Searching for an ideal vaccine candidate among different MERS coronavirus receptor-binding fragments—The importance of immunofocusing in subunit vaccine design

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ABSTRACT

The newly emerged Middle East respiratory syndrome coronavirus (MERS-CoV) is currently spreading among humans, making development of effective MERS vaccines a high priority. A defined receptor-binding domain (RBD) in MERS-CoV spike protein can potentially serve as a subunit vaccine candidate against MERS-CoV infections. To identify an ideal vaccine candidate, we have constructed five different versions of RBD fragments, S350-588-Fc, S358-588-Fc, S367-588-Fc, S367-606-Fc, and S377-588-Fc (their names indicate their residue range in the spike protein and their C-terminal Fc tag), and further investigated their receptor binding affinity, antigenicity, immunogenicity, and neutralizing potential. The results showed that S377-588-Fc is among the RBD fragments that demonstrated the highest DPP4-binding affinity and induced the highest-titer IgG antibodies in mice. In addition, S377-588-Fc elicited higher-titer neutralizing antibodies than all the other RBD fragments in mice, and also induced high-titer neutralizing antibodies in immunized rabbits. Structural analysis suggests that S377-588-Fc contains the stably folded RBD structure, the full receptor-binding site, and major neutralizing epitopes, such that additional structures to this fragment introduce non-neutralizing epitopes and may also alter the tertiary structure of the RBD. Taken together, our data suggest that the RBD fragment encompassing spike residues 377-588 is a critical neutralizing receptor-binding fragment and an ideal candidate for development of effective MERS vaccines, and that adding non-neutralizing structures to this RBD fragment diminishes its neutralizing potential. Therefore, in viral vaccine design, it is important to identify the most stable and neutralizing viral RBD fragment, while eliminating unnecessary and non-neutralizing structures, as a means of "immunofocusing".

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1. Introduction

An emerging infectious disease, Middle East respiratory syndrome (MERS) caused by MERS coronavirus (MERS-CoV), was first identified in 2012 in Saudi Arabia [1], and has since spread to other countries, including the United States. As of July 14, 2014, there have been 834 laboratory-confirmed cases, including 288 deaths, (http://www.who.int/csr/don/2014_07_14_mers/en/), raising serious concerns over its pandemic potential [2,3]. With bats and dromedary camels as its likely natural reservoir and intermediate transmission host, respectively [4–11], MERS-CoV poses a long-term threat to human health [12,13]. Thus, the need for the development of effective prophylactic strategies, such as vaccines, to control the further spread of MERS-CoV is urgent.

The spike (S) protein of MERS-CoV plays important roles in mediating viral entry to host cells [14]. As the first step of cell entry, a defined receptor-binding domain (RBD) in the spike proteins binds to its functional receptor, dipeptidyl peptidase 4 (DPP4),

on the host cell surface for viral attachment [15]. Several versions of MERS-CoV RBD fragments have been identified by different groups. These RBD fragments encompass spike residues 358-588, 367-588, 377-588, and 367-606, respectively [16–20]. Extensive studies have found that the spike RBD of SARS coronavirus (SARS-CoV), which caused the SARS epidemic in 2002–2003 [21,22], is a critical neutralizing receptor-binding domain and an attractive subunit vaccine candidate against SARS-CoV infection [23–28]. It is likely that the MERS-CoV RBD could also serve as a subunit vaccine candidate against MERS-CoV infection. Indeed, it was previously shown that some of these MERS-CoV RBD fragments are immunogenic in animals, resulting in neutralizing antibody responses [17,18]. However, it is not clear which one of these RBD fragments represents an ideal vaccine candidate and what is the mechanism behind the potential differences in the neutralizing abilities of these RBD fragments.

In this study, we have expressed each of these MERS-CoV RBD fragments that were fused with Fc fragment of human IgG, and investigated their receptor binding affinity, antigenicity, immunogenicity, and neutralizing potential. We have found the RBD fragment with the most neutralizing potential, and explained the mechanism behind it. Overall, this study has identified an ideal vaccine candidate for controlling MERS-CoV infections, and enhanced understanding of design strategies for vial subunit vaccines.

2. Materials and methods

2.1. Ethics statement

Four- to six-week-old female BALB/c mice and four- to five-month-old female NZW rabbits were used in the study. The animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved by the Committee on the Ethics of Animal Experiments of the New York Blood Center (Permit Number: 194.15).

