RESEARCH PAPER

Human Vaccines & Immunotherapeutics 11:5, 1244–1250; May 2015; © 2015 Taylor & Francis Group, LLC

Optimization of antigen dose for a receptor-binding domain-based subunit vaccine against MERS coronavirus

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Keywords: antigen doses, MERS, MERS-CoV, receptor-binding domain, subunit vaccines

Middle East respiratory syndrome (MERS) is an emerging infectious disease caused by MERS coronavirus (MERS-CoV). The continuous increase of MERS cases has posed a serious threat to public health worldwide, calling for development of safe and effective MERS vaccines. We have previously shown that a recombinant protein containing residues 377–588 of MERS-CoV receptor-binding domain (RBD) fused with human Fc (S377-588-Fc) induced highly potent anti-MERS-CoV neutralizing antibodies in the presence of MF59 adjuvant. Here we optimized the doses of S377-588-Fc using MF59 as an adjuvant in order to elicit strong immune responses with minimal amount of antigen. Our results showed that S377-588-Fc at 1 μg was able to induce in the immunized mice potent humoral and cellular immune responses. Particularly, S377-588-Fc at 1 μg elicited strong neutralizing antibody responses against both pseudotyped and live MERS-CoV similar to those induced at 5 and 20 μg, respectively. These results suggest that this RBD-based subunit MERS vaccine candidate at the dose as low as one μg is sufficiently potent to induce strong humoral and cellular immune responses, including neutralizing antibodies, against MERS-CoV infection, thus providing guidance for determining the optimal dosage of RBD-based MERS vaccines in the future clinical trials and for applying the dose-sparing strategy in other subunit vaccine trials.

Introduction

Middle East respiratory syndrome (MERS) is a newly emerged infectious disease caused by MERS coronavirus (MERS-CoV).[1,2] First reported in Saudi Arabia in 2012,[3] the virus has now been identified in 20 other countries of the world and has led to the infection of 965 individuals with 357 deaths worldwide (a mortality rate ~37%) (http://www.who.int/csr/don/03-february-2015-mers/en/). Studies have indicated bats and camels as the natural reservoirs and intermediate transmission hosts of MERS-CoV, respectively, and they have, moreover, elucidated the bat-to-human transmission mechanism of MERS-CoV.[4-9] MERS-CoV has caused diseases in several family clusters and healthcare workers.[10-13] With continuous increase of human cases, MERS-CoV has posed a serious threat to public health worldwide, demonstrating the need to develop safe and effective vaccines against virus infection.

MERS-CoV spike (S) protein plays significant roles in mediating virus entry into target cells expressing viral receptor dipeptidyl peptidase 4 (DPP4) and subsequent fusion of virus and cell membranes.[14-16] To accomplish this, MERS-CoV depends on the receptor-binding domain (RBD) in the S1 subunit to bind host cellular receptors.[17-19] As such, RBD is an important target for the development of MERS vaccines.[20-24] Previous studies have mapped the RBD to the regions containing residues 358–588, 367–588, 377–588, and 367–606 of MERS-CoV S protein.[17–19,22,23,25]

It is known that a fragment containing residues 377–588 of MERS-CoV RBD is a critical neutralizing domain.[22-23,26] After comparing 5 different RBD fragments respectively containing residues 350–588, 358–588, 367–588, 367–606, and 377–588 of MERS-CoV S protein fused with Fc of human IgG, namely S350-588-Fc, S358-588-Fc, S367-588-Fc, S367-606-Fc, S377-588-Fc, we found that S377-588-Fc induced the highest antibody responses and neutralizing antibodies in immunized animals.[22–23] We have further compared the effects of several commercially available adjuvants, such as Freund's, aluminum, Monophosphoryl lipid A, Montanide ISA51, and MF59, in the

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Submitted: 01/05/2015; Revised: 02/04/2015; Accepted: 02/17/2015
http://dx.doi.org/10.1080/21645515.2015.1021527

promotion of immunogenicity of the aforementioned S377-588-Fc, and demonstrated that MF59 is an ideal adjuvant for use with this protein.[27] However, the minimal dose of the RBD protein required to induce sufficient immune responses against MERS-CoV infection remains to be elucidated. This calls for further optimization of the antigen dose for MERS subunit vaccines.

In this study, we examined the immunization potential of different doses of S377-588-Fc and compared their ability to induce specific humoral and cellular immune responses, particularly neutralizing antibodies against infection of MERS-CoV, using S377-588-Fc as a model antigen and MF59 as a selected adjuvant.

Results

S377-588-Fc at 1 μg was able to induce strong humoral immune responses

To optimize the dose of S377-588-Fc required to induce sufficient antibody responses, we immunized mice with S377-588-Fc at 1, 5, and 20 μg, respectively, and detected specific IgG antibody, as well as IgG1 and IgG2a subtypes, in immunized mouse sera.

As shown in **Figure 1A**, S377-588-Fc at all 3 test doses was able to induce MERS-CoV S1-specific IgG antibody response, with the antibody rapidly reaching a high level after the 2nd immunization and maintaining similar levels thereafter, suggesting that 2 doses of S377-588-Fc formulated with MF59 adjuvant are sufficient to induce strong antibody responses. As expected, only a background level of IgG antibody response was induced in the adjuvant only group (S377-588-Fc at 0 μg).

We then calculated and compared the endpoint IgG titers from the 3rd sera of mice immunized with the 3 RBD doses. Results, as shown in **Figure 1B**, revealed that S377-588-Fc at 1 μg induced high levels of IgG antibody response, but such response was significantly increased when the mice received 5 and 20 μg of the S377-588-Fc. Nevertheless, no significant difference was observed for IgG titers in the 5 and 20 μg immunization groups. As expected, mice receiving adjuvant only (S377-588-Fc at 0 μg) failed to induce MERS-CoV S1-specific IgG antibody response (**Fig. 1B**).

