Structural Definition of a Unique Neutralization Epitope on the Receptor-Binding Domain of MERS-CoV Spike Glycoprotein

Highlights

- MERS-4 binds RBD from the outside of the RBD–DPP4 binding interface
- MERS-4 favors binding to the RBD in the “up” position in the S trimer
- MERS-4 neutralizes MERS-CoV by indirect rather than direct competition with DPP4
- MERS-4 is a valuable addition to the combined use of MERS-CoV antibodies

In Brief

Zhang et al. report the structural and functional analysis of the potent MERS-CoV neutralizing antibody MERS-4. MERS-4 recognizes a unique epitope and indirectly disrupts interaction between the receptor binding domain and the receptor DPP4. This mechanism provides a valuable addition for the combined use of antibodies against MERS-CoV infection.

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Structural Definition of a Unique Neutralization Epitope on the Receptor-Binding Domain of MERS-CoV Spike Glycoprotein

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SUMMARY

The major mechanism of antibody-mediated neutralization of the Middle East respiratory syndrome coronavirus (MERS-CoV) involves competition with the cellular receptor dipeptidyl peptidase 4 (DPP4) for binding to the receptor-binding domain (RBD) of the spike (S) glycoprotein. Here, we report a unique epitope and unusual neutralizing mechanism of the isolated human antibody MERS-4. Structurally, MERS-4 approached the RBD from the outside of the RBD-DPP4 binding interface. Such binding resulted in the folding of the β5–β6 loop toward a shallow groove on the RBD interface critical for accommodating DPP4. The key residues for binding are identified through site-directed mutagenesis. Structural modeling revealed that MERS-4 binds to RBD only in the “up” position in the S trimer. Furthermore, MERS-4 demonstrated synergy with several reported antibodies. These results indicate that MERS-4 neutralizes MERS-CoV by indirect rather than direct competition with DPP4. This mechanism provides a valuable addition for the combined use of antibodies against MERS-CoV infection.

INTRODUCTION

The 2012 emergence of the Middle East respiratory syndrome coronavirus (MERS-CoV) in Saudi Arabia was the second major introduction of a highly pathogenic coronavirus into the human population since the outbreak of SARS-CoV in China in 2003 (Assiri et al., 2013; Zaki et al., 2012). The global spread of SARS-CoV resulted in more than 8,000 infections and nearly 800 deaths worldwide (Peiris et al., 2004). Fortunately, the SARS-CoV epidemic rapidly died off because of conventional public health measures, even if the exact mechanism of its introduction into, and disappearance from, the human population still remains a mystery (Graham et al., 2013). By contrast, the MERS-CoV epidemic has persisted for more than 5 years with no signs of abating (http://www.who.int/csr/don/10-november-2017-mers-oman/en/). Apart from the sudden initial outbreak in Saudi Arabia, MERS-CoV has spread to other countries outside the Arabian Peninsula carried by infected travelers (Bermingham et al., 2012), leading to an outbreak in South Korea in 2015 (Choi, 2015). These are continuous reports of human MERS-CoV infection in affected regions, largely due to contact with dromedary camels, which are believed to be a major reservoir host for MERS-CoV and the immediate source of human infection (Hemida et al., 2014). Compared with SARS-CoV with a fatality rate of approximately 10% (Peiris et al., 2004), MERS-CoV appears to be more deadly, with its fatality rate reaching as high as 35% (de Wit et al., 2016). All of these facts indicate that MERS-CoV will remain as a severe and long-term threat to global health and highlight the urgent need for effective prophylactic and therapeutic measures.

