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Receptor-binding domain-based subunit vaccines against MERS-CoV

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ABSTRACT

Development of effective vaccines, in particular, subunit-based vaccines, against emerging Middle East respiratory syndrome (MERS) caused by the MERS coronavirus (MERS-CoV) will provide the safest means of preventing the continuous spread of MERS in humans and camels. This review briefly describes the structure of the MERS-CoV spike (S) protein and its receptor-binding domain (RBD), discusses the current status of MERS vaccine development and illustrates the strategies used to develop RBD-based subunit vaccines against MERS. It also summarizes currently available animal models for MERS-CoV and proposes a future direction for MERS vaccines. Taken together, this review will assist researchers working to develop effective and safe subunit vaccines against MERS-CoV and any other emerging coronaviruses that might cause future pandemics.

1. Introduction

Middle East respiratory syndrome (MERS) is a newly emerged infectious disease caused by a novel β-coronavirus (β-CoV), MERS coronavirus (MERS-CoV). MERS-CoV was first identified in humans in Saudi Arabia on June 12, 2012 (Zaki et al., 2012). Although the spread of MERS-CoV among humans has been limited, MERS-CoV infection has been linked to several family clusters and healthcare workers (Assiri et al., 2013; Memish et al., 2013b, 2013c), providing evidence for human-to-human transmissibility of the virus. MERS-CoV has demonstrated an increasing trend of infection since its first identification. Sporadic MERS cases have been reported in at least 21 countries in the Middle East, Africa, Europe, Asia and North America. As of November 7, 2014, a total of 909 laboratory-confirmed cases including 331 deaths (mortality rate ~36%) were reported (http://www.who.int/csr/don/07-november-2014-mers/en/). The geographic spread and rapid increase of MERS cases during the past several months have raised concerns of pandemic potential, even though the consequences of such pandemic might be less severe than those caused by severe acute respiratory syndrome coronavirus (SARS-CoV), another β-CoV which led to a worldwide outbreak in 2003 (Peiris et al., 2003). As a practical control strategy against the potential outbreak of MERS-CoV-caused emerging infectious disease, development of effective vaccines has become a high priority.

A number of studies have pointed out the transmission hosts for MERS-CoV. It is reported that bat-derived coronaviruses, particularly bat coronavirus HKU4, have close phylogenetic relationship with MERS-CoV and that dipeptidyl peptidase 4 (DPP4), the receptor for MERS-CoV, is also the receptor for HKU4. While HKU4 prefers bat DPP4 over human DPP4, MERS-CoV has adapted to use human DPP4, in addition to bind bat DPP4, suggesting that (1) bats are potential natural reservoirs for MERS-CoV; and (2) bat coronaviruses remain a threat to human health because of their potential for cross-species transmission (Annan et al., 2013; Cui et al., 2013; Ithete et al., 2013; Memish et al., 2013a; Yang et al., 2014). In addition to bats, camels have recently become a focus for the study of MERS-CoV transmission since MERS-CoV neutralizing antibodies and MERS-CoV gene fragments have been identified in dromedary camels, and infectious MERS-CoV has been recovered from infected camels (Briese et al., 2014; Chu et al., 2014; Drosten et al., 2014; Haagmans et al., 2014; Meyer et al., 2014; Nowotny and Kolodziejek, 2014; Reusken et al., 2013). The fact that humans were infected with MERS-CoV after exposure to infected camels suggests that camels are the most likely intermediate transmission hosts of MERS-CoV (Memish et al., 2014). Most recently, MERS-CoV RNA fragments were

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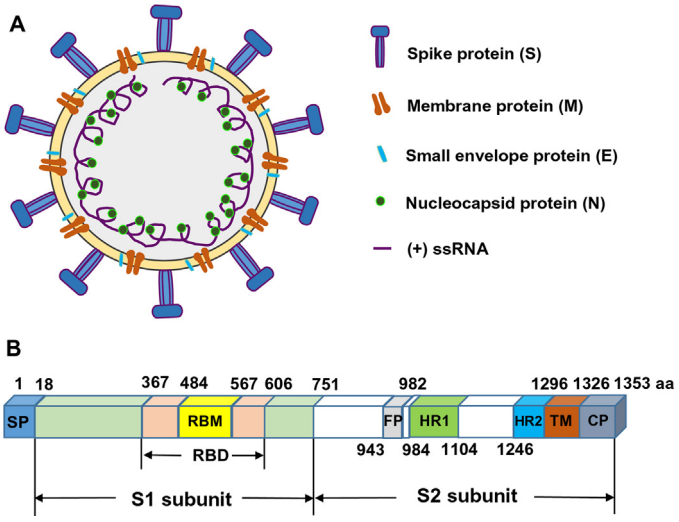

Fig. 1. Schematic structures of MERS-CoV and its spike protein RBD. (A) Schematic structure of MERS-CoV. MERS-CoV contains a positive, single-stranded RNA and four structural proteins, including S, M, E and N. (B) Spike protein of MERS-CoV and its RBD. MERS-CoV S protein contains S1 and S2 subunits, and their functional regions with specific amino acid residues are shown. SP, signal peptide. RBD, receptor-binding domain. RBM, receptor-binding motif within RBD. FP, fusion peptide. HR1 and HR2, heptad repeats 1 and 2. TM, transmembrane domain. CP, cytoplasmic tail.

detected in an air sample collected from the barn that sheltered MERS-CoV-infected camels (Azhar et al., 2014), indicating possible airborne transmission of MERS-CoV between animals and humans. Although the spread of MERS-CoV among humans is limited and inefficient (Drosten et al., 2014), the increased infection rate among healthcare workers during the month of April 2014 (http://www.who.int/csr/disease/coronavirus_infections/archive_updates/en/) has raised concerns of future epidemic potential, which call for the development of effective and safe vaccines to prevent and control MERS (Hotez et al., 2014).

