Antigenicity and immunogenicity of SARS-CoV S protein receptor-binding domain stably expressed in CHO cells

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ABSTRACT

The receptor-binding domain (RBD) of SARS coronavirus (SARS-CoV) spike (S) protein contains multiple conformation-dependent epitopes that induce neutralizing antibody responses. Here we used CHO-K1 cells to establish a cell line for stable expression of a 193-mer (residues 318–510) RBD (RBD193-CHO) and determined its antigenicity and immunogenicity. We found that RBD193-CHO reacted strongly with a panel of six monoclonal antibodies recognizing various conformational and linear epitopes in RBD, suggesting that this recombinant protein maintains intact conformation and good antigenicity. Immunization of mice with RBD193-CHO resulted in induction of high titers of RBD-specific neutralizing antibodies and potent IL-4-expressing T cell responses. RBD193-CHO induced immunity that protected a majority of the vaccinated mice from SARS-CoV challenge. These results suggest that the recombinant RBD produced in an established stable cell line maintains strong immunogenicity with high potential for use as an effective and economic subunit SARS vaccine.

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The global outbreak of severe acute respiratory syndrome (SARS) in 2003 led to severe health and economic consequences worldwide. To prevent the re-emergence of this infectious disease from potential animal reservoirs [1–3], it is necessary to develop effective vaccines and antiviral agents against SARS coronavirus (SARS-CoV), the causative agent of SARS [4,5]. Compared with other antiviral strategies, vaccines, particularly those based on the spike (S) protein, demonstrate greater efficacy in the prevention of SARS [6]. The receptor-binding domain (RBD) in S protein contains multiple conformational-dependent epitopes that induce potent neutralizing antibodies against SARS-CoV infection [7,8], suggesting an attractive target for development of SARS vaccine. Therefore, establishment of an approach for producing large quantity of recombinant RBD with appropriate antigenicity and immunogenicity is needed.

The Chinese hamster ovary (CHO) cell line has been widely used to express recombinant proteins and biopharmaceutical products in a stable and large-scale manner. As such, the selected CHO-K1 stable cell lines have been established to express persistently recombinant secreted proteins, such as dust mite allergen, Blo t 5 [9], human MUC1 mucin [10] and transforming growth factor-β (TGF-β) [11]. Furthermore, when adapted to serum-free medium, recombinant proteins can be highly expressed in suspension culture, making it possible to purify proteins from the suspension in large quantity [9]. Proteins obtained in this way have been shown to maintain biological activity and proper conformation [9,11].

Therefore, the successful expression and purification of a number of recombinant proteins in CHO-K1 cells provides a rational basis for developing effective and economic subunit SARS vaccines by continuous production of recombinant proteins containing the functional domains of SARS-CoV S protein. Based on this reasoning, the present study aimed to express a 193-mer (residues 318–510) recombinant RBD of SARS-CoV S protein (RBD193-CHO) in an established stable CHO-K1 cell line and further determine its antigenicity, immunogenicity and protective immunity in a mouse model.

Materials and methods

Plasmid construction, protein transient expression and purification. Genes encoding a 193-amino acid (residues 318–510) RBD of SARS-CoV S protein were amplified by PCR using the plasmid containing the codon-optimized full-length S protein as the template [12,13], followed by insertion into the GS gene expression vector PEE14.1. The recombinant RBD plasmid was transiently transfected using FuGENE 6 transfection reagents (Roche Applied Science, Indianapolis, IN) into CHO-K1 cells (ATCC, Manassas, VA). Transfected F-12K medium (ATCC) was replaced by fresh OPTI-MEM I Reduced-Serum Medium (Invitrogen, Carlsbad, CA) 10 h post-trans-

fection, and culture supernatant was collected 72 h later, followed by purifying recombinant proteins using His columns (Promega, Madison, WI) according to the manufacturer's protocol. The purified protein was designated as RBD193-CHO.

