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Functional reconstitution of the MERS CoV receptor binding motif

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ABSTRACT

In the early 1960's the first human coronaviruses (designated 229E and OC43) were identified as etiologic agents of the common cold, to be followed by the subsequent isolation of three more human coronaviruses similarly associated with cold-like diseases. In contrast to these "mild" coronaviruses, over the last 20 years there have been three independent events of emergence of pandemic severe and acute life-threatening respiratory diseases caused by three novel beta-coronaviruses, SARS CoV, MERS CoV and most recently SARS CoV2. Whereas the first SARS CoV appeared in November 2002 and spontaneously disappeared by the summer of 2003, MERS CoV has continued persistently to spill over to humans via an intermediary camel vector, causing tens of cases annually. Although human-to-human transmission is rare, the fatality rate of MERS CoV disease is remarkably higher than 30%. COVID-19 however, is fortunately much less fatal, despite that its etiologic agent, SARS CoV2, is tremendously infectious, particularly with the recent evolution of the Omicron variants of concern (BA.1 and BA.2). Of note, MERS CoV prevalence in camel populations in Africa and the Middle East is extremely high. Moreover, MERS CoV and SARS CoV2 co-exist in the Middle East and especially in Saudi Arabia and the UAE, where sporadic incidences of co-infection have already been reported. Co-infection, either due to reverse spill-over of SARS CoV2 to camels or in double infected humans could lead to recombination between the two viruses, rendering either SARS CoV2 more lethal or MERS CoV more transmittable. In an attempt to prepare for what could develop into a catastrophic event, we have focused on developing a novel epitope-based immunogen for MERS CoV. Implementing combinatorial phage-display conformer libraries, the Receptor Binding Motif (RBM) of the MERS CoV Spike protein has been successfully reconstituted and shown to be recognized by a panel of seven neutralizing monoclonal antibodies.

1. Introduction

On November 16, 2002, a 64-year-old man from the Guangdong province in China died from an unknown respiratory disease (Peiris et al., 2004; Rota et al., 2003). In the fullness of time he would be known as "Patient Zero" of what would be the first of three beta-coronavirus outbreaks in the last 20 years. Severe Acute Respiratory Syndrome (SARS) is caused by what had been a previously unknown coronavirus (SARS CoV) (Rota et al., 2003). The natural reservoir of the virus is bats (Hu et al., 2017); however, an intermediate vector, masked civets, was initially identified (Wang et al., 2006). These predators populated the food markets frequently and so in an effort to curb the spread of the

Abbreviations: MERS CoV, Middle East Respiratory Syndrome Corona Virus; SARS CoV, Severe Acute Respiratory Syndrome Corona Virus; ECDC, European Centre for Disease Prevention and Control; IACUC, Institutional Animal Care and Use Committee; FAO, Food and Agriculture Organization of the United Nations; UAE, United Arab Emirates; RBM, Receptor Binding Motif; mAbs, monoclonal antibodies; MBP, maltose binding protein; GST, gutathione-S-transferase; TBS, Tris Buffered Saline; TBST, Tris Buffered Saline with Tween-20; PCR, Polymerase Chain Reaction; NGS, Next Generation Sequencing; ACE2, angiotensin converting enzyme 2; huDPP4, human dipeptidyl peptidase 4; gBlock, gene block.

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Table 1
MERS CoV cases in humans by country and dates. Data were obtained from Ministries of Agriculture or Livestock, Ministry of Health; Centers for Disease Prevention and Control (CDC) and World Health Organization (WHO), World Organization for Animal Health (OIE).

18 August 2021,	Country	MERS-CoV infections in human	First observation	Last Observation	Covid-19 on 5/02/22
Middle East	Saudi Arabia	**2184**	13/06/2012	29/12/2021	708,650
	United Arab Emirates	**93**	19/03/2013	06/11/2021	857,633
	Jordan	28	02/04/2012	26/09/2015	1326,993
	Qatar	23	15/08/2013	18/02/2020	349,027
	Oman	24	26/10/2013	20/02/2019	351,641
	Iran (Islamic Republic of)	6	11/05/2014	18/03/2015	6566,967
	Kuwait	4	30/10/2013	08/09/2015	579,032
	Lebanon	2	22/04/2014	08/06/2017	974,099
	Yemen	1	17/03/2014	17/03/2014	11,167
	Bahrain (the Kingdom of)	1	04/04/2016	04/04/2016	420,806
Asia	Republic of Korea	185	11/05/2015	28/08/2018	1007,380
	Philippines	2	15/04/2014	30/06/2015	3608,632
	Thailand	3	10/06/2015	25/07/2016	2496,612
	China	1	21/05/2015	21/05/2015	106,324
	Malaysia	2	08/04/2014	24/12/2017	2913,248
Europe	United Kingdom	5	03/09/2012	16/08/2018	17,810,577
	Germany	3	05/10/2012	07/03/2015	11,112,069
	Netherlands	2	01/05/2014	05/05/2014	4837,279
	France	2	23/04/2013	27/04/2013	20,735,309
	Austria	2	22/09/2014	08/09/2016	2059,862
	Turkey	1	25/09/2014	25/09/2014	12,249,282
	Italy	1	25/05/2013	25/05/2013	11,635,950
	Greece	1	08/04/2014	08/04/2014	2052,892
America	United States of America	2	14/04/2014	01/05/2014	78,071,713
Africa	Tunisia	3	01/05/2013	17/06/2013	945,734
	Algeria	2	23/05/2014	23/05/2014	258,390
	Egypt	1	22/04/2014	22/04/2014	493,648

epidemic, 10,000 civets were destroyed in a massive culling action (Parry, 2004). By July 2003, 8096 cases of SARS CoV were reported in 37 countries with a fatality rate of 9.6%. No new cases of SARS CoV infection have been reported since 2004 (Low, 2004; Stockman et al., 2006).

A second coronavirus outbreak occurred in 2012, in the Saudi peninsula leading to Middle East Respiratory Syndrome (MERS) which is caused by MERS CoV (Al-Abdallat et al., 2014; Killerby et al., 2020; Zaki et al., 2012).[1] Similar to the SARS epidemic, MERS CoV apparently transfer from bats to an intermediate vector, this time dromedary camels, and from camels spill-over to humans (Durai et al., 2015). The latest update from the European Centre for Disease Prevention and Control (ECDC) on the MERS CoV threat (31 December, 2021) is that a total of 2600 cases in 27 countries have been reported with 943 deaths (fatality rate of 36%) (ECDC, 2022). Since the emergence of MERS CoV, every year there have been multiple cases and deaths reported, mainly from the Saudi Peninsula, Table 1 (FAO, 2022; Robert Carlson, 2021). Of note was an isolated, but serious, focused outbreak that occurred in 2015 in South Korea where 185 cases (and one more in China) were detected leading to 38 deaths (WHO, 2021). In response, over 16,900 close contacts were quarantined for two weeks to contain the virus and since then the Korean epidemic seems to be under control (Oh et al., 2018). Since January 2021, 19 new MERS CoV cases have been reported of which 8 patients died (ECDC, 2022).

Although MERS CoV causes a very severe respiratory disease with a very high fatality rate, the human-to-human transmission of this virus, via droplets, is limited to very close contacts, primarily in the hospital - patient setting. Thus, the overall pandemic reflects more multiple zoonotic spill-over events, rather than human-to-human transmission (Berruga-Fernández et al., 2021; A.-R. Zhang et al., 2021). Fortunately, thus far, the virus seems not to have adapted for efficient human transmission.

In total contrast to MERS CoV, in December 2019 a third pandemic event emerged in Wuhan, China with the appearance of a new beta-coronavirus, SARS CoV2 (Zhou et al., 2020). Once again, the virus apparently originates in bats, transferring to humans via an intermediate vector, possibly pangolins (Y. Zhang et al., 2021). In marked contrast however, with the two previous outbreaks, SARS CoV2 has evolved rapidly to become an extremely contagious virus, serially adapting to humans in multiple waves of infections, each time with the appearance of new variants of concern (Chan et al., 2020; Li et al., 2021; Salleh et al., 2021; Volz et al., 2021). To date (February 2022) over 390 million people worldwide have been infected with SARS CoV2, killing over 5.7 million in its wake (ca. 1.5% fatality rate). As a result, unprecedented vaccination campaigns have been promoted globally in an attempt to curb the pandemic and keep SARS CoV2 in check.

