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Identification and characterization of novel neutralizing epitopes in the receptor-binding domain of SARS-CoV spike protein: Revealing the critical antigenic determinants in inactivated SARS-CoV vaccine

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Abstract

The spike (S) protein of severe acute respiratory syndrome coronavirus (SARS-CoV) is considered as a major antigen for vaccine design. We previously demonstrated that the receptor-binding domain (RBD: residues 318–510) of S protein contains multiple conformation-dependent neutralizing epitopes (Conf I to VI) and serves as a major target of SARS-CoV neutralization. Here, we further characterized the antigenic structure in the RBD by a panel of novel mAbs isolated from the mice immunized with an inactivated SARS-CoV vaccine. Ten of the RBD-specific mAbs were mapped to four distinct groups of conformational epitopes (designated Group A to D), and all of which had potent neutralizing activity against S protein-pseudotyped SARS viruses. Group A, B, C mAbs target the epitopes that may overlap with the previously characterized Conf I, III, and VI respectively, but they display different capacity to block the receptor binding. Group D mAb (S25) was directed against a unique epitope by its competitive binding. Two anti-RBD mAbs recognizing the linear epitopes (Group E) were mapped to the RBD residues 335–352 and 442–458 respectively, and none of them inhibited the receptor binding and virus entry. Surprisingly, most neutralizing epitopes (Groups A to C) could be completely disrupted by single amino acid substitutions (e.g., D429A, R441A or D454A) or by deletions of several amino acids at the N-terminal or C-terminal region of the RBD; however, the Group D epitope was not sensitive to the mutations, highlighting its importance for vaccine development. These data provide important information for understanding the antigenicity and immunogenicity of SARS-CoV, and this panel of novel mAbs can be used as tools for studying the structure of S protein and for guiding SARS vaccine design.

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1. Introduction

Severe acute respiratory syndrome (SARS) is a fatal emerging infectious disease caused by a novel coronavirus (SARS-CoV) [1–5]. Recent studies suggest that SARS-CoV is zoonotic and may have a broad host range besides humans [6–9]. Although there are no recent SARS outbreaks, serious concerns remain over its re-emergence from animal reservoirs and its potential application as a bioterrorism agent. The need to develop an effective vaccine against SARS-CoV remains of high importance. Since the emergency of SARS, a number of candidate vaccines, using a variety of approaches, are under development [10–12]. As a starting point, inactivated SARS-CoV has been considered as one of the major vaccine candidates [12]. Several inactivated SARS vaccines prepared by conventional protocols, e.g., beta-propiolactone, formaldehyde or UV light, have been tested in preclinical studies [13–19]. These killed whole virus vaccines are effective in terms of their capac-
ity to induce neutralizing antibodies and protective immunity in several animal models. However, the antigenic and immunogenic properties of inactivated SARS-CoV remain to be characterized. Specially, identifying and characterizing the antigenic epitopes that induce protective immunity is a priority for developing a safe and effective SARS vaccine.

Similar to other coronaviruses, SARS-CoV is an enveloped positive-strand RNA virus, featuring a large viral genome that encodes viral replicase proteins and major structural proteins consisting of spike (S), nucleocapsid (N), membrane (M), and small envelope protein (E), and several small proteins with unknown functions [1,3,5]. Although all viral proteins are immunogenic to elicit immune responses in vaccinated animals, the S protein of SARS-CoV, a type I transmembrane glycoprotein providing virion with a corona-like appearance, is considered a major antigen for vaccine development. Several live virus and DNA vaccines expressing the S protein can induce neutralizing immunity against SARS-CoV [20–24].

Infection of SARS-CoV is initiated by binding of its S protein to the functional receptor angiotensin-converting enzyme 2 (ACE2) expressed on the target cells [25,26]. A 193-amino acid sequence of SARS-CoV strain BJ01 (accession number AY278488) showed that ACE2 exhibits a high degree of sequence similarity with ACE2 in other species [27,28]. In the present study, S1 (residues 12–466) and S2 (residues 467–672) domains of SARS-CoV Urbani (accession number AY278741) were expressed in expresSF+® insect cells with recombinant baculovirus D3252 by the Protein Sciences Corporation (Bridgeport, CT). Both proteins were expressed in a serum-free cell line and purified to apparent homogeneity with >90% purity, and were shown to bind to soluble ACE2 [36].

2.2. Recombinant S proteins and synthetic peptides

The plasmids encoding truncated S fragments corresponding to the S1 subunit (residues 12–672), and the receptor-binding domain (residues 318–510) of SARS-CoV were expressed in expresSF+® insect cells with recombinant baculovirus D3252 by the Protein Sciences Corporation (Bridgeport, CT). Both proteins were expressed in a serum-free cell line and purified to apparent homogeneity with >90% purity, and were shown to bind to soluble ACE2 [36].