Establishment of stable cell lines expressing RBD193-CHO protein. The recombinant RBD plasmid was transfected into CHO-K1 cells as above. Twenty-four hours later, culture medium was changed into fresh glutamine-free DMEM (SAFC Biosciences, Lenexa, KS) containing 10% dialyzed FBS (dFBS, Invitrogen), 1× glutamine synthetase (GS) supplement (SAFC Biosciences) and 25 μM L-methionine sulfoximine (MSX) selective agent (Chemicon, Billerica, MA). Fresh selective medium was replaced every 3–4 days thereafter until the appearance of resistant colonies, which were then picked and expanded to culture for protein expression detection by Western blot and ELISA. The established CHO-K1 stable cell line expressing RBD193-CHO protein was adapted to EX-CELL302 CHO serum-free medium according to the manufacturer's protocol (SAFC Biosciences) to facilitate large-scale recombinant protein expression. Further purification of the RBD protein was performed using the conditioned suspension culture medium.

Western blot. The purified RBD193-CHO protein was tested for expression by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot according to the protocol previously described [14]. The blots were detected by a panel of RBD-specific monoclonal antibodies (mAbs) generated in our laboratory [7] at the final concentration of 0.5 μg/ml.

Mouse vaccination and sample collection. Each of five female BALB/c mice was subcutaneously (s.c.) vaccinated with RBD193-CHO protein according to the protocol previously described [15], with 20 μg/mouse of purified protein containing Freund's complete adjuvant (FCA, Sigma) for the prime and 10 μg/mouse of immunogen containing Freund's incomplete adjuvant (FIA, Sigma) for two subsequent boosts at 3-week intervals. PBS was used as the negative control. Ten days post-last vaccination, mouse sera were collected and assayed to detect specific IgG and neutralizing antibody responses, while splenocytes were assayed to determine cellular responses. Ten days post-last vaccination, mice were also challenged with live SARS-CoV, and at 5 days post-challenge, detection for viral replication was performed.

ELISA. A previously described ELISA protocol [15] was used for the selection of CHO-K1 cells stably expressing RBD193-CHO protein and for the detection of RBD-specific IgG antibody in protein-vaccinated mouse sera. For protein expression detection, 96-well microtiter plates were pre-coated with CHO-K1 cell culture supernatant, and SARS-CoV RBD-specific mAb 33G4 (1:2000 dilution) [7] was added after washing with PBST. For sera IgG antibody detection, plates were first coated with expressed RBD193-CHO protein, followed by addition of a serial dilution of mouse sera after washes; then, detection was conducted as before [15].

Neutralization assay. The neutralization assay against SARS pseudovirus infection was performed according to the method established in our laboratory, i.e., using HEK293T cells expressing the receptor angiotensin-converting enzyme 2 (ACE2/293T) [7]. The neutralization of SARS pseudovirus was calculated as before [16] and indicated as 50% neutralizing antibody titer (NT_{50}). The neutralization assay against live SARS-CoV (GZ50 strain; GenBank Accession No. AY304495) infection was performed as previously described [17,18], and neutralizing titers that completely prevented cytopathic effect (CPE) in 50% of the wells (NT_{50}) were calculated by the Reed–Muench method.

ELISPOT assay. The assay was performed using an ELISPOT mouse kit (Mabtech, Mariemont, OH) according to the manufacturer's protocol and our previously described method [17,18]. Ninety-six-well ELISPOT plates were coated with anti-IFN-γ, -IL-2, -IL-4 and -IL-10 mAbs. Cells were incubated at 37 °C for 24 h in the presence of an identified MHC-H-2^d restricted SARS-CoV-

specific cytotoxic T lymphocyte (CTL) peptide (N50: S365–374, KCYGVSATKL) or Th peptide (N60: S435–444, NYNYKYRYLR) [19]. Plates were incubated with biotinylated labeled anti-mouse IFN-γ, IL-2, IL-4 and IL-10 mAbs at 1:1000 dilution. Spots of cytokine-producing T cells were counted and expressed as the number of spot-forming cells (SFC) per 10^6 input cells.