2.2. Construction, expression and purification of recombinant proteins

The construction, expression and purification of recombinant MERS-CoV RBD fragments with Fc of human IgG were done as previously described with some modifications [18,29]. Briefly, genes encoding residues 350-588, 358-588, 367-588, 367-606, and 377-588 of MERS-CoV S protein were respectively amplified by PCR using codon-optimized MERS-CoV S sequences (GenBank: AFS88936.1) as template and inserted into the pFUSE-hIgG1-Fc2 expression vector (hereinafter named Fc; InvivoGen, San Diego, CA). MERS-CoV S1 (residues 18-725) plus a C-terminal His$_6$ (S1-His) was amplified and inserted into pJW4303 expression vector (Jiangsu Taizhou Haiyuan Protein Biotech, Co., Ltd., China). The recombinant plasmids were transfected into 293T cells (ATCC, Manassas, VA), changed into fresh serum-free DMEM (Invitrogen, Carlsbad, CA) 8 h later, and collected for supernatant containing expressed proteins 72 h post-transfection. The recombinant proteins were purified by Protein A affinity chromatography (GE Healthcare, Piscataway, NJ) for proteins with Fc or Ni-NTA Superflow (Qiagen, Valencia, CA) for proteins with His tag, according to the manufacturers' instructions.

Human DPP4 ectodomain (residues 39-766) was expressed and purified as previously described [19]. Briefly, recombinant human DPP4 ectodomain containing an N-terminal honeybee melittin signal peptide and a C-terminal His$_6$ was expressed in insect cells using the Bac-to-Bac expression system (Invitrogen), secreted into cell culture medium, and subsequently purified by Ni-NTA affinity column and Superdex200 gel filtration column (GE Healthcare).

2.3. SDS-PAGE and Western blot

The purified MERS-CoV RBD fragments were analyzed by SDS-PAGE and Western blot as previously described [29,30]. Briefly, the boiled and nonboiled proteins were separated by 10% Tris–Glycine SDS-PAGE gels, followed by transferring to nitrocellulose membranes. After blocking overnight at 4 °C using 5% non-fat milk in PBST, the blots were incubated for 1 h at room temperature with MERS-CoV S-specific polyclonal antibodies (1:1000). After three washes, the blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:3000, Invitrogen) for 1 h at room temperature, and the signals were visualized using ECL Western blot substrate reagents and Amersham Hyperfilm (GE Healthcare).

2.4. Co-immunoprecipitation assay

The binding of MERS-CoV RBD fragments with DPP4 was performed by co-immunoprecipitation assay as previously described [18]. Briefly, recombinant soluble DPP4 (sDPP4) (10 μg) or DPP4-expressing Huh-7 cell lysates (5×10^7/ml) were respectively incubated with MERS-CoV RBD fragments (10 μg) plus Protein A Sepharose Beads at 4 °C for 1 h, followed by washing with lysis buffer and PBS, and boiling for 10 min. The samples were subjected to SDS-PAGE and Western blot analysis, followed by detection using anti-DPP4 monoclonal antibody (1 μg/ml, R&D Systems, Minneapolis, MN) and MERS-CoV S1-specific polyclonal antibodies (1:1000), respectively.

2.5. Animal vaccination and sample collection

Mice were immunized with MERS-CoV RBD fragments following a previously described protocol [18,29]. Briefly, mice were prime-vaccinated (subcutaneously, s.c.) with 10 μg/mouse of recombinant MERS-CoV RBD fragments in the presence of Montanide ISA 51 adjuvant (SEPPIC, Fairfield, NJ), and boosted twice with the same immunogen and adjuvant at 3-week (21 days) intervals. Sera from 10 days post-last vaccination (52 days) were heat-inactivated at 56 °C for 30 min, and detected for MERS-CoV S-specific antibody response and neutralizing antibodies. Sera from 0, 10, 31, and 136 days post-initial immunization of S377-588-Fc were also tested for MERS-CoV S-specific antibody response.

The rabbit immunization (Covance Research Products Inc., Denver, PA) followed a protocol similar to that of mice with some modifications. Briefly, rabbits were prime-vaccinated with MERS-CoV S377-588-Fc fragment (250 μg/rabbit) plus Freund's complete adjuvant and then boosted twice at 3-week intervals with the same immunogen (125 μg/rabbit) plus Freund's incomplete adjuvant. Sera were collected and tested as above.