To elucidate the IgG subtypes induced by different doses of S377-588-Fc, we detected IgG1 and IgG2a production using mouse sera from the 3rd immunization. As shown in **Figure 2**, high titers of IgG1 (Th2) and IgG2a (Th1) antibodies were induced by 1, 5, and 20 μg of S377-588-Fc. However, S377-588-Fc at 5 and 20 μg induced a significantly higher level of IgG1 antibody than that at 1 μg (**Fig. 2A**). Notably, while no significant difference was revealed between titers of IgG1 and IgG2a antibodies induced by 5 and 20 μg of S377-588-Fc, 5 μg of S377-588-Fc appears to elicit stronger IgG2a antibodies than either 1 or 20 μg (**Fig. 2B**). As expected, no IgG subtypes were induced in the adjuvant control group (**Fig. 2**).

The above results suggest that 1 μg of S377-588-Fc is sufficient to induce high titers of RBD-specific antibody responses. Although S377-588-Fc at 5 and 20 μg could induce higher titers of total IgG and IgG1 subtype than those at 1 μg, the increased level of IgG and IgG1 may not necessarily provide stronger neutralizing antibody response that is required for protecting animals from MERS-CoV infection.

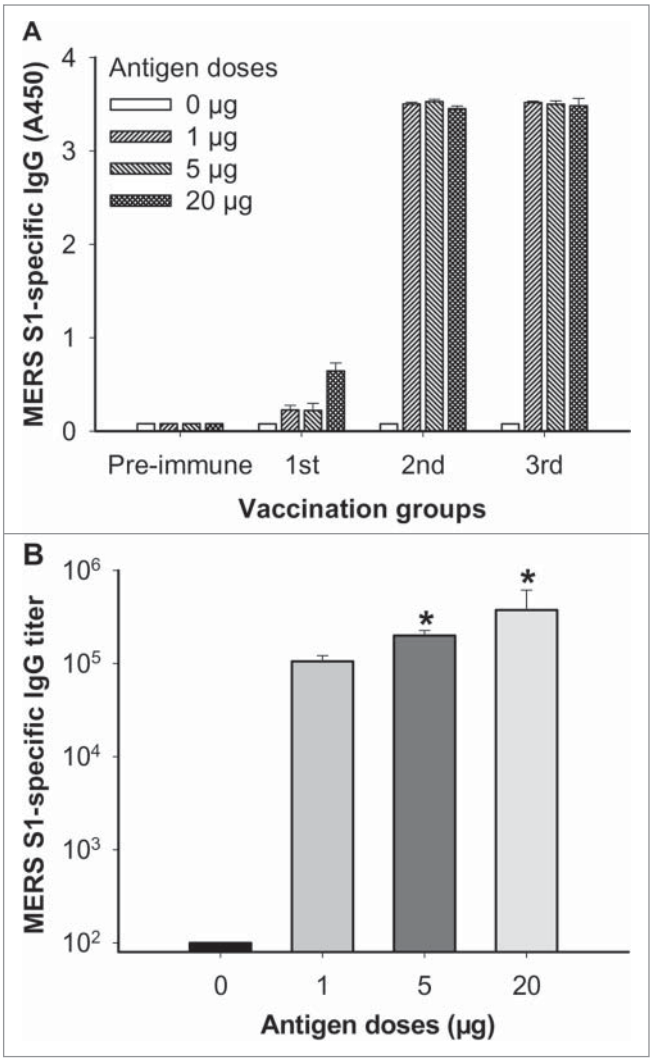

Figure 1. Comparison of IgG antibodies induced by S377-588-Fc at different antigen doses. Mice were immunized without (0 μg) or with 1, 5, or 20 μg of S377-588-Fc, and sera were assessed for MERS-CoV S1-specific IgG antibody by ELISA. (**A**) IgG was tested in sera (1:3,200 dilution) of mice before (pre-immune) and 10 d post-each immunization, and the data are presented as mean A450 ± standard deviation (SD) from 5 mice in each group. (**B**) Sera from 10 d post-last immunization were tested for S1-specific IgG titers and expressed as the endpoint dilutions that remain positively detectable. The data are presented as mean titers ± SD from 5 mice in each group. Significant differences were noted between the groups immunized with 5 or 20 μg, respectively, and 1 μg of MERS-CoV RBD (*).

S377-588-Fc at 1 μg induced high levels of neutralizing antibody responses, similar to those induced by 5 and 20 μg in immunized mice

To elucidate the neutralizing potential induced by different doses of S377-588-Fc and determine the minimal dose of such