SARS-CoV challenge. RBD193-CHO protein-vaccinated mice were intranasally challenged with SARS-CoV (GZ50 strain, 100 $TCID_{50}$) as previously described [17,18]. The challenged mice were sacrificed 5 days post-challenge, and the lungs were collected for further virological tests.

Viral load and viral replication detection. The viral load (RNA copies) in the challenged mouse lung tissues was determined by quantitative reverse-transcriptase polymerase chain reaction (Q-RT-PCR) according to our previously described protocols [15]. The SARS-CoV replication was measured by using cytopathic effect (CPE)-based assay in order to determine the titration of the virus in the lung tissues of the SARS-CoV challenged mice. The viral titers were calculated as described before [15].

Results

RBD193-CHO protein that was highly and persistently expressed in CHO-K1 stable cell lines reacted with a majority of a panel of mAbs that recognize various conformational and linear epitopes in RBD

To establish a stable cell line that persistently expresses the recombinant RBD protein, CHO-K1 cells were transiently transfected with plasmid encoding the RBD193-CHO protein. The purified protein from the CHO-K1 cell culture exhibited antigenicity similar to the recombinant RBD193 that was expressed in 293T cells, as determined by ELISA and Western blot assays (data not shown). The culture medium of CHO-K1 cells was replaced with fresh selective medium every 3–4 days until resistant colonies were identified. One of these colonies was selected as a stable CHO-K1 cell
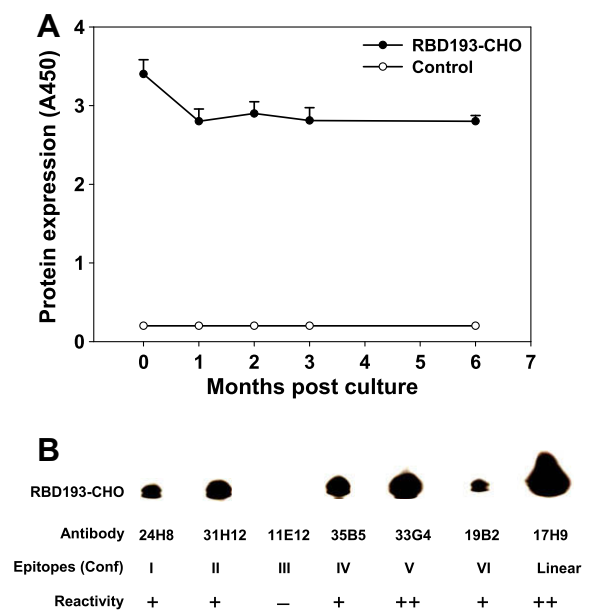

Fig. 1. RBD193-CHO protein expression and reactivity detection. (A) ELISA detection of the expression of RBD193-CHO protein in culture supernatant of CHO-K1 stable cell lines. Supernatant from normal CHO-K1 cells was used as the negative control (Control). The data are presented as mean A450 + standard error (SE) from three parallel wells. (B) Expressed RBD193-CHO protein was detected by Western blot for reactivity with RBD mAbs containing conformational and linear epitopes. One mAb from each epitope was selected for the detection, which was labeled as − (no reactivity), + (reactivity), and ++ (high reactivity).

line expressing RBD193-CHO protein and was adapted to EX-CELL302 CHO serum-free medium. The expression of RBD193-CHO protein was then monitored by ELISA using RBD-specific mAb 33G4. As shown in Fig. 1A, RBD193-CHO was continuously expressed at a high level in the culture supernatants of the CHO-K1 stable cell line for at least six months, suggesting that this CHO-K1 stable cell line could be used for large-quantity production of RBD-based vaccine, if needed.