2.6. ELISA

ELISA was carried out to test the binding of MERS-CoV RBD fragments to sDPP4 as previously described [18]. Briefly, 96-well ELISA plates were precoated with sDPP4 (4 μg/ml) overnight at 4 °C and blocked with 2% non-fat milk at 37 °C for 2 h. Diluted MERS-CoV RBD fragments were added to the plates and incubated at 37 °C for 1 h, followed by four washes. Bound antibodies were incubated with HRP-conjugated anti-human IgG (1:3000, Invitrogen) at 37 °C for 1 h. The reaction was visualized by substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Invitrogen) and stopped by 1 N H_2SO_4. The absorbance at 450 nm (A450) was measured by ELISA plate reader (Tecan, San Jose, CA).
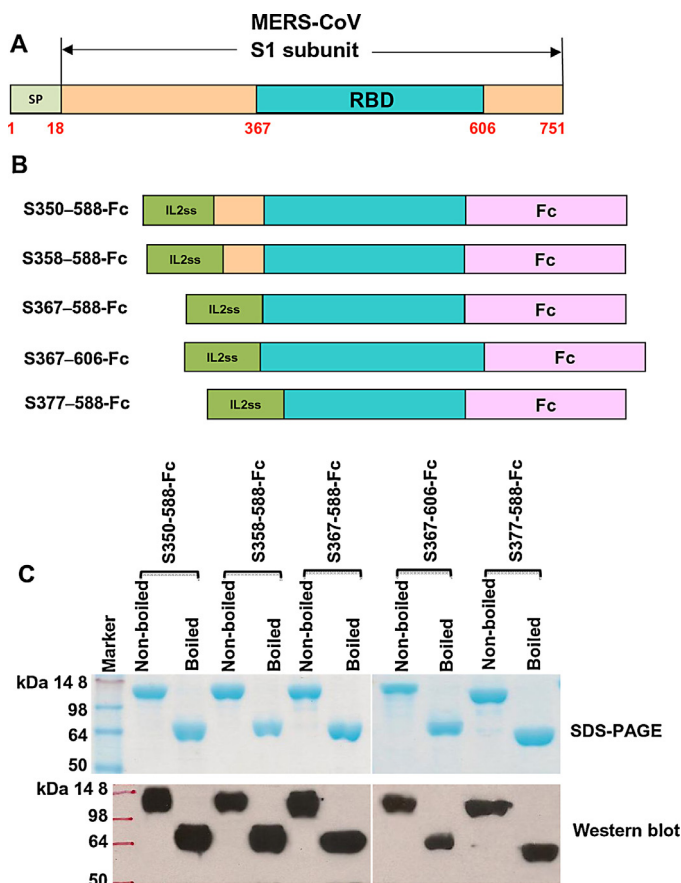
Fig. 1. Construction and characterization of MERS-CoV RBD protein fragments. (A) Schematic structure of MERS-CoV S1 subunit. SP, signal peptide. (B) Constructed plasmids encoding MERS-CoV RBD fragments fused with human IgG Fc. IL2ss: IL2 signal sequence that directs the secretion of the expressed protein into the culture supernatant. (C) SDS-PAGE and Western blot analysis of the purified RBD fragments. Denatured (boiled) or non-denatured (nonboiled) samples (5 μg) were subjected to SDS-PAGE (top) and Western blot analysis (bottom) using anti-MERS-CoV S1 antibodies. The molecular weight marker (kDa) is indicated on the left.

Fig. 2. Receptor-binding affinity of MERS-CoV RBD fragments. (A) Co-immunoprecipitation assays, followed by Western blot analyses, were performed to determine the binding affinity between the RBD fragments and DPP4 receptor. Briefly, purified recombinant RBD fragments were incubated with either 10 μg sDPP4 (left) or DPP4-expressing Huh-7 cells (right) in the presence of Protein A Sepharose beads before subjecting to Western blot analysis with either anti-DPP4 (1 μg/ml, top) or anti-MERS-CoV S1 antibodies (1:1000, bottom). (B) ELISA was carried out to measure the binding affinity between the RBD fragments and recombinant sDPP4. Recombinant human IgG Fc (hIgG-Fc) protein was included as the negative control. There were significant differences between three of the RBD fragments (S358-588-Fc, S367-588-Fc, and S377-588-Fc) and the other two (S350-588-Fc and S367-606-Fc) both at 50 (*) and 12.5 (#) μg/ml, respectively.