SARS CoV2 thus illustrates the serious potential of coronaviruses to adapt and gradually improve their ability for human-to-human transmission. Clearly, all efforts to prevent such dramatic transmissibility for MERS CoV and to counter act virus evolution should be made (Du et al., 2016; A.-R. Zhang et al., 2021). No less concerning is the fact that co-infections of both viruses have been reported (Al-Abdallat et al., 2014; Baddal and Cakir, 2020; Lai et al., 2020). Such situations could possibly lead to genetic recombination (FAO, 2021; Funk et al., 2016; Hu et al., 2017; Kiambi et al., 2018; Li et al., 2020; Peiris and Perlman, 2022; So et al., 2019; Su et al., 2016; Te et al., 2021; Teng et al., 2021; Xiong et al., 2022; Zhang et al., 2019, 2005), creating novel MERS CoV isolates that gain improved transmissibility (Baddal and Cakir, 2020; Elhazmi et al., 2021; Funk et al., 2016; Kiambi et al., 2018; Su et al., 2016; Te et al., 2021; Xiong et al., 2022; Zhang et al., 2019) or produce markedly more lethal versions of SARS CoV2 (Chen et al., 2021; Dimonaco et al., 2020; Safari and Elahi, 2021). In this regard, it should be noted that the prevalence of MERS CoV in camel populations in Africa and the Middle East is extremely high (Ababneh et al., 2021; Al-Tawfiq and Memish, 2020; Funk et al., 2016; Te et al., 2021; Zhang et al., 2019). Moreover, there have been numerous reports of the ability of SARS CoV2 to successfully back spill-over into a variety of mammals including dogs, cats, tigers, minks and ferrets (Banerjee et al., 2021; Hobbs and Reid, 2021; Oude Munnink et al., 2021; Schlottau et al., 2020; Sharun et al., 2021; Shi et al., 2020; Starr et al., 2022). It would seem that it is just a

[1] In a retrospective study, an earlier case of MERS was identified in Jordan, April 2012 (Al-Abdallat et al., 2014).

matter of time that SARS CoV2 might infect camels as well. This is especially concerning for countries such as Saudi Arabia and in the UAE with over 700,000 and 850,000 reported cases of SARS CoV2 respectively, two countries that have large MERS CoV-infected camel populations.

With this in mind, here we describe the functional reconstitution of the MERS CoV Receptor Binding Motif (RBM) which is the main neutralizing epitope for beta-coronaviruses and thus could provide an effective immunogen for the development of a MERS CoV epitope-based vaccine, to be considered for humans and camels alike.

2. Materials and methods

2.1. Vectors

The fth1 vector was developed at Tel Aviv University as previously described (Enshell-Seijffers et al., 2001) and subsequently modified for expression of recombinant peptides as fusions at the N-terminus of the infectivity protein, P3, of fd filamentous bacteriophage (Smelyanski and Gershoni, 2011). The pMALc vector system was provided by N. T. Freund, Tel Aviv University. The pET30a-N6xHis-GST vector was adapted from the commercial pET-30a (Novagen, Sigma-Aldrich; 69909, USA) by G. Kaplan, Tel Aviv University.

2.2. Antibodies and Receptor

Seven neutralizing mAbs that target the MERS CoV RBM were used in this study: human mAbs m-336, m-337, m-338 were kindly provided by D. S. Dimitrov and T. Ying. The human mAb CDC-C2, mouse mAbs F-11 and C-12 and rhesus macaque mAb JC57–11 were kindly provided by B. S. Graham (see Lingshu et al., 2021; Wang et al., 2015; Ying et al., 2014). Recombinant human CD26 protein (Fc-chimera) was purchased from Abcam (ab 155730), Zotal, Israel. Polyclonal rabbit anti-M13 serum was produced in the Gershoni Laboratory in-house at Tel Aviv University. HRP-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA, USA).

2.3. Construction of the MERS CoV RBM conformer library

Reconstitution of the SARS CoV RBM was previously described (Freund et al., 2015) and served as the basis for the reconstitution of the MERS CoV RBM. In principle, segments of the natural MERS CoV RBM were connected via combinatorial linkers thus, yielding a diversity of conformers. In view of the fact that the MERS CoV RBM is larger than that of SARS CoV, the libraries were expressed and displayed on Protein, P3 of the fd filamentous bacteriophage (5 copies per phage). Thus, virus specific combinatorial phage-display conformer libraries were produced. For this, the modified fth1 vector (Smelyanski and Gershoni, 2011), was used for the construction of the MERS CoV RBM conformer libraries.

Specifically, a gBlock of double stranded DNA sequences corresponding to the MERS CoV RBM (K493 – E565, the "full length RBM") (Raj et al., 2013), containing fth1 *BstXI* overlapping sequences on both 5' and 3' ends was purchased from Integrated DNA Technologies (IDT, Israel). This was then used as a PCR-template to generate two segments corresponding to:

Segment A, amino acid residues K493-P515 of the MERS CoV RBM followed by a series of linkers of 3, 4, 5, 6 and 7 random amino acids in length (using sense Primer #1 paired against antisense Primers #3–7) or no linker at all (antisense Primer #2).

Segment B, residues C526-E565 of the MERS CoV RBM (using the sense Primer #8 and antisense Primer #9) of note, residue cysteine 526 was retained as part of the RBM in order to maintain the disulfide C526-C503 that stabilizes the RBM.

Primers: (note, Primers #3–7 are antisense and thus contain "MNN" antisense codons that complement NNK sense codons)**:**

1: 5'CCTTTCTATTCTCACTCCGCTC 3'.

2: 5'GGATGGGACAATGGATACACAAGGTACTTCAGTAACGATCATCAGA 3'.

3: 5'GGATGGGACAATGGATACACAMNNMNNMNNAGGTACTTCAGTACGATCATCAGA 3'.

4: 5'GGATGGGACAATGGATACACAMNNMNNMNNMNNAGGTACTTCAGTACGATCATCAGA 3'.

5: 5'GGATGGGACAATGGATACACAMNNMNNMNNMNNMNNAGGTACTTCAGTACGATCATCAGA 3'.

6: 5'GGATGGGACAATGGATACACAMNNMNNMNNMNNMNNMNNAGGTACTTCAGTACGATCATCAGA 3'.

7: 5'GGATGGGACAATGGATACACAMNNMNNMNNMNNMNNMNNMNNAGGTACTTCAGTACGATCATCAGA 3'.

8: 5'TGTGTATCCATTGTCCCATCC 3'.

9: 5'CTTTCAACAGTTTCCCAGACG 3'.

All PCR products were purified using AMPure beads (Beckman Coulter, Indianapolis, IN, USA) and cloned into *BstXI* precut vector by Gibson assembly reactions (Gibson et al., 2010). Thus, the following constructs were generated:

- "Full Length" RBM (FL), residues 493–565 including the "anchor loop" (residues Q516-P525);
- "Loopless" RBM (LL) in which residues P515 and C526 are connected directly to one another, with no amino acids in place of the deleted loop;
- RBM random conformers containing any one of 5 random linkers (3, 4, 5, 6 and 7 amino acids), in place of the deleted "anchor loop".

Ethanol-purified Gibson reaction products were used to electroporate *E. coli* ER2738 electro-competent cells (cat. no. 60522–1, Lucigen, Middleton, WI, USA) and clones were isolated and confirmed for correct sequence by standard Sanger's sequencing. Bacteria were cultured (shaking at 225 rpm, 37 ^{o}C, overnight), and phages were precipitated from culture media using polyethylene glycol 6000/NaCl and resuspended in Tris buffered saline (50 mM Tris-HCl pH 7.5, 150 mM NaCl (TBS)).

2.4. Screening of the conformer libraries

The conformer libraries were screened against MERS CoV neutralizing mAbs. The following mAbs were used: human neutralizing mAbs m-336, m-337, m-338 and CDC-C2; macaque mAb JC57–11; and murine mAbs F-11 and C-12 (Wang et al., 2018; Xu et al., 2019; Ying et al., 2014; Zhang et al., 2018).

In general, screening was performed as previously described. Briefly, 2 μg of mAb were added to 10^{11} phages suspended in 3% bovine serum albumin in TBS, in total volume of 100 μl and incubated at room temperature for 1 h on a rotator. 30 μl of magnetic Protein-G beads (Dynabeads™ Protein G, Cat. No. 10009D, Invitrogen by Thermo Fisher Scientific, CA, USA) were added to the mAb-phage suspension and incubated for 30 min on a rotator. Unbound mAb and phages were removed from the beads by three rounds of washing with TBST (0.5% Tween-20 in TBS) using a magnetic stand. The mAb-bound phages were eluted with glycine-HCl pH 2.2 and neutralized with Tris-HCl pH 9.1. The eluted phages were used to infect DH5αF+ cells for amplification as previously described (Freund et al., 2015). Three additional rounds of amplification and screening were carried out for each mAb. In order to confirm mAb binding to affinity-selected phages, single colonies were picked and grown as mini-cultures from which dot-blots on nitrocellulose membrane filters were prepared. The filters were blocked using 5% skim milk in TBS for 1 h at room temperature, probed with mAbs (2 μg/ml) overnight at 4 ^{o}C and subsequently detected using HRP-conjugated antibodies (1:5000, Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). Signals were developed using the enhanced chemo-luminescence (ECL) reaction (Rhenium, Israel).