The RBD193-CHO protein purified from CHO-K1 cell culture media was analyzed with SDS–PAGE and Western blot using a panel of six mAbs that specifically recognize various conformational epitopes (Conf I–VI) and one mAb that reacts with linear epitopes (17H9) in RBD. As Fig. 1B indicates, the expressed RBD193-CHO protein reacted with five of six conformational epitope-specific mAbs and one linear epitope-specific mAb. These results suggest that RBD193-CHO maintains native conformation and authentic antigenicity.

RBD193-CHO induced potent RBD-specific antibodies that neutralize both SARS pseudovirus and live SARS-CoV infection in vitro

A good vaccine antigen should first be able to induce humoral immune responses, which are mainly represented by the induction of specific antibodies in vaccinated individuals. To assess this, BALB/c mice were immunized with purified RBD193-CHO protein as described in Materials and methods, and humoral immune responses and neutralizing activity were detected in the vaccinated mouse sera. Fig. 2A indicates that all five mice in the RBD193-CHO vaccination group presented a high titer of RBD-specific IgG
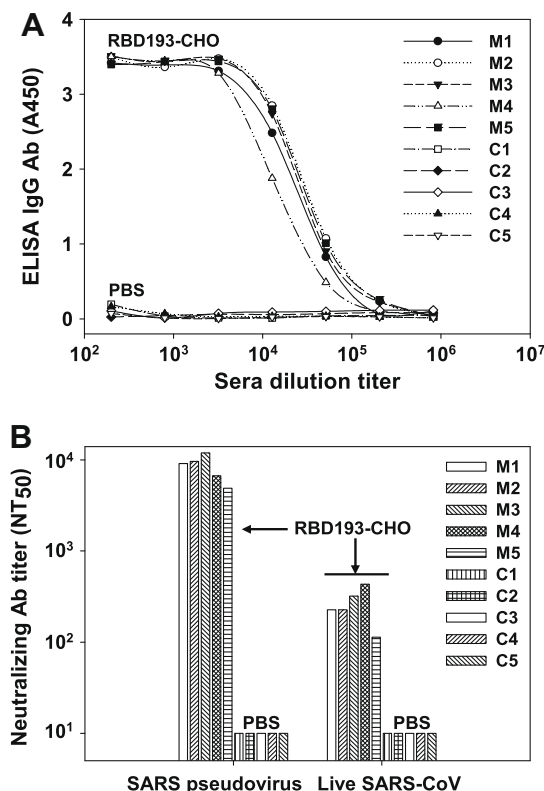
antibody responses in their sera, with the mean end-point titer reaching $1:8.2 \times 10^5$ at 10 days post-last vaccination. These RBD-specific antibodies significantly neutralized infection by both SARS pseudovirus and live SARS-CoV in ACE2/293T and Vero E6 cells, respectively (Fig. 2B). These results indicate that RBD193-CHO was able to induce strong humoral immune responses and neutralizing antibodies in protein-vaccinated mice.

RBD193-CHO induced RBD-specific T cell responses mainly represented by increasing number of IL-4 T cells in vaccinated mouse splenocytes

To evaluate the ability of RBD193-CHO protein to induce cellular immune responses, splenocytes were collected from vaccinated mice 10 days post-last vaccination and stimulated, respectively, with a RBD-specific CD8+ peptide N50, CD4+ peptide N60, and the purified RBD protein. As shown in Fig. 3, RBD193-CHO elicited both CTL and Th responses, represented by production of a relatively higher level of IL-4-secreting CD8+ and Th2 T cells than that of IFN-γ- and IL-2-representing CTL and Th1 T cells under the stimulation of the CTL peptide N50 and Th peptide N60, respectively. In comparison, stronger T cell responses, which were represented by induction of a high level of IL-2-, IL-4- and IL-10-presenting T cells, could be observed in the vaccinated mouse splenocytes with the stimulation of the recombinant RBD protein. In addition, IFN-γ-expressing T cells induced by RBD protein are also relatively higher than those stimulated by N50 or N60 peptide alone (Fig. 3). However, an undetectable level of cellular immune responses was found in the splenocytes of PBS control mice. Overall, these data confirm the ability of RBD193-CHO protein to elicit cellular immune responses in the vaccinated mice.