A set of 27 peptides overlapping the S protein RBDD sequence of SARS-CoV strain Tor2 (each peptide contains 17 residues with nine residues overlapping with the adjacent peptides) were synthesized at Gene Gateway, LLC (Hayward, CA) as previously described [31]. A standard solid-phase FMOC method was used for peptide synthesis. Peptides were purified to homogeneity (purity >90%) by high-performance liquid chromatography (HPLC) and identified by laser desorption mass spectrometry.

2.3. Immunization of mice and production of mAbs

Four BALB/c mice (6 weeks old) were subcutaneously immunized with 20 μg of inactivated SARS-CoV vaccine resuspended in PBS plus MLP+TDM Adjuvant (Sigma, Saint Louis, MI) and boosted with 10 μg of the same antigen plus the MLP+TDM adjuvant at 3-week intervals. Preimmune sera were collected before starting the immunization and antiserum were collected 7 days after each boost. Sera were kept at 4 °C before use.

Hybridomas producing anti-RBD mAbs were generated using standard protocol as previously described [34]. Briefly, the spleenocytes from the immunized mice were harvested and fused with SP2/0 myeloma cells. Cell culture supernatants from the wells containing hybridoma colonies were screened by enzyme-linked immunosorbent assay (ELISA) using the RBD-Fc as a coating antigen. Cells from positive
wells were expanded and retested. Cultures that remained positive were subcloned to generate stable hybridoma cell lines. All mAbs were purified from culture supernatants by the Protein A Sepharose 4 Fast Flow (Amersham Biosciences). The isotypes of mAbs were determined with Mouse Monoclonal Antibody Isotyping Kit (Amersham Biosciences).

2.4. ELISA and binding competition

Reactivity of mouse sera or mAbs with various S proteins was determined by ELISA. Briefly, 0.5 or 1 μg/ml recombinant protein was used to coat 96-well microtiter plates (Corning Costar, Acton, MA) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% non-fat milk, serially diluted mouse sera or mAbs were added and incubated at 37 °C for 1 h, followed by three washes with PBS containing 0.1% Tween 20. Bound antibodies were detected with HRP-conjugated goat anti-mouse IgG (Zymed Laboratories) at 37 °C for 1 h, followed by washes. The reaction was visualized by addition of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) and absorbance at 450 nm was measured by an ELISA plate reader (Tecan US, Research Triangle Park, NC). To determine the effect of disulfide bond reduction on the binding of anti-RBD mAbs, ELISA plate was coated with recombinant RBD-Fc at a concentration of 1 μg/ml and then treated for 1 h at 37 °C with dithiothreitol (DTT) at a concentration of 10 mM, followed by washes. Then the wells were treated with 50 mM iodoacetamide for 1 h at 37 °C. After washes, a standard ELISA was performed as described above.

A competitive ELISA was performed to determine the inhibitory activity of the anti-RBD mAb on binding of the biotinylated mAbs to the RBD-Fc. Briefly, the wells of ELISA plates were coated with the RBD-Fc as described above. A mixture containing 100 μg/ml of an unlabelled mAb and 1 μg/ml of a biotinylated mAb was added, followed by incubation at 37 °C for 1 h. Binding of the biotinylated mAbs was detected after addition of HRP-conjugated streptavidin (Zymed Laboratories) and TMB sequentially. Biotinylation was detected after addition of HRP-conjugated streptavidin (Zymed Laboratories) at 37 °C with dithiothreitol (DTT) at a concentration of 10 mM, followed by washes. Then the wells were treated with 50 mM iodoacetamide for 1 h at 37 °C. After washes, a standard ELISA was performed as described above.

2.5. Neutralization of SARS pseudoviruses

SARS-CoV pseudovirus system was developed in our laboratory as previously described [35,37]. In brief, HEK293T cells were co-transfected with a plasmid encoding the S protein and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE) by using Fugene 6 reagents (Boehringer Mannheim). Supernatants containing SARS pseudoviruses were harvested 48 h post-transfection and used for single-cycle infection of human or civet ACE2-transfected 293T (293T/ACE2) cells. Briefly, 293T/ACE2 cells were plated at 10⁴ cells/well in 96-well tissue-culture plates and grown overnight. The supernatants containing pseudovirus were preincubated with serially diluted mAbs at 37 °C for 1 h before addition to cells. The culture was re-fed with fresh medium 24 h later and incubated for an additional 48 h. Cells were washed with PBS and lysed using lysis reagent included in a luciferase kit (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar, Corning, NY), followed by addition of luciferase substrate (Promega). Relative light units (RLU) were determined immediately in the Ultra 384 luminometer (Tecan US).

