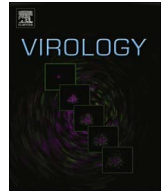




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A recombinant receptor-binding domain of MERS-CoV in trimeric form protects human dipeptidyl peptidase 4 (hDPP4) transgenic mice from MERS-CoV infection

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ABSTRACT

Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) was first identified in 2012, and it continues to threaten human health worldwide. No MERS vaccines are licensed for human use, reinforcing the urgency to develop safe and efficacious vaccines to prevent MERS. MERS-CoV spike protein forms a trimer, and its receptor-binding domain (RBD) serves as a vaccine target. Nevertheless, the protective efficacy of RBD in its native trimeric form has never been evaluated. In this study, a trimeric protein, RBD-Fd, was generated by fusing RBD with foldon trimerization motif. It bound strongly to the receptor of MERS-CoV, dipeptidyl peptidase 4 (DPP4), and elicited robust RBD-specific neutralizing antibodies in mice, maintaining long-term neutralizing activity against MERS-CoV infection. RBD-Fd potentially protected hDPP4 transgenic mice from lethal MERS-CoV challenge. These results suggest that MERS-CoV RBD in its trimeric form maintains native conformation and induces protective neutralizing antibodies, making it a candidate for further therapeutic development.

1. Introduction

Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) was first identified in Saudi Arabia in 2012, and since then, it has resulted in increased incidence of human infection (Zaki et al., 2012; Chan et al., 2012; Rha et al., 2015). As of October 04, 2016, a total of 1806 laboratory-confirmed MERS cases, including 643 deaths (mortality rate ~36%), have been reported worldwide (<http://www.who.int/emergencies/mers-cov/en/>). Bats are regarded as a natural reservoir of MERS-CoV, and the potential mechanisms driving bat-to-human transmission of MERS-CoV are being investigated by comparing MERS-CoV with a closely related bat coronavirus, HKU-4 (Yang et al., 2014; Memish et al., 2013; Ithete et al., 2013; Cui et al., 2013; Wang et al., 2014). Dromedary camels are shown to be an important intermediate host for MERS-CoV, and, as such, they are considered the key to stopping MERS-CoV transmission (Adney et al., 2014; Yusof

et al., 2015; Gossner et al., 2016). The severe outbreak of MERS-CoV in South Korea in 2015 demonstrated the ability of this virus to cause human-to-human transmission (Khan et al., 2015; Ki, 2015). The continuing spread of MERS-CoV and increase of MERS cases have highlighted the urgent need to develop effective and safe vaccines against MERS-CoV.

The native spike (S) of MERS-CoV is an envelope glycoprotein presented as a trimer on the virus surface. It is cleaved during virus infection by host cell proteases into S1 and S2 subunits. S1 is responsible for MERS-CoV binding to host cells expressing viral receptor dipeptidyl peptidase 4 (DPP4) via the receptor-binding domain (RBD) (Raj et al., 2013; Lu et al., 2013; Wang et al., 2013; Chen et al., 2013; Li, 2015). Meanwhile, S2 is engaged in fusion between virus and cell surface membranes, thus mediating the entry of MERS-CoV into target cells (J. Gao et al., 2013; Lu et al., 2014). These activities make MERS-CoV S protein, in particular its RBD, an

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important vaccine target (Zhang et al., 2014; Du et al., 2013b; Du et al., 2013a; Ma et al., 2014a, 2014b). A number of fragments have been identified as constituents of MERS-CoV RBD, but the fragment containing residues 377–588 in the viral S protein is the critical neutralizing domain (CND) responsible for the induction of neutralizing antibodies in immunized animals (Ma et al., 2014a, 2014b; Zhang et al., 2015). Nevertheless, the RBD in its native trimeric form has never been evaluated for protective efficacy against MERS-CoV infection.

Foldon (Fd), a 27-amino acid phage T4 fibrin, is a trimerization motif which is now being constructed as a fusion partner to guide formation of ultra-stable protein fibers or promote trimerization of recombinant fusion proteins (Bhardwaj et al., 2008; Papanikolopoulou et al., 2004; Sissoeff et al., 2005). For example, influenza virus hemagglutinins (HAs) with an Fd-formed trimerization domain have increased binding affinity to neutralizing antibodies targeting the stalk region of viral HAs (Krammer et al., 2012). In addition, recombinant HIV-1 trimeric envelope glycoproteins induced more efficient and broad-spectrum neutralizing antibodies with increased potency than monomeric gp120 protein against divergent HIV-1 strains (Grundner et al., 2005). These reports reinforce the importance of mimicking native viral trimeric structures in the design of envelope glycoprotein-based subunit vaccines. However, imitating conformational structures of MERS-CoV S protein has never been attempted in MERS vaccine design.

In this study, we expressed a recombinant trimeric RBD protein by fusing the MERS-CoV RBD sequence (residues 377–588) with Fd trimerization motif (RBD-Fd). We then investigated its antigenicity, receptor binding affinity, immunogenicity, neutralization potential, and protective efficacy against MERS-CoV challenge in a human DPP4-transgenic (hDPP4-Tg) mouse model. The outcomes of this study indicate the potential of developing MERS subunit vaccines based on the trimeric RBD of MERS-CoV S protein.