The detection of MERS-CoV S-specific antibody responses in collected mouse sera was performed following a protocol similar to that described above, except for replacing the coating antigen with MERS-CoV S1-His protein (1 μg/ml), the primary antibody with serially diluted mouse sera, and the secondary antibody with anti-mouse IgG-HRP (1:3000, Invitrogen) [31].

2.7. Neutralization assay

The standard micro-neutralization assay was used to quantify the neutralizing potential of each of the RBD fragments, as previously described [18,31,32]. Briefly, serum samples were diluted at serial 2-fold in 96-well tissue culture plates, and incubated for 1 h at room temperature with ~100 infectious MERS-CoV/EMC-2012/well before transferring to duplicate wells of Vero E6 cells grown in 96-well tissue culture plates. After 72 h of incubation, when the virus control wells exhibited advanced virus-induced cytopathic effect (CPE), the neutralizing capacity of individual serum samples was assessed by determining the presence or absence of CPE. Neutralizing antibody titers were expressed as the reciprocal of the highest dilution of serum that completely inhibited virus-induced CPE in at least 50% of the wells (NT_{50}).
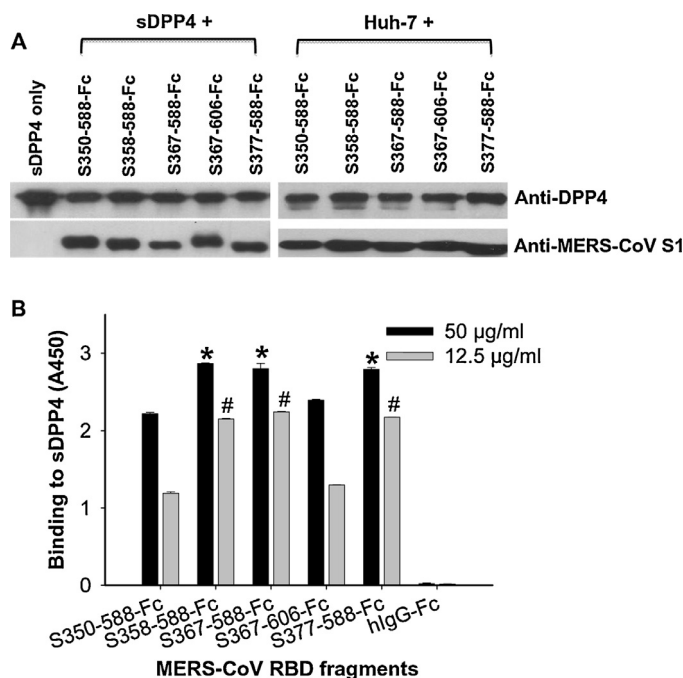
2.8. Statistical analysis

Values are presented as mean with standard deviation (SD). Statistical significance among different vaccination groups was calculated by Student's t-test using Stata statistical software. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Characterization of MERS-CoV RBD protein fragments

Five human IgG Fc-fused fragments representing the defined RBD residues 358-588, 367-588, 367-606, and 377-588 of MERS-CoV spike protein and the RBD residues 350-588 control were constructed, expressed, and subjected to antigenicity measurement, using the procedures as previously described (Fig. 1B) [18]. All five RBD fragments were expressed at high levels from culture supernatants of transfected 293 T cells and purified to high homogeneity. The C-terminal Fc tag strongly promoted formation of RBD dimers, as evidenced by the almost twice of the molecular weight of non-denatured versus denatured proteins (Fig. 1C, top). These recombinant RBD fragments reacted strongly with polyclonal antibodies specifically raised in mice against recombinant S1 protein of MERS-CoV (Fig. 1C, bottom), suggesting that they are in their native and antigenic conformation.

3.2. Receptor binding affinity of MERS-CoV RBD fragments

Two alternative assays, co-immunoprecipitation and ELISA, were carried out to measure the receptor-binding activities of

MERS-CoV RBD fragments. Results from co-immunoprecipitation assay showed that the five MERS-CoV RBD fragments were co-immunoprecipitated with recombinant sDPP4 protein. Two clear bands corresponding to the sizes of DPP4 and respective Fc-fused MERS-CoV RBD fragments were readily revealed with anti-DPP4- and anti-MERS-CoV S1-specific antibodies, whereas a single band with a molecular weight corresponding to DPP4 was detected in the sample containing only sDPP4 (Fig. 2A, left). In addition, each of the five MERS-CoV RBD fragments co-immunoprecipitated cell-associated DPP4 anchored on Huh-7 cells, in which target proteins with anticipated sizes were identified using anti-DPP4- and anti-MERS-CoV S1-specific antibodies, respectively (Fig. 2A, right). Thus, co-immunoprecipitation assay demonstrated that all of the five RBD fragments specifically interact with MERS-CoV's receptor DPP4.

