Recombinant Receptor Binding Domain Protein Induces Partial Protective Immunity in Rhesus Macaques Against Middle East Respiratory Syndrome Coronavirus Challenge

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Background: Development an effective vaccine against Middle East respiratory syndrome coronavirus (MERS-CoV) is urgent and limited information is available on vaccination in nonhuman primate (NHP) model. We herein report of evaluating a recombinant receptor-binding domain (rRBD) protein vaccine in a rhesus macaque model.

Methods: Nine monkeys were randomly assigned to high-dose, low-dose and mock groups, which were immunized with different doses of rRBD plus alum adjuvant or adjuvant alone at different time points (0, 8, 25 weeks). Immunological analysis was conducted after each immunisation. Monkeys were challenged with MERS-CoV at 14 days after the final immunisation followed by observation for clinical signs and chest X-rays. Nasal, oropharyngeal and rectal swabs were also collected for analyses. Monkeys were euthanized 3 days after challenge and multiple specimens from tissues were collected for pathological, virological and immunological tests.

Conclusion: Robust and sustained immunological responses (including neutralisation antibody) were elicited by the rRBD vaccination. Besides, rRBD vaccination alleviated pneumonia with evidence of reduced tissue impairment