2. Materials and methods

2.1. Ethics statement

Four- to six-week-old female BALB/c mice and four-month-old male and female hDPP4-Tg mice were used in the study. The animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocols were approved by the Committee on the Ethics of Animal Experiments of the New York Blood Center (Permit Number: 194.17) and the Beijing Institute of Microbiology and Epidemiology (Permit Number: PMB15-0012).

2.2. Construction, expression and purification of recombinant proteins

The construction, expression and purification of the recombinant proteins were carried out as previously described (Du et al., 2013a; Ma et al., 2014a, 2014b; Du et al., 2013b). Briefly, recombinant RBD-Fd (residues 377–588 of MERS-CoV RBD) containing a C-terminal Fd and a His₆ tag was constructed using hIgG-Fc vector (InvivoGen), and expressed in the transfected 293T cell culture supernatants. Recombinant MERS-CoV S1 (residues 18–725) containing a C-terminal His₆ (S1-His) was constructed using pJW4303 expression vector, and expressed as described above. Recombinant human DPP4 ectodomain (residues 39–766) containing a C-terminal His₆ (sDPP4) was expressed in insect cells using the Bac-to-Bac expression system (Invitrogen), and secreted into cell culture medium (Chen et al., 2013). The aforementioned His-tag proteins were purified using Ni-NTA Superflow (Qiagen).

2.3. SDS-PAGE and western blot

RBD-Fd was analyzed by SDS-PAGE and Western blot as previously described (Ma et al., 2014a; Du et al., 2013b). Briefly, the boiled and non-boiled proteins were separated by 10% Tris-Glycine SDS-PAGE gels and then transferred to nitrocellulose membranes. After blocking overnight at 4 °C with 5% non-fat milk in PBST, the blots were incubated for 1 h at room temperature with MERS-CoV S1-specific antibody (1:1000). After three washes, the blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:3000) (GE Healthcare) for 1 h at room temperature, and the signals were visualized using ECL Western blot substrate reagents and Amersham Hyperfilm (GE Healthcare).

2.4. Size-exclusion chromatography with multi-angle light scattering (SEC-MALS)

RBD-Fd protein was concentrated to 2 mg/ml in buffer containing 50 mM PBS and 150 mM NaCl, and analyzed in-line using tandem miniDAWN TREOS MALS and Optilab rEX differential refractive index detectors (Wyatt Technologies) at the PEPCC Facility of Case Western Reserve University. The protein was loaded for molar mass determinations, and the data were analyzed using the ASTRA 6.1 software package (Wyatt Technologies). The size distribution of the protein was measured by DynaPro NanoStar (Wyatt Technologies).

2.5. Co-immunoprecipitation assay

The binding between RBD-Fd protein and DPP4 receptor was carried out by Co-immunoprecipitation (Co-IP) assay and Western blot as previously described (Du et al., 2013a). Briefly, RBD-Fd (10 µg) was incubated with DPP4-expressing Huh-7 cell lysates (5×10⁷/ml) plus Ni-NTA Superflow at 4 °C for 1 h, followed by washing with wash buffer. The eluted proteins were boiled for 10 min and subjected to SDS-PAGE and Western blot analysis to detect the binding using anti-DPP4 antibody (0.2 µg/ml) (R & D Systems) and anti-MERS-CoV RBD antibody (1:5000), respectively.

2.6. Animal immunization and sample collection

BALB/c mice were immunized with RBD-Fd as previously described (Ma et al., 2014a; Ma et al., 2014b). Briefly, mice were subcutaneously (s.c.) vaccinated with RBD-Fd (10 µg/mouse) in the presence of MF59 adjuvant (Schultze et al., 2008) and boosted with the same immunogen and adjuvant at 3 weeks, 6 weeks, and 6 months post-immunization. PBS plus adjuvant was included as the negative control. Sera were collected monthly post-first dose for 6 months to detect MERS-CoV S1-specific antibody responses and/or neutralizing antibodies.

2.7. ELISA

ELISA was carried out to detect the binding between RBD-Fd and sDPP4 proteins as previously described (Du et al., 2013a). Briefly, ELISA plates were precoated with RBD-Fd (2 µg/ml) overnight at 4 °C and blocked with 2% non-fat milk at 37 °C for 2 h. Serially diluted sDPP4 was added to the plates and incubated at 37 °C for 1 h, followed by four washes. Bound antibodies were sequentially incubated with goat anti-DPP4 (0.2 µg/ml) and HRP-conjugated anti-goat IgG (1:5000) (R & D Systems) antibodies at 37 °C for 1 h, followed by addition of substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Invitrogen) and H₂SO₄ (1 N) sequentially. The absorbance at 450 nm (A₄₅₀) was measured by an ELISA plate reader (Tecan).

The binding between RBD-Fd protein and MERS-CoV RBD-specific mouse mAb Mersmab1 and human mAbs m336, m337 and m338 (Du et al., 2014; Ying et al., 2014) was carried out using a protocol similar

to that described above, but replacing the primary antibody with serially diluted mAbs. The HRP-conjugated anti-mouse (1:3000) (GE Healthcare) or anti-human (1:5000) (Sigma) IgG antibodies were used as secondary antibodies.

For detecting MERS-CoV S1-specific antibodies in mouse sera, plates were coated with MERS-CoV S1-His protein (1 μ g/ml), followed by sequential addition of serially diluted mouse sera and HRP-conjugated anti-mouse IgG (1:3000), IgG1 (1:2000), or IgG2a (1:5000) antibodies (Invitrogen).