2.6. Inhibition of RBD-Fc binding to the receptor ACE2 by anti-RBD mAbs

Inhibition of anti-RBD mAbs on the RBD-Fc binding to ACE2-expressing cells was measured by flow cytometry as previously described [32,37]. Briefly, 10⁶ 293T/ACE2 cells were detached, collected and washed with Hank’s balanced salt solution (HBSS) (Sigma, St. Louis, MO). RBD-Fc was added to the cells to a final concentration of 1 μg/ml in the presence or absence of 50 μg/ml mAbs, followed by incubation at room temperature for 30 min. Cells were washed with HBSS and incubated with anti-human IgG-FITC conjugate (Zymed) at 1:50 dilution at room temperature for an additional 30 min. After washing, cells were fixed with 1% formaldehyde in PBS and analyzed on a Facs Canto flow cytometer (BD Biosciences, Mountain View, CA) using Facs Diva software.

3. Results

3.1. Inactivated SARS-CoV vaccine induces high titers of antibodies against the RBD of S protein

Inactivated SARS-CoV was prepared with beta-propiolactone and purified to >95% purity as described. Four BALB/c mice were subcutaneously immunized with the inactivated viruses in the presence of MLP + TDM adjuvant. We used recombinant FL-S and RBD-Fc as antigens in ELISA to measure antibody responses specific for S protein and its RBD, respectively. As showed in Fig. 1A and B, all four mice developed significant antibodies against the S protein and its RBD after first boosting immunization, and their serum reactive titers apparently increased with subsequent boosts. The titers of RBD-specific antibodies were further determined by ELISA. As shown in Fig. 1C, the anti-RBD antibodies in the mouse antisera collected 7 days after the third boost reached a mean end-point titer of 1/64,000. This result further confirmed that the RBD of S protein in the inactivated SARS vaccine is highly immunogenic to elicit specific antibodies, consistent with our previous finding [35].
Fig. 1. Induction of anti-RBD antibodies in mice immunized with inactivated SARS-CoV vaccine. (A) Antibody responses against the full-length S protein. Sera were tested by ELISA at 1/100 dilution. (B) Antibody responses against the RBD of S protein. Sera were tested by ELISA at 1/100 dilution. (C) Titers of RBD-specific antibodies in the mouse sera collected after the third boost.

### Table 1

| mAb   | Isotype | Antigen  | RBD-Fc | S1-Fc | EC-S | FL-S |
|-------|---------|----------|--------|-------|------|------|
| S6    | IgG1/k  | 2.89     | 1.14   | 2.62  | 2.53 |
| S9    | IgG1/k  | 2.25     | 1.33   | 2.07  | 2.28 |
| S20   | IgG1/k  | 2.92     | 2.23   | 2.49  | 2.50 |
| S25   | IgG1/k  | 2.82     | 1.97   | 2.27  | 2.46 |
| S29   | IgG2k   | 2.50     | 0.87   | 0.40  | 1.62 |
| S33   | IgG2k   | 2.53     | 0.87   | 0.58  | 1.81 |
| S37   | IgG1/k  | 2.84     | 1.28   | 2.19  | 2.40 |
| S38   | IgG1/k  | 2.69     | 2.12   | 2.48  | 2.44 |
| S40   | IgG1/k  | 2.85     | 1.97   | 2.59  | 2.62 |
| S44   | IgG1/k  | 2.85     | 2.63   | 2.42  | 2.50 |
| S50   | IgG1/k  | 2.85     | 1.48   | 2.33  | 2.52 |
| S53   | IgG2k   | 2.39     | 1.20   | 1.48  | 2.08 |
| 102D7 | IgG1/k  | 0.01     | 0.01   | 2.00  | 2.75 |

*Antigens were coated to ELISA plates at 0.5 μg/ml and mAbs were tested at 10 μg/ml. OD450 value >0.2 was considered positive reaction and highlighted in boldface.

102D7 is a control mAb targeting the S2 domain.

3.2. Isolation and initial characterization of mAbs specific for the RBD of S protein

To characterize RBD-based antigenic epitopes presented in the inactivated vaccine preparation, we generated a panel of 12 RBD-specific mAbs from the immunized mice by fusing mouse splenocytes with Sp20/myeloma cells, and then screening hybridomas using RBD-Fc as an antigen (Table 1). The specificity of anti-RBD mAbs was determined by ELISA with a set of SARS-CoV antigens, including recombinant S1 subunit (S1-Fc), truncated extracellular domain (EC-S), and full-length S protein (FL-S). All anti-RBD mAbs reacted with not only the RBD-Fc, but also the S1-Fc, EC-S, and FL-S (Table 1), suggesting that they may target the epitopes presented on the native S protein. Some mAbs bound to RBD, EC-S and FL-S effectively, but not S1-Fc. The reduced binding could be due to alteration of the epitope conformation resulted from lack of trimetic association in the S protein. It was of interest to note that two mAbs (S29 and S33) had relatively weaker reactivity with S1-Fc and EC-S, implying that their epitopes may not be well exposed on these two proteins.