ELISA was then performed by adding MERS-CoV RBD fragments to the plates coated with a fixed amount of sDPP4, followed by identification of the binding affinity based on the OD values at 450 nm. While all of the tested MERS-CoV RBDs were capable of binding to sDPP4, we noted the existence of their differential binding capacity, as shown in Fig. 2B. Among the five RBD fragments, S358-588-Fc, S367-588-Fc and S377-588-Fc possessed significantly higher DPP4-binding affinity than S350-588-Fc and S367-606-Fc at concentrations of 50 and 12.5 µg/ml, respectively. As expected, the control hIgG-Fc protein did not show any specific binding to sDPP4 (Fig. 2B). Therefore, the extended N- or C-terminus of MERS-CoV RBD fragments appears to have negative effect on the receptor-binding activity.

3.3. Immunogenicity of MERS-CoV RBD fragments

To evaluate the immunogenicity of the five MERS-CoV RBD fragments, we immunized mice with each of the RBD fragments according to the dosing strategy described in Materials and Methods, and detected specific responses of total IgG antibody, along with IgG1 and IgG2a subtypes, in serum specimens of immunized mice. ELISA results showed that all five RBD fragments were immunogenic in mice, and induced production of IgG antibodies that specifically bind to MERS-CoV S1 protein without the fusion of Fc. Particularly, sera from mice immunized with S367-588-Fc or S377-588-Fc acquired a stronger binding to MERS-CoV S1 protein than those immunized with the other three RBD fragments, whereas the control samples from PBS-immunized mice revealed negligible binding activity (Fig. 3A), confirming the specificity of such IgG antibody response elicited by these RBD fragments. In addition, the endpoint titration of IgG antibody response revealed that S367-588-Fc and S377-588-Fc were more immunogenic than S350-588-Fc, S358-588-Fc or S367-606-Fc in eliciting MERS-CoV S1-specific IgG antibodies in mice after 3 vaccinations (Fig. 3B). Thus, the extended N- or C-terminus of the MERS-CoV RBD also reduces the immunogenicity of the RBD fragments.
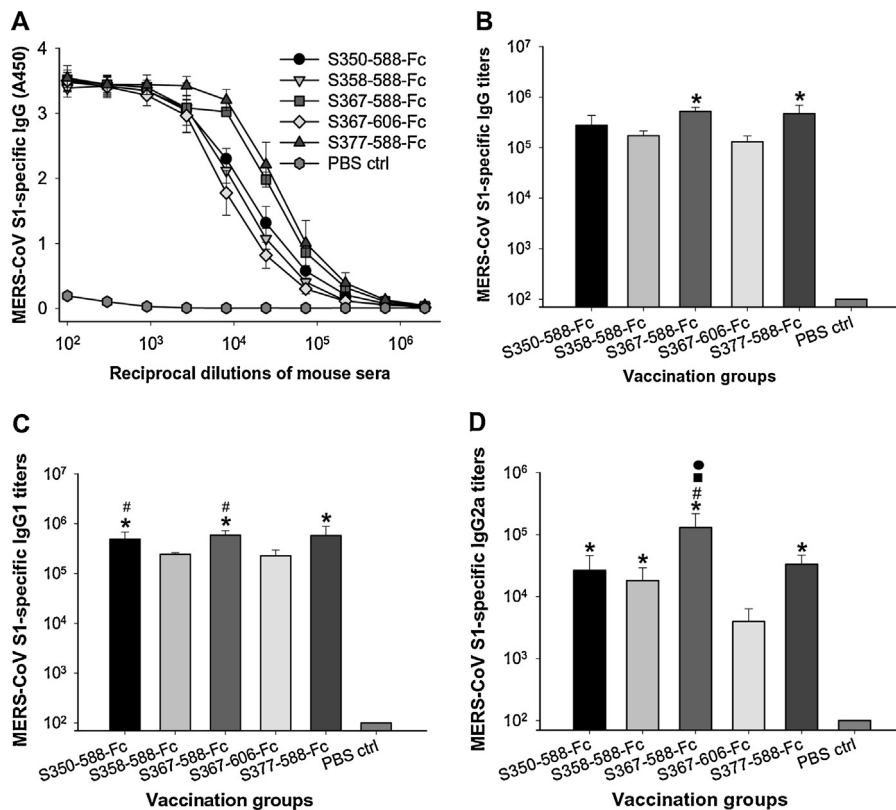