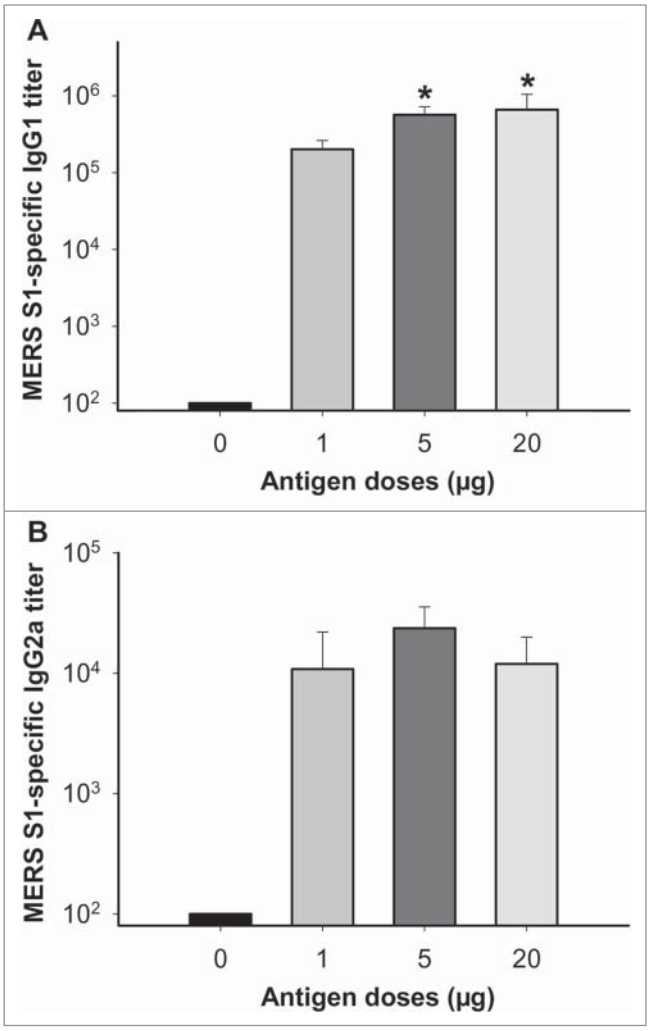

Figure 2. Comparison of antibody subtypes induced by S377-588-Fc at different antigen doses. Mice were immunized without (0 μg) or with 1, 5, or 20 μg of S377-588-Fc, and sera from 10 d post-last immunization were tested for MERS-CoV S1-specific IgG1 and IgG2a subtypes by ELISA. The titers were expressed as the endpoint dilutions that remained positively detectable, and the values are presented as mean ± SD from 5 mice in each group. Significant differences were noted in the IgG1 responses between the groups immunized with 5 or 20 μg, respectively, and 1 μg of MERS-CoV RBD (*).

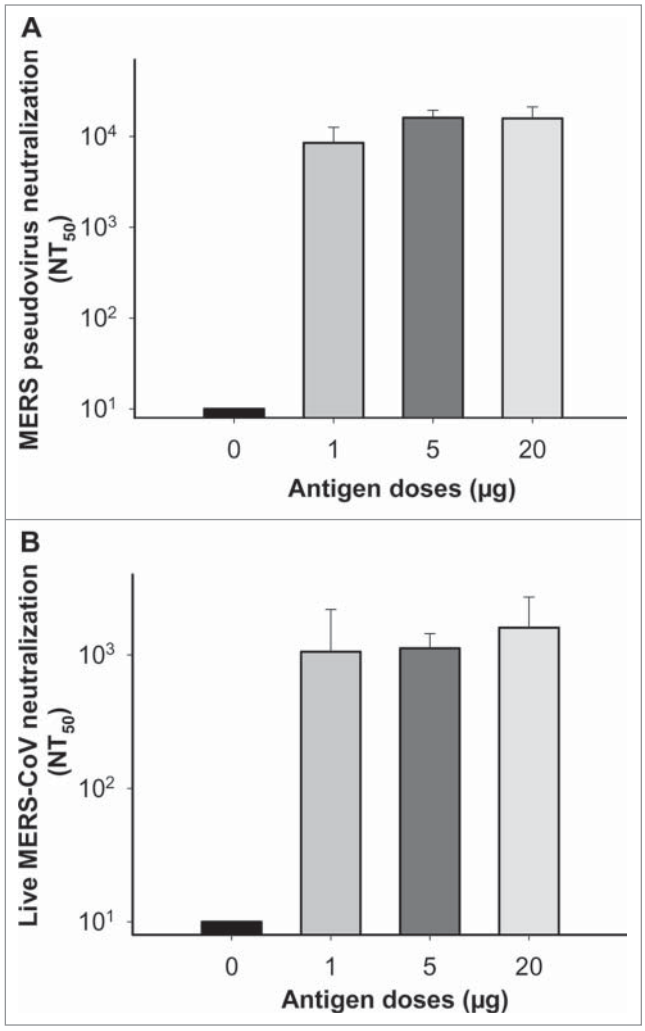

Figure 3. Comparison of neutralization induced by S377-588-Fc at different antigen doses. Mice were immunized without (0 μg) or with 1, 5, or 20 μg of S377-588-Fc, and sera from 10 d post-last immunization were tested for neutralization against MERS pseudovirus (**A**) and live MERS-CoV infection (**B**) in DPP4-expressing Huh-7 and Vero E6 cells, respectively. MERS pseudovirus neutralization data were expressed as 50% neutralizing antibody titers (NT_{50}), while live MERS-CoV-based neutralizing antibody titers were presented as the reciprocal of the highest dilution of sera that resulted in a complete inhibition of virus-induced CPE in at least 50% of the wells (NT_{50}). The data are presented as mean ± SD from 5 mice in each group.

an antigen required to elicit strong neutralization against MERS-CoV infection, we investigated neutralizing antibodies in mouse sera from the 3rd immunization based on MERS pseudovirus and live MERS-CoV neutralization assays. Results, as shown in **Figure 3**, demonstrated that S377-588-Fc at all 3 doses tested was able to induce potent neutralizing antibody titers against infections of MERS pseudovirus in Huh-7 cells (**Fig. 3A**) and live MERS-CoV in Vero E6 cells (**Fig. 3B**). There were no significant differences in the neutralizing activity among the sera of mice immunized with S377-588-Fc at 1, 5, and 20 μg, respectively, in the presence of MF59 adjuvant, while the sera from the adjuvant control mice (S377-588-Fc at 0 μg) showed no neutralizing activity (**Fig. 3**). These data suggest that S377-588-Fc at 1 μg concentration is able to induce sufficient MERS-CoV neutralizing antibodies in the immunized mice.