A majority of the mAbs (9 of 12) was reactive with native RBD-Fc, but not DTT-reduced RBD-Fc, indicating that they target disulfide bond-dependent conformational epitopes expressed on the RBD of S protein (Fig. 2). Interestingly, the mAb S25 bound strongly to the native and weakly to the reduced RBD-Fc, suggesting that its epitope is conformation-dependent but a linear sequence may be involved. The mAbs S29 and S33, which had lower reactivity with recombinant S1 and extracellular domain, recognized both the native and reduced RBD-Fc equally, suggesting that they target linear epitopes within the RBD.

### 3.3. Potent neutralization of S-pseudotyped SARS viruses by the anti-RBD mAbs

We used S protein-pseudotyped SARS pseudoviruses in a sensitive and quantitative single-cycle infection assay to measure the neutralizing activity of these newly isolated anti-RBD mAbs. As shown in Fig. 3, all mAbs specific for the conformational epitopes could neutralize SARS pseudoviruses with 50% neutralization dose (ND50) ranging from...
Fig. 2. Reactivity of anti-RBD mAbs with the native and DTT-reduced RBD-Fc measured by ELISA. Antigens were coated at 1 μg/ml, and the mAbs were tested at 10 μg/ml.

Fig. 3. Neutralization of SARS pseudovirus by anti-RBD mAbs. Infection of HEK293T cells expressing human ACE2 by SARS pseudovirus Tor2 was determined in the presence of serially diluted anti-RBD mAb. Percent neutralization was calculated and plotted. Numbers in parentheses indicate 50% neutralization dose (ND50) at μg/ml.

Fig. 4. Localization of epitopes for the anti-RBD mAbs by overlapping peptides

A set of 27 overlapping peptides that cover the RBD sequence of the S protein were used as antigens in ELISA to localize the epitopes of the newly isolated anti-RBD mAbs. As expected, none of the conformation-dependant mAbs, including S25, reacted with any of the tested peptides (data not shown). Two mAbs (S29 and S33) that recognized the DTT-reduced RBD-Fc reacted with one of the peptides, respectively (Fig. 4). S29 reacted with the peptide 442-458 (YLRHGKLRPFERDISNV), which overlaps with the previously characterized epitope for the non-neutralizing mAb 17H9 isolated from the RBD-Fc-immunized mice [34], suggesting that this linear site is immunogenic in both inactivated virus and recombinant RBD-Fc immunogens. S33 reacted with the peptide 335-352 (SVYAWERKKISNC-VADY), which locates at the N-terminal region of the RBD. Thus, the linear epitopes of S29 and S33 were finely localized by the overlapping peptides.

3.5. Characterization of epitopes for the anti-RBD mAbs by binding competition assays

The epitope specificity of this panel of anti-RBD mAbs was further mapped by binding competition assays. Four mAbs (S6, S20, S37, and S38) were biotinylated and their binding activity to the RBD-Fc was respectively measured in the presence or absence of an unlabeled anti-RBD mAb. As shown in Table 2, five of the mAbs (S6, S9, S37, S40, and S50) were divided into a competitive Group A since they competed with two biotinylated mAbs (S6 and S37) with a similar pattern. Three mAbs (S20, S38, and S53) were grouped into Group B by their ability of competing with biotinylated S20 and S38. The mAb S44 competed with three biotinylated mAbs (S6, S20, and S37) and was designated as a Group C mAb. Interestingly, the mAb S25 was able to inhibit the binding of all four biotinylated mAbs to the RBD-Fc, suggesting that it recognizes a distinct epitope (Group D). Two linear epitope-specific mAbs (S29 and S33) could not compete with any of conformation-specific mAbs and were thereby designated as Group E mAbs. These results suggest that the RBD of S protein presented in the inactivated SARS-CoV
vaccine contains several different antigenic epitopes capable of inducing specific antibodies in the immunized mice.