2. MERS-CoV and its spike protein receptor-binding domain

MERS-CoV belongs to lineage C of β-CoV with a close relationship to the bat coronaviruses HKU4 and HKU5 and is the first known lineage C β-CoV associated with human infections (Chan et al., 2013b; Woo et al., 2012). MERS-CoV is a positive-sense, single-stranded RNA virus whose genome encodes four major structural proteins, including spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N), each with unique functions (Fig. 1A). The E protein is a transmembrane protein which forms an ion channel on the viral surface, while the N protein interactively functions with the M protein and other N molecules encapsulating genomic RNA (Hurst et al., 2010).

Among the four structural proteins of MERS-CoV, the S protein plays the most important roles in virus infection and pathogenesis. It displays as a trimer on the viral membrane surface. The precursor S protein is cleaved into two noncovalently associated subunits: the distal subunit S1 and the membrane-anchored subunit S2. The MERS-CoV S1 subunit contains the receptor-binding domain (RBD), including a core structure and an accessory subdomain receptor-binding motif (RBM), while the S2 subunit consists of a putative fusion peptide, transmembrane domain and two heptad repeat regions, termed heptad repeats 1 and 2 (HR1 and HR2) (Fig. 1B) (Chen et al., 2013b; Lu et al., 2013a; Wang et al., 2013).

Although MERS-CoV and SARS-CoV share similar core structures to maintain conformational integrity, their RBMs are different (Chen et al., 2013b; Li et al., 2005). As such, the receptors recognized by both coronaviruses are distinctively different. Angiotensin-converting enzyme 2 (ACE2) is a functional receptor for SARS-CoV, while DPP4 (also known as CD26) is an identified receptor for MERS-CoV (Li et al., 2003; Raj et al., 2013). The crystal structures of SARS-CoV RBD complexed with its receptor ACE2 have identified the RBD of SARS-CoV as residues 306–527 and the RBM as residues 424–494 (Li et al., 2005). Crystal structure analysis of MERS-CoV RBD alone or RBD/DPP4 complex has mapped the RBD to residues 367–588 and 367–606, respectively, in the S protein of MERS-CoV, with the RBM spanning residues 484–567 (Chen et al., 2013b; Lu et al., 2013a; Wang et al., 2013). Fig. 2 lists the crystal structures of SARS-CoV and MERS-CoV RBDs and their complexes with respective receptors.

MERS-CoV undergoes two major processes to infect target cells. First, the virus binds to the cellular receptor DPP4 via the RBD in the S1 subunit. Second, the HR1/HR2 complex in the S2 subunit forms a fusion core, leading to cell-virus membrane fusion, thereby mediating MERS-CoV entry into the target cells (Gao et al., 2013; Lu et al., 2014). Therefore, like SARS-CoV S protein, the S protein of MERS-CoV plays an essential role in receptor binding, membrane fusion and cell entry. We have previously demonstrated that the S protein, or, more specifically, the RBD, of SARS-CoV played an essential role in developing SARS vaccines (Du et al., 2009a; Jiang et al., 2012b). It is thus expected that the RBD of MERS-CoV S protein will be an important target for developing vaccines against MERS (Lu et al., 2013b; Ma et al., 2014b).

3. Current status in developing MERS vaccines

Antibodies induced by SARS-CoV RBD do not cross-neutralize MERS-CoV infection since different receptors are recognized by the two coronaviruses, suggesting that developing a safe and effective MERS vaccine is a high priority (Du et al., 2013b, 2013c). Since MERS is a newly-emerged viral disease, no vaccines have been developed for clinical use. However, a number of vaccine candidates have been tested and/or proven effective in *in vitro* preclinical studies. Current updates on MERS vaccine development, including the possibility for developing certain vaccine types as candidates against MERS, are discussed below.

It was revealed that a recombinant modified vaccinia virus Ankara (MVA) expressing the full-length MERS-CoV S protein, MVA-MERS-S, produced neutralizing antibodies in immunized mice against infections from MERS-CoV in cell cultures *in vitro* (Song et al., 2013), providing a basis for developing viral vector-based MERS vaccines. In addition, full-length infectious cDNA clones of MERS-CoV have been constructed using reverse genetics systems, and relevant infectious viruses could be rescued and propagated in Vero A66 and Huh-7 (human liver) cells (Almazán et al., 2013; Scobey et al., 2013). Reports have also shown that a full-genome sequence of MERS-CoV (Jordan-N3/2012 strain) exhibited stability after sequential passages in two mammalian cell lines: Vero (African green monkey kidney) and MRC5 (human lung) (Frey et al., 2014). The above studies indicate the potential for developing live-attenuated viruses as MERS vaccine candidates. Moreover, it was reported that high titers of specific antibodies with neutralizing activity can be generated in mice through vaccination with nanoparticles expressing the full-length MERS-CoV S protein, suggesting the possibility of developing nanoparticle-based MERS vaccines (Coleman et al., 2014a).