2.8. MERS pseudovirus inhibition and neutralization assays

These assays were carried out as previously described with some modifications (Zhao et al., 2013; Du et al., 2010). Briefly, 293T cells were co-transfected with a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc. RE) and a plasmid encoding S protein of MERS-CoV prototypic strain (EMC-2012), or each of the representing strains isolated in the 2012–2015 outbreaks that carry respective mutations in the RBD (Qiu et al., 2016), using the calcium phosphate method. The pseudovirus-containing supernatants were harvested 72 h post-transfection for single-cycle infection of Huh-7 cells. For the inhibition assay, Huh-7 cells were incubated with serially diluted RBD-Fd protein at 37 °C for 1 h, followed by removal of the protein-containing media and then infection with MERS pseudovirus. For the neutralization assay, MERS pseudovirus was incubated with serially diluted mouse sera at 37 °C for 1 h, followed by addition of the mixture to the cells. The cells were refed with fresh medium 24 h later, lysed at 72 h with cell lysis buffer (Promega), and transferred into 96-well luminometer plates. Luciferase substrate (Promega) was added to the plates, and relative luciferase activity was determined in an Infinite 200 PRO Luminator (Tecan). MERS pseudovirus inhibition and neutralization were calculated as previously described (Chou, 2006), and expressed as 50% inhibition dose (ID₅₀) and neutralizing antibody titer (NT₅₀), respectively.

2.9. Live MERS-CoV neutralization assay

A micro-neutralization assay was carried out to detect neutralizing antibodies against live MERS-CoV infection as previously described (Tao et al., 2013; Ma et al., 2014b). Briefly, mouse sera were diluted at a serial 2-fold in 96-well tissue culture plates, followed by incubation for 1 h at room temperature with ~100 infectious MERS-CoV (EMC-2012) before transferring to Vero E6 cells. After 72 h, cells were observed for the presence or absence of virus-induced cytopathic effect (CPE), and the neutralizing antibody titers were determined as the reciprocal of the highest dilution of sera that completely inhibited virus-induced CPE in at least 50% of the wells (NT₅₀).

2.10. Animal challenge studies

The challenge studies were carried out in hDPP4-Tg mice using our previously optimized protocols (Zhao et al., 2015). Briefly, mice were intramuscularly (i.m.) immunized with RBD-Fd (5 μ g/mouse) plus aluminum adjuvant and boosted once at 4 weeks. Immunized mice were challenged with MERS-CoV (EMC-2012 strain, 10⁴ TCID₅₀) 12 weeks later, and observed for 3 weeks post-challenge to detect survival rate, body weight, and pathological changes.

3. Results

3.1. Characterization and antigenicity of RBD-Fd protein

To determine whether MERS-CoV RBD containing the CND (residues 377–588) would form a native trimeric structure, we fused its C-terminus to Fd trimerization motif (Fig. 1A), expressed RBD-Fd protein in 293T cell culture supernatants, and further characterized the protein by SDS-PAGE and Western blot analysis. A high molecular-weight band (trimer) was shown in the non-denatured (non-boiled) sample, which was 3 times that of the denatured (boiled) protein