RBD193-CHO protected a majority of the vaccinated mice from SARS-CoV challenge

To determine the protective efficacy of RBD193-CHO protein, vaccinated mice were challenged with SARS-CoV, and lung tissues were collected 5 days post-challenge to detect viral load (RNA copies) and viral replication (Log_{10} $TCID_{50}$). No SARS-CoV was detected in the mice vaccinated with RBD193-CHO protein, with undetectable viral RNA copies in challenged mouse lungs. In contrast, a high level of virus was found in the lung tissues of all five PBS control mice, with viral load ranging from 1.5×10^3 to 2.2×10^3 RNA copies/μg of lung tissues (Fig. 4A). Consistently, no virus replication was detected in three out of the five vaccinated mice challenged

Fig. 2. Humoral immune response detection in RBD193-CHO protein-vaccinated mouse sera. M1–M5 and C1–C5 indicate five mice, respectively, from RBD193-CHO vaccination group and PBS control group. (A) ELISA detection of RBD-specific IgG antibody responses. The data are, respectively, presented as absorbance at OD 450 nm (A450) of five mice per group. (B) Neutralizing antibody titer to SARS pseudovirus and live SARS-CoV infection. The data are, respectively, presented as 50% neutralizing titer (NT50) of five mice per group.
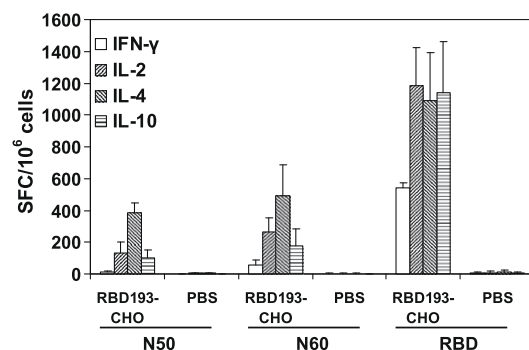

Fig. 3. ELISPOT detection of cellular immune responses in RBD193-CHO protein-vaccinated mouse splenocytes. Frequencies of cytokine-producing cells are expressed as mean + SE of cytokine spot-forming cells (SFC)/10^6 cells of five mice per group. CTL and Th responses were detected, respectively, under the stimulation of a RBD-specific CD8+ T cell peptide, N50, and a CD4+ T cell peptide, N60. Cellular immune responses were also detected by the stimulation of the expressed RBD193-CHO (RBD) protein.
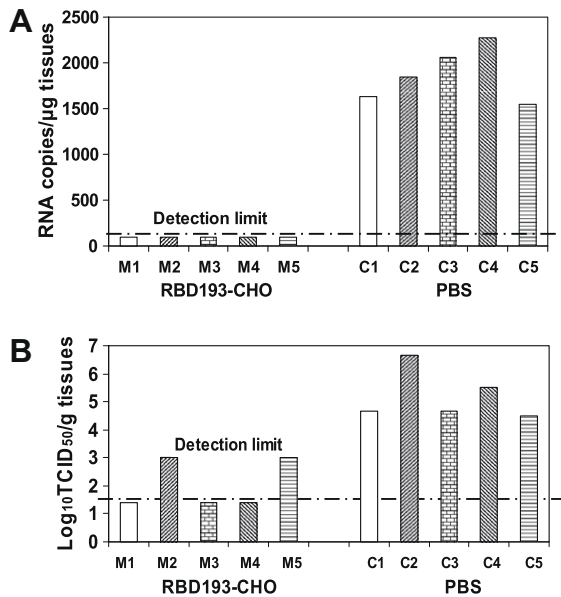
Fig. 4. Protection of the RBD193-CHO protein-vaccinated mice from SARS-CoV challenge. M1–M5 and C1–C5 indicate five mice in the vaccination and control groups, respectively. (A) Viral load was detected by Q-RT-PCR and expressed as RNA copies/μg of lung tissues. The detection limit was 1×10^2 copies/μg tissues. (B) SARS-CoV replication was determined by a CPE-based assay and expressed as Log_{10} $TCID_{50}$/g of tissues. The detection limit was 1.5 Log_{10} $TCID_{50}$/g of lung tissues.