In addition to the aforementioned vaccine types, epitope-based and subunit vaccines also show promise against MERS-CoV infection or are under investigation for their efficacy. For example, recent studies in sequence analysis and computational prediction have identified an immunogenic and conserved epitope, WDYPKCDRA, in the RNA-directed RNA polymerase protein of human coronaviruses, supporting the concept of designing and

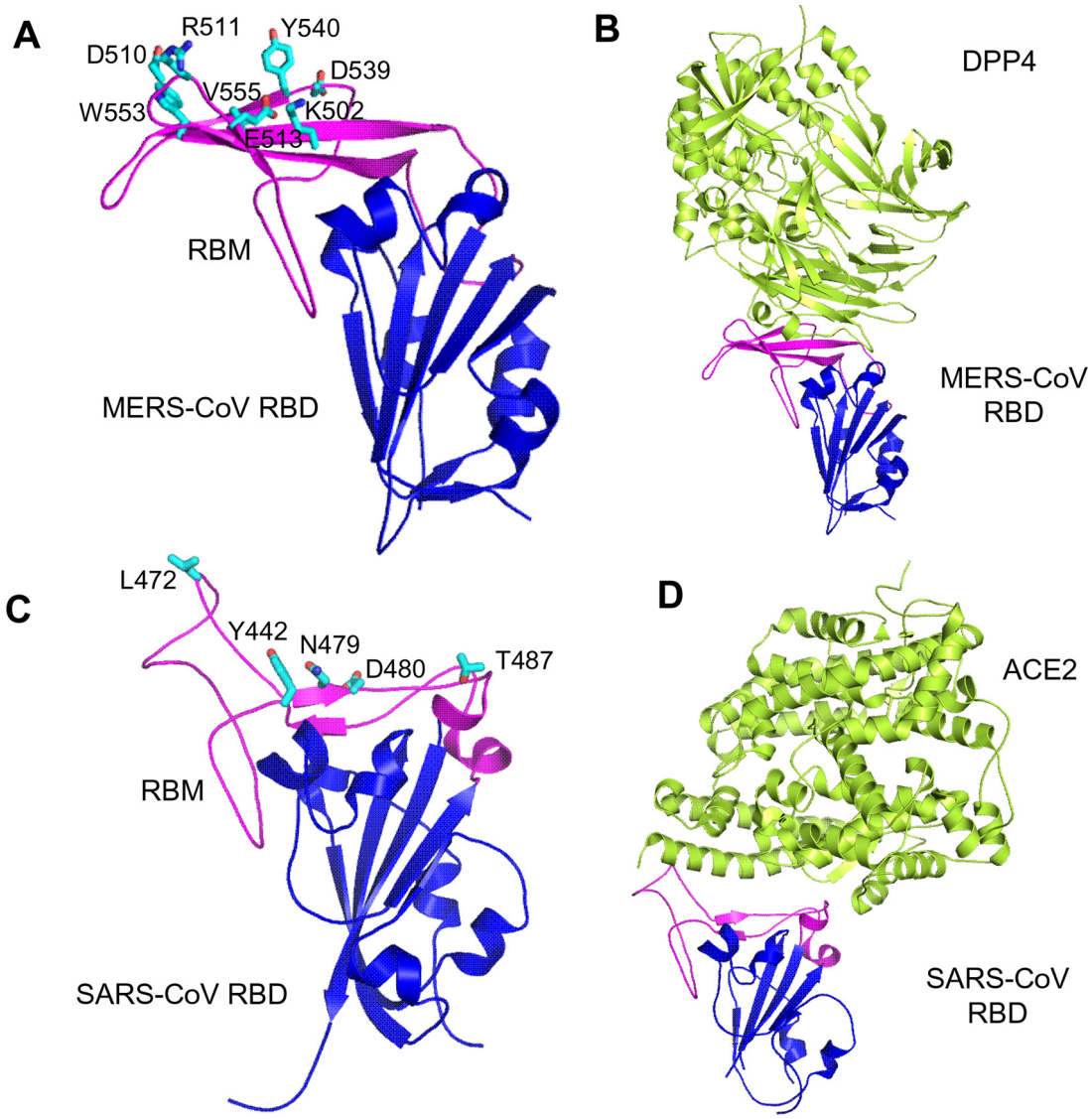

Fig. 2. Crystal structures of MERS-CoV and SARS-CoV RBDs and their complexes with respective receptors. (A) Crystal structure of MERS-CoV RBD. The receptor binding motif (RBM) is in magenta. Critical residues in the RBD-DPP4 binding interface are shown in cyan (PDB ID: 4KQZ). The residues are selected based on their direct and strong interactions with receptor residues. (B) Crystal structure of MERS-CoV RBD (blue) complexed with its receptor human DPP4 (lemon) (PDB ID: 4KR0). (C) Crystal structure of SARS-CoV RBD. The receptor binding motif (RBM) is in magenta. Critical residues in the RBD-ACE2 binding interface are shown in cyan (PDB ID: 2GHW). The residues are selected based on their direct and strong interactions with receptor residues. (D) Crystal structure of SARS-CoV RBD (blue) complexed with its receptor human ACE2 (lemon) (PDB ID: 2AJF). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

developing epitope-based universal vaccines against MERS (Sharmin and Islam, 2014). Additionally, recombinant proteins containing RBD of MERS-CoV S protein are able to elicit strong neutralizing antibodies in vaccinated rabbits and mice, respectively (Du et al., 2013a, 2013c; Ma et al., 2014a, 2014b; Mou et al., 2013), reinforcing the significance of developing protein-based subunit MERS vaccines. These candidate vaccines represent the first step in the control and prevention of MERS-CoV infection.