2.5. Sample preparation for NGS

Phages from the 4th eluate (and the naïve library) were used as templates for PCR using:

1: sense primer: containing Illumina "Adapter A" sequence followed by a 5-base barcode (barcode = NNNNN) and then the 4 codons corresponding to residues 512–515 of Segment A, just preceding the linker sequence:

5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNACTGAAGTACCT 3'.

2: antisense primer: containing Illumina "Adapter B" sequence followed by the codons corresponding to the residues 525–528 in Segment B:

5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTAATGGATACACA 3'.

The thermal profile was:

1. 94 °C 5 min
2. 94 °C 1 min
3. 53 °C 1 min
4. 72 °C 20 s
5. steps 2–4 × 35 cycles
6. 72 °C 5 min

The amplified PCR products were validated for size in a 2% agarose gel. PCR samples were purified on AMPure beads (Beckman Coulter, Indianapolis, IN, USA) diluted to give 10 nM, pooled into a single tube and sent for NGS (Illumina HiSeq High Output, SR60-V4).

2.6. Peptide linker Motif Analysis

We used the multiple peptide linkers as input for the Motif Elicitation (MEME, "zoop mode") algorithm (Bailey and Elkan, 1994) for linker motif discovery.

2.7. Construction of reconstituted MERS CoV RBMs as MBP/GST conjugates

The reconstituted MERS CoV RBMs were amplified by PCR from fth1 constructs using the sense primer #10 and antisense primer #11 containing overlapping sequences for *NdeI, HindIII* for maltose binding protein (MBP), and the sense primer #12 and antisense primer #13 containing overlapping sequences for *BamHI* and *HindIII* for glutathione-S-transferase (GST).

Primers #10–13:

10: 5'TTCCAGGGCATTTCACATATGAAGCCTCTTAAGTACAGCT 3'.

11: 5'GAGCCTTTCGTTTTATTTGAAGCTTATTTACTCAGTCATGGCAACAGT 3'.

12: 5'GATCTGGTTCCGCGTGGATCCAAGCCTCTTAAGTACAGCTAT 3'.

13: 5'GAGTGCGGCCGCAAGCTTTCACTCAGTCATGGCAACAGTTGA 3'.

The thermal profile was:

1. 94 °C 5 min
2. 94 °C 30 s
3. 50 °C 30 s
4. 72 °C 30 s
5. steps 2–4 × 35 cycles
6. 72 °C 5 min

The amplified PCR products were validated for size in a 2% agarose gel. All PCR products were purified using AMPure beads and cloned into *NdeI/HindIII* precut pMAL-p5x-MBP vector for MBP constructs and *BamHI/HindIII* precut pET30a-N6xHis-GST vector for GST constructs using Gibson assembly reaction.

For both expression systems, the vectors were transformed into *E. coli* BL21 Rosetta (DE3) bacteria (Novagen Merck, Darmstadt, Germany) and used to produce MBP and GST constructs using 100 mM IPTG for 5 h induction at 37 °C.

2.8. Protein production and purification

E. coli BL21 Rosetta (DE3) cells were transformed with recombinant plasmids coding for MERS CoV RBMs conjugated with either MBP or GST. The transformed cells were grown in 200 ml LB medium + 100 μg/ml ampicillin (for pMAL-p5x-MBP) or 50 μg/ml kanamycin (for pET30a-N6xHis-GST) at 37 °C. When the culture reached $OD_{600\ nm} = 0.6$, 1 ml of 100 mM isopropyl β-d-1-thiogalactopyranoside (IPTG, Bio-Lab, 162423) was added and incubated for additional 5 h at 37 °C.

The saturated bacterial cultures were harvested by centrifugation at 5000 rpm for 10 min. The bacterial pellet was resuspended in Lysis buffer (PBS containing 0.1% Triton X-100, 0.2 M NaCl) and lysed by sonication. The lysates were clarified by centrifugation at 14,000 rpm for 30 min, the supernatant was discarded. The pelleted inclusion bodies were resuspended by sonication in 40 ml lysis buffer containing 8 M urea (ice cold), then incubated with 200 μl of Nickel beads overnight at 4 °C on a rotator to extract protein from inclusion bodies.

The fusion proteins were affinity purified by metal ion affinity chromatography on Sepharose-nickel beads according to the supplier's instructions (Ni Sepharose 6 Fast Flow, GE Healthcare Life Sciences, 17–5318–06) using gravity columns. The buffer exchange following elution was done using Amicon Ultra-15 centrifugal filter 30k for MBP and 10k for GST fusion proteins (Merck; UFC903024, Ireland). Protein purity was estimated using polyacrylamide gel electrophoresis (12%). The yields ranged from 1 to 7 mg of purified protein per 200 ml of culture.

2.9. Immunizations

Six New Zealand female white rabbits were purchased from Envigo, Tel Aviv, Israel. Rabbits were housed under specific pathogen free conditions. All procedures used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Tel Aviv University, Israel.

Two rabbits (body weight 1.4 – 1.8 kg) per each antigen: E5 and E9, and two rabbits for control scaffolds (MBP and GST) were immunized with 0.5 mg/ml of each E5-MBP, E9-MBP and MBP scaffold emulsified with complete Freund's Adjuvant via subcutaneous injections at 5 different sites. Subsequently, three booster doses of antigens using alternate scaffolds of MBP or GST were administered with incomplete Freund's Adjuvant at 18 days of dose intervals. Pre-immune and immune blood samples (5mls) were collected from the marginal ear-vein of each animal a day before each immunization or boost.

2.10. Enzyme-linked immunosorbent assay (ELISA)

ELISA tests were conducted to evaluate antibody binding to the various RBM constructs and for the measure of the antibody response in immunized animals.

2.10.1. RBM capture assays

ELISA plate wells were coated overnight with 5 μg/ml of the different neutralizing mAbs in TBS. Next, the plates were washed with TBS, blocked with 5% skim milk in TBS and incubated with phages displaying the RBM constructs (10^{10} phages/well). Wells were washed with TBST (0.05% Tween-20) and incubated with polyclonal rabbit anti-M13 serum (1:10,000). Next, wells were washed and incubated with HRP-conjugated goat anti-rabbit antibody (1:5000, Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). Following an additional round of washing, wells were reacted with the TMB/E ELISA substrate (Merck Millipore; ES001, USA). Absorbance was measured at