We previously identified six groups of conformation-dependent epitopes (Conf I–VI) in the RBD by a panel of 27 mAbs isolated from the mice immunized with the RBD-Fc [34]. The epitope specificity of the newly isolated 12 anti-RBD mAbs was further characterized by binding competition assays to measure their inhibitory activities on the binding of the previously biotinylated mAbs (10E7, 11E12, 33G4, 45B5) (Table 3). All of the five Group A mAbs (S6, S9, S37, S40, and S50) characterized above competed with the biotinylated Conf I mAb (10E7), suggesting that their epitopes may overlap with the Conf I epitope. All three Group B mAbs (S20, S38, and S53) were classified into Conf III-like mAbs since they competed with the biotinylated Conf III mAb 11E12. The mAb S44 (Group C) was able to compete with Conf VI mAb 45B5. Outstandingly, the mAb S25 (Group D) that competed with each of the above biotinylated mAbs did not compete with any of the previously characterized biotinylated mAbs, suggesting that it recognizes a unique novel epitope (designated Conf VII). Similarly, two mAbs specific for the linear epitopes (S29 and S33) did not significantly compete with any of the biotinylated reagents. Furthermore, none of 12 new anti-RBD mAbs had a binding competition pattern similar to the previously characterized Conf II, IV, or V mAbs, suggesting that these three groups of epitopes may not be immunogenic in the inactivated virus-immunized mice.

Table 2
Mapping of anti-RBD mAbs by binding competition assay

| Group | Competing mAb | % Inhibition of binding by biotinylated mAb |
|-------|---------------|---------------------------------------------|
|       |               | S6  | S20 | S37 | S38 |
| A     | S6            | 92.22| 13.41| 71.41| -1.52|
|       | S9            | 95.78| 19.47| 97.85| 8.05 |
|       | S37           | 98.71| 7.07 | 95.47| 11.11|
|       | S40           | 99.77| 19.83| 99.58| 10.13|
|       | S50           | 99.92| 19.83| 99.82| 10.13|
| B     | S20           | 23.45| 24.45| 99.20| 85.78|
|       | S38           | 23.45| 24.45| 99.20| 85.78|
|       | S53           | 23.45| 24.45| 99.20| 85.78|
| C     | S44           | 63.31| 52.70| 56.94| 16.85|
|       | S25           | 95.06| 83.29| 93.56| 85.78|
| D     | S29           | 13.07| 11.64| 24.47| 10.19|

* Competing mAbs were tested at 100 µg/ml for the ability to block binding of the biotinylated mAbs to the RBD-Fc in ELISA. Greater than 40% inhibition was considered positive competition (values in bold). Negative numbers indicate increased binding of the biotinylated reagent.
### Table 3: Mapping of anti-RBD mAbs by binding competition assays

| Group | Competing mAb | % Inhibition of binding by biotinylated mAb | Epitope |
|-------|---------------|--------------------------------------------|---------|
|       | 1E 7 (I)      | 11E 12 (II) | 3G4 (V) | 45B5 (VI) |          |
| A     | S6            | 58.2        | 1.8     | –2.4     | 0.5      | Conf I-like |
|       | S9            | 64.2        | 6.1     | –1.1     | 3.0      | Conf I-like |
|       | S37           | 67.9        | 3.7     | –1.8     | 5.2      | Conf I-like |
|       | S40           | 92.2        | 7.4     | 2.5      | 19.7     | Conf I-like |
|       | S50           | 78.8        | 2.3     | 2.3      | 5.6      | Conf I-like |
| B     | S20           | 6.4         | 91.8    | –3.6     | 9.0      | Conf III-like |
|       | S38           | –2.0        | 94.2    | –2.1     | 6.8      | Conf III-like |
|       | S53           | 20.3        | 90.8    | 1.9      | –0.8     | Conf III-like |
| C     | S44           | 6.9         | –17.8   | –1.2     | 94.7     | Conf IV-like |
| D     | S25           | 38.9        | 8.5     | –1.5     | 20.1     | Conf VII   |
|       | S29           | 3.6         | 14.9    | –1.3     | 8.0      | Linear     |
|       | S33           | 6.2         | 15.6    | –2.9     | 13.7     | Linear     |

* Competing mAbs were tested at 100 μg/ml for the ability to block binding of the biotinylated mAbs to the RBD-Fc in ELISA. Greater than 40% inhibition was considered positive competition (values in bold). Negative numbers indicate increased binding of the biotinylated reagent.

#### 3.6. Characterization of the mAbs that may target the receptor-binding motif (RBM) in the RBD

Crystal structure of the RBD bound to ACE2 reveals that the residues 424 to 494 in the RBD form the receptor-binding motif (RBM) [38]. We previously showed that Conf III to VI mAbs could efficiently or partially block the receptor-binding but Conf I to II not [34]. To determine whether the newly isolated anti-RBD mAbs had inhibitory activity on the receptor binding, a similar flow cytometry-based assay was used to measure the binding of RBD-Fc to human ACE2 expressed on 293T cells (293T/ACE2) in the presence of the anti-RBD mAbs. As shown in Fig. 5, RBD-Fc was able to bind to ACE2-expressed 293T cells, but the binding could be efficiently or partially blocked by representative anti-RBD mAbs. Group B (S20, S38, and S53) and Group C (S44) mAb had higher potency compared with the corresponding Conf III (11E12 and 18D9) and VI (13B6 and 19B2) control mAbs, respecti-
Fig. 6. % Inhibition of RBD-Fc binding to cell-associated human ACE2 expressed on 293T/ACE2 cells.