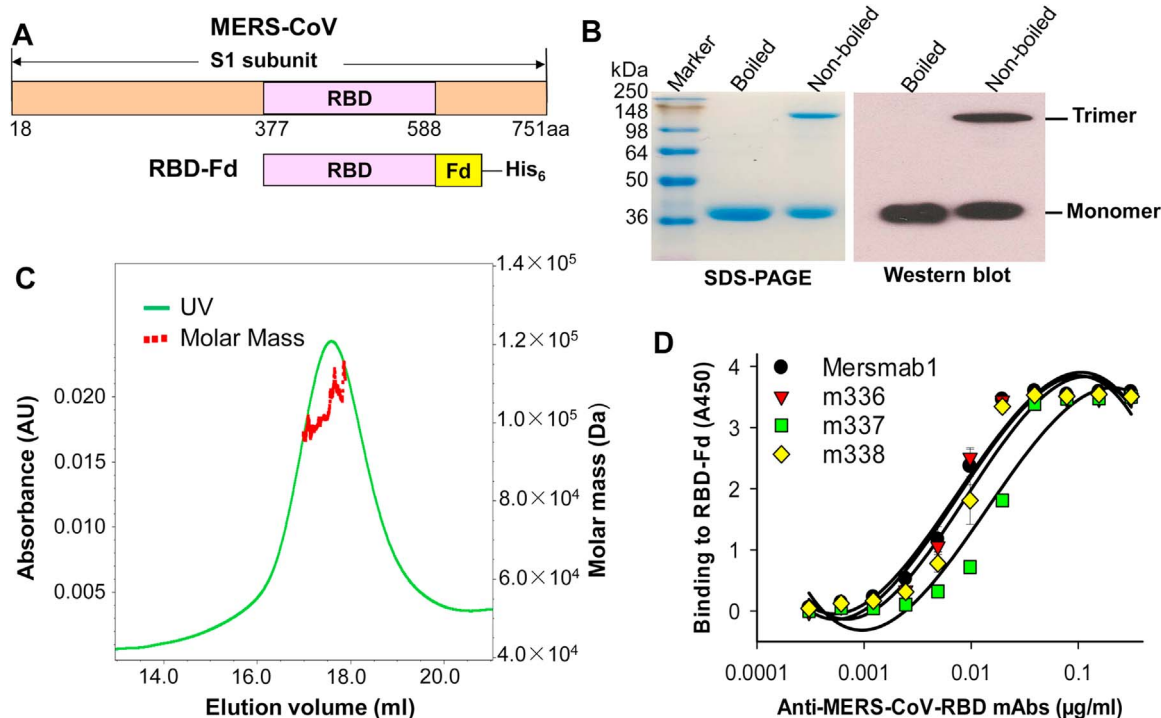


Fig. 1. Construction, characterization and antigenicity evaluation of MERS-CoV RBD-Fd protein. (A) Schematic structure of MERS-CoV S1 subunit and construction of RBD-Fd. A His₆ tag was added at the C-terminus of RBD-Fd for easy purification. (B) SDS-PAGE and Western blot analysis of purified RBD-Fd protein. Denatured (boiled) or non-denatured (non-boiled) samples (5 μ g) were subjected to SDS-PAGE (left) and Western blot analysis (right) using MERS-CoV S1-specific antibody. The molecular weight marker (kDa) is indicated on the left. (C) SEC-MALS analysis of purified RBD-Fd protein. RBD-Fd (2 mg/ml, 100 μ l) was subjected to the analysis. The data are presented as mean molar mass (Da). (D) ELISA detection of the binding between RBD-Fd and RBD-specific neutralizing mAbs. RBD-Fd (2 μ g/ml)-coated ELISA plates were incubated with serially diluted mAbs for the test.

(monomer) (Fig. 1B, left). The trimeric molecule of the RBD-Fd protein was further confirmed by SEC-MALS analysis, indicating the molar mass of about 1.043×10^5 ($\pm 0.036 \times 10^5$) Da (Fig. 1C). These results demonstrate that recombinant RBD formed a trimeric conformation in the presence of Fd trimerization motif. In addition, RBD-Fd had a strong reactivity with antibodies against a recombinant S1 protein of MERS-CoV (Fig. 1B, right), suggesting that the trimeric RBD is derived from MERS-CoV S protein.

We further investigated the antigenicity of RBD-Fd protein by testing its binding affinity with neutralizing mAbs specific to the conformational epitopes in MERS-CoV RBD (Ying et al., 2014; Du et al., 2014). ELISA data revealed that RBD-Fd bound strongly to these antibodies and that the binding occurred in a dose-dependent manner (Fig. 1D), suggesting that trimeric MERS-CoV RBD maintains the antigenicity required to recognize RBD-specific antibodies that target conformational epitopes.

3.2. Binding affinity of RBD-Fd protein to DPP4, the receptor of MERS-CoV

Co-IP assay and ELISA were performed to determine the affinity of RBD-Fd binding to cell-associated and recombinant DPP4, respectively. Co-IP results revealed two clear bands in the pull-down samples containing Huh-7 cell lysates and RBD-Fd protein, which correspond to the size of DPP4 and RBD-Fd monomers, and were identified by anti-DPP4 (Fig. 2A) and anti-MERS-CoV-RBD (Fig. 2B) antibodies, respectively. However, soluble DPP4 (sDPP4) or RBD-Fd protein alone could only be recognized by either anti-DPP4 or anti-MERS-CoV-RBD, but not by both antibodies (Fig. 2A and B). The results from ELISA further revealed a strong binding between RBD-Fd and sDPP4 proteins in a dose-dependent manner (Fig. 2C). These data confirm the binding affinity of the trimeric MERS-CoV RBD protein to the cell-associated and recombinant DPP4 proteins.

3.3. RBD-Fd protein strongly inhibited MERS-CoV infection and induced long-term potent antibody responses with cross-neutralizing activity against MERS-CoV infection

To test the ability of trimeric RBD-Fd to inhibit MERS-CoV infection, we incubated this protein with Huh-7 cells, and calculated the corresponding inhibition of infection as ID_{50} . The data demonstrated that RBD-Fd strongly blocked MERS pseudovirus entry into the target cells, with the ID_{50} as low as 1.5 $\mu\text{g}/\text{ml}$, whereas the control protein had no inhibitory activity even at the highest concentration (Fig. 3A). These data suggest the potential application of RBD-Fd as an effective therapeutic agent against MERS-CoV infection.

To investigate the immunogenicity of trimeric RBD-Fd, we used this protein to s.c. immunize BALB/c mice in the presence of MF59 adjuvant since this protocol has been previously optimized for RBD-based vaccines in the test mouse strain (Zhang et al., 2016). We then measured the generation of MERS-CoV S1-specific IgG antibodies, as well as IgG1 and IgG2a subtypes, using ELISA in the immunized mouse sera. As shown in Fig. 3B, RBD-Fd induced highly potent MERS-CoV S1-specific IgG antibodies, which maintained for at least 6 months during the detection period. As expected, only a background level of IgG antibody responses was induced in the control mice. Evaluation of IgG1 and IgG2a subtypes further demonstrated that both Th1 (IgG2a) and Th2 (IgG1) antibody responses could be elicited by RBD-Fd and for at least the next six months, all maintained titers similar to, or higher than, those induced at 2 months post-immunization (Fig. 3C and D). In contrast, no specific IgG1 and IgG2a antibodies were induced in the control mice injected with PBS. These data confirm high immunogenicity of trimeric MERS-CoV RBD in inducing long-term, strong humoral immune responses in mice.