4. Development of RBD-based subunit vaccines against MERS-CoV

Subunit vaccines are defined as those based on purified proteins or peptides consisting of major antigenic fragments of pathogens (Hansson et al ., 2000). Subunit vaccines possess a variety of advantages, including high safety profile, minimal side effects at the injection sites and constant immune effects for the well-defined pathogenic fragments (Du et al., 2008; Zhang et al., 2014). Although reports on MERS-CoV RBD-based subunit vaccines are limited, subunit vaccines based on SARS-CoV RBD have been extensively studied and tested since the occurrence of SARS in 2002, showing sufficient efficacy and strong protection against SARS-CoV infections in various animal models (Du et al., 2007, 2009b; He et al., 2004; Zakhartchouk et al., 2007). Therefore, a summary of SARS-CoV RBD-based subunit vaccines will provide useful information and specific guidance on the design of effective RBD-based subunit vaccines against MERS-CoV.

4.1. Previous studies on the development of SARS-CoV S protein RBD-based subunit vaccines

Considerable evidence has shown that the SARS-CoV RBD contains multiple conformation-dependent epitopes that induce highly potent neutralizing antibodies and is, therefore, a critical neutralization determinant for developing SARS subunit vaccines (He et al., 2005a, 2005b).

It is believed that a recombinant fusion protein (RBD-Fc) containing the RBD (residues 318–510) of SARS-CoV S protein fused with human IgG1 Fc fragment induced strong antibody responses with neutralizing activity and elicited long-term protective immunity in immunized rabbits and mice, respectively, completely protecting immunized mice from SARS-CoV challenge (Du et al., 2007; He et al., 2004). We have also identified that recombinant RBD proteins (residues 318–510) expressed in mammalian cells 293T and CHO, insect cell sf9 and *Escherichia coli*, respectively, were able to elicit sufficient neutralizing antibodies and protective immunity against SARS-CoV challenge in immunized mice, among which the mammalian 293T cell-expressed RBD induced higher neutralizing antibody responses than those expressed in insect cell and *E. coli* systems (Du et al., 2009b, 2009c). Moreover, the 293T-expressing RBD was capable of inducing high titers of protective anti-RBD antibody response in immunized nonhuman primates, strongly neutralizing S protein-mediated SARS pseudovirus infection in ACE2-expressing target cells (Wang et al., 2012). Furthermore, we have shown that a CHO-expressing SARS-CoV RBD protein containing residues 318–536 elicited potent neutralizing antibody response in immunized mice with complete protective immunity (Du et al., 2010), and that a yeast-expressed RBD219N-1 protein induced strong RBD-specific neutralizing antibody responses against pseudovirus and live SARS-CoV infections (Chen et al., 2013a).

Interestingly, the recombinant RBDs from the S proteins of Tor2, GD03 and SZ3, the representative strains of human 2002–2003 and 2003–2004 SARS-CoV and palm civet SARS-CoV, respectively, elicited strong cross-neutralizing antibodies in immunized mice and rabbits against pseudoviruses expressing S proteins of these virus strains. The cross-neutralization of human and palm civet by SARS-CoV RBD-specific antibodies suggests that the RBD-based SARS-CoV subunit vaccines are able to induce broad cross-protective immunity against human and animal SARS-CoV variants (He et al., 2006). It is noted that high titers of RBD-specific neutralizing antibodies persistently existed in recovered patients in a three-year follow-up study, indicating that the RBD of SARS-CoV is highly immunogenic in humans (Cao et al., 2010), thus providing the basis for the development of effective subunit vaccines to prevent SARS-CoV infections in humans. Currently, this RBD-based subunit vaccine is in preclinical testing in small animal and primate animal challenge models, in anticipation of manufacture for clinical evaluation in the next five years (Jiang et al., 2012a).

The above summary suggests a paradigm that supports the development of a MERS-CoV subunit vaccine, indicating that the RBD of MERS-CoV is an important target for the development of MERS-CoV subunit vaccines and will, like its SARS-CoV counterpart, induce strong neutralizing antibody responses against MERS-CoV infection (Du et al., 2009a; Zhu et al., 2013).

4.2. Current studies on the development of MERS-CoV S protein RBD-based subunit vaccines

Based on our previous experience in developing SARS-CoV RBD-based subunit vaccines, we rapidly identified the RBD of MERS-CoV as an essential vaccine target for MERS. We have optimized the MERS-CoV RBD region and identified a critical neutralizing domain, among the five RBD fragments tested that induces the highest neutralizing antibodies against MERS-CoV infection (Ma et al., 2014b). Our progress in the development of MERS-CoV RBD-based subunit vaccines is summarized below.

4.2.1. Identification of a RBD of MERS-CoV S protein as an essential target for MERS vaccine development

To identify the RBD of MERS-CoV, we initially aligned the sequences of MERS-CoV S protein with those of SARS-CoV S protein and found that residues 377–662 in the MERS-CoV S protein shared similar core structures with SARS-CoV RBD, increasing the likelihood that the RBD of MERS-CoV might be located in this region (Jiang et al., 2013). A recombinant protein (S377-662-Fc) containing residues 377–662 of MERS-CoV S protein fused with Fc fragment of human IgG was expressed in the culture supernatant of mammalian cell 293T for the purpose of forming conformational structures and, thus, increasing immunogenicity (Du et al., 2013c).