Unexpectedly, most of Group A mAbs (4 out of 5) was also able to partially inhibit the receptor-binding, except that S40, which had highest capacity to compete with Conf I mAb, had no significant inhibitory activity, similar to the Conf I mAbs (10E7 and 12B11). These results imply that Group A–C epitopes may not be identical with those of the previously characterized, rather than may their epitopes be overlapped in terms of their competitive binding. Therefore, the mAbs of the Groups A, B, C were also named as Conf I, III, VI-like mAbs, respectively (Table 3). Similar to the Group A mAbs, the Group D mAb (S25) also weakly inhibited the binding of RBD-Fc and ACE2 (Figs. 5 and 6). In contrast, none of two linear epitope-dependant mAbs (S29 and S33) blocked the association between RBD-Fc and ACE2. These data suggest that inactivated SARS-CoV vaccine can induce RBD-specific antibodies that primarily target the receptor-binding motif (RBM).

3.7. Mapping of the anti-RBD mAb epitopes by recombinant RBD-Fc mutants

We then used a panel of RBD-Fc mutants to map the conformational epitopes in the RBD. First, we assessed the reactivity of all 12 anti-RBD mAbs with two truncated RBD-Fc. Strikingly, a majority of neutralizing epitopes (Group A–C) was completely disrupted by the deletion of several amino acids at the N-terminus (residue 318–326) or the C-terminus (residues 491–510) of the RBD (Table 4), indicating that both N- and C-terminal sequences are essential for maintaining the proper conformation of these neutralizing epitopes. However, the S25 epitope (Group D) was not affected by the N- and C-terminal deletions, suggesting that this epitope resides within residues 327-490 in the RBD, and is not constrained by the conformation of full-length RBD.

Further, a set of RBD-Fc fusion proteins bearing specific point mutations of the conserved basic or acidic residues were produced and used in ELISA to map the epitopes of these anti-RBD mAbs. Interestingly, even single amino acid substitutions of some residues (D429A, R441A or D454A) could abolish Group A, B, and C epitopes (Table 4). E452A and D463A substitutions severely damaged the epitopes for Group A mAbs, which reacted weakly or did not react with these two mutants. Moreover, the epitope for mAb S53 (Group B) was sensitive to the D463A substitution. Regardless, the S25 epitope was not sensitive to any of these single mutations, further indicating that it is a highly stable and conserved motif in the RBD. Noticeably, the linear epitope for S29 and 17H9 (residues 442–458) was knocked out by D454A substitution. In comparison, K390A substitution did not impact any of the conformational and linear epitopes in RBD.

We recently found that Conf I to IV and VI mAbs had a similar reaction pattern with Group A to C mAbs to the RBD mutants, whereas the reactivity of Conf V mAbs (24F4, 33G4, and 38D4), similar to that of the mAb S25, was not significantly affected by the mutations (Table 4). We therefore determined whether S25 could compete with the Conf V mAbs in binding. Interestingly, S25 could not inhibit the binding of biotinylated Conf V mAbs to the RBD-Fc; however, the binding of S25 to the RBD-Fc could be dramatically enhanced by the Conf V mAbs (Fig. 7). In contrast, three Conf V mAbs were able to efficiently block the binding by two biotinylated Conf V mAbs. This result further indicates that S25 and Conf V mAbs target two independent conformational epitopes, but binding of the Conf V mAbs may result in increased exposure of S25 epitope.

4. Discussion

Since the sudden appearance of SARS, we have focused our studies to characterize the antigenicity and immunogenicity of SARS-CoV in efforts to develop a safe and effective
vaccine. We previously demonstrated that the SARS-CoV inactivated by β-propiolactone could induce high titers of neutralizing antibodies that primarily target the receptor-binding region (RBD) of the S protein [32,35]. To finely map the neutralizing epitopes in the RBD, we isolated a panel of novel RBD-specific mAbs from the mice immunized with inactivated SARS-CoV vaccine and used these mAbs as probes for the study. We found that the majority of neutralizing antibodies that primarily target the receptor-binding motif (RBM) as the corresponding mAbs can efficiently or partially block the receptor binding. With similar binding competition assays, the conformation-specific anti-RBD mAbs isolated from the mice immunized with the inactivated SARS-CoV vaccine have been mapped to four distinct epitopes. Half of the conformation-specific mAbs (5 out of 10) contain multiple conformation-dependent neutralizing epitopes. The RBD of the S protein in the inactivated SARS-CoV vaccine retains native conformation and can induce predominantly neutralizing antibodies.