S377-588-Fc at 1 μg induced high levels of IFN-γ-expressing T cell responses in immunized mice

To compare the cellular immune responses induced by different doses of S377-588-Fc, we immunized mice with the protein at 1, 5, and 20 μg, respectively, and detected MERS-CoV S1-specific IL-2-and IFN-γ-expressing T cells in mouse splenocytes collected from the 3rd immunization. As shown in **Figure 4**, S377-588-Fc at 1 μg was capable of inducing strong IFN-

γ-expressing T cells in both $CD4^+$ (**Fig. 4A**) and $CD8^+$ (**Fig. 4B**) populations, while S377-588-Fc at the increased doses (5 or 20 μg) did not induce higher T cell responses. In addition, S377-588-Fc at 1, 5, or 20 μg failed to elicit strong IL-2-expressing $CD4^+$ and $CD8^+$ T cells. As expected, the adjuvant control group (S377-588-Fc at 0 μg) induced a background level of specific T cell responses. These results suggest that 1 μg of S377-588-Fc is sufficient to induce potent IFN-γ-expressing T cell responses in immunized mice.

Discussion

Development of effective vaccines is urgently needed to prevent continuous epidemic of MERS. Currently, several MERS vaccine candidates have been tested in preclinical settings, some of which show immunogenicity.[21,23,24,28–30] It was reported that a modified vaccinia virus Ankara (MVA) expressing full-length S protein and adenoviruses encoding full-length S protein or S1 subunit induced S-specific antibody responses that neutralized MERS-CoV infection *in vitro*,[28,29] indicating the potential of developing viral vector-based MERS vaccines. The nanoparticle-conjugated full-length S protein elicited neutralizing antibodies in mice, bringing some hopes for developing nanoparticle-based MERS vaccine.[31] An engineered replication-competent, propagation-defective MERS-CoV provides a platform to develop attenuated viruses as MERS vaccine candidates.[30] We and others have identified several protein fragments, including residues 350-588, 358-588, 367-588, 367-606, 377-588 and 377-622, of the RBD of MERS-CoV S protein that induced neutralizing antibodies in mice or rabbits,[21-23,25] suggesting the potential to develop subunit vaccines against MERS-CoV.

Among various vaccine types, such as those based on inactivated and live-attenuated viruses and viral vectors, recombinant protein-based subunit vaccines are considered to be the safest type of vaccines against virus infection.[20,26,32] However, the efficacy of subunit vaccines largely depends on the identification of suitable antigens and selection of appropriate adjuvants.[20,32] We have shown that a recombinant protein containing residues 377-588 of MERS-CoV RBD elicited the highest neutralizing antibodies among the 5 RBD fragments tested against MERS-CoV infection.[22,23,27]In addition, we have demonstrated that MF59 is most potent among the 5 adjuvants, including Freund's, aluminum, Monophosphoryl lipid A, MF59, and Montanide ISA51, to augment the immunogenicity of S377-588-Fc to induce strong antibody responses, neutralizing antibodies, and protection against MERS-CoV infection, suggesting it an ideal adjuvant for MERS-CoV RBD-based subunit vaccines.[27] Apart from

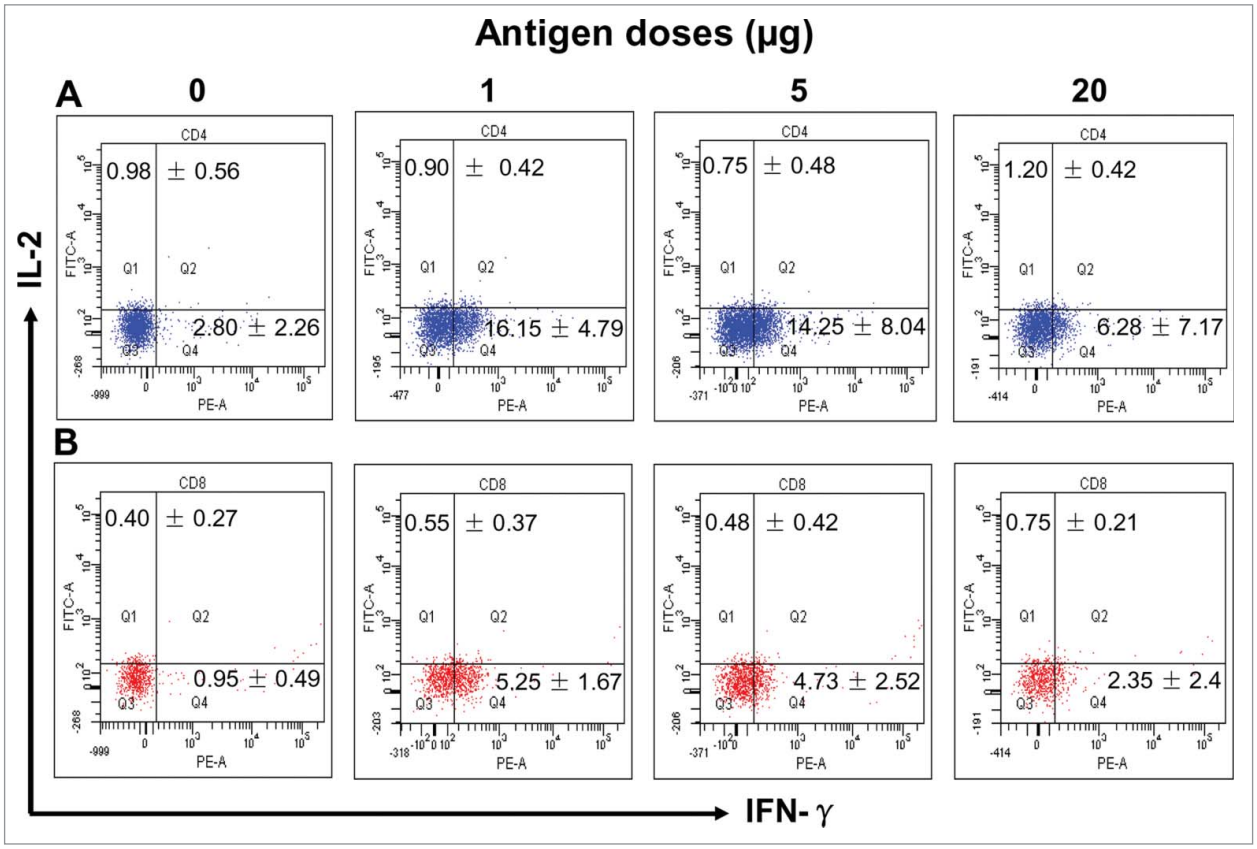

Figure 4. Comparison of cellular immune responses induced by S377-588-Fc at different antigen doses. Mice were immunized without (0 μg) or with 1, 5, or 20 μg of S377-588-Fc, and splenocytes from 10 d post-last immunization were tested for MERS-CoV S1-specific T cell responses by flow cytometric analysis. The frequencies of IL-2-(upper left corner) and IFN-γ-(bottom right corner) producing cells were expressed as percentages of $CD4^+$ (**A**) or $CD8^+$ (**B**) T cells. The samples were tested in triplicate and presented as mean ± SD from 5 mice in each group.

antigens and adjuvants, an equally crucial, but often neglected, aspect of immunization is the optimization of antigen dosage to find the minimal antigen dose able to induce strong immune responses.[33,34]

In this study, we compared the levels of immune responses in mice immunized with S377-588-Fc at 1, 5, and 20 μg, respectively, formulated with MF59 adjuvant. This range of doses was selected based on our previous studies showing that 10 μg of this protein induced strong immune responses with neutralizing activity that protected all of the vaccinated mice from challenge of MERS-CoV.[22,23] Here, we found that S377-588-Fc at 1 μg was able to elicit strong humoral and cellular immune responses in the immunized mice. Particularly, this protein at 1 μg induced high levels of neutralizing antibodies against infections of both MERS pseudovirus and live MERS-CoV, similar to those induced at 5 and 20 μg, respectively, suggesting that 1 μg of S377-588-Fc formulated with MF59 is sufficient to induce strong neutralizing antibody response capable of protecting mice from MERS-CoV challenge, as that was induced by 10 μg of S377-588-Fc formulated with MF59 in our previous study.[27] Therefore, only about 10% of S377-588-Fc that we tested before is actually needed to achieve the efficacy for prevention of MERS-CoV infection in vaccinated mice, based on the results from the present study.

Application of the lowest possible amount of the antigen and fewer injections is an important dose sparing strategy for a vaccine with low productivity (e.g., a subunit vaccine), especially during a pandemic or epidemic of an emerging infectious disease, like MERS. This study provides important information for rational design of optimal dosages of vaccines against MERS and other emerging infectious diseases for their future clinical trials.

Materials and Methods

Ethics statement

Four-to six-week-old female BALB/c 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 protocol was approved by the Committee on the Ethics of Animal Experiments of the New York Blood Center (Permit Number: 194.15).

Recombinant MERS-CoV RBD and S1 proteins

The recombinant S377-588-Fc containing residues 377-588 of MERS-CoV spike plus a C-terminal Fc tag (S377-588-Fc, hereinafter named RBD) was used as the antigen to optimize antigen doses for RBD-based subunit vaccines. The construction, expression and purification of the S377-588-Fc were described previously by fusing the RBD gene with human IgG Fc (InvivoGen, San Diego, CA), expressing the S377-588-Fc protein in 293T cell culture supernatant, and purifying it by Protein A affinity chromatography (GE Healthcare, Piscataway, NJ).[22,23] MERS-CoV S1 protein (residues 18–725) plus a C-terminal His_6 (S1-His) was constructed using the pJW4303 expression vector (Jiangsu Taizhou Haiyuan Protein Biotech, Co., Ltd., China), expressed in 293T cell culture supernatant, and purified using Ni-NTA Superflow (Qiagen, Valencia, CA).[23,24]

Animal immunization and sample collection

Mice were immunized with S377-588-Fc as previously described with some modifications.[21,23,35] Briefly, mice were subcutaneously prime-immunized with S377-588-Fc at 1, 5, and 20 μg, respectively, in the presence of MF59 adjuvant,[36] and boosted twice with the same immunogen and adjuvant at 3-week intervals. Adjuvant only without antigen (0 μg) was included as the negative control. Sera were collected before immunization and 10 d post-each vaccination to assess MERS-CoV S1-specific antibody responses and neutralizing antibodies. Immunized mice were sacrificed at 10 d after the last immunization, and splenocytes were collected to detect MERS-CoV S1-specific T cell responses.

ELISA

ELISA was used to test MERS-CoV S1-specific antibody responses in mouse sera based on our previously described protocols.[23,37] Briefly, ELISA plates were precoated with MERS-CoV S1-His protein overnight at 4°C, followed by addition of serially diluted sera and incubation at 37°C for 1 h. After four washes with PBST, the plates were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:3000, GE Healthcare), IgG1 (1:2000), or IgG2a (1:5000) (Invitrogen, Carlsbad, CA), respectively, at 37°C for 1 h, and washed 4 times. The reaction was visualized by substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Invitrogen, Carlsbad, CA) and stopped with 1 N H_2SO_4. The absorbance at 450 nm (A450) was measured by ELISA plate reader (Tecan, San Jose, CA).

MERS pseudovirus neutralization assay

This was done as previously described with some modifications.[38,39] 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 MERS-CoV (EMC-2012 strain) S protein using the calcium phosphate method. Cells were changed into fresh DMEM 8 h later, and pseudovirus-containing supernatants were harvested 72 h post-transfection for single-cycle infection of Huh-7 cells. The pseudovirus was incubated with serially diluted mouse sera at 37°C for 1 h before adding to the cells preplated in 96-well culture plates. Twenty-four hours later, cells were refed with fresh medium, which was followed by lysing cells 72 h later using cell lysis buffer (Promega, Madison, WI) and transferring the lysates 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 neutralization was calculated and expressed as 50% neutralizing antibody titer, NT_{50}.[40]

Live MERS-CoV neutralization assay

A standard micro-neutralization assay was used to confirm the anti-MERS-CoV neutralizing antibodies as previously

described.[23,41] Briefly, mouse sera were diluted at serial 2-fold in 96-well tissue culture plates and incubated for 1 h at room temperature with ~100 infectious MERS-CoV (EMC-2012) before transfer to Vero E6 cells. Seventy-two hours later, the neutralizing capacity of serum samples was assessed by determining the presence or absence of virus-induced cytopathic effect (CPE). Neutralizing antibody titers were expressed as the reciprocal of the highest dilution of sera that completely inhibited virus-induced CPE in at least 50% of the wells (NT_{50}).

Intracellular cytokine staining and flow cytometry analysis

MERS-CoV S1-specific cellular immune responses were evaluated in immunized mice by intracellular cytokine staining followed by flow cytometric analysis as previously described.[21,42,43] Briefly, mouse splenocytes (2×10^6) were stimulated with MERS-CoV S1-His protein for 5 h at 37°C with 5% CO_2 in the presence of GolgiPlug™ containing brefeldin A (1 μl/ml; BD Biosciences, San Jose, CA). The cells were stained with conjugated anti-mouse-CD4 (APC) and -CD8 (P-Cy5-5) antibodies for 30 min at 4°C. After washes, the cells were fixed using the Cytofix/Cytoperm™ Kit (BD Biosciences) and stained with anti-mouse-IL-2 (FITC) and -IFN-γ (PE) (BD Biosciences) antibodies for 30 min at 4°C. The stained cells were analyzed using a FACSCalibur (BD Biosciences) and FACSDiva software v.6.1.2 (BD Biosciences).

Statistical analysis

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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This study was supported by China National Project of Infectious Diseases grant (2014ZX10004001004), NIH grant (R21AI109094), and an intramural fund of New York Blood Center (NYB000068).