Scientific progress achieved since the SARS-CoV epidemic has greatly increased our capacity to respond to MERS-CoV. Like that of SARS-CoV, the spike (S) glycoprotein of MERS-CoV plays a critical role in mediating viral entry and in inducing a protective antibody response in infected individuals (Raj et al., 2013; Zumla et al., 2016). The isolation of potent neutralizing monoclonal antibodies has been reported shortly after the identification of MERS-CoV. This was achieved by using various technological platforms such as phage or yeast display of antibody library (Jiang et al., 2014; Tang et al., 2014; Ying et al., 2014), immunization of experimental animals (Chen et al., 2017a; Du et al., 2014; Li et al., 2015; Pascal et al., 2015; Wang et al., 2015a), and direct isolation from human survivors of MERS-CoV infection (Chen et al., 2017b; Corti et al., 2015; Wang et al., 2018). Currently, close to 20 different neutralizing monoclonal antibodies have been characterized in cell culture and experimental animal models. A large majority target the receptor-binding domain (RBD) of the MERS-CoV S glycoprotein.
and interfere with the binding of the cellular receptor dipeptidyl peptidase 4 (DPP4). We previously reported the isolation of the two neutralizing antibodies MERS-4 and MERS-27 via screening of a yeast-displayed library of human scFv (single-chain variable domain fragment) using the MERS-CoV RBD as bait (Jiang et al., 2014). Both antibodies demonstrated potent neutralizing activity against live and pseudotyped MERS-CoV, and a high level of synergy when used together.

Structural determination of antibodies and their epitope specificities provides a critical foundation for a better understanding of their mechanisms of neutralization. We and others have demonstrated that MERS-CoV RBD possesses core and receptor-binding subdomains (Chen et al., 2013; Lu et al., 2013; Wang et al., 2013, 2015a). A large majority of neutralizing antibodies against MERS-CoV, including MERS-27, 4C2, D12, JC57-14, m336, MCA1, and CDC-C2, have been found to target the receptor-binding subdomain and overlap with the DPP4 binding surface (Chen et al., 2017b; Li et al., 2015; Wang et al., 2015a, 2018; Ying et al., 2015; Yu et al., 2015). Therefore, these antibodies share at least one similar aspect of neutralization by directly competing with the cellular receptor DPP4 for binding to RBD.

Here, we report the structural and functional analysis of our previously isolated potent neutralizing antibody MERS-4 and its variant MERS-4V2, which reveals their unique epitope specificity and unusual mechanism of action. In contrast to all the reported RBD-targeting neutralizing antibodies that compete with DPP4 for binding to the RBD, MERS-4 and MERS-4V2 approached the RBD from the outside of the RBD-DPP4 binding interface. Site-directed mutagenesis identified several key residues critical for binding and the neutralizing activity of MERS-4 and MERS-4V2. Structural comparisons of the RBD in unbound, DPP4-bound, and antibody-bound states revealed significant conformational changes in the RBD when bound to MERS-4 or MERS-4V2. To the best of our knowledge, this is the first report demonstrating that a MERS-CoV neutralizing antibody can indirectly interfere with DPP4 binding through conformational changes. Its unique epitope specificity and unusual mechanism of action enable MERS-4 to synergize with other antibodies, providing a valuable addition for the combined use of antibodies against MERS-CoV infection.

RESULTS

Overall Structure of MERS-4 and Its MERS-4V2 Variant Bound to the RBD

We previously reported that the human monoclonal antibody MERS-4 targets the RBD of the spike glycoprotein and exhibits strong neutralization activity against live and pseudotyped MERS-CoV infection (Jiang et al., 2014). However, the yield of MERS-4 from the transfected HEK293F cells was rather low (<1 mg/L), hampering our efforts toward detailed structural analysis. To identify a variant with improved productivity, we generated a library of mutant MERS-4 comprising random replacements in the 5-residue-long CDR3 region, displayed on the surface of yeast, and selected for binding to the RBD through fluorescence-activated cell sorting (FACS)-based sorting as described previously (Jiang et al., 2014). Among a total of 17 selected mutants, only the variant MERS-4V2 demonstrated a substantial improvement of productivity (>10-fold) while maintaining potency as strong as that of the original MERS-4 in neutralizing pseudotyped virus and binding the S trimer (Figure S1A). Sequence analysis showed that the original CDR3 residues Ala-Gly-Asn-Asp (AGND) of MERS-4 were replaced by Thr-Asn-Thr-Tyr (TNTY) in MERS-4V2 (Figure S1B).