Fig. 3. Immunogenicity of MERS-CoV RBD fragments. Sera collected at 10 days post-last immunization with each of the RBD fragments were the subjects to immunogenicity study, with the serum specimens of sham/PBS-immunized mice included as the negative control. The data are presented as mean ±SD from five mice in each group. (A) Total IgG antibodies specific to MERS-CoV S1 protein were assessed by ELISA. (B) MERS-CoV S1-specific IgG antibodies elicited by groups of differentially immunized mice were determined by ELISA, and the titers were expressed as the endpoint dilutions that remain positively detectable. There were significant differences between two of the RBD fragments (S367-588-Fc and S377-588-Fc) and the other three (S350-588-Fc, S358-588-Fc, and S367-606-Fc). (C) MERS-CoV S1-specific IgG1 subtype antibody titers of groups of mice immunized with indicated RBD fragments were compared, based on the endpoint analysis by ELISA. There were significant differences between three of the RBD fragments (S350-588-Fc, S367-588-Fc, and S377-588-Fc), and S367-606-Fc (*), and between two of the RBD fragments (S350-588-Fc and S367-588-Fc) and S358-588-Fc (#). (D) The endpoint titers of MERS-CoV S1-specific IgG2a subtype antibodies derived from mice immunized with different RBD fragments were compared. There were significant differences between four of the RBD fragments (S350-588-Fc, S358-588-Fc, S367-588-Fc, and S377-588-Fc) and S367-606-Fc (*), or between S367-588-Fc and three of the RBD fragments (S350-588-Fc (#), S358-588-Fc (■), and S377-588-Fc (●)), respectively.

Observation of IgG subtypes revealed that S350-588-Fc, S367-588-Fc and S377-588-Fc were capable of inducing a significantly higher level of IgG1 (Th2) antibody response than S367-606-Fc, while S350-588-Fc and S367-588-Fc elicited IgG1 antibody significantly higher than S358-588-Fc (Fig. 3C). In addition, S350-588-Fc, S358-588-Fc, S367-588-Fc and S377-588-Fc promoted a significantly higher level of IgG2a (Th1) antibody response than S367-606-Fc, whereas the IgG2a antibodies induced by S367-588-Fc were also significantly higher than those induced by S350-588-Fc, S358-588-Fc and S377-588-Fc, respectively (Fig. 3D). The above results suggest that S377-588-Fc is among the RBD fragments that induced high titers of Th1 and Th2 antibody response.

3.4. S377-588-Fc having the best potential to induce highly potent neutralizing antibody responses in vaccinated animals

Neutralizing potentials of the five RBD fragments were initially evaluated in immunized mice. Results from Vero E6-based micro-neutralization showed that each of the five RBD fragments was capable of inducing neutralization antibodies. Among them, S377-588-Fc induced the highest level of neutralizing antibody response on the 10th day after the third immunization (Fig. 4A). As expected, sera from PBS-immunized mice did not show neutralizing activity. Thus, S377-588-Fc has the highest neutralizing potential among the five RBD fragments. Furthermore, S377-588-Fc was used to immunize rabbits to confirm its neutralizing potential in different animal species. Vero E6-based micro-neutralization assay demonstrated that on the 10th day after the third immunization with S377-588-Fc, high titers of neutralizing antibody responses were generated in rabbits (Fig. 4B). Therefore, S377-588-Fc, the shortest among the five RBD fragments, can produce high-titer neutralizing antibody responses in both mice and rabbits, suggesting that the fragment containing residues 377-588 of MERS-CoV spike protein is a critical neutralizing receptor-binding domain.

To elucidate the potential of S377-588-Fc as a vaccine candidate, we immunized mice with this protein for a period of 136 days, and observed the kinetics of antibody responses. As expected, increasing titers of IgG antibodies were found in mouse sera after initial immunization, and reached the highest titers during 31 and 52 days post-immunization. The antibody titers decreased slightly afterwards, but still maintained at relative high levels (Fig. 4C). These data demonstrated that the fragment containing residues 377-588 of MERS-CoV spike protein is able to induce and maintain RBD-specific immune responses in the vaccinated animals for at least four months, confirming its good potential to be further developed as a subunit MERS vaccine.