As expected, the expressed recombinant protein was able to form a high molecular-weight molecule, as shown in nonboiled samples in SDS-PAGE (Fig. 3A, left), which was recognized by MERS-CoV S1-specific antibodies (Fig. 3A, right). Detection of the binding of S377-662-Fc with the receptor of MERS-CoV, DPP4, by co-immunoprecipitation assay revealed that this protein bound significantly to soluble DPP4 (sDPP4) and cell-associated DPP4 in DPP4-expressing Huh-7 cells, being recognized by anti-DPP4 and anti-MERS-CoV-S1 antibodies, respectively (Fig. 3B). ELISA and flow cytometry analyses indicated that S377-662-Fc bound to sDPP4 and Huh-7 in a dose-dependent manner (Du et al., 2013c). These data suggest that the expressed recombinant protein formed conformational structures and that the predicted 286-amino acid fragment contained MERS-CoV RBD.

Further evaluation of the immunogenicity of the identified MERS-CoV RBD demonstrated that the expressed recombinant S377-662-Fc protein induced MERS-CoV RBD-specific antibodies in mice subcutaneously (s.c.) immunized after two vaccinations, resulting in the effective neutralization of MERS-CoV infection in Vero E6 cells *in vitro* (Du et al., 2013c), indicating that this region may serve as an essential target for developing MERS subunit vaccines.

4.2.2. The identified MERS-CoV RBD-based vaccine is an effective and safe mucosal candidate for prevention of MERS-CoV infection

As MERS-CoV is a mucosal pathogen, any vaccination that induces the production of strong mucosal immune responses represented by mucosal IgA would have increased potential for the prevention of MERS-CoV at the site of virus infection. To test the ability of the identified MERS-CoV RBD as a potential mucosal vaccine, we immunized mice using MERS-CoV S377-662-Fc protein via the intranasal (i.n.) pathway and then compared the systemic and mucosal immune responses and neutralizing antibodies induced by the i.n. route with those elicited by the s.c. route.

Evaluation of the humoral immune response demonstrated that intranasal boost vaccination of S377-662-Fc induced levels of MERS-CoV S1-specific IgG antibodies, as well as IgG1 (Th2) and IgG2a (Th1) subtypes, as high as those induced via the s.c. route, although IgG antibody elicited through the i.n. pathway proved to be relatively lower than that elicited through the s.c. route after a single vaccination (Ma et al., 2014a). Similar to immunization by the s.c. route, intranasal vaccination of S377-662-Fc was also capable of eliciting effective long-term IgG antibody responses, particularly after multiple vaccinations (Fig. 3C) (Ma et al., 2014a), confirming the ability of S377-662-Fc to induce strong humoral immune responses via the mucosal route.

Comparison of the mucosal immune response illustrated that intranasal vaccination of S377-622-Fc induced a significantly higher level of MERS-CoV S1-specific IgA antibodies than that induced through the s.c. route in immunized mouse lungs (Fig. 3D), although the IgA level was only slightly higher in immunized mouse sera via the i.n. route compared to the s.c. route (Ma et al., 2014a). These data suggest that immunization of MERS-CoV S377-662-Fc protein induced strong local mucosal immune responses by the i.n. route, rather than the s.c. route.

Serum neutralizing antibody titers from intranasal vaccination of S377-662-Fc protein maintained levels similar to those of the