To study the overall structure of antibodies bound to the RBD, we expressed MERS-4 and MERS-4V2 in HEK293F cells and obtained the corresponding Fab fragments. The antibody and RBD complexes were formed by mixing the MERS-4 or MERS-4V2 Fab with the RBD. However, despite our repeated efforts in optimizing and screening more than 200 crystals, we were only able to obtain X-ray diffraction data of RBD in complex with MERS-4 Fab at 4.5 Å and with MERS-4V2 Fab at 7 Å. Nevertheless, we solved the structure of the MERS-4 Fab/RBD complex (PDB: 5SZX) and refined it to $R_{work}$ and $R_{free}$ factors of 30.2% and 34.4%, respectively (Figure S2A; Table S1). We went further to construct an scFv version of MERS-4V2 and replaced the MERS-4V2 Fab during complex formation with RBD. The structure of the MERS-4V2 scFv/RBD complex (PDB: 5YY5) was successfully solved to a resolution of 2.8 Å with $R_{work}$ and $R_{free}$ factors of 24.7% and 27.7%, respectively (Figure S2B; Table S1).

As shown in Figure 1A, MERS-4 Fab and MERS-4V2 scFv shared the same mode of binding to the RBD. A superimposition of MERS-4 Fab with MERS-4V2 scFv revealed that their respective recognition was largely mediated through interactions with the β5-β6, β6-β7, and β7-β8 loops in the receptor-binding subdomain of the RBD (Figure 1B). Negligible structural differences were found between the two, with an overall root-mean-square deviation (RMSD) of 0.6 Å for 204 aligned Ca atoms. At the binding interface, the β7-β8 loop of RBD inserted into the cavity between the antibody heavy and light chains, forming interactions with all CDR loops of the antibody except for HCDR3 (Figures 1C and 1D). In particular, the short β5-β6 loop interacted with the HCDR1 and HCDR2 loops of the antibody heavy chain, and the long β6-β7 loop predominately interacted with the LCRD2 loop of the antibody light chain (Figures 1C and 1D).

Structural Features at the Binding Interface between MERS-4V2 and the RBD

We conducted a detailed analysis of structural features at the binding interface derived from the MERS-4V2 scFv/RBD complex (Figures 2 and S2C). At the binding interface, 16 MERS-4V2 scFv residues from all 6 CDR except for HCDR3 formed contacts with 15 residues from the β5-β6, β6-β7, and β7-β8 loops of the receptor-binding subdomain of the RBD (Figure 2A; Table S2). Specifically, the RBD residues Leu507 and Ser508 from the β5-β6 loop interacted with Ser30, Asn31, and Tyr53 from the heavy chain (Figure 2B; Table S2). The RBD residues Gin516, Asn519, Asn521, Gln522, Tyr523, and Pro525 from the β6-β7 loop interacted with Tyr50, Thr51, Asp53, Gln54, Arg55, and Asp61 from the antibody light chain (Figures 2C and 2D; Table S2). Furthermore, the RBD residues Lys543, and Leu545 to Gly550 from the β7-β8 loop interacted with Ala33, Thr35, Tyr53, and Tyr59 from the heavy chain, as well as Asn32, Tyr33, Thr35, Thrp51, and Thrp92 from the light chain (Figures 2B and 2D; Table S2).
and 2E; Table S2). Among all interactions at the interface, one example of hydrophobic interactions involved Leu548 (RBD \(\beta_7-\beta_8\) loop), Tyr35 (heavy chain), Tyr35 (light chain), and Trp92 (light chain) (Figures 2B and 2E; Table S2). Examples of hydrogen bonds included Ser508 (RBD \(\beta_5-\beta_6\) loop) to Tyr53 (heavy chain), Asn519 (RBD \(\beta_6-\beta_7\) loop) to Tyr50 (light chain), Asn521 (RBD \(\beta_6-\beta_7\) loop) to Asp61 (light chain), and Gln522 (RBD \(\beta_6-\beta_7\) loop) to Arg55 (light chain) (Figures 2B–2D; Table S2). As indicated above, the HCDR3 appeared not to be involved in the RBD interaction within a distance cutoff of 4.0 Å. This may explain the unchanged neutralizing and binding activities of MERS-4V2 compared with the parental MERS-4 despite the four-residue replacement in the HCDR3 (Figures S1A and S1B).