4. Discussion

The increasing number of MERS cases indicates urgency for the development of effective vaccines against MERS-CoV infection and disease. Among all of the MERS-CoV structural proteins, the spike protein, particularly its RBD, is an important target for such an effort, due to its proven ability to elicit potent neutralizing antibody response in immunized laboratory animals [33]. Indeed, recombinant RBD fragments containing residues 358-588 and 377-662 of MERS-CoV S have been shown to induce neutralizing antibody response in immunized rabbits and mice, respectively [17,29]. Moreover, a modified vaccinia virus Ankara (MVA) expressing the full-length S protein of MERS-CoV was also proven effective in vaccinated mice to produce potent neutralizing antibodies and restricted MERS-CoV infection in vivo [34]. In addition to the aforementioned recombinant proteins and viral vectors, nanoparticles and Venezuelan Equine Encephalitis Virus Replicon Particles
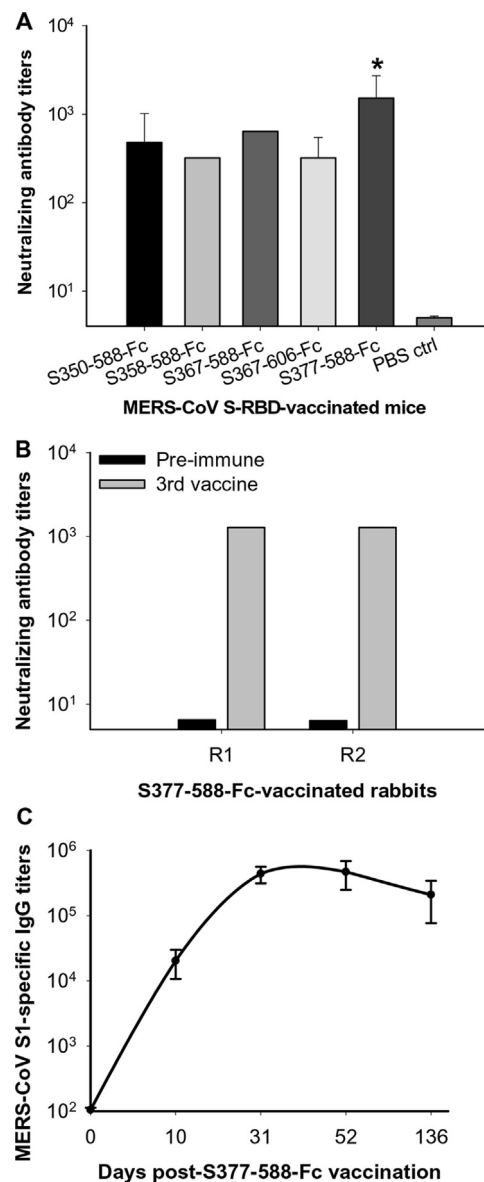

Fig. 4. Neutralization potentials of MERS-CoV RBD fragments. The aforementioned serum specimens were subjected to Vero E6-based micro-neutralization assays against live MERS-CoV infection. Neutralizing antibody titers are expressed as the reciprocal of the highest dilutions of specimens tested that resulted in a complete inhibition of virus-induced CPE in at least 50% of the wells (NT$_{50}$). (A) Neutralizing antibody titers in sera of mice immunized with MERS-CoV RBD fragments. Sera from 10 days post-3rd immunization were used for the test and the data are presented as mean ± SD from five mice in each group. There were significant differences between S377-588-Fc and the other four RBD fragments. (B) Neutralizing antibody titers in sera of rabbits (R1 and R2) immunized with S377-588-Fc protein. The sera from pre-immunization and 10 days post-3rd immunization were used for the test. The data are presented as mean ± SD from duplicate wells. (C) The endpoint titers of MERS-CoV S1-specific IgG antibodies by ELISA in S377-588-Fc-immunized mouse sera at 0, 10, 31, 52, and 136 days post-immunization. The data are presented as mean ± SD from five mice in each group.

(VRPs)-expressing spike protein of MERS-CoV have also shown great potentials as MERS vaccines [35,36].