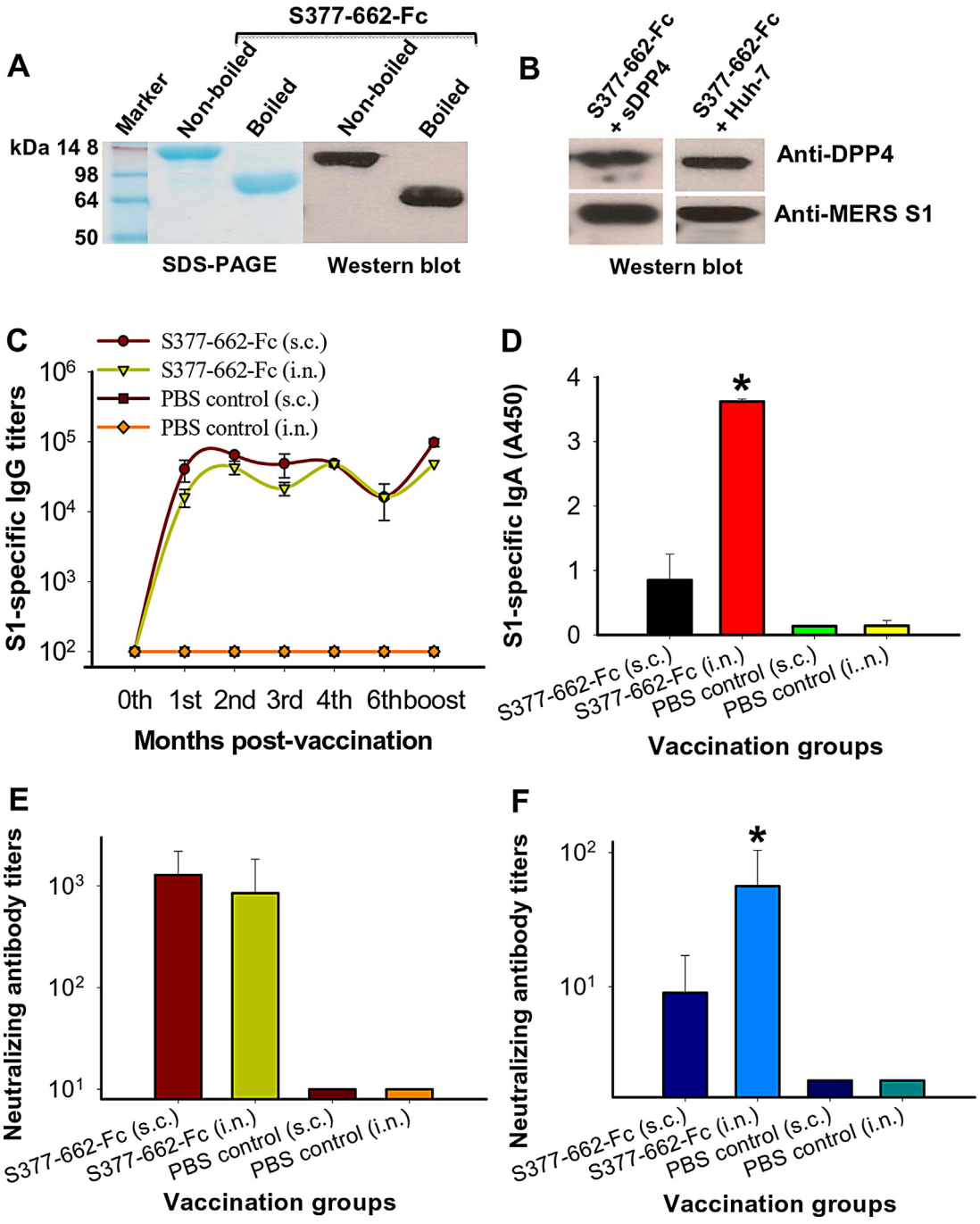

Fig. 3. Characterization of MERS-CoV S377-662-Fc protein for its antigenicity and receptor binding affinity, and comparison of its immunogenicity via s.c. and i.n. routes. (A) SDS-PAGE (left) and Western blot (right) detection of S377-662-Fc protein. Samples (5 μg), either boiled (denatured) or nonboiled (nondenatured), were subjected to SDS-PAGE, followed by Coomassie Blue staining or Western blot, using anti-MERS-CoV-S1 antibodies (1: 1000) developed in our laboratories. The protein molecular weight marker (kDa) is indicated on the left. (B) Co-immunoprecipitation and Western blot analysis of S377-662-Fc binding sDPP4, the receptor for MERS-CoV (left), and cell-associated DPP4 in Huh-7 cells (right). The binding affinity was tested using anti-human DPP4 monoclonal antibody (1 μg/ml, R&D Systems, Minneapolis, MN) and anti-MERS-CoV-S1 polyclonal antibodies (1: 1000). (C) Comparison of long-term humoral immune responses induced by S377-662-Fc protein in s.c.- and i.n.-immunized mice. The mouse sera were collected at 0, 1, 2, 3, 4 and 6 months and 10 days post-last boost, and the data are presented as mean (IgG endpoint titers) ± standard deviation (SD) of five mice per group. (D) Comparison of mucosal immune responses induced by S377-662-Fc protein in s.c.- and i.n.-immunized mice. The mouse lung washes were collected at 10 days post-last boost, and the data are presented as mean (IgA A450) ± SD of five mice per group.* indicates significant differences between S377-662-Fc i.n. group and the other groups ($P < 0.05$). Comparison of neutralizing antibody response against MERS-CoV infection in Vero E6 cells in sera (E) and lung washes (F) of mice s.c.- and i.n.-vaccinated with S377-662-Fc protein. Neutralizing antibody titers are expressed as the reciprocal of the highest dilutions of samples that completely inhibit virus-induced cytopathic effect (CPE) in at least 50% of the wells (NT_{50}). Samples were collected at 10 days post-last boost, and the data are presented as mean (neutralizing antibody titers) ± SD of five mice per group.* indicates significant differences between the S377-662-Fc i.n. group and the other groups ($P < 0.05$). For C–F, mice injected with PBS plus respective adjuvants (Montanide ISA51 for s.c. and Poly (I:C) for i.n.) were included as the control.