The neutralizing antibodies in the sera of mice immunized with trimeric RBD-Fd were detected by MERS pseudovirus and live MERS-

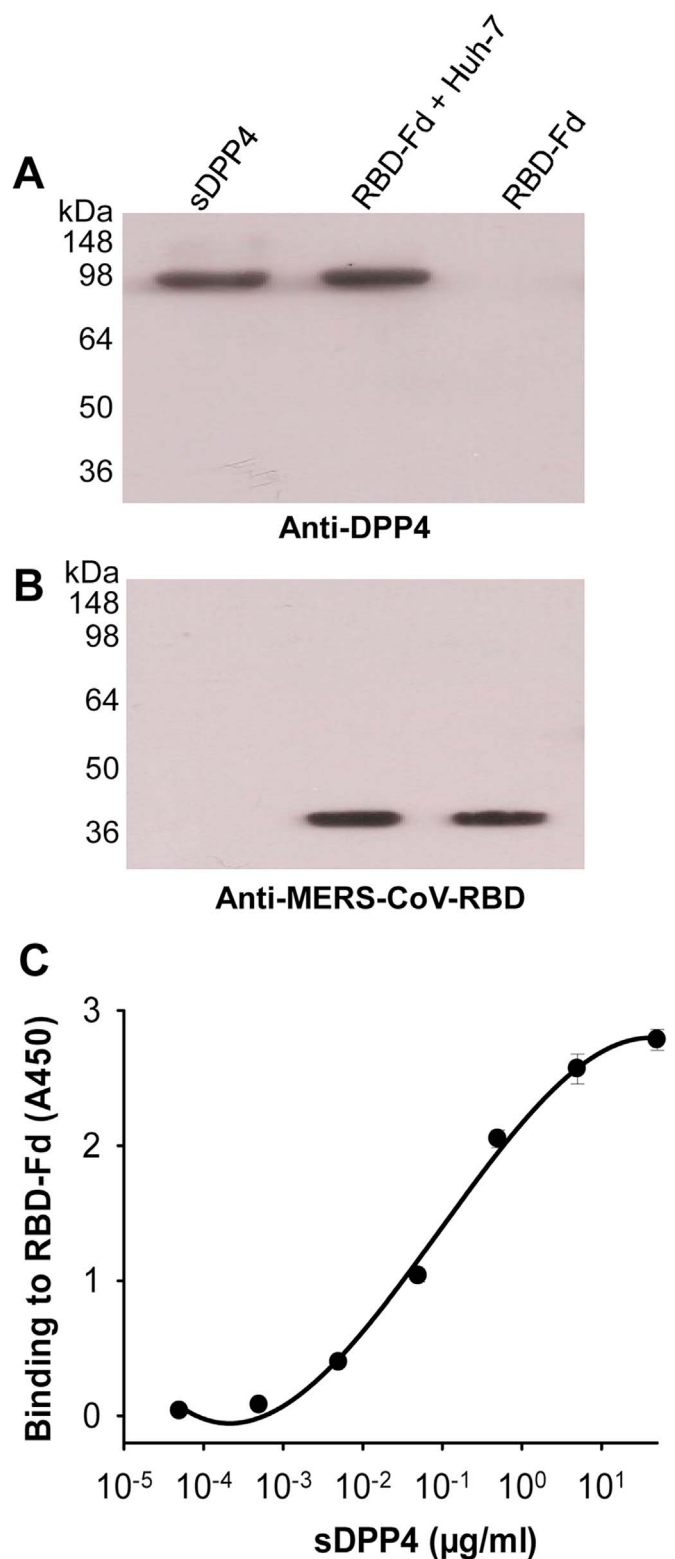


Fig. 2. Functionality of RBD-Fd protein in binding to the DPP4 receptor of MERS-CoV. (A-B) Co-IP and Western blot analysis of the binding between RBD-Fd and cell-associated DPP4. RBD-Fd was incubated with DPP4-expressing Huh-7 cell lysates in the presence of Ni-NTA Superflow, followed by detection of the binding by Western blot using DPP4- (0.2 $\mu\text{g}/\text{ml}$) (A) or MERS-CoV RBD-specific antibody (B) (1:5000). sDPP4 and RBD-Fd proteins only were included as the controls. The molecular weight marker (kDa) is indicated on the left. (C) ELISA measurement of the binding between RBD-Fd and sDPP4 proteins. RBD-Fd (2 $\mu\text{g}/\text{ml}$)-coated ELISA plates were incubated with serially diluted sDPP4 for the test.