Figure 1. Crystal Structures of MERS-CoV RBD in Complex with MERS-4 and Its Variant MERS-4V2

(A) Overall structures of the RBD/MERS-4 Fab and the RBD/MERS-4V2 scFv (right) complexes. The RBD core subdomain was in blue, while the receptor-binding subdomain was in green, the MERS-4 light chain in magenta, the MERS-4 heavy chain in cyan, the MERS-4V2 VL in orange, and the MERS-4V2 VH in red.

(B) Structural superimposition of the RBD/MERS-4 and the RBD/MERS-4V2 complexes and schematic illustration of the MERS-CoV RBD (right).

(C and D) MERS-4 (C) and MERS-4V2 (D) interact with the \(\beta_5-\beta_6\), \(\beta_6-\beta_7\), and \(\beta_7-\beta_8\) loops of the RBD. See also Figure S1 and Table S1.

Structural Alterations in the RBD Bound to MERS-4 and MERS-4V2

We have previously shown that MERS-4 interfered with the interaction between soluble RBD and the cellular receptor DPP4 expressed on the surface of Huh7 cells (Jiang et al., 2014). Surface plasmon resonance (SPR) analysis showed that the binding of soluble RBD to chip-coupled DPP4 was reduced in the presence of increasing concentrations of MERS-4 in a dose-dependent manner (Figure S3). However, structural superimposition demonstrated that the epitope targeted by MERS-4 and MERS-4V2 is located outside the DPP4 binding surface on the RBD (Figure 3). This apparent disconnect prompted us to compare all of the available RBD structures in the unbound (PDB: 4ZPW, 4KQZ, and 4L3N) (Chen et al., 2013; Lu et al., 2013; Wang et al., 2015a), DPP4-bound (PDB: 4L72 and 4KR0) (Lu et al., 2013; Wang et al., 2013), MERS-4-bound, and MERS-4V2-bound states (Figure 3A). While the overall structure of RBD remained relatively stable in all the different states, the MERS-4- or MERS-4V2-bound RBD was found to have a conformational change of the \(\beta_5-\beta_6\) loop (Leu506 to Glu513) (Figure 3A). This particular change involved the folding of the \(\beta_5-\beta_6\) loop toward a shallow groove on the RBD interface critical for accommodating a short helix of DPP4 (Figure 3A). The maximum distance change occurred at Asp510, whose Cα atom moved more than 3 Å into the groove (Figure 3B). Our previous study revealed that the \(\beta_5-\beta_6\) loop participates in the formation of a shallow groove to accommodate the docking of a short helix of DPP4 in patch 2 of the binding interface (Figure 3C) (Wang et al., 2013). Residues Leu506, Asp510, and Glu513 from this loop were found to be involved in forming the core hydrophobic and peripheral hydrophilic interactions in patch 2 (Figure 3C), and mutations of these residues...
Interactions between the RBD residues from the loop, and MERS-4V2 heavy chain. The working hypothesis involving the “up” or “down” positions of RBD (Gui et al., 2017; We and others have recently demonstrated that MERS-CoV venting the docking of the short helix of DPP4 into the shallow and Ser292 and Arg317 from DPP4 (Figure 3D), thereby pre-
expected to bring steric clashes between Asp510 from RBD significantly. The conformational change identified here is therefore decreased the binding affinity between RBD and DPP4 signifi-
cantly. The SPR analysis showed that mutations at Leu507, Ser546, Pro547, and Glu549 in the RBD significantly decreased the binding affinity to MERS-4 (Figures 5A and S4). We next measured the neutralization activity of MERS-4 against pseudotyped viruses bearing wild-type or mutant S glycoprotein (Leu507Ala, Leu545Ala, Ser546Ala, and Pro547Ala) in the cell entry assay. Consistent with the binding changes, pseudotyped viruses bearing these mutations became less sensitive to MERS-4 neutralization (Figure 5B). Those bearing Ser508Ala, Leu548Ala, or Glu549Ala mutations failed to produce detectable amounts of infectious viral particles and therefore were excluded from our experiments. Lastly, we went further to study whether the binding of MERS-4 to the mutant S glycoprotein was also reduced when the protein was expressed on the surface of cells. Specifically, transfected HEK293T cells expressing either the wild-type or mutant S glycoprotein were harvested, stained with MERS-4 or antibody 5F9 specific for the NTD of S glycoprotein as a control (Chen et al., 2017a), and analyzed by FACS. As shown in Figure 5C, MERS-4 demonstrated variable levels of reduction in its binding to the mutant S glycoprotein, while the control antibody 5F9 remained virtually unchanged. Furthermore, the L545A mutation completely abolished the binding activity of MERS-4, which was consistent with the results obtained using the pseudotyped viruses. These
results suggest that reduced binding affinity of MERS-4 to the mutant S glycoprotein is one of the major contributors to viral resistance. Collectively, we confirmed the unique epitope targeted by MERS-4 and MERS-4V2 observed in the crystal structures and highlighted the unusual mechanism of neutralization by these antibodies.

