouse antibody heavy chain and kappa chain Ig super family (not shown). They have the basic structure of scFv antibodies, where the framework regions are highly conserved parts of the variable component of the antibody, while CDRs are hypervariable regions, and they show the coherent sequences of the created scFv to the other V libraries from the mouse IgG. Six complementary-determining regions (CDRs) were exposed, three in vH and three in vL. Sequences of the CDRs’ residues have been found and are shown in Table 1. Furthermore, the three-dimensional (3D) structure (Fig. 7) showed clearly the proper and normal structure of the three selected scFv sequences (the whole length and accurate vH-linker-vL structure).

### 3.5. Periplasmic expression and purification of scFv recombinant antibodies

The three selected scFv sequences were separately transformed into a non-suppressive amber codon E. coli strain (SS32). After induction, periplasmic protein extracts were separated on SDS-PAGE. All extracts
showed a distinct protein band at an apparent molecular weight of ~27KDa corresponding to the expected size of recombinant scFv (Fig. 8). As the expressed scFv proteins were tagged with 6 His amino acids, they were recognized with an anti-His antibody in Western blot analysis. Ni-NTA affinity resins, which have the ability to specifically bind to 6 His tag polypeptides were used to purify the recombinant scFv proteins. Approximately 4 mg of each scFv recombinant protein was purified from one liter of E. coli culture.

3.6. Verification of the scFv detection activity by ELISA against rRBD and SARS-CoV-2

To confirm that the purified scFv are still as biologically active as keeping their binding affinity to the rRBD protein, the later was used as coating antigen in an indirect-ELISA and serial dilutions of each scFv was used as a first antibody. Results showed that all of the three scFv antibodies are still able to recognize and react with the rRBD protein (Fig. 9A). To check the specificity of developed scFv antibodies, the suitable dilution of each scFv was used against SARS-CoV-2, in addition to other different unrelated antigens (N, 3AB, and VP2) and against the rRBD as a positive control (Fig. 9B).

4. Discussion

SARS-CoV-2 has spread dramatically all over the world due to its easy transmission between persons, resulting in an unexpected pandemic, with over 42.5 million confirmed cases and more than 1.1 million deaths (WHO, 2020). This makes a necessity for developing fast detection tools to assist in disease controlling (Nardell and Nathavitharan, 2020). Monoclonal antibodies (mAbs) have been approved as...
foremost particles in virus detection, and they are quickly gaining popularity as an emergency or supplemental treatment near vaccinations (Renn et al., 2020; Jin et al., 2017; Zhou and Zhao, 2020; Jiang et al., 2020).

Basically, the SARS-CoV-2 infection occurring via recognition of ACE-2 receptor on lung epithelial cells by the receptor binding domain (RBD) of viral transmembrane spike protein (Chung et al., 2020) empowering virus infectivity (Wang et al., 2020).

Here we have generated and characterized a group of recombinant monoclonal antibodies, namely scFvs, targeting the SARS-CoV-2 RBD protein. Firstly, to ensure that the RBD recombinant protein could be effectively expressed and maintained its native conformation, we used the Baculovirus Expression Vector System (BEVS), as it has been broadly used for the synthesis of recombinant proteins (El-Gaied et al., 2017; Elmenofy et al., 2020), and vaccine manufacturing (Cox, 2012; Van Oers, 2006). Furthermore, we have used the BEVS as it involves protein processing and modification that was in accordance with Yang et al., 2020 who selected BEVS system for preparation of recombinant RBD for SARS-CoV-2. Additionally, BEVS is a helper-independent viral system used to infect insect cells for high-level expression of genes from many different sources (Junzhi Wang, 2021).

Our RBD coding sequence was correctly expressed in insect cells, as expected. The antigenic and immunogenic properties of recombinant RBD (rRBD) protein were also finely characterized by generating polyclonal antibodies in mice, as the prepared rRBD was immunogenic and capable of inducing antibody response in injected mice. That outcome was supported by ELISA and western blotting as rRBD proved its integrity, antigenicity and specific reactivity. Furthermore, the rRBD merely reacted with the convalescent sera from SARS-CoV-2-infected...
patients, not with sera from healthy non-infected persons.

Despite the international nonstop attempts to end COVID-19, the pandemic continues to spread worldwide. Vaccines are possibly the most promising approach of controlling emerged coronavirus. Helpfully, monoclonal antibodies (mAbs) are swiftly gaining favor as a complement to vaccinations (Renn et al., 2020; Jin et al., 2017; Zhou and Zhao, 2020; Jiang and Hillyer, 2020). Yang et al. (2020) found that a recombinant RBD protein of SARS-CoV-2 produced from insect cells exhibited protective immunity in non-human primates. In the same context, He et al. (2004) has discovered five linear immunogenic epitopes in original conformation of the S protein, four of them were located in the S1 (containing RBD) as an external fragment of the viral envelope. The RBD has well exposure to the immune system response, while the S2 protein is hidden, resulting in its lower immunogenicity. As a result, the S1 component of the S protein is a key antigen for eliciting immunological responses. MAbs targeting the RBD demonstrated substantial neutralizing efficacy against the SARS-CoV, by inhibiting the receptor attachment in the virus neutralization. Chen et al. (2020) discovered that a recombinant viral vector harboring S protein of SARS-CoV-2 develops neutralizing antibodies that are largely directed against the RBD (Li, 2012). Thus, the majority of neutralizing action might be lost if RBD-specific antibodies were removed from the immunological serum of SARS-CoV-2 patients or vaccinated animals (Li, 2012; Yang et al., 2020). Additionally, the majority of the identified SARS-CoV-2-neutralizing MAbs targeted the S protein’s RBD (Yang et al., 2020; Smith, 1985; Hoogenboom, 2005; Bradbury et al., 2011). Basically, all proteins of SARS-CoV-2 can theoretically be exploited as targets for anti-SARS-CoV-2 antibody screening. However, research has showed that the RBD protein is involved in viral entry into host cells and interacts directly with the angiotensin-converting enzyme 2 (ACE2) host receptor (Lan et al., 2020).