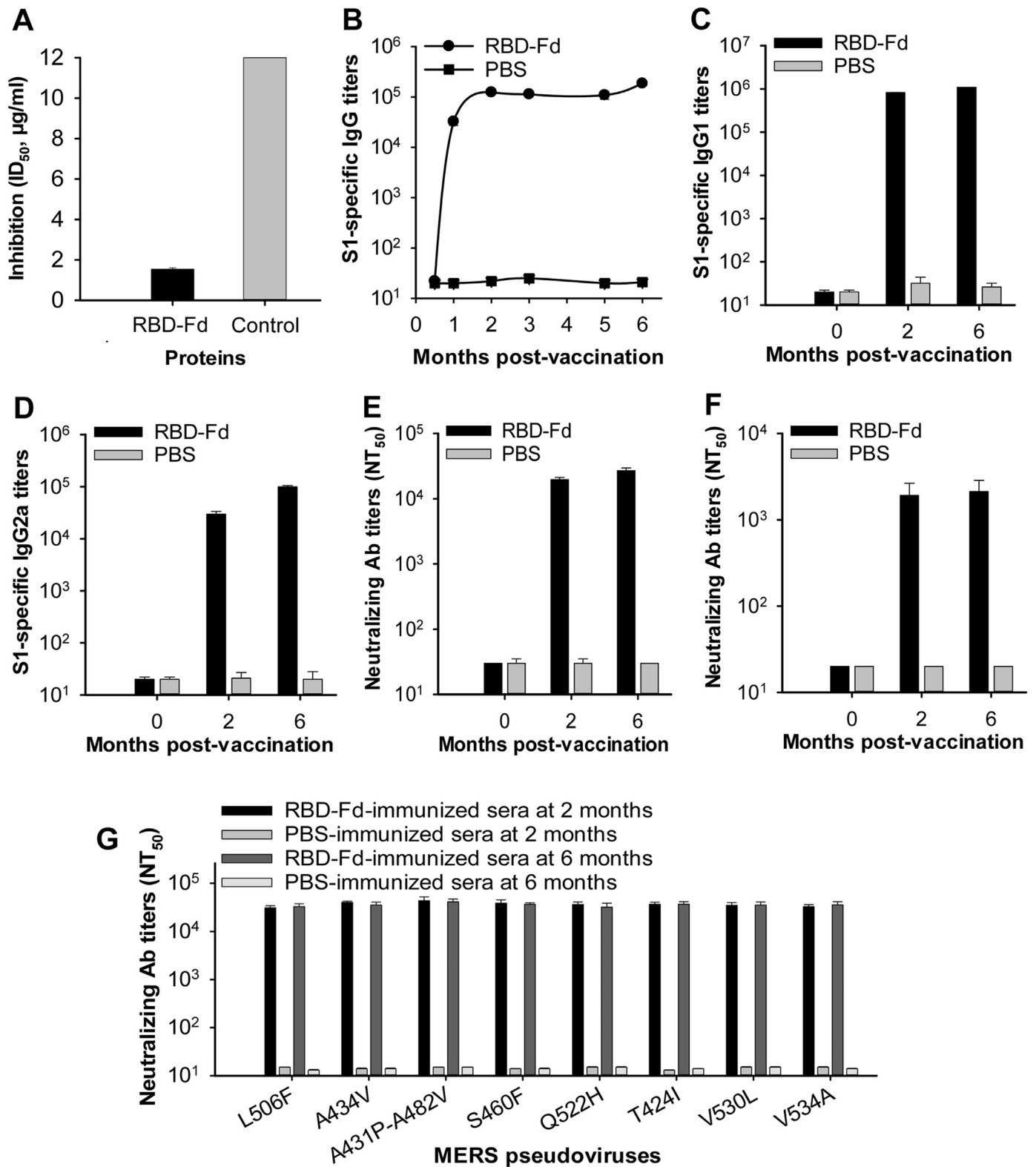


Fig. 3. Inhibitory activity of RBD-Fd and its immunogenicity and neutralizing ability in immunized mice. (A) Inhibitory activity of RBD-Fd protein against infections of pseudotyped MERS-CoV (EMC2012) in Huh-7 cells. An unrelated SARS-CoV RBD protein was included as the control. The data are presented as 50% inhibition dose (ID₅₀). (B) MERS-CoV S1-specific IgG antibody titers. Mouse sera from different months were used for the test. MERS-CoV S1-specific IgG1 (C) and IgG2a (D) antibody titers. Mouse sera from 2 and 6 months post-1st immunization were used for the test. The antibody titers are expressed as the endpoint dilution that remains positively detectable, and presented as mean titers ± SD of five mice in each group. RBD-Fd-induced neutralizing antibodies against infections of pseudotyped (E) and live (F) MERS-CoV of EMC2012 strain, as well as pseudotyped MERS-CoV of representative strains isolated from the 2012 (L506F), 2013 (A434V, A431P-A482V, S460F), 2014 (Q522H, T424I), and 2015 (V530L, V534A) outbreaks, respectively (G). Shown here are respective mutations in the RBD of S protein, as compared with those of EMC2012 strain (Qiu et al., 2016). Neutralizing antibody titers were calculated as the reciprocal of the highest dilution of sera that resulted in a complete inhibition of MERS pseudovirus infection (E and G) or MERS-CoV-induced CPE (F) in at least 50% of the wells (NT₅₀). Mouse sera from 2 and 6 months were used for the test, and the data are presented as mean titers ± SD of five mice in each group. For (B)–(G), PBS was included as the control.

CoV-based neutralization assays. Indeed, RBD-Fd elicited highly potent neutralizing antibodies against infections of both pseudotyped and live MERS-CoV of prototypic EMC2012 strain in susceptible Huh-7 and Vero E6 cells, respectively, maintaining similar levels at 2 and 6 months. Importantly, these antibodies strongly cross-neutralized infections of MERS pseudoviruses expressing S proteins of divergent strains isolated in the 2012–2015 outbreaks, demonstrating their cross-neutralizing activity. However, no specific neutralizing antibody was induced in the PBS control mice (Fig. 3E–G). The above results suggest that trimeric MERS-CoV RBD has ability to elicit long-term and potent neutralizing antibodies in mice.