**Binding of MERS-4 and MERS-27 to the RBD**

The epitope of MERS-4 is different from those of other reported antibodies including MERS-27, which we isolated and defined previously (Figure S5). It prompted us to study the combined binding of MERS-4 and MERS-27 to the RBD. Gel filtration analysis showed that the peak of the mixture comprising RBD, MERS-4 Fab, and MERS-27 Fab had a forward shift compared with that of the RBD/MERS-4 Fab complex (Figure S6A), indicating that MERS-4 and MERS-27 could bind to the RBD at the same time. To confirm this, we used small-angle X-ray scattering (SAXS) study on the purified ternary complex in solution. We initially built a monomeric model of the RBD/MERS-4/MERS-27 ternary complex, which was generated by superimposing the RBD/MERS-4 and RBD/MERS-27 crystal structures. However, the monomeric model fits poorly to the SAXS data with a $c$ value of 7.0 (Figure 6). To investigate the assembly of the complex, we built a dimeric model of the ternary complex using the interface observed in the RBD/MERS-27 crystal structure. To understand the dynamic assembly, MultiFoXS was applied to define the population of the models in solution (Pelikan et al., 2009). SAXS fitting with multi-state models (31% monomer model, 18% dimer model of the RBD/MERS-4/MERS-27, and 51% RBD/MERS-27) significantly improved the goodness of the fit with a $c$ value of 2.0 (Figure 6), revealing the transient complexation of Fabs. The transient behavior was also observed from the SEC-SAXS (size exclusion chromatography in line with SAXS) (Figure S6). The broad distribution of radius of gyration (Rg) values across the SEC-SAXS peak, ranging from 47 to 37 Å (Figure S6B), suggests a multi-state mixture of the complex, which was further confirmed by the good fits obtained for various sections of the SEC-SAXS peak using ensemble models (Figures S6C and S6D). These results collectively showed that MERS-4 and MERS-27 are capable of binding to RBD at different epitopes to form a ternary complex.
Synergistic Neutralization Effects of MERS-4 with Other Antibodies against RBD and NTD

We have previously shown that MERS-4 and MERS-27 exhibited a synergistic effect by titrating the neutralizing potency of an equimolar mixture of the two antibodies and comparing the dose response with that of neutralization assays performed with the individual antibody alone (Chou, 2010; Chou and Talalay, 1984; Keck et al., 2013). The synergy between them was consistent with the unique epitopes of MERS-4 and MERS-27 on the RBD (Figure 7C). Here, we further tested whether MERS-4 could synergize with additional antibodies targeting either the RBD (m336) or NTD (5F9) of the S glycoprotein. As shown in Figure 7A, the percent neutralization obtained using combined MERS-4 and m336 demonstrated a 2.60-fold reduction of half maximal inhibitory concentration (IC50) and 2.73-fold reduction of IC80 compared with that of either MERS-4 or m336 alone. Furthermore, the combination index (CI) values of combined MERS-4 and m336 at fractional effect values of effective dose 50%, 75%, 90%, and 95% (ED50, ED75, ED90, and ED95) were 0.48, 0.38, 0.30, and 0.26, respectively. As a CI value of 1 indicates an additive effect, <1 indicates synergism, and >1 indicates antagonism, the combination of MERS-4 and m336 works in a clearly synergistic manner. Furthermore, the combination of MERS-4 and 5F9 demonstrated better synergy in
particular at relatively lower concentrations (Figure 7B). The percent neutralization of combined MERS-4 and 5F9 demonstrated a 15.21-fold reduction in IC50 and 52.7-fold reduction in IC80 compared with that of either antibody alone. Furthermore, the CI values of combined MERS-4 and 5F9 at fractional effect values of ED50, ED75, ED90, and ED95 were 0.06, 0.05, 0.06, and 0.06, respectively. These results showed that MERS-4 can act in synergy with RBD-specific m336 as well as NTD-specific 5F9 antibodies.