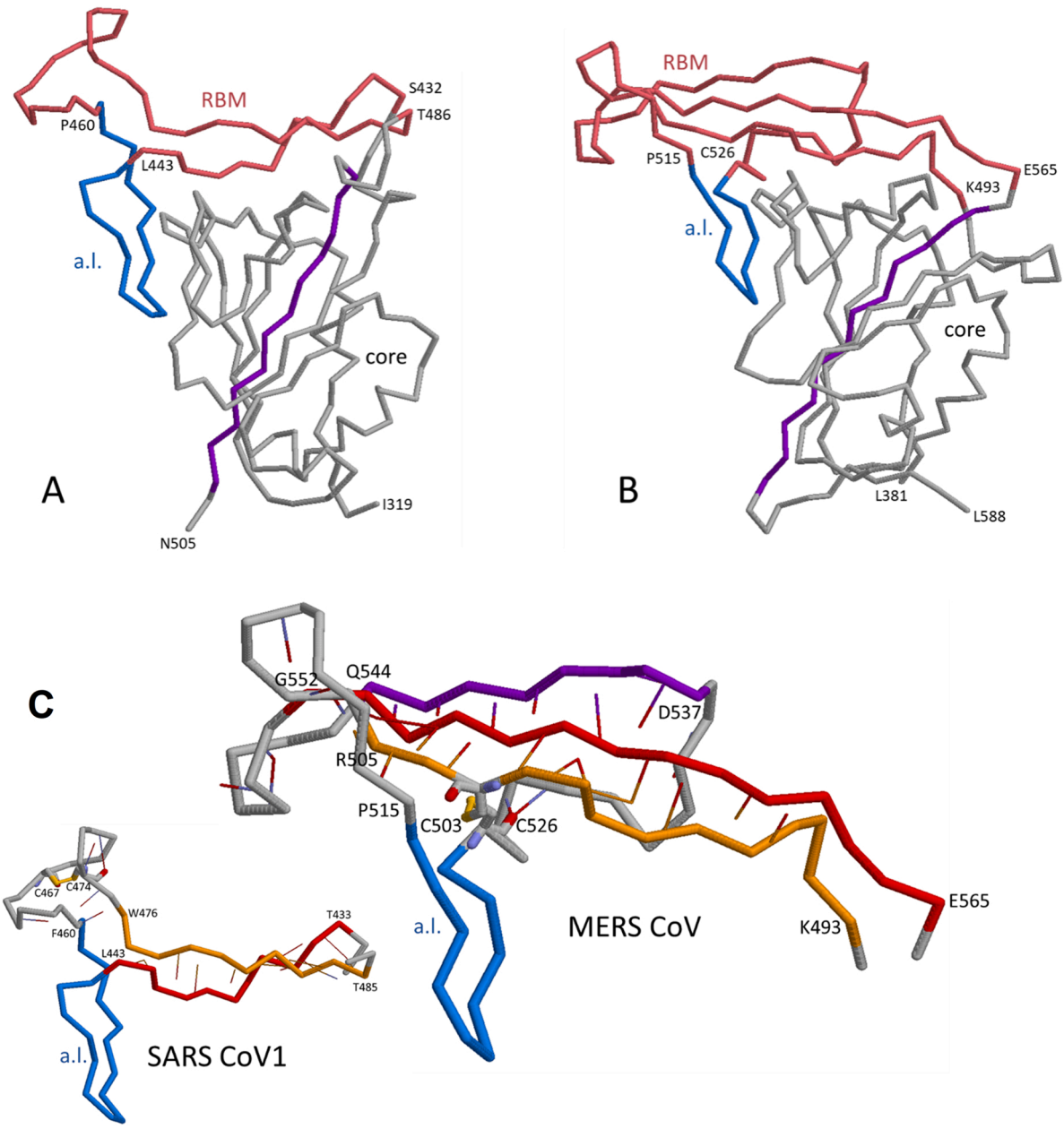


Fig. 1. Receptor Binding Domains (RBDs) of SARS CoV and MERS CoV.Backbone representations of the RBDs of SARS CoV (A, PDBID:2GHV) and MERS CoV (B, PDBID:4KQZ) are shown. Both structures contain a central core harboring 5 beta-strands. An excursion leaves the core (pink), from beta-strand 4, forming an extended surface that contacts the virus receptor and is the target of neutralizing mAbs thus forming the RBM as has been confirmed for SARS CoV (Li et al., 2005) and MERS CoV (Lu et al., 2013). The fifth core beta strand (purple), serves as a lynch-pin that stabilizes the structure. The RBM excursion is held in place via an "anchor loop" (a.l., blue) that forms hydrogen bonds with the RBD core. In C more detailed comparison of the two RBMs is given. Here the markedly more complex MERS CoV RBM is illustrated showing an additional beta strand (purple) complicates the folding and conformation compared to the simple RBM of SARS CoV that contains only two beta strands (red and orange) also shared in the RBM of MERS CoV. Note also the position of the C503-C526 disulphide at the rim of the anchor loop of the MERS CoV RBM.

650 nm using a micro-plate reader (Bio Tek, Winooski, VT, USA). All samples were tested in duplicate and experiments were repeated at least three times.

2.10.2. Measure of antibody binding to MBP and GST constructs

The MBP and GST fusion proteins displaying the RBM constructs were used to coat wells overnight (10 μg/ml in TBS), washed with TBS, blocked with 5% skim milk in TBS and incubated with 2 μg/ml neutralizing mAbs, overnight at 4 ^{o}C. The secondary antibodies (HRP conjugates) corresponding to each of the neutralizing antibodies were added 1:5000 in skim milk and incubated for 45 min at room temperature (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). Following an additional round of washing, wells were reacted with the TMB/E ELISA substrate (Merck Millipore; ES001, USA). Absorbance was measured at 650 nm using a micro-plate reader (Bio Tek, Winooski, VT, USA). All samples were tested in duplicate and experiments were repeated at least three times.

2.10.3. ELISA tests of rabbit sera

The sera samples were analyzed first against phage displayed immunogens (E5, E9) and control phage (fth1) directly used to coat the ELISA wells and followed the general protocol above probing with

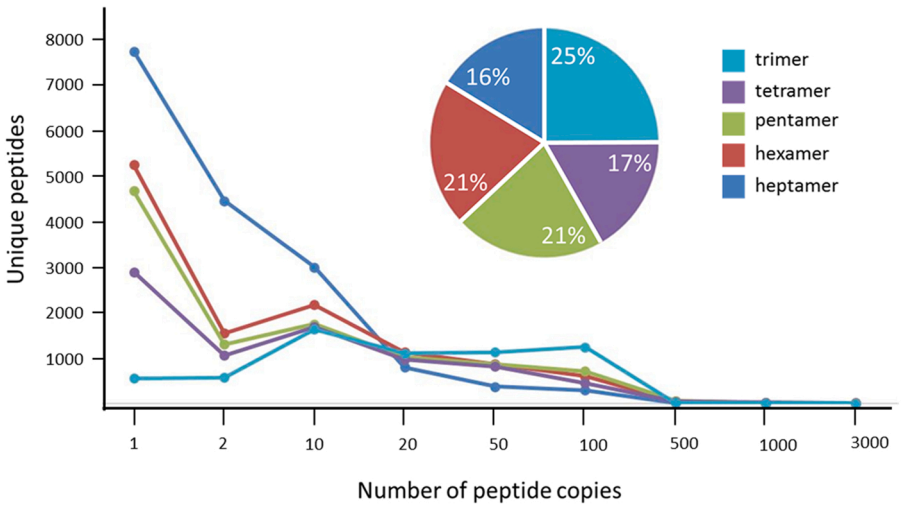


Fig. 2. NGS analysis of the MERS CoV conformer library. The phage-displayed conformer library constructed for the MERS CoV was analyzed by NGS. The "anchor loop" was replaced by combinatorial linkers ranging from 3–7 amino acids in length. The pie-chart shows the distribution of peptide-linkers of different lengths. The graph gives the number of unique peptide-linkers per million reads that are found once, twice or 10–3000 times for each linker length. Thus, for example, close to 8000 different heptamer linkers appear once and close to 2000 different trimer linkers are detected 3–10 times.

serum samples diluted 1:10,000 in milk. Subsequently, the reaction against recombinant MERS CoV spike S1 protein antigen was tested, using the commercial anti-MERS CoV ELISA (IgG) kit, (EI 2604–9601 G, EUROIMMUN Medizinische Labordiagnostika AG, Lübeck). Wells were washed with TBST and incubated with the polyclonal rabbit pre-immune or immune sera (as per kit instructions) followed by washes and incubation with secondary antibody HRP-conjugate as described above.

3. Results and Discussion

The 2002 SARS CoV Receptor Binding Domain (RBD, 193 amino acids) was functionally identified two years after the death of Patient Zero (Li et al., 2003). The atomic structure of the RBD was solved by X-ray diffraction analysis of its co-crystal with the corresponding viral receptor, angiotensin converting enzyme 2 (ACE2) (Li et al., 2005) and then with the neutralizing mAb 80 R (Sui et al., 2004). These co-crystals revealed a Receptor Binding Motif (RBM, residues S432-T486); an extended excursion that runs along the surface of the central core of the RBD and presents the active surface that directly contacts the receptor as well as the competing neutralizing mAb (Fig. 1 A). We were able to reconstitute an isolated RBM of SARS CoV (42 amino acids) that binds both the neutralizing mAb and the receptor (Freund et al., 2015).