3.4. RBD-Fd protein elicited long-term protective immunity in hDPP4-Tg mice against lethal infection of MERS-CoV

To investigate the ability of trimeric RBD-Fd to induce protective immunity against MERS-CoV challenge, we utilized this protein to i.m. immunize hDPP4-Tg mice, a small animal model on the C57BL/6 background that is susceptible to MERS-CoV infection (Zhao et al., 2015), in the presence of aluminum adjuvant. The above immunization protocol was selected because that it is optimized for hDPP4-Tg mice (unpublished data), and that aluminum is an approved adjuvant for using with human vaccines (Lee and Nguyen, 2015). We then challenged the immunized mice with MERS-CoV at a lethal dose, and evaluated for their survivals, weight, and pathological changes.

The data demonstrated that 83% of the RBD-Fd-immunized mice survived from lethal MERS-CoV infection (Fig. 4A). These mice had neutralizing antibody titers ranging from 1:100 to 1:673 (average 1:312) against live MERS-CoV infection before virus challenge (Fig. 4B). Although the challenged mice slightly reduced weight during day 8–14 after MERS-CoV challenge, they rapidly recovered to the normal weight (Fig. 4C). In contrast, the control mice with a background level of neutralizing antibodies in their sera kept continual reduction of the weight after virus challenge, and all mice (100%) died

at day 11 (Fig. 4A–C). Observation of pathological changes in the challenged mouse lungs revealed almost normal lung tissues in the RBD-Fd-immunized mice (Fig. 4Da). These lung tissues were similar to those of normal mice (Fig. 4Db), with only slightly thickened alveolar wall (Fig. 4Da). By comparison, the PBS control mice challenged with MERS-CoV had marked interstitial pneumonia, including inflammatory cell infiltration, alveolar septal thickening, focal exudation, and hemorrhage (Fig. 4Dc). The hDPP4-Tg mice were immunized with RBD-Fd at 4 months old, boosted once 4 weeks later, challenged with MERS-CoV at 12 weeks post-boost, and survived for 3 weeks post-challenge before being sacrificed for evaluating pathological changes. Thus, the above results demonstrate the ability of RBD-Fd to induce long-term protective immunity in aging mice against MERS-CoV infection.

4. Discussion

Rapid development of a safe and efficacious vaccine with high potency has become the first priority to prevent continuous infection of MERS-CoV in humans. We have previously demonstrated that a RBD fragment containing residues 377–588 of MERS-CoV S protein is a critical neutralizing domain (CND) capable of inducing neutralizing antibodies against MERS-CoV infection (Ma et al., 2014b; Du et al., 2013a, 2016) and, hence, an important target for the development of subunit vaccines. It is known that native MERS-CoV S protein forms a trimer and is cleaved to form functional S1 and S2 subunits (Wang et al., 2013; Lu et al., 2013; Du et al., 2016), but it is still unknown whether the trimeric form of RBD (CND) of MERS-CoV S1 protein can induce protective immunity against MERS-CoV infection.

Fd, a trimerization motif, has been previously fused with recombinant proteins, such as influenza virus HA and HIV-1 glycoproteins and promotes them to form trimeric structures, subsequently stabilizing the proteins to broaden and increase their immunogenicity against infections from divergent virus strains (Grundner et al., 2005; G. Gao et al.,