**DISCUSSION**

We report the structural and functional analysis of the potent neutralizing antibody MERS-4 and its variant MERS-4V2, which revealed a unique epitope specificity and novel mechanism of neutralization. The structure of MERS-4 Fab bound to RBD was determined at a resolution of 4.5 Å, and that of MERS-4V2 scFv bound to RBD was solved at 2.8 Å. MERS-4 and MERS-4V2 demonstrated the same binding mode and epitope specificity. In contrast to all other RBD-targeting neutralizing antibodies, which directly compete with DPP4 for binding to the RBD, MERS-4 and MERS-4V2 approached the RBD outside the RBD-DPP4 binding interface by targeting the \(\beta_5-\beta_6\), \(\beta_6-\beta_7\), and \(\beta_7-\beta_8\) loops. MERS-4- and MERS-4V2-bound RBD demonstrated significant conformational changes largely involving the folding of the \(\beta_5-\beta_6\) loop toward a shallow groove on the RBD interface critical for accommodating a short helix of DPP4. In the context of the S trimer, MERS-4 prefers binding to the RBD in the “up” rather than the “down” position when virus becomes partially activated. Site-directed mutagenesis confirmed the key residues critical for binding and neutralizing activities of MERS-4 and MERS-4V2. Reduced affinity for the RBD appeared to be the major contributor to the compromised neutralizing activities against the mutant viruses. A synergistic effect was observed between MERS-4 and RBD-specific (m336) as well as NTD-specific (5F9) antibodies, although the exact mechanism remains unknown. Taken together, our study reveals that MERS-4 and MERS-4V2 recognize a unique neutralizing epitope with an unusual mechanism of action. Such special features will enable...
MERS-4 to synergize with other antibodies and provide a valuable addition for the combined use of antibodies against MERS-CoV infection.

Since the identification of the highly pathogenic MERS-CoV in 2012 (Birmingham et al., 2012; Zaki et al., 2012), great efforts have been made to develop prophylactic and therapeutic interventions against this virus. In particular, monoclonal antibodies and vaccines targeting the S glycoprotein are a major research focus due to its critical role in mediating viral entry and its great potency in inducing protective antibody response in infected and naïve individuals (Chen et al., 2009; Zumla et al., 2016). Among a total of 16 interactive binding residues, 14 from the β6-β7 and β7-β8 loops interacted with the light chain, whereas 2 in the β5-β6 loop participated in binding with the heavy chain. The interactions between the β6-β7 and β7-β8 loops of the RBD and the light chain of MERS-4V2 are therefore the most likely the driving force of antigen-antibody binding. However, optimal binding of MERS-4 would require the heavy chain to overcome the steric obstruction by pushing and folding the β5-β6 loop toward the binding interface, resulting in a distorted conformation as shown in the crystal structure. Regardless of the exact process, the observed folding of the β5-β6 loop toward the binding interface would be expected to disrupt the docking of the short helix of DPP4 onto the binding surface of RBD, thereby blocking virus entry. Of note, such unique mechanism of action has not been reported for other viruses. Antibodies against the receptor binding site (RBS) of influenza virus and the CD4 binding site (CD4bs) of HIV type I (HIV-1) exert their neutralizing activities largely through direct competition with their respective receptors (Wu and Kong, 2016; Ren and Zhou, 2016). Perhaps the closest scenario to MERS-4-mediated inhibition is found in antibodies targeting the V3 region of the HIV-1 envelope where binding may capture or induce conformational changes that block the subsequent interaction between the V3 region and the co-receptor CCR5 or CXCR4. Certainly, such a hypothesis would have to be verified in the future (Barnes et al., 2018; Mouquet et al., 2012; Pejchal et al., 2011).