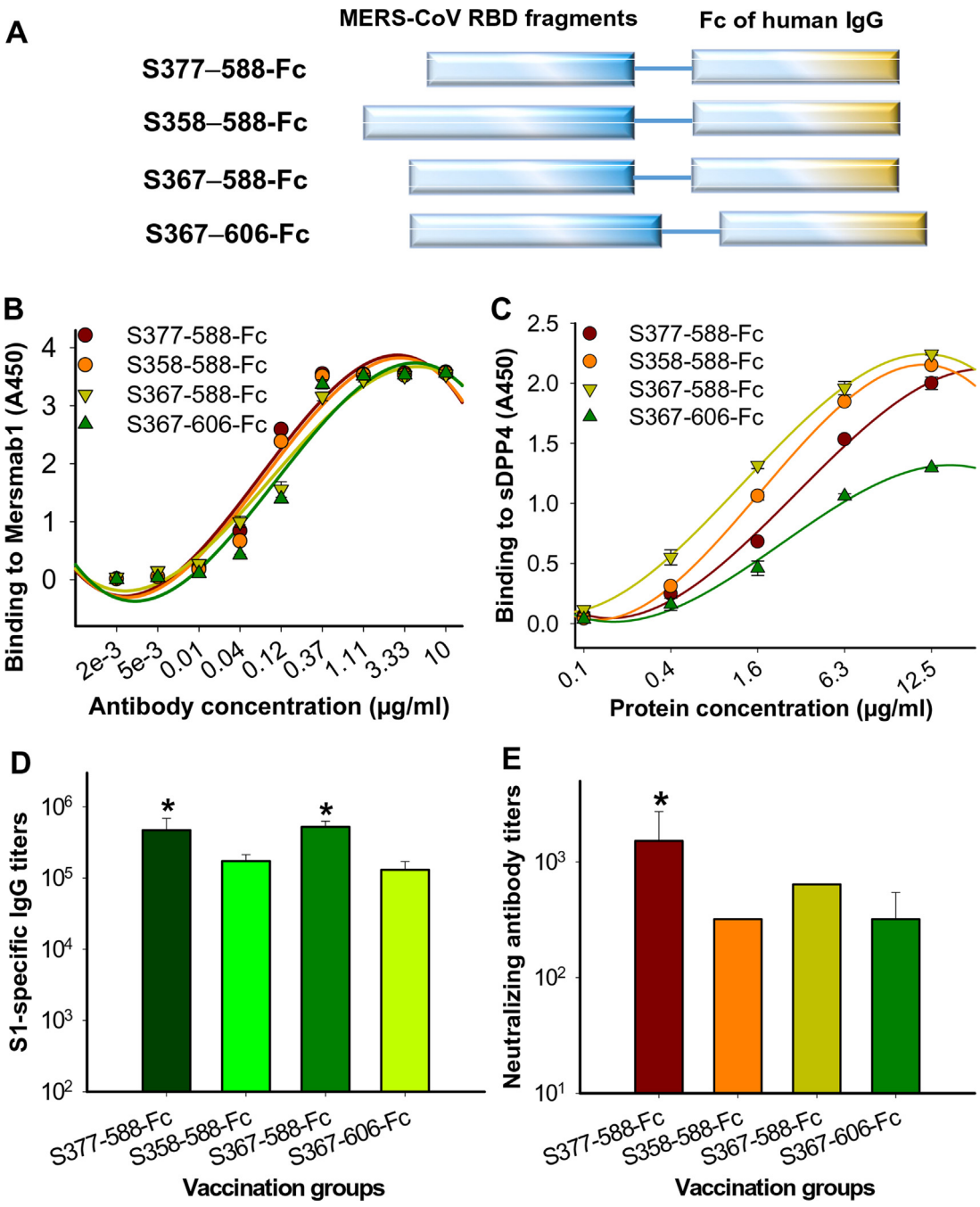

Fig. 4. Comparison of four MERS-CoV RBD protein fragments for their antigenicity, receptor binding affinity, immunogenicity and neutralizing potential. (A) Construction of S377-588-Fc, S358-588-Fc, S367-588-Fc and S367-606-Fc by fusing RBD fragments containing corresponding residues of MERS-CoV S protein with Fc of human IgG. (B) Comparison of binding of RBD fragments with MERS-CoV RBD-specific monoclonal antibody Mersmab1 by ELISA. The data are presented as mean (IgG A450) ± SD of duplicate wells. (C) Comparison of receptor binding affinity of RBD fragments with sDPP4 by ELISA. The binding shows dose dependency. The data are presented as mean (IgG A450) ± SD of duplicate wells. (D) Comparison of immunogenicity of RBD fragments in immunized mice. Mouse sera were collected at 10 days post-3rd vaccination, and the data are presented as mean (IgG endpoint titers) ± SD of five mice per group.* indicates significant differences between S377-588-Fc and S367-588-Fc with other groups ($P < 0.05$). (E) Comparison of neutralizing antibody response against MERS-CoV infection in Vero E6 cells in RBD fragment-immunized mice. Neutralizing antibody titers are expressed as the reciprocal of the highest dilutions of sera that completely inhibit virus-induced CPE in at least 50% of the wells (NT_{50}). Mouse sera were collected at 10 days post-3rd vaccination, and the data are presented as mean (neutralizing antibody titers) ± SD of five mice per group.* indicates significant differences between S377-588-Fc and other groups ($P < 0.05$).

s.c. route after boost vaccination (Fig. 3E). However, it is worth noting that these neutralizing antibodies were significantly higher in immunized mouse lungs in neutralizing MERS-CoV infections in target Vero E6 cells via the i.n. route, rather than the s.c. route (Fig. 3F), potentially because of the higher titers of mucosal IgA antibodies induced in the i.n.-immunized mouse lungs (Ma et al., 2014a).

It should be noted that S377-662-Fc protein was also able to elicit MERS-CoV S1-specific cellular immune responses in immunized mouse spleens (Ma et al., 2014a), indicating that cellular immune response might also play a role in the prevention of MERS-CoV infection. Importantly, since RBD-induced systemic humoral and mucosal immune responses, neutralizing antibodies in particular, correspond to the protection against SARS-CoV infection (Du

et al., 2007), it is expected that neutralizing antibodies induced by MERS-CoV RBD protein will correlate well with inhibition of MERS-CoV infection. Nevertheless, based on the ability of MERS-CoV RBD protein to induce strong systemic and mucosal immune responses with neutralizing activity, it is expected that MERS-CoV RBD-based vaccine is an effective and safe mucosal candidate for prevention of MERS-CoV infection.

4.2.3. Identification of a critical neutralizing domain of MERS-CoV RBD as an ideal vaccine candidate for developing effective MERS subunit vaccines

In addition to the above identified 286-amino acid fragment containing residues 377–662, three additional fragments, respectively, spanning residues 358–588, 367–588 and 367–606 of MERS-CoV S protein, were defined as MERS-CoV RBD by other groups (Chen et al., 2013b; Lu et al., 2013a; Mou et al., 2013; Wang et al., 2013). We have also shown that a truncated 212-amino acid fragment spanning S protein residues 377–588 of MERS-CoV served as a RBD of MERS-CoV (Du et al., 2013a). To identify a critical neutralizing domain in the MERS-CoV RBD as an ideal MERS vaccine candidate, we compared these identified RBD fragments in terms of their antigenicity, DPP4 receptor binding, immunogenicity and neutralizing activity. In order for the recombinant proteins to form suitable conformational structures and maintain sufficient bioactivity, we fused each of these MERS-CoV RBD fragments to the Fc of human IgG and expressed the proteins in a mammalian cell expression system (Fig. 4A). Previously, this same strategy was implemented and proved to be successful in expressing RBD proteins of SARS-CoV and influenza virus (Du et al., 2011, 2013d; Li et al., 2013).