With the emergence of MERS CoV in 2012, its RBD was co-crystallized along with its corresponding receptor, human dipeptidyl peptidase 4 (huDPP4) (Lu et al., 2013), revealing a conformational layout which generally follows the structure elucidated previously for SARS CoV. The MERS CoV RBD contains a central core stabilized with 5 beta strands and an excursion, stemming from the fourth beta strand, that runs along the surface of the core and returns, inserting the fifth

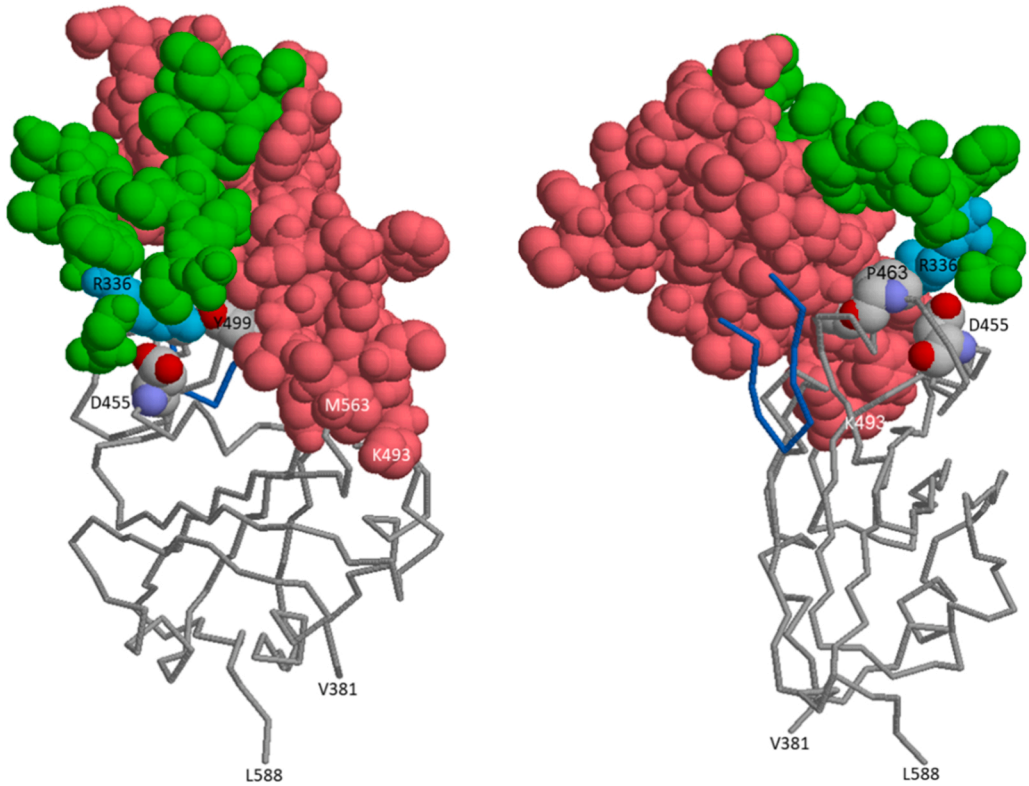


Fig. 3. RBD contact-residues with residue R336 of huDPP4. Two views of the MERS CoV RBD showing a backbone presentation of the core and space-fill (pink) of the RBM (the blue anchor loop is shown as backbone). The bound segments of huDPP4 are shown in green. Residue R336 of huDPP4 is shown in cyan. Y499 of the RBM contacts R336, however two critical residues, P463 and D455 of the core (shown in space-fill, colored CPK) contact R336 and are essential for huDPP4 binding to the MERS CoV RBD.

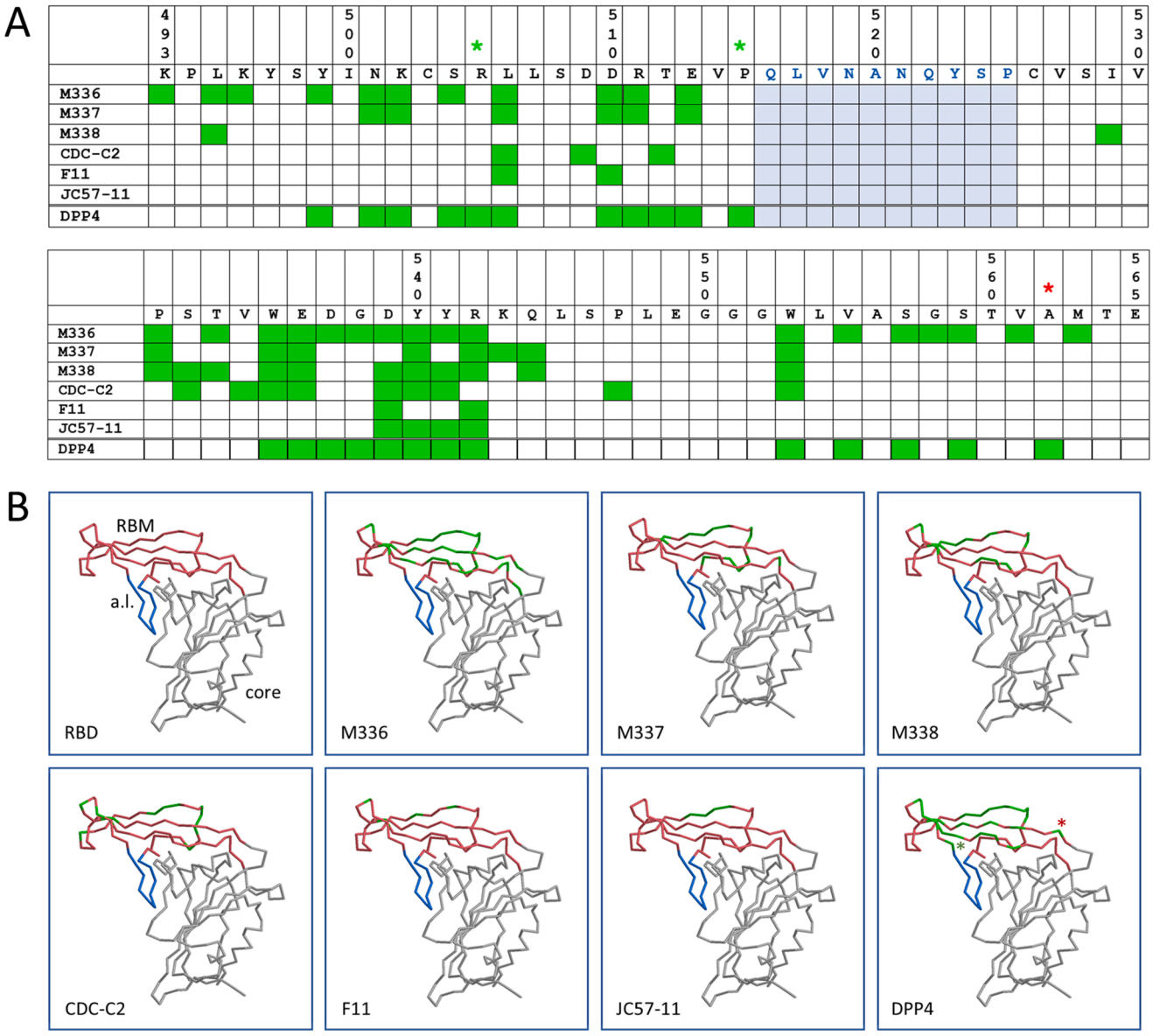


Fig. 4. Contact residues used by MERS CoV neutralizing mAbs. A) The contact residues given in Fig. 3 are shown in backbone depictions of the MERS CoV RBD. The RBM is colored pink, the anchor loop blue. The residues that contact huDPP4 are green. The exclusive contacts of the RBM that contact huDPP4 (right-bottom) are shown with asterisks (see also Fig. 3). B) The MERS CoV RBM extends from K493 through E565 (73 amino acids) and contains an "anchor loop" (from Q516 through P525, shaded light-blue). Six neutralizing mAbs have been co-crystallized with MERS CoV RBD, and their atomic structures have been solved (Lingshu et al., 2021; Xu et al., 2019; Ying et al., 2015; Zhang et al., 2018). As is illustrated, each mAb uses an array of contacts to bind the virus RBM, none of which are in the "anchor loop". For comparison, the contacts with the MERS CoV receptor, huDPP4, are given. Note that three huDPP4 contact residues (R505, P515 – green asterisks, and A562 – red asterisk) do not directly contribute to mAb recognition.

beta strand that serves as a lynch-pin stabilizing the compact RBD structure (Fig. 1B).

Examination of the MERS-CoV RBD co-crystals with its receptor or various neutralizing mAbs (Ying et al., 2014; Zhang et al., 2018) confirms the RBM which, however, is markedly larger and more complex compared to that of SARS CoV. The MERS CoV RBM (residues K493-E565) consists of 73 amino acids and contains three formidable beta-strands in addition to an "anchor loop" (residues P516-C525, Fig. 1 C). Although, reconstitution of the MERS CoV RBM followed the same general scheme previously used for SARS CoV, because of its greater size and complexity, it was not obvious that reconstitution would be successful. Ultimately, reconstitution of the MERS CoV RBM entailed:

- Production of a comprehensive combinatorial phage-display conformer library expressing the RBM excursion in which the "anchor loop" was replaced with a series of combinatorial linkers (3–7 random amino acids, see Fig. 1B). It was necessary to construct the library as Protein 3 fusions (compared to Protein 8 for the expression of the SARS CoV RBM). Moreover, residue C526 was retained as part of the RBM so to ensure the disulphide with C503 that stabilized the RBM.
- Affinity-selection of functional conformers by screening the phage library against a panel of 7 neutralizing mAbs.