The MERS-CoV spike glycoprotein showed limited sequence variation among different strains, especially in the RBD that is responsible for receptor binding. However, this does not mean the spike glycoprotein will remain unchanged as the virus continues to spread among multiple animal species and to probe...
and adapt in human population. For stronger and broader protective effect against MERS-CoV, a combined use of two or more antibodies will provide a superior alternative (Wang et al., 2018). However, any effective combination would require the candidate antibodies to recognize distinct epitopes for additive or synergistic effect. The unique epitope of MERS-4 and MERS-4V2 therefore makes them good candidates for combination use with those reported elsewhere (Chen et al., 2017b; Li et al., 2015; Wang et al., 2015a, 2018; Ying et al., 2015; Yu et al., 2015). Indeed, combinations of MERS-4 and MERS-27, MERS-4 and m336, and MERS-4 and 5F9 demonstrated impressive synergy in the pseudotyped MERS-CoV assay. The exact mechanism in achieving the synergy, however, is uncertain, particularly for those sharing the same mechanism in disrupting interaction between RBD and DPP4 (MERS-4 and MERS-27, and MERS-4 and m336). Presumably, the two antibodies may preferentially act on RBD at the different spatial and temporal points during interaction with the receptor DPP4, allowing better

Figure 7. Effects of MERS-4 Combined with m336, 5F9, and MERS-27, Respectively, in Neutralizing Pseudotyped MERS-CoV

(A) Effects of MERS-4 combined with m336 in neutralizing pseudotyped MERS-CoV. Percent neutralization was calculated for serial 3-fold dilutions of each antibody alone and in combination at constant ratios in a range of concentrations from 27 times to 1/81 of IC50s. The constant ratios of the combined antibodies were their IC50s. On the x axis, a dose of 1 was at the IC50 concentration. Fractional effect (FA) plots generated by the CompuSyn program for MERS-4, m336, and their combination showing dosage versus effect. Median effect plot of calculated CI values (logarithmic) versus FA values, in which a log CI of <0 is synergism and a log CI of >0 is antagonism. Data shown are average values from four independent experiments.

(B and C) The percent neutralization, fractional effect, and CI values for MERS-4 combined with 5F9 (B) and MERS-4 combined with MERS-27 (C) were calculated and generated using the same method.

See also Figures S5 and S7.
exposure of otherwise less accessible epitopes. If this is the case, the observed synergy would be most likely derived from the recognitions of distinct epitopes rather than the same neutralization mechanism. Obviously, antibodies with distinct mechanisms and binding at disparate epitopes would be more likely to have synergy than those shared mechanism and overlapped epitopes. Synergy showed here between MERS-4 and 5F9 is a good example. Nevertheless, the exact mechanisms underlying synergy must be complex and should be treated differently from case to case. The preliminary results presented here do offer some rationales for MERS-4 as a valuable addition for the combined use of antibodies against MERS-CoV infection.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**

The MERS-CoV RBD (residues 367–588) and the ectodomain of human DPP4 (residues 93–768) were expressed in Sf9 insect cells. The purified RBD was digested with endoglycosidase F1 and F3 at room temperature overnight and was then further purified through gel-filtration chromatography. The MERS-4 and MERS-4V2 IgG3 were expressed in HEK293F cells. The purified MERS-4 and MERS-4V2 were digested with endoproteinase Lys-C at 37°C, and the Fab and Fc fragments were separated by loading samples onto a diethylaminoethyl (DEAE) ion-exchange column. The genes encoding the scFv of MERS-4V2 were cloned with a C-terminal His-tag in the heavy chain variable region of G4S3. The MERS-4V2 scFv was expressed in HEK293F cells through transient transfection. The scFv was captured by nickel beads and purified through gel-filtration chromatography.

**Crystallization and Data Collection**

Crystals of the RBD/MERS-4 Fab complex were successfully grown at 18°C using the sitting drop vapor diffusion method, which involved mixing equal volumes of protein and reservoir solution containing 2% v/v tacsimate (pH 5.0), 0.1 M sodium citrate tribasic dihydrate (pH 5.6), 16% w/v polyethylene glycol 3350, and 2 M sodium thiocyanate. Crystals of the RBD/MERS-4V2 scFv complex were successfully grown at 18°C using the sitting drop vapor diffusion method, which involved mixing equal volumes of protein and reservoir solution containing 0.1 M Tris (pH 7.5), 10% w/v polyethylene glycol 8000, and 8% (v/v) ethylene glycol. Diffraction data were collected on the BL17U beamline at Shanghai Synchrotron Research Facility (Wang et al., 2015b) and processed with HKL2000 (Otwinowski and Minor, 1997). All data collection and processing statistics are listed in Table S1.