As expected, all four MERS-CoV RBD proteins, designated S377-588-Fc, S358-588-Fc, S367-588-Fc and S367-606-Fc, respectively, formed dimeric conformational structures and maintained good antigenicity (Ma et al., 2014b), reacting strongly with MERS-CoV RBD-specific monoclonal antibody Mersmab1 developed in our laboratories (Fig. 4B) (Du et al., 2014). Although all proteins bound efficiently to sDPP4- and DPP4-expressing Huh-7 cells, their binding affinities were notably different, with S377-588-Fc as one of the proteins having the highest DPP4-binding activity (Fig. 4C). Notably, S377-588-Fc also maintained stronger immunogenicity than the other proteins tested, inducing significantly higher IgG antibody responses in immunized mice (Fig. 4D), as well as IgG1 (Th2) and IgG2a (Th1) subtypes. Importantly, S377-588-Fc protein was shown to elicit the highest neutralizing antibody titers against infections from MERS-CoV (Fig. 4E) (Ma et al., 2014b).

Our study identified a truncated fragment spanning residues 377–588 of MERS-CoV RBD as a critical neutralizing domain, thus making it an ideal target for the development of subunit vaccines against infections from MERS-CoV (Ma et al., 2014b). The identification of this critical neutralizing RBD fragment provides a foundation for the rapid development of effective and safe subunit vaccines to prevent the spread of MERS-CoV in humans or the transmission from infected camels to humans.

5. Current animal models for MERS-CoV infections and vaccine evaluation

Vaccine candidates that have proved effective against MERS-CoV infections in *in vitro* culture conditions need to be evaluated in effective animal challenge models to confirm protective immunity. MERS-CoV has been shown to cause transient lower respiratory tract infection in rhesus macaques, and infected monkeys demonstrated clinical signs of diseases, virus replication, histological lesions and neutralizing antibody generation (de Wit et al., 2013b; Yao et al., 2014), demonstrating that monkeys can be used for testing the efficacy of MERS candidate vaccines. However, this MERS-CoV animal model is not affordable for most researchers based on the high cost for purchasing and maintaining monkeys, making it particularly urgent to develop small animal models of MERS-CoV infection (Zhang et al., 2014). Studies have shown that MERS-CoV is susceptible to human and nonhuman primate cell types, including Huh-7, A549, Calu-3, Caco-2 (human) and Vero (African green monkey), but it is not susceptible to BHK (hamster), MEFC57B16 and 3T3 (mouse), or ferret primary kidney cells (Chan et al., 2013a; Tao et al., 2013; van Doremalen et al., 2014). These findings correspond to the observation that MERS-CoV infects human and nonhuman primates, but it does not replicate in small animals, such as Syrian hamsters, mice and ferrets (Coleman et al., 2014b; de Wit et al., 2013a; Devitt, 2013; Raj et al., 2014; van Doremalen et al., 2014).

Although small animal models for MERS-CoV are still lacking (Devitt, 2013), researchers have made great progress towards the identification and development of an effective small animal model for MERS-CoV infections. In particular, it has been shown that mice are susceptible to MERS-CoV infection after prior transduction with an adenoviral vector expressing the receptor of MERS-CoV, human DPP4, with infected mice developing pneumonia accompanied by inflammatory cell infiltration and histopathological changes (Zhao et al., 2014). Thus, such small animal models can provide a practical platform and an affordable means for evaluating the efficacy of current MERS-CoV vaccines. Accordingly, transgenic DPP4 mouse models that integrate human DPP4 genes into the mouse genome are being established in several laboratories (http://www.nature.com/news/biologists-make-first-mouse-model-for-mers-1.14634) and are expected to be tested for vaccine efficacy in the near future.

6. Conclusions and perspectives

Development of effective vaccines is urgently needed to control the spread of emerging MERS-CoV infections. While viral vector-based and live-attenuated virus-based vaccines have potential for providing effective immunity, subunit vaccines will offer the safest means for prevention. Although subunit-based vaccines might induce less immunogenicity than the other vaccine types, their efficacy can be significantly improved by rational design based on the structural analysis of RBD of MERS-CoV S protein and by identification of the most stable and critical neutralizing fragment of RBD, while eliminating non-neutralizing epitopes through immunofocusing (Ma et al., 2014b). Overall, our strategies in the development of MERS-CoV RBD-based subunit vaccines will provide useful information for the design of effective vaccines against MERS-CoV and any other emerging coronaviruses that might cause future pandemics. It is expected that such developed MERS candidate vaccines will be tested for efficacy in preventing MERS-CoV infections *in vivo* once affordable small animal models for MERS-CoV have become available. Because serologic and neutralization responses against S proteins of MERS-CoV, SARS-CoV and other coronaviruses share no, to low, levels of cross-reactivity within and/or across subgroups, it is advisable that MERS-CoV and other coronavirus vaccines be designed by considering construction of chimeric S protein containing neutralizing epitopes from variant virus strains (Agnihothram et al., 2014).

Conflict of interest statement

The authors declared no conflict of interest.

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