We previously identified six groups of conformation-dependent neutralizing epitopes (Conf I to VI) in the RBD by using a set of 27 RBD-specific mAbs isolated from the mice immunized with an independently folded-RBD [34]. The Conf III to VI epitopes may overlap the receptor-binding motif (RBM) as the corresponding mAbs can efficiently or partially block the receptor binding. With similar binding}

### Table 4

Mapping of anti-RBD mAb epitopes with RBD mutants by ELISA

| Group | MAb | Deletion of residues | Single-point mutation |
|-------|-----|----------------------|----------------------|
|       |     | 318–326 | 301–310 | K390A | D429A | B441A | E452A | D454A | D456A |
| New mAbs |     |          |          |       |       |       |       |       |       |
| A     | S6  | 0.00       | 0.02     | 2.33  | 0.04  | 0.02  | 0.03  | 0.01  | 0.35  |
|       | S9  | 0.00       | 0.04     | 2.27  | 0.06  | 0.06  | 0.08  | 0.01  | 0.39  |
|       | S37 | 0.00       | 0.03     | 2.39  | 0.06  | 0.05  | 0.06  | 0.02  | 0.40  |
|       | S40 | 0.00       | 0.06     | 2.62  | 0.14  | 0.12  | 0.94  | 0.01  | 1.08  |
|       | S50 | 0.00       | 0.02     | 2.30  | 0.05  | 0.01  | 0.04  | 0.01  | 0.40  |
| B     | S20 | 0.00       | 0.05     | 2.43  | 0.02  | 0.04  | 1.34  | 0.01  | 1.41  |
|       | S38 | 0.00       | 0.04     | 2.32  | 0.02  | 0.04  | 1.44  | 0.01  | 1.24  |
|       | S55 | 0.00       | 0.00     | 2.13  | 0.01  | 0.02  | 0.21  | 0.01  | 0.04  |
| C     | S44 | 0.02       | 0.18     | 2.71  | 0.19  | 0.04  | 1.99  | 0.06  | 2.38  |
| D     | S25 | 1.68       | 2.06     | 2.43  | 1.27  | 0.96  | 3.00  | 2.24  | 1.23  |
| E     | S29 | 2.12       | 2.57     | 1.79  | 1.87  | 2.23  | 2.93  | 0.02  | 3.34  |
|       | S33 | 1.48       | 2.19     | 1.96  | 1.18  | 1.05  | 2.59  | 1.73  | 1.30  |
| Control mAbs |     |          |          |       |       |       |       |       |       |
| Conf I | 10E7 | 0.00       | 0.11     | 2.39  | 0.19  | 0.01  | 1.44  | 0.03  | 1.71  |
|       | 12B11 | 0.00      | 0.03     | 2.40  | 0.09  | 0.03  | 0.19  | 0.00  | 0.45  |
| Conf II | 20E7 | 0.01      | 0.02     | 2.36  | 0.12  | 0.02  | 0.65  | 0.00  | 1.24  |
|       | 26A4 | 0.00      | 0.02     | 2.42  | 0.14  | 0.02  | 0.65  | 0.00  | 1.08  |
| Conf III | 11E12 | 0.01     | 0.01     | 2.05  | 0.01  | 0.02  | 0.25  | 0.01  | 0.05  |
|       | 18D9 | 0.00      | 0.01     | 2.18  | 0.01  | 0.02  | 0.39  | 0.00  | 0.44  |
| Conf IV | 28D6 | 0.00      | 0.07     | 2.37  | 0.07  | 0.02  | 1.69  | 0.00  | 1.49  |
|       | 30F9 | 0.00      | 0.06     | 2.43  | 0.05  | 0.02  | 1.59  | 0.00  | 1.37  |
| Conf V | 24F4 | 1.22     | 1.73     | 2.75  | 1.45  | 0.73  | 2.59  | 1.95  | 0.86  |
|       | 33G4 | 1.96     | 2.03     | 2.90  | 1.71  | 1.05  | 2.56  | 2.06  | 2.92  |
|       | 38D4 | 1.06     | 1.27     | 2.49  | 0.77  | 0.50  | 2.41  | 1.30  | 0.77  |
| Conf VI | 15B6 | 0.01      | 0.00     | 2.32  | 0.15  | 0.02  | 1.40  | 0.00  | 1.28  |
|       | 19B2 | 0.01      | 0.04     | 2.36  | 0.00  | 0.01  | 1.15  | 0.00  | 0.86  |
| Linear | 4D5 | 2.20      | 2.70     | 1.75  | 0.89  | 0.83  | 3.03  | 2.44  | 0.82  |
|       | 17H9 | 3.62      | 2.95     | 2.63  | 2.78  | 3.59  | 3.23  | 0.19  | 4.18  |