3.1. Construction of the conformer combinatorial linker library

The RBM oligonucleotide sequence (corresponding to residues K493-

Table. 2
NGS analysis of 25 RBM clones. The MERS CoV conformer library was screened with 7 mAbs and the affinity-selected phages were sent to NGS analysis. The sequences for the various linkers used to replace the "anchor loop" were determined and are ranked for the SUM of reads scored for all seven mAbs. Also shown is the number of mAbs (#mAbs) for which reads were detected. Linkers for which a "Name" is provided (highlighted rows) are those that were also manually picked and confirmed as binders as is illustrated (see text). Note, the 5 linkers that were further characterized in Fig. 7 are given in bold.

#	Name	Linkers	m-336	m-337	m-338	C-12	F-11	JC57-11	CDC-C2	mAbs	SUM
1	D8	LALSA	828,420	936,441	705,321	236,722	787,062	881,111	875,784	7	5250,861
2	A11	QPPTE	6421	8833	15,310	35,710	93,455	33,310	25,934	7	218,973
3	E5	LHPDHPD	19,234	12,271	33,687	2428	5061	20,025	10,440	7	103,146
4		LRT	22,934	889	4707	48,882	8058	1730	15,589	7	102,789
5		NRG	13,415	–	4600	34,760	36	1111	900	6	54,822
6	BC10	MAPQT	2619	347	2729	30,823	3827	1958	2090	7	44,393
7	G3	LSKEY	3327	213	13,942	1656	1266	2051	184	7	22,639
8	BA5	LPRKE	10,266	1330	1758	4470	5	1	3165	7	20,995
9	ED2	YDLFVKE	2331	96	335	162	13,174	4659	177	7	20,934
10	BB2	LNHAE	2690	94	12,733	3293	42	108	224	7	19,184
11		LSRDS	1061	185	12,009	3387	–	967	0	5	17,609
12		LEMHE	1247	–	8402	1960	230	2311	1	6	14,151
13		MNRLSDA	461	–	12,068	–	–	1	0	3	12,530
14	EC2	LPPEE	380	305	4862	278	4668	1224	728	7	12,445
15	AA5	VPVHR	2074	484	4387	101	4926	125	170	7	12,267
16		LSAHSPE	183	–	11,781	–	–	–	90	3	12,054
17		SNQ	–	90	238	–	–	10,648	941	4	11,917
18	BC3	LPRHN	3290	1588	2979	63	–	1916	540	6	10,376
19	EC6	YSVPVST	616	–	417	5113	2712	–	213	5	9071
20		ETIPSYN	3999	163	366	1457	19	235	2512	7	8751
21		MFPTDPK	129	–	7882	–	–	–	0	2	8011
22	BB6	LSPTE	2503	202	3360	–	385	2	840	6	7292
23	BA3	LSNEA	1412	4	4463	2	–	574	0	5	6455
24		LSATNSA	53	–	6118	–	–	–	0	2	6171
25	E9	LPLQA	133	267	321	14	36	302	2	7	1075

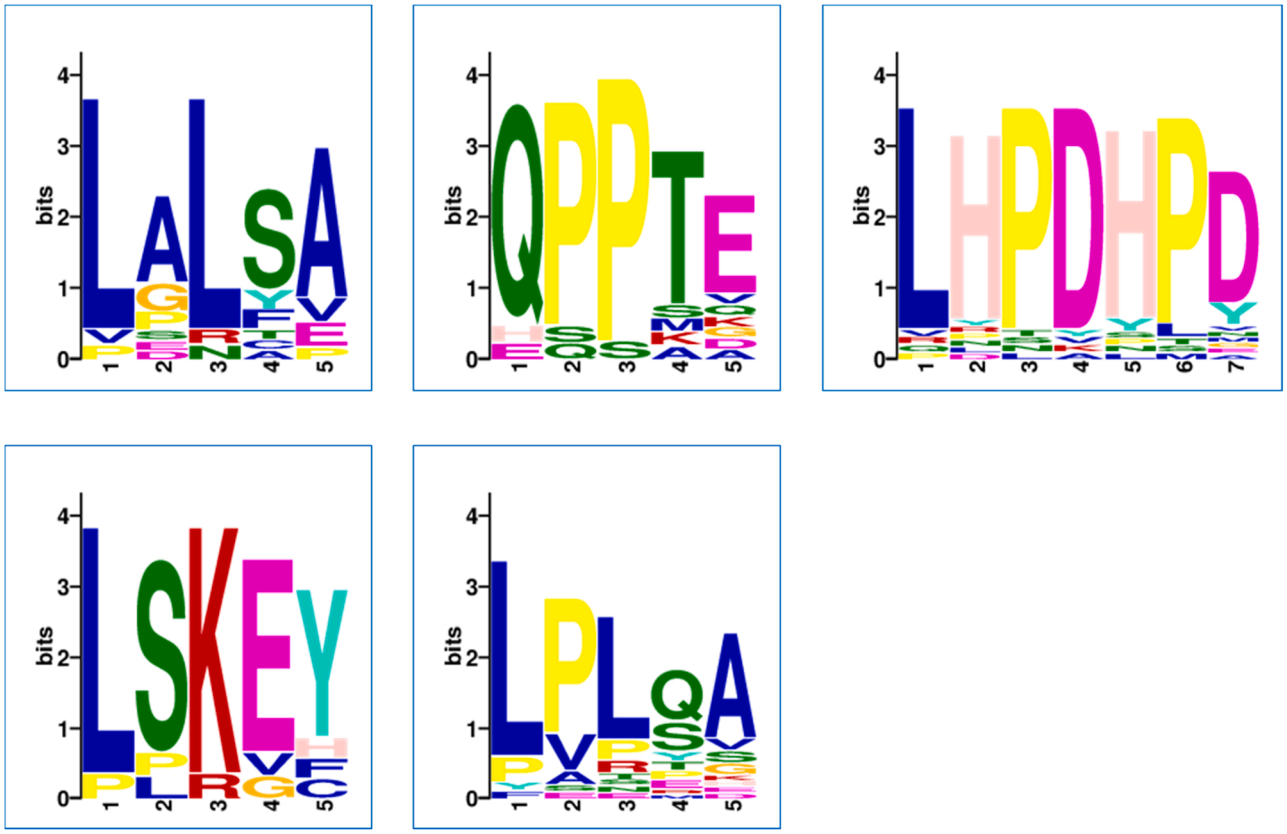


Fig. 5. Assignment of MEME motifs to cross reactive linkers. Over 1000 clones from the screening of the conformer library were manually picked and tested for each of the 7 neutralizing mAbs used in this study. The following linkers supported binding of at least two different mAbs: LALSA, QPPTE, LSKEY, LPLQA, and LHPDHPD. Furthermore, affinity-selected peptides from these screens were sent to NGS analysis. 1415 pentameric linkers and 242 heptameric linkers were found to have more than 1000 copies and to cross-react with at least two mAbs. Using the MEME algorithm (REF), 17, 15, 10, 28 and 33 linkers could be assigned to the LALSA, QPPTE, LSKEY, LPLQA, and LHPDHPD linkers, respectively. The motifs defined by MEME using the 1657 peptides (pentamers + heptamers) correspond well with the 5 linkers obtained manually.

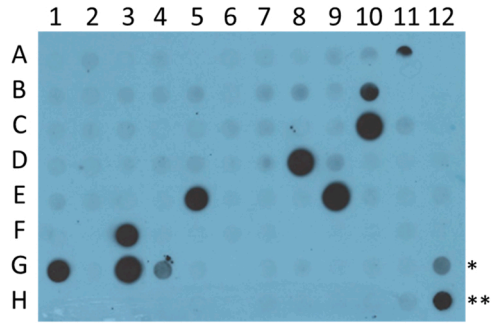


Fig. 6. Dot-blot detection of MERS CoV RBM clones recognized by mAb m336. For each of the different mAbs, 1000 phage-displayed conformer clones were manually picked and tested for mAb binding by dot-blots on nitrocellulose membrane filters. An example of such an analysis for mAb m336 is shown illustrating the detection of 9 positive clones (for details see Methods). The fth1 wild type phage (*) shows the level of background binding while a "dot" of m336 mAb is given as a positive control (**).