with SARS-CoV, and only a low level of replicable virus (about 3 Log_{10} $TCID_{50}$/g of tissues, which is slightly above the detection limit of 1.5) was detected in the lungs of the remaining two mice (Fig. 4B). These suggest that RBD193-CHO could induce strong immunity that protects a majority of the vaccinated mice against SARS-CoV challenge. But further evaluation of the *in vivo* efficacy of RBD193-CHO vaccine in a lethal mouse model [28,29] is warrented.

Discussion

The mammalian cell expression system is a relatively mature eukaryotic system for expression of recombinant proteins. It can either transiently or stably express recombinant antigens, promote signal synthesis, and correctly fold, secrete and glycosylate synthesized proteins [20–22]. Recombinant proteins expressed in this way may maintain native conformation and good antigenicity and immunogenicity. Therefore, the mammalian cell expression system has been recently used for expressing a variety of heterologous proteins, including viral structural proteins and bioactive peptides, for specific functional analysis [23,24].

We have previously shown that the recombinant RBD of SARS-CoV S protein expressed in 293T cells could induce highly potent neutralizing antibody responses and protective immunity in immunized mice, suggesting that it could be developed as an effective and safe SARS subunit vaccine for the prevention of SARS-CoV infection [6,25]. However, to obtain highly expressed RBD protein with biological functions in a convenient and economic way, we sought to establish a stable cell line for long-term and high volume production of the recombinant RBD. Therefore, CHO-K1 cells, rather than 293T cells, were selected because CHO-K1 cells have the ability to express the protein of interest persistently at a high level [22,26,27]. Based on this rationale, the plasmid for transient expression of a 193-amino acid (residues 318–510) RBD (RBD193-CHO) of SARS-CoV S protein in CHO-K1 cells was constructed first. The purified protein was characterized, and its function was confirmed. Then, a stable CHO-K1 cell line was

successfully established, and it was demonstrated that this cell line could highly and persistently express RBD193-CHO protein in the culture supernatant of conditioned medium (Fig. 1A). The purified protein was proven to exhibit good antigenicity since it significantly reacted with five of six mAbs that recognize the conformational epitopes in RBD and one mAb specific for the linear RBD epitope (Fig. 1B), suggesting that RBD193-CHO protein maintains intact conformation. The analysis of the immunogenicity of RBD193-CHO protein in the mouse model indicated that the expressed protein induced potent RBD-specific antibody responses with neutralizing antibody activity (Fig. 2) and strong T cell immune responses (Fig. 3).

Compared with our previously-developed adeno-associated virus-expressing RBD (RBD-rAAV) vaccine candidate [17], the RBD193-CHO protein expressed in CHO-K1 cells could induce stronger protective immunity in the vaccinated mice against subsequent SARS-CoV challenge, as shown by the undetectable viral load (RNA copies) in lungs of the challenged mice (Fig. 4A). Although virus replication was detected in two of five vaccinated mice, the level was only slightly above the detection limit and significantly below that of PBS control (Fig. 4B), suggesting that expressed RBD193-CHO protein could induce protection against SARS-CoV infection in a majority of the vaccinated mice. Considering the feasibility of high expression of the recombinant RBD protein in CHO-K1 cells on a large scale at relatively low cost, this study provides a suitable method for developing subunit SARS vaccines for the prevention of future SARS outbreak.

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