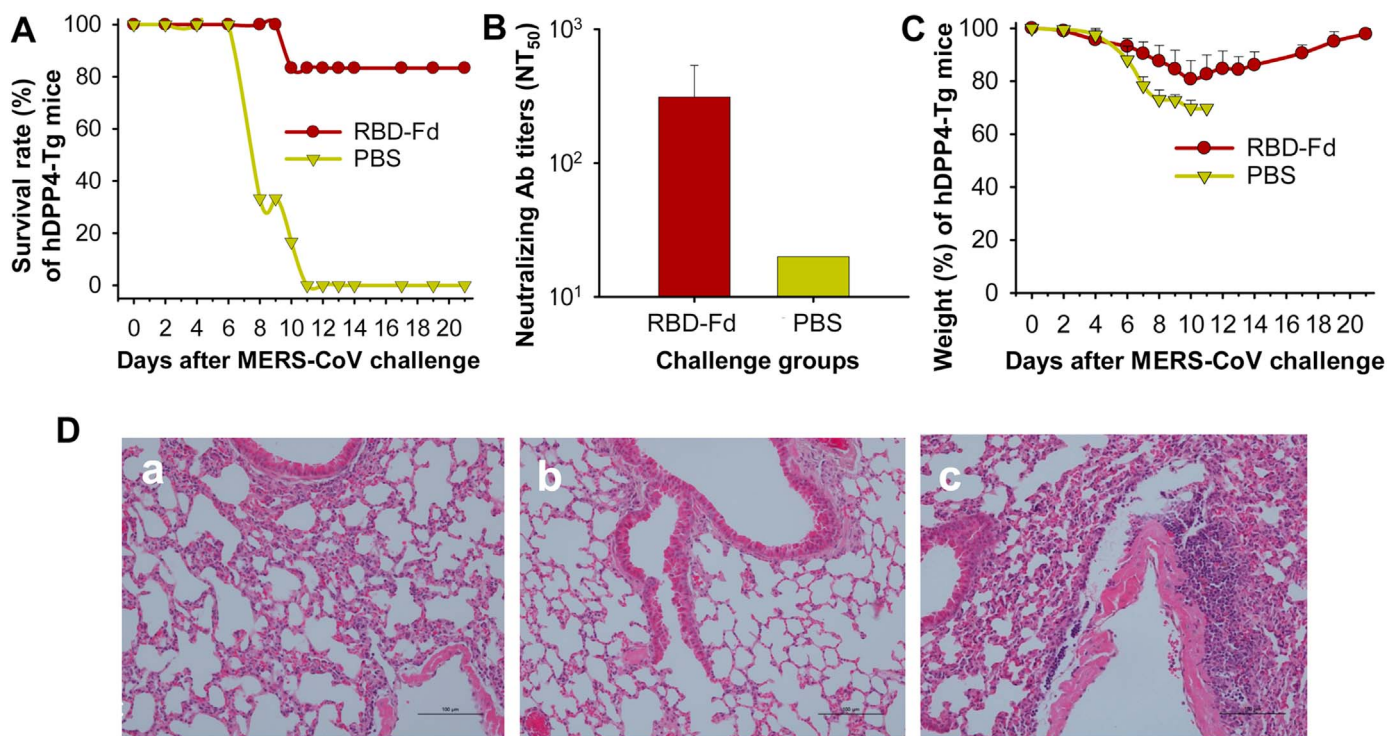


Fig. 4. Protective efficacy of RBD-Fd in hDPP4-Tg mice. Groups of 6 mice were challenged with MERS-CoV at 12 weeks post-last dose and then observed for 3 weeks for survival rate (A) and body weight changes (C). The data are presented as mean with SD. (B) Neutralizing antibodies were detected in the sera of immunized mice before MERS-CoV challenge. Neutralizing antibody titers are presented as mean $NT_{50} \pm SD$ of six mice in each group. For (A)–(C), PBS was included as the control. (D) Evaluation of pathological changes in lung tissues of challenged mice. Representative images from mice immunized with RBD-Fd (a) and PBS (c) challenged with MERS-CoV (EMC2012) and normal mice (b) are shown. Lung tissue sections were stained with hematoxylin and eosin (H & E) and observed under light microscopy (100 \times magnification).

2013; Nkolola et al., 2010; Du et al., 2013c; Li et al., 2013). Using MERS-CoV RBD fused with Fd (RBD-Fd) as a model antigen, this study attempts to generate a MERS-CoV RBD trimer, and then evaluate its antigenicity, receptor binding affinity, immunogenicity, neutralizing potential, and protective efficacy against MERS-CoV infection.

Indeed, RBD-Fd protein formed a trimeric structure that bound strongly to MERS-CoV RBD-specific, conformational epitope-targeting mAbs, as well as MERS-CoV's receptor DPP4, demonstrating its capacity to elicit highly efficacious IgG antibodies, along with Th2-based IgG1 and Th1-based IgG2 antibody responses. These results suggest that the trimeric MERS-CoV RBD maintains potent antigenicity, strong receptor-binding affinity, and high immunogenicity to induce robust antibody responses in the immunized animals. Particularly, RBD-Fd protein was able to induce long-term and highly potent neutralizing antibodies against infections of live and pseudotyped MERS-CoV of divergent strains isolated in the 2012–2015 outbreaks, possibly because RBD-Fd forms a trimeric structure to mimic the native conformation of the CND in MERS-CoV S protein, thus eliciting vigorous cross-neutralizing antibodies.

Due to the low expression of RBD protein monomer in the mammalian cell expression system, we were unable to obtain sufficient monomeric RBD protein to include it as a control in this study. Nevertheless, 83% of the survival rate and minimal pathological changes in the RBD-Fd-immunized hDPP4-Tg mice demonstrate its high protective efficacy against lethal MERS-CoV challenge. To further improve its protective immunity, RBD-trimer could be optimized by stabilizing its trimeric structure with disulfide bonds, adding other trimeric motifs, such as GCN4, adjusting linker sequences between the RBD and Fd, or combining these approaches, as reported for other viral proteins (Yang et al., 2000a, 2000b; Ringe et al., 2015). Since RBD-Fd only induced a background level of T cell responses (data not shown), its protective efficacy is largely associated with neutralizing antibodies. This is evidenced by that all of the mice survived from MERS-CoV infection had relatively high serum neutralizing antibody titers, suggesting that neutralizing antibodies, rather than cellular immune responses, may play a key role to prevent MERS-CoV infection in trimeric RBD-based subunit vaccines.

Overall, this report confirms the capability of MERS-CoV RBD's critical neutralizing epitopes to form a trimeric structure, resulting in the induction of strong humoral immune responses, potent neutralizing antibodies, and significant protective efficacy. It further emphasizes the importance of maintaining the native conformational structure of S protein in developing MERS subunit vaccines. In this context, our study provides valuable guidance for the rational design of efficacious subunit vaccines against MERS-CoV and other viruses with class I membrane fusion proteins.

Conflict of interest statement

The authors declared no conflict of interest.

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