E565, 73 amino acids) was cloned into the phage *protein 3* gene in two segments, A: K493-P515 and B: C526-E565, connected via a series of 5 combinatorial linkers ranging from 3 to 7 amino acids in length (see Materials and Methods). A sample of the conformer library was analyzed by Next Generation Sequencing (NGS) and is summarized in Fig. 2, confirming that linkers of all sizes are well represented. Moreover, for linkers of 4 residues and more, most of the linkers were unique, i.e., appeared once in the sequenced sample. The total theoretical complexity of 3 residue-linkers is only 8000 (20^3) and consequently most trimer linkers appear as multiple copies.

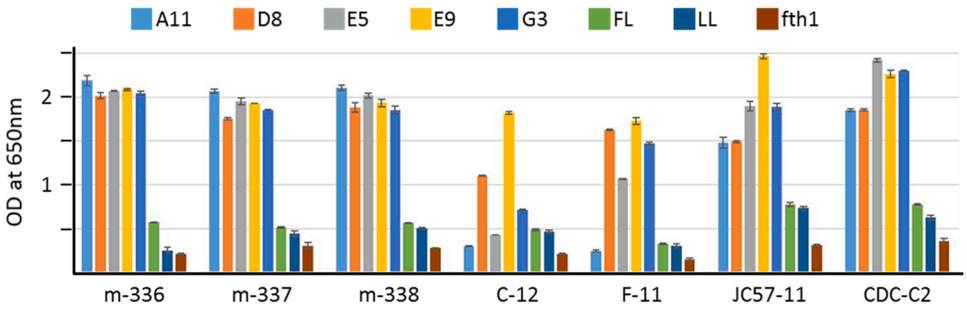
Initially, we screened the conformer library against the MERS CoV receptor protein, huDPP4, but without success, no constructs were affinity selected. Detailed examination of the MERS CoV RBD complexed with huDPP4, reveals that a critical residue of the receptor, residue R336, makes contacts with the RBM residue Y499. However, additional essential residues of the RBD, residues L450-Q466, are situated in the core of the RBD, and thus are not included in the RBM constructs in our library. These residues have been demonstrated to be especially important for receptor binding (see Fig. 3). Moreover, the receptor residue, R336, has been determined to be indispensable for RBD binding (Peck et al., 2017; Song et al., 2014; van Doremalen et al., 2016; Wang et al., 2013). Contacts of R336 with the two RBD residues, D455 and P463, are of particular importance (van Doremalen et al., 2014). As demonstrated, mutagenesis of anyone of these three residues (D455 and P463 of the RBD or R336 of huDPP4) knocks out receptor binding completely (Lu et al., 2013). The fact that the two critical RBD amino acid residues (D455 and P465) are excluded from our RBM library would explain the lack of success in isolating receptor-binding RBM constructs.

3.2. *Screening the library and affinity selection of reconstituted conformers*

We proceeded to reconstitute the RBM by screening the library against neutralizing mAbs. A panel of 7 neutralizing mAbs was collected which bind the MERS CoV RBM through a variety of contact residues. The rationale being that a functionally folded RBM that assumes the correct physiological conformation, should be able to be recognized by at least two different conformation-sensitive neutralizing mAbs (Gorny et al., 2002).

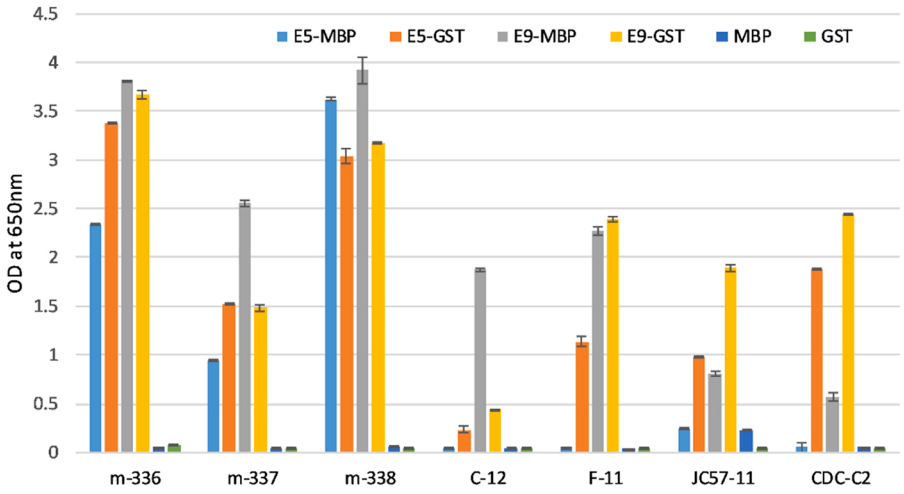
Fig. 4 A shows the distribution of contact residues on either side of the anchor loop for each of the six mAbs for which co-crystals were available and for comparison the contacts for huDPP4. The mAbs differ from each other in their requirement of contact residues within the RBM. Thus, for example, mAb m336 makes 28 contacts with the RBM distributed on either side of the "anchor loop", while m338 and CDC-C2 make a majority of contacts only downstream to the "anchor loop". Hence, each mAb presents a different set of conformational requirements for RBM binding (further illustrated in Fig. 4B). Consequently, a construct that cross-reacts with multiple mAbs should be viewed as satisfying a broad set of conformational constraints that are important for antibody recognition. Each of the 7 mAbs was used to screen the conformer library in triplicate and the enriched phages were sent to NGS. As is illustrated in Table 2, numerous linkers were found for RBM constructs that were affinity enriched by all 7 mAbs. Although some short, 3 amino acid and 4 amino acid linkers were selected; the majority of effective linkers were 5 and 7 residues long. Considering variations in linker compositions one can easily assign many of the linkers to the 5 motifs shown in Fig. 5. Note that a Leucine residue is dominant for the first position in many linkers as is a Leucine residue in the third position, Proline is prevalent in positions two and three.

In order to confirm that the enriched linker-sequences actually produce functional reconstituted RBMs that physically bind the mAbs, we manually screened the library against the mAbs, picking over 1000 clones for each and testing mAb binding by dot blot overlay as is illustrated in Fig. 6. The linkers of the positive clones were determined and found to correspond to the most enriched linkers seen previously *in silico* by the NGS analyses, thus confirming their ability to actually support conformations recognized by the panel of neutralizing antibodies (see Table 2). Fig. 7 shows the binding of five selected clones to all 7 mAbs.



clone	Linker	m-336	m-337	m-338	C-12	F-11	JC57-11	CDC-C2
A11	QPPTE	+++	+++	+++	–	–	++	+++
E9	LPLQA	+++	+++	+++	+++	+++	+++	+++
D8	LALSA	+++	+++	+++	–	++	++	+++
G3	LSKEY	+++	+++	+++	+	+++	++	+++
E5	LHPDHPD	+++	+++	+++	+	+++	+++	+++

Fig. 7. Reconstituted MERS CoV RBMs bind neutralizing mAbs. Seven neutralizing mAbs, used in this study, show different levels of cross-reactive binding to five reconstituted RBMs, as measured by ELISA. No substantial binding is found for the "Full Length" RBM (residues K493-E65, **FL**) or the "Loopless" (**LL**) form of the RBM in which the "anchor loop" (Q516-P525) has been deleted. The linkers that connect residues 515–526 are given in Table 2 along with their relative binding to the different mAbs. All the constructs were phage-displayed; fth1 wild type phage provides a negative control baseline. The values shown are the average of three independent experiments and their standard errors.



Construct	Linker	m-336	m-337	m-338	C-12	F-11	JC57-11	CDC-C2
E5-MBP	LHPDHPD	++	+	+++	-	-	-	-
E5-GST	LHPDHPD	+++	+	+++	-	+	+	++
E9-MBP	LPLQA	+++	+++	+++	++	++	+	-
E9-GST	LPLQA	+++	+	+++	-	++	++	+++

Fig. 8. Reconstituted MERS CoV RBM fusion proteins bind neutralizing mAbs. Seven neutralizing mAbs, used in this study, show different levels of cross-reactive binding to the most cross-reactive reconstituted RBMs (E5 and E9), as measured by ELISA. The two constructs were conjugated with MBP and GST; unconjugated MBP and GST provide negative controls to establish baseline binding. The values shown are the average of three independent experiments and their standard errors.