* Antigens were coated to ELISA plates at 1 μg/ml and mAbs were tested at 10 μg/ml. OD450 value >0.2 was considered as positive reaction and highlighted in boldface.
ing to the unique binding competition pattern, the Group D mAb, S25, recognized a novel conformational neutralizing epitope in RBD. These data suggested that the RBD in the inactivated virus and the recombinant S protein might display different immunogenicity, and thus allowed the isolation of neutralizing mAbs that recognize different conformation-dependent epitopes. However, none of the mAbs targeting the Conf IL, IV, and V epitopes were isolated from the inactivated SARS-CoV-immunized mice, suggesting that these epitopes might not be immunodominant or even not be functional in the inactivated vaccine. Conf IV and V epitopes may overlap the receptor-binding motif in RBD and induce more potent neutralizing antibodies [34]. Their functional presentation on the immunogens is critical for SARS vaccines. It is possible that viral inactivation procedures may affect the antigenic structures of the neutralizing epitopes in the S protein of SARS-CoV, thus limiting their ability to induce neutralizing antibodies. Consistent with our previous finding [34], the mAbs targeting the linear epitopes in the RBD had no neutralizing activity. The RBD of SARS-CoV S protein is well exposed on the surface of virion and is responsible for attachment and binding with receptor ACE2. However, we recently found that the linear epitopes in the RBD, unlike the conformational epitopes, were not well exposed on the S protein expressed on the transfected-cells as shown by flow cytometry assays (data not shown), providing evidence to explain why the linear epitope-specific mAbs can not neutralize SARS-CoV.

The RBD presented on the S protein is a 193-amino-acids fragment and shows a high degree of structural complexity. It contains seven cysteines and five of them are essential for receptor ACE2 binding [29]. Several critical residues in the RBD have been characterized to be determinants for SARS-CoV receptor adaptation from palm civets to humans [39,40]. Crystal structure of the RBD bound with the human ACE2 reveals that several disulfide bonds connect cysteines 323 to 348, 366 to 419, and 467 to 474 to form the loops [38], and the residues 424 to 494 constitute the receptor-binding motif (RBM) [38]. The conformation-dependent epitopes in RBD may be determined by its complex structures. Recently, it was reported that the ability of the full-length S protein to induce neutralizing antibodies could be abolished by single-point mutations of the conserved amino acid residues in the RBD (e.g. R441A) [41], indicating that the immunogenicity of S protein is highly determined by the RBD configuration. To further map the critical residues for the RBD-based neutralizing epitopes, we generated a panel of RBD mutants with substitutions or deletions of conserved amino acid residues. It was surprising to find that Group A to C conformational epitopes, similar to the Conf I to IV and V epitopes, could be disrupted by single amino acid substitutions (e.g. D429A, R441A or D454A) or by deletions of several amino acids at the N- or C-terminal region of the RBD. However, the Group D epitope, like the Conf V epitope, is not sensitive to the deletions and mutations. It was of interest to note that K390A substitution could not did not impact any of the antigenic epitopes significantly even though it abolishes the functionality of ACE2 binding [42]. These results suggest that some particularly conserved RBD residues are essential to maintain the antigenic integrity of RBD, and that the S25 epitope within RBD is unique. Although Group D and Conf V mAbs have a similar pattern in the reactions with the mutants, they are apparently directed against different epitopes. First, they do not compete with each other in binding; Second, Conf V mAbs can completely block the receptor-binding but S25 only has marginal inhibitory activity. Further localization and characterization of both Group D and Conf V epitopes will provide important information for rationally designing SARS vaccines.

As first generation of SARS vaccines, the inactivated SARS-CoV vaccine has been tested in preclinical and clinical studies, and shown its capacity to elicit sterilizing immunity in several animal models [13–18]. Our results here indicate that although the major neutralizing domain of SARS-CoV is functional in the inactivated vaccine preparation, some important neutralizing epitopes (e.g. Conf IV and V) may not be properly presented compared with those presented on the recombinant RBD molecules. Vigilance should be taken for application of the inactivated vaccines in humans since viral particle contains a number of components that may induce harmful immune and/or inflammatory responses. We believe that the RBD of SARS-CoV S protein may serve as a better candidate for developing safe and effective vaccines since it is a functional region that mediates the receptor-binding and virus entry, and contains multiple epitopes that induce highly potent neutralizing antibodies [10,11]. In addition, RBD-based vaccine can be safely manufactured comparing with the inactivated SARS-CoV.

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