References

1. Drosten C, Seilmaier M, Corman VM, Hartmann W, Scheible G, Sack S, Guggemos W, Kallies R, Muth D, Junglen S, et al. Clinical features and virological analysis of a case of Middle East respiratory syndrome coronavirus infection. Lancet Infect Dis 2013; 13: 745-51; PMID:23782859; http://dx.doi.org/10.1016/S1473-3099(13)70154-3
2. Assiri A, Al-Tawfiq JA, Al-Rabeeah AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, Flemban H, Al-Nassir WN, Balkhy HH, Al-Hakeem RF, et al. Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. Lancet Infect Dis 2013; 13:752-61; PMID:23891402; http://dx.doi.org/10.1016/S1473-3099(13)70204-4
3. Zaki AM, van BS, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med 2012; 367:1814-20; PMID:23075143; http://dx.doi.org/10.1056/NEJMoa1211721
4. Yang Y, Du L, Liu C, Wang L, Ma C, Tang J, Baric RS, Jiang S, Li F. Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus. Proc Natl Acad Sci U S A 2014; 111:12516-21; PMID:25114257; http://dx.doi.org/10.1073/pnas.1405889111
5. Ithete NL, Stoffberg S, Corman VM, Cottontail VM, Richards LR, Schoeman MC, Drosten C, Drexler JF, Preiser W. Close relative of human Middle East respiratory syndrome coronavirus in bat, South Africa. Emerg Infect Dis 2013; 19:1697-9; PMID:24050621; http://dx.doi.org/10.3201/eid1910.130946
6. Lelli D, Papetti A, Sabelli C, Rosti E, Moreno A, Boniotti MB. Detection of coronaviruses in bats of various species in Italy. Viruses 2013; 5:2679-89; PMID:24184965; http://dx.doi.org/10.3390/v5112679
7. Azhar EI, Hashem AM, El-Kafrawy SA, Sohrab SS, Aburizaiza AS, Farraj SA, Hassan AM, Al-Saeed MS, Jamjoom GA, Madani TA. Detection of the middle East respiratory syndrome coronavirus genome in an air sample originating from a camel barn owned by an infected patient. MBio 2014; 5:e01450-14; PMID:25053787; http://dx.doi.org/10.1128/mBio.01450-14
8. Azhar EI, El-Kafrawy SA, Farraj SA, Hassan AM, Al-Saeed MS, Hashem AM, Madani TA. Evidence for camel-to-human transmission of MERS coronavirus. N Engl J Med 2014; 370:2499-505; PMID:24896817; http://dx.doi.org/10.1056/NEJMoa1401505
9. Reusken CB, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, Muth D, Raj VS, Smits-De VL, Corman VM, et al. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. Lancet Infect Dis 2013; 13:859-66; PMID:23933067; http://dx.doi.org/10.1016/S1473-3099(13)70164-6
10. Memish ZA, Zumla AI, Al-Hakeem RF, Al-Rabeeah AA, Stephens GM. Family cluster of Middle East respiratory syndrome coronavirus infections. N Engl J Med 2013; 368:2487-94; PMID:23718156; http://dx.doi.org/10.1056/NEJMoa1303729
11. Omrani AS, Matin MA, Haddad Q, Al-Nakhli D, Memish ZA, Albarrak AM. A family cluster of Middle East respiratory syndrome coronavirus infections related to a likely unrecognized asymptomatic or mild case. Int J Infect Dis 2013; 17:e668-72; PMID:23916548; http://dx.doi.org/10.1016/j.ijid.2013.07.001
12. Harriman K, Brosseau L, Trivedi K. Hospital-associated Middle East respiratory syndrome coronavirus infections. N Engl J Med 2013; 369:1761; PMID:24171525; http://dx.doi.org/10.1056/NEJMc1311004
13. Assiri A, McGeer A, Perl TM, Price CS, Al Rabeeah AA, Cummings DA, Alabdullatif ZN, Assad M, Almulhim A, Makhdoom H, et al. Hospital outbreak of Middle East respiratory syndrome coronavirus. N Engl J Med 2013; 369:407-16; PMID:23782161; http://dx.doi.org/10.1056/NEJMoa1306742
14. Lu L, Liu Q, Zhu Y, Chan KH, Qin L, Li Y, Wang Q, Chan JF, Du L, Yu F, et al. Structure-based discovery of Middle East respiratory syndrome coronavirus fusion inhibitor. Nat Commun 2014; 5:3067; PMID:24473083
15. Raj VS, Mou H, Smits SL, Dekkers DH, Muller MA, Dijkman R, Muth D, Demmers JA, Zaki A, Fouchier RA, et al. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature 2013; 495:251-4; PMID:23486063; http://dx.doi.org/10.1038/nature12005
16. Gao J, Lu G, Qi J, Li Y, Wu Y, Deng Y, Geng H, Li H, Wang Q, Xiao H, et al. Structure of the fusion core and inhibition of fusion by a heptad-repeat peptide derived from the S protein of MERS-CoV. J Virol 2013; 87:13134-40; PMID:24067982; http://dx.doi.org/10.1128/JVI.02433-13
17. Lu G, Hu Y, Wang Q, Qi J, Gao F, Li Y, Zhang Y, Zhang W, Yuan Y, Bao J, et al. Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. Nature 2013; 500:227-31; PMID:23831647; http://dx.doi.org/10.1038/nature12328
18. Chen Y, Rajashankar KR, Yang Y, Agnihothram SS, Liu C, Lin YL, Baric RS, Li F. Crystal structure of the receptor-binding domain from newly emerged Middle East respiratory syndrome coronavirus. J Virol 2013; 87:10777-83; PMID:23903833; http://dx.doi.org/10.1128/JVI.01756-13
19. Wang N, Shi X, Jiang L, Zhang S, Wang D, Tong P, Guo D, Fu L, Cui Y, Liu X, et al. Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4. Cell Res 2013; 23:986-93; PMID:23835475; http://dx.doi.org/10.1038/cr.2013.92
20. Zhang N, Jiang S, Du L. Current advancements and potential strategies in the development of MERS-CoV vaccines. Expert Rev Vaccines 2014; 13:761-74; PMID:24766432; http://dx.doi.org/10.1586/14760584.2014.912134
21. Ma C, Li Y, Wang L, Zhao G, Tao X, Tseng CT, Zhou Y, Du L, Jiang S. Intranasal vaccination with recombinant receptor-binding domain of MERS-CoV spike protein induces much stronger local mucosal immune responses than subcutaneous immunization: Implication for designing novel mucosal MERS vaccines. Vaccine 2014; 32:2100-8; PMID:24560617; http://dx.doi.org/10.1016/j.vaccine.2014.02.004
22. Du L, Kou Z, Ma C, Tao X, Wang L, Zhao G, Chen Y, Yu F, Tseng CT, Zhou Y, et al. A truncated receptor-binding domain of MERS-CoV spike protein potently inhibits MERS-CoV infection and induces strong neutralizing antibody responses: implication for developing therapeutics and vaccines. PLoS One 2013; 8:e81587;