In this study, we expressed five different versions of MERS-CoV RBD fragments based on the previously published studies [16–19], and further investigated and compared their receptor binding ability, antigenicity, immunogenicity, and neutralizing potential. The Fc of human IgG was fused to these RBD fragments because the Fc tag can enhance the expression, purification, and immunogenicity of viral RBD proteins [37–42]. Our results show that the abilities
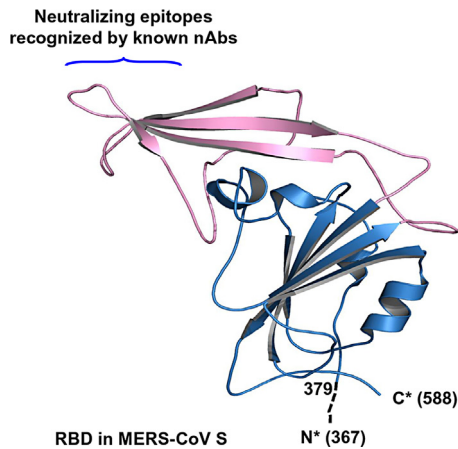
Fig. 5. Crystal structure of MERS-CoV spike RBD (PDB 4L3N) [19]. MERS-CoV spike RBD is a well-folded structure, and contains a core structure (in blue) and a receptor-binding motif (RBM; in magenta). Helices are drawn as cylinders, and strands as arrows. The crystallized RBD fragment encompasses residues 367 to 588, with the N- and C-terminus labeled as N* and C*, respectively. The N-terminal region of the RBD fragment (from residues 369 to 378) is disordered, as indicated by a dashed line. Neutralizing epitopes recognized by known nAbs are indicated in RBM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of these RBD fragments to induce immune responses and neutralizing antibodies differ significantly. Among these RBD fragments, S377-588-Fc demonstrated high receptor-binding affinity, induced high titer IgG antibody in mice, elicited the highest titer neutralizing antibodies in mice, and also induced high titer neutralizing antibodies in rabbits.

Crystal structure of MERS-CoV RBD reveals that RBD fragment encompassing residues 377-588 of MERS-CoV spike protein has a stably folded structure with ordered residues on the N- and C-termini. It contains the receptor-binding motif (RBM, residues 484-567) (Fig. 5) and almost all of the major neutralizing epitopes identified in MERS-CoV RBD, many of which are specifically recognized by neutralizing monoclonal antibodies (nAbs) [31,43–45]. These epitopes are clustered on a protruding ridge in the RBM region of the RBD, and overlap with the DPP4-binding site (Fig. 5) [16,19,20]. Although not yet experimentally confirmed, other regions in the RBM that overlap with the DPP4-binding site may also contain neutralizing epitopes. On the other hand, the regions in the MERS-CoV spike that do not overlap with the DPP4-binding site likely contain non-neutralizing epitopes. These non-neutralizing epitopes might hinder the production of neutralizing antibodies and may also induce harmful immune responses [46–48]. The regions in the RBD core structures contribute to the folding and the stability of the whole RBD, and thus they are essential. However, the regions flanking the RBD do not contribute to protein folding or receptor binding. Therefore, it is necessary to identify the critical neutralizing epitopes in RBD in the MERS-CoV spike protein, and exclude non-neutralizing epitopes, without the expense of domain integrity and stability, as a means of "immunofocusing" for vaccine development.

It is important to further test the immune efficacy of S377-588-Fc in an animal model challenged by MERS-CoV. Non-human primates, such as rhesus macaques, can be infected by MERS-CoV, showing clinical signs of the disease and histopathological changes in lungs [49,50]. However, the high cost of purchasing and maintaining the monkeys and performing the experiments in ABSL-3 facility have prevented us from using this animal model. Small animals such as mice, Syrian hamsters, and ferrets that can be infected by SARS-CoV are unsusceptible to MERS-CoV infection [51–54]. Although mice transduced with adenoviral vectors

expressing human DPP4 can be infected by MERS-CoV, they did not show significant clinical symptoms of MERS [55]. Therefore, the in vivo efficacy of this MERS-CoV RBD-based vaccine candidate will be evaluated in a more sophisticated small animal model once it becomes available.

Overall, the RBD fragment encompassing residues 377-588 of MERS-CoV S protein is the ideal subunit vaccine candidate with the capability of inducing strong neutralizing antibodies in different species of animals. Therefore, it can be the focus of future development of MERS vaccines. Furthermore, this study suggests that in vaccine design it maybe helpful to eliminate non-neutralizing epitopes from the RBD fragments to keep the RBD fragments in their most physiological relevant conformation, and also allow the host immune responses to focus on the neutralizing epitopes.

Conflict of interest statement

The authors declared no conflict of interest.

Acknowledgements

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