Consequently, the rRBD was utilized to inject mice and produce a set of recombinant scFv antibodies in this enterprise. The top three antibody clones’ variable heavy (vH) and variable light (vL) sequences were determined. In this study, we chose specific recombinant scFv antibodies against the rRBD of SARS-CoV-2 from a murine phage display library, which will serve as a foundation for future researches and early diagnosis of SARS-CoV-2. Hereby, three distinctive recombinant scFv MAbs against SARS-CoV-2-RBD were generated in this study, using the phage display technique to provide a quick and simple diagnostic tool. Related significant efforts have been made to explore MAbs against the RBD of SARS-CoV-2 through a number of approaches, such as phage display technique (Li et al., 2020; Sun et al., 2020; Noy-Porat, 2020; Zeng et al., 2020); immunization of animals (Hansen et al., 2020; Wang et al., 2020; Esparza et al., 2020; Cheong et al., 2020); plasma or memory B cells of infected donors (Hansen et al., 2020; Chi et al., 2020; Pinto et al., 2020; Cao et al., 2020; Chen et al., 2020; Zost et al., 2020).

In brief, biopanning was used to screen a library of recombinant phage expressing and displaying scFv coding sequences for specific binding to recombinant SARS-CoV-2-RBD. As a result, three of those scFv were chosen and validated for SARS-CoV-2 detection. The RBD protein was injected intraperitoneally, followed by three intravenous booster injections. Seven days after four injections, sera have been gathered, mice were killed, and spleens were obtained for RNA isolation, and cDNA synthesis. Following that, the repertoires of vL and vH coding sequences were PCR-amplified. Using the SOE-PCR method, the resulting amplicons were assembled by joining the vH and vL amplicons. Cloning the assembled scFv repertoire into the pMIL-22c plasmid vector resulted in the creation of a phage library. Following six rounds of panning against rRBD and SARS-CoV-2, three distinct scFv clones were chosen and exposed to characterization. For expression, selected scFv clones were transformed into a non-suppressor amber codon (UAG) E. coli SS320. By switching to a non-suppressor strain of E. coli SS320, when the UAG stop codon was introduced in the recombinant phagemid to turn off expression of the PmIL fusion protein, allowing only the production of soluble scFv without further subcloning. Plasmidic proteins were purified and separated on SDS–PAGE after induction. All clones produced a clearly new polypeptide with an apparent molecular weight of approximately 27KDa, which was not visible in the control. The 27KDa polypeptide represented a fusion polypeptide made up of the His tag and a scFv polypeptide. The identification of the putative 27KDa protein was confirmed using a Western blot assay with an anti-His tag antibody. Furthermore, recombinant His-scFv proteins were purified from periplasmic extract using Ni-NTA affinity resins. Furthermore, we have been capable of recovering scFv with a near-homogeneity. Following purification, it really was crucial to analyze the appropriate folding of scFv as an antibody and to make sure that the proteins didn’t even lose their recognizing and binding characteristics to the rRBD during the purification process. Indirect ELISA revealed that almost all clones developed extremely strong signals.

5. Conclusion

To summarize, this endeavor describes generation of murine derived recombinant single-chain fragment variable (scFv) as a monoclonal antibody (mAb) platform targeting the receptor binding domain (RBD) of Spike protein of SARS-CoV-2. Moreover, the obtained scFv antibodies showed authentic immunoreactivity and specificity to both rRBD and SARS-CoV-2. Accordingly, these antibodies will be helpful in research laboratories and have the potential to be used in the future for quick, precise, and low-cost diagnostics for COVID-19 patients. These antibodies could be used in future studies to determine the structure of RBD protein, diagnostic advancement, intracellular interactions, and possible future therapeutics. Ultimately, we wish that such findings would be beneficial to the research community in combating the continuous COVID-19 pandemic. Further mAb refinement could also allow the development of promising anti-SARS-CoV-2 therapeutics as well as diagnostic reagents.

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**Compliance with ethical standards**

**Ethics statement:** All serum samples were collected after written informed consent from the patients, from November 2020 to January 2021. Cases clinical history was collected in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee. Immunized mice were treated in compliance with the principles and policies of the American Physiological Society’s Guiding Principles in the Care and Use of Animals and after the approval of Animal Care and Use Committee in Faculty of the Veterinary Medicine, Cairo University (Vet CU16072020181).

**Author statement**

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Molecular Immunology.

**Data availability**

Data will be made available on request. No data was used for the research described in the article.
The authors declare that they have no conflict of interest.

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