PMID:24324708; http://dx.doi.org/10.1371/journal.pone.0081587
23. Ma C, Wang L, Tao X, Zhang N, Yang Y, Tseng CT, Li F, Zhou Y, Jiang S, Du L. Searching for an ideal vaccine candidate among different MERS coronavirus receptor-binding fragments - the importance of immunofocusing in subunit vaccine design. Vaccine 2014; 32:6170-6; PMID:25240756; http://dx.doi.org/10.1016/j.vaccine.2014.08.086
24. Du L, Zhao G, Kou Z, Ma C, Sun S, Poon VK, Lu L, Wang L, Debnath AK, Zheng BJ, et al. Identification of a receptor-binding domain in the S protein of the novel human coronavirus Middle East respiratory syndrome coronavirus as an essential target for vaccine development. J Virol 2013; 87:9939-42; PMID:23824801; http://dx.doi.org/10.1128/JVI.01048-13
25. Mou H, Raj VS, van Kuppeveld FJ, Rottier PJ, Haagmans BL, Bosch BJ. The receptor binding domain of the new Middle East respiratory syndrome coronavirus maps to a 231-residue region in the spike protein that efficiently elicits neutralizing antibodies. J Virol 2013; 87:9379-83; PMID:23785207; http://dx.doi.org/10.1128/JVI.01277-13
26. Zhang N, Tang J, Lu L, Jiang S, Du L. Receptor-binding domain-based subunit vaccines against MERS-CoV. Virus Res 2014; S0168-1702:00473-0; PMID:25445336; http://dx.doi.org/10.1016/j.virusres.2014.11.013
27. Zhang N, Channappanavar R, Ma C, Wang L, Tang J, Garron T, Tao X, Tasneem S, Lu L, Tseng CT, et al. Identification of an ideal adjuvant for receptor-binding domain-based subunit vaccines against Middle East respiratory syndrome coronavirus. Cell Mol Immunol 2015; PMID:25640653; http://dx.doi.org/10.1038/cmi.2015.03
28. Song F, Fux R, Provacia LB, Volz A, Eickmann M, Becker S, Osterhaus AD, Haagmans BL, Sutter G. Middle East respiratory syndrome coronavirus spike protein delivered by modified vaccinia virus ankara efficiently induces virus-neutralizing antibodies. J Virol 2013; 87:11950-4; PMID:23986586; http://dx.doi.org/10.1128/JVI.01672-13
29. Kim E, Okada K, Kenniston T, Raj VS, AlHajri MM, Farag EA, AlHajri F, Osterhaus AD, Haagmans BL, Gambotto A. Immunogenicity of an adenoviral-based Middle East Respiratory Syndrome coronavirus vaccine in BALB/c mice. Vaccine 2014; 32:5975-82; PMID:25192975; http://dx.doi.org/10.1016/j.vaccine.2014.08.058
30. Almazan F, DeDiego ML, Sola I, Zuniga S, Nieto-Torres JL, Marquez-Jurado S, Andres G, Enjuanes L. Engineering a replication-competent, propagation-defective Middle East respiratory syndrome coronavirus as a vaccine candidate. MBio 2013; 4:e00650-13; PMID:24023385; http://dx.doi.org/10.1128/mBio.00650-13
31. Coleman CM, Liu YV, Mu H, Taylor JK, Massare M, Flyer DC, Glenn GM, Smith GE, Frieman MB. Purified coronavirus spike protein nanoparticles induce coronavirus neutralizing antibodies in mice. Vaccine 2014; 32:3169-74; PMID:24736006; http://dx.doi.org/10.1016/j.vaccine.2014.04.016
32. Zhang N, Zheng B, Lu L, Zhou Y, Jiang S, Du L. Advancements in the development of subunit influenza vaccines. Microbes Infect 2014; 17:123-34; PMID:25529753; http://dx.doi.org/10.1016/j.micinf.2014.12.006
33. Belshe RB, Frey SE, Graham I, Mulligan MJ, Edupuganti S, Jackson LA, Wald A, Poland G, Jacobson R, Keyserling HL, et al. Safety and immunogenicity of influenza A H5 subunit vaccines: effect of vaccine schedule and antigenic variant. J Infect Dis 2011; 203:666-73; PMID:21282194; http://dx.doi.org/10.1093/infdis/jiq093
34. Frey SE, Harrison C, Pass RF, Yang E, Boken D, Sekulovich RE, Percell S, Izu AE, Hirabayashi S, Burke RL, et al. Effects of antigen dose and immunization regimens on antibody responses to a cytomegalovirus glycoprotein B subunit vaccine. J Infect Dis 1999; 180:1700-3; PMID:10515836; http://dx.doi.org/10.1086/315060
35. Du L, Zhao G, Sun S, Zhang X, Zhou X, Guo Y, Li Y, Zhou Y, Jiang S. A critical HA1 neutralizing domain of H5N1 influenza in an optimal conformation induces strong cross-protection. PLoS One 2013; 8:e53568; PMID:23320093; http://dx.doi.org/10.1371/journal.pone.0053568
36. Schultze V, D'Agosto V, Wack A, Novicki D, Zorn J, Hennig R. Safety of MF59 adjuvant. Vaccine 2008; 26:3209-22; PMID:18462843; http://dx.doi.org/10.1016/j.vaccine.2008.03.093
37. Du L, Zhao G, Yang Y, Qiu H, Wang L, Kou Z, Tao X, Yu H, Sun S, Tseng CT, et al. A conformation-dependent neutralizing monoclonal antibody specifically targeting receptor-binding domain in Middle East respiratory syndrome coronavirus spike protein. J Virol 2014; 88:7045-53; PMID:24719424; http://dx.doi.org/10.1128/JVI.00433-14
38. Zhao G, Du L, Ma C, Li Y, Li L, Poon VK, Wang L, Yu F, Zheng BJ, Jiang S, et al. A safe and convenient pseudovirus-based inhibition assay to detect neutralizing antibodies and screen for viral entry inhibitors against the novel human coronavirus MERS-CoV. Virol J 2013; 10:266; PMID:23978242; http://dx.doi.org/10.1186/1743-422X-10-266
39. Du L, Zhao G, Zhang X, Liu Z, Yu H, Zheng BJ, Zhou Y, Jiang S. Development of a safe and convenient neutralization assay for rapid screening of influenza HA-specific neutralizing monoclonal antibodies. Biochem Biophys Res Commun 2010; 397:580-5; PMID:20617558; http://dx.doi.org/10.1016/j.bbrc.2010.05.161
40. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006; 58:621-81; PMID:16968952; http://dx.doi.org/10.1124/pr.58.3.10
41. Tao X, Hill TE, Morimoto C, Peters CJ, Ksiazek TG, Tseng CT. Bilateral entry and release of Middle East respiratory syndrome coronavirus induces profound apoptosis of human bronchial epithelial cells. J Virol 2013; 87:9953-8; PMID:23824802; http://dx.doi.org/10.1128/JVI.01562-13
42. Du L, Zhao G, Lin Y, Sui H, Chan C, Ma S, He Y, Jiang S, Wu C, Yuen KY, et al. Intranasal vaccination of recombinant adeno-associated virus encoding receptor-binding domain of severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein induces strong mucosal immune responses and provides long-term protection against SARS-CoV infection. J Immunol 2008; 180:948-56; PMID:18178835; http://dx.doi.org/10.4049/jimmunol.180.2.948
43. Du L, Zhao G, Lin Y, Chan C, He Y, Jiang S, Wu C, Jin DY, Yuen KY, Zhou Y, et al. Priming with rAAV encoding RBD of SARS-CoV S protein and boosting with RBD-specific peptides for T cell epitopes elevated humoral and cellular immune responses against SARS-CoV infection. Vaccine 2008; 26:1644-51; PMID:18289745; http://dx.doi.org/10.1016/j.vaccine.2008.01.025