**Structural Determination and Refinement**

The structure was determined by molecular replacement with the crystallographic software Phaser (McCoy et al., 2007). The search models are the highest sequence identities. Iterative refinement with the program PHENIX (Adams et al., 2002) and constant domain of the heavy and light chains available in the PDB with MODELLER to construct the missing loops and linkers (Sali and Blundell, 1993). A multistate model of complexes coexisting in solution was selected based on the level of improvement in the model quality. The size of the multistate model was selected based on the level of improvement in the SAXS fit.

**SAXS Data Collection and Analysis**

SAXS data were collected at the SIBLys beamline 12.3.1 of the Advanced Light Source at the Lawrence Berkeley National Laboratory using 1.0Å wave-length and Pilatus 2M detector at a 1.5-m sample-to-detector distance (Cassen et al., 2013), resulting in scattering vectors ranging from 0.01 to 0.5 Å⁻¹. The scattering vector is defined as q = 4πsinθ/λ, where 2θ is the scattering angle. SEC in line with SEC-SAXS was performed to ensure the aggregation-free state of the sample. The SEC column was equilibrated with running buffer (50 mM Tris-HCl [pH 7.3], 100 mM NaCl, 3% glycerol, and 0.01% sodium azide) with a flow rate of 0.5mL/min. The 50-μL sample was run through the SEC and 2-5 X-ray exposures were collected continuously during an ~25-min elution. The SAXS frames recorded prior to the protein elution peak were used to subtract all other frames. The subtracted frames were investigated by Rg and scattering intensity at q = 0 Å⁻¹ (I(0)) derived by the Guinier approximation I(q) = I(0)exp(-qRg²/3) with the limits qRg < 1.5 (Guiner and Fournet, 1955). I(0) and Rg values were compared for each collected SAXS curve across the entire elution peak. The elution peak was mapped by plotting the scattering intensity at q = 0 Å⁻¹ (I(0)) relative to the recorded frame. Graduate decreasing of Rg values across an elution peak indicates transient sample behavior. SAXS was also acquired in the high-throughput modality at sample concentrations between 1 and 5 mg/mL to compare with the SEC–SAXS profiles (Hura et al., 2009). The full atomic model was built with the program MODELLER to construct the missing loops and linkers (Sali and Blundell, 1993). The theoretical SAXS profile and the corresponding fit to the experimental data were calculated using the program FOXS (Schneidman-Duhovny et al., 2013). A multistate model of complexes coexisting in solution was selected by MultiFOXS (Schneidman-Duhovny et al., 2016). The size of the multistate model was selected based on the level of improvement in the SAXS fit.
DATA AND SOFTWARE AVAILABILITY

The accession numbers for the atomic coordinates reported in this paper are PDB: 5ZXV and 5YY5.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.06.041.

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AUTHOR CONTRIBUTIONS

S.Z., P.W., P.Z., and L.J. expressed, purified, and crystallized the protein, and carried out the SPR analysis with help from D.W.; P.Z., Y.L., W.J., H.W., and X.S. carried out the pseudotyped virus entry and antibody neutralization analyses; S.Z. and P.W. collected and processed the diffraction data; X.W. carried out structural determination and refinement; M.H. collected the X-ray crystallography beamline at the Advanced Light Source in Berkeley, CA. The manuscript was written with help from S.Z., P.W., P.Z., and L.J. expressed, purified, and crystallized the protein, and carried out the SPR analysis with help from D.W.; P.Z., Y.L., W.J., H.W., and X.S. carried out structural determination and refinement; M.H. collected and analyzed the SAXS data with input from S.W. and X.F.; X.W. and L.Z. supervised the project and wrote the manuscript with help from S.Z., P.W., P.Z., and A.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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