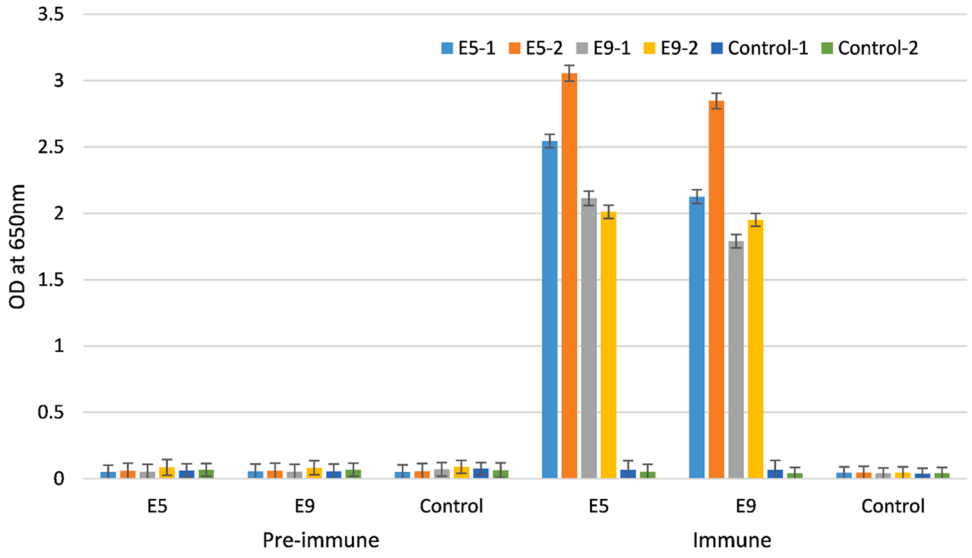


Fig. 9. Immunogenicity of Reconstituted MERS CoV RBMs. Sera samples of two rabbits immunized with E5-MBP or E9-MBP show different levels of cross-reactive binding to the phage displayed E5 and E9 reconstituted MERS-CoV RBM, as measured by ELISA. Pre-immune sera and control sera (derived from animals immunized with scaffolds alone) provide negative control scores. The values shown are the average of three independent experiments and their standard errors.

Two clones in particular, clones E5 and E9, were found to be broadly cross-reactive. These two conformers were then expressed as fusion proteins of maltose binding protein (MBP) and glutathione-S-transferase (GST), thus testing their binding capacity as monovalent epitopes as compared to their polyvalent presentations (5 Protein-3 copies per phage) in the context of the phages. Fig. 8 depicts the binding of the four constructs against all 7 mAbs. Generally, the MBP construct of the E9 conformer showed the highest cross reactive mAb binding. GST also supported functional representation of the two conformers.

3.3. Testing the immunogenicity of the constructs

The next question we addressed is whether or not the MBP or GST E5 and E9 constructs were immunogenic. For this, rabbits were immunized with E5 and E9 MBP constructs and then followed with boosts alternating between MBP or GST constructs. Control rabbits were immunized and boosted with the MBP/GST scaffolds alone. Pre-immune and final immune sera were collected and tested for their ability to specifically bind the E5 and E9 constructs expressed on phages. As is illustrated in Fig. 9, the E5 and E9 immunized rabbits mounted strong antibody responses to the reconstituted RBMs as compared to negligible background by sera of the control animals. We then tested whether or not the RBM specific antibodies cross-reacted with genuine MERS CoV spike protein. For this the sera were tested using the commercial Euroimmun Anti-MERS CoV ELISA (IgG) kit in which recombinant MERS CoV spike protein is provided as the reference antigen. As is demonstrated in

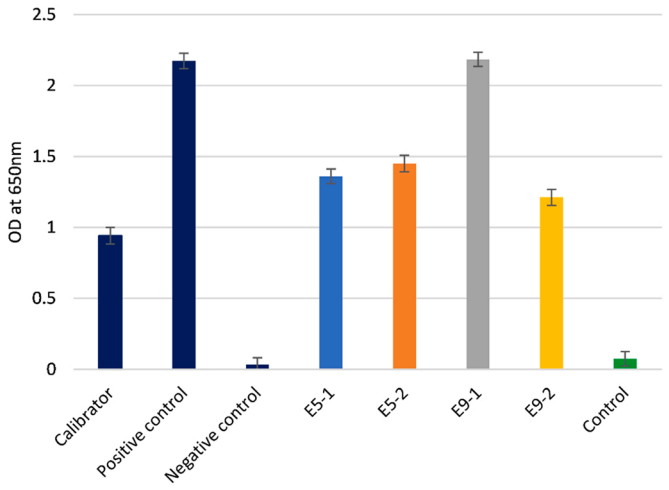


Fig. 10. Cross-reactive binding of sera to recombinant spike S1 MERS CoV antigen. Sera samples of two rabbits immunized with E5-MBP or E9-MBP show cross-reactive binding to the recombinant spike S1 MERS CoV antigen as compared to a Control rabbit (immunized with scaffolds alone). The EUROIMMUN kit used provides three control wells (dark blue): a "Calibrator", Positive and Negative controls. The Calibrator represents the upper limit of what could be a negative response. Thus, values above the Calibrator represent bona fide positive binding while below the Calibrator should be regarded as negative. The level of serum binding and cross-reactivity is higher than the binding to the Calibrator. Error bars are shown.

Fig. 10, both the E5 and E9 constructs elicited strong cross-reactive spike specific antibodies.

The coronavirus receptor binding domain (RBD, ca. 200 amino acids) represents only 18% of the viral spike protein, yet it is the critical component that defines the viral tropism. Further examination of the receptor:RBD interactions has led to the identification of the RBM, an excursion of some 50–70 amino acids that provide a series of contacts with the receptor. The RBM is held in place via a set of hydrogen bonds formed between the core of the RBD and an "anchor loop" that projects from the receptor binding surface. Previously, we discovered that the replacement of the "anchor loop" of the SARS-CoV, with a series of combinatorial linkers allows the expression and selection of functional reconstituted RBMs that bind a SARS-specific neutralizing antibody as well as ACE2. Thus, the RBM represents a target immunogen, a neutralizing surface for the production of an epitope-based vaccine (Gershoni, 2019; Gershoni et al., 2007).

Here we describe the implementation of the lessons learned from SARS CoV to the reconstitution of the MERS CoV RBM. Although the general structural hallmarks of the RBD defined for SARS exist for MERS as well, the reconstitution of the RBM of MERS CoV is markedly more challenging. The MERS CoV RBM binds a totally different receptor and its conformation is structurally more complex as the receptor binding surface is comprised of three anti-parallel beta-strands and is markedly larger than that found for SARS CoV (55 amino acids vs 73 amino acids). Reconstitution required the replacement of the "anchor loop" with a comprehensive library of combinatorial linkers which allowed the selection of reconstituted RBMs that cross-reacted with a panel of 7 neutralizing mAbs. Two of these conformers, E5 and E9, have been demonstrated to continue to present a functional cross-reactive RBM as MBP and GST fusion proteins. Moreover, these two modalities were demonstrated to be immunogenic in rabbits and able to elicit the production of RBM specific antibodies that cross-react with genuine MERS CoV spike protein. The E5 and E9 reconstituted RBMs are now being further developed as epitope-based vaccines. For this, multimeric polyvalent constructs (Correia et al., 2014; He and Zhu, 2015; Kulp and Schief, 2013; Marcandalli et al., 2019; Sesterhenn et al., 2020) and alternative scaffolds (Morris et al., 2017; Negahdaripour et al., 2017; Saylor et al., 2020) are being considered.

4. Conclusion

The need to produce a vaccine modality for MERS CoV is relevant in view of the extremely high fatality rate for MERS CoV disease. The very low global prevalence of MERS CoV is not a reason to dismiss this concern. The annual cases of MERS CoV combined with an ever-increasing rise in SARS CoV2 infections in the Middle East, taken together with the high prevalence of MERS CoV in camel populations, could lead to genetic recombination of these two beta-coronaviruses. The consequence of such an event would be devastating. Hence, we believe that the pursuit of the MERS CoV RBM-based immunogen described in this study, could provide a preemptive answer should such a development occur.

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CRediT authorship contribution statement

J.M.G. developed the concept and design of Coronavirus RBM reconstitution and L.U. designed and executed the MERS CoV RBM reconstitution experiments, D.S.D. and T.Y. provided neutralizing MERS CoV mAbs and discussion of the RBM reconstitution for MERS CoV, A.R-B. and Y.W-O. assisted throughout the project and the writing of the manuscript. All authors commented on the manuscript at all stages.

Data and materials availability

All data is available in the manuscript.

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Competing interests

There are no conflicting interests for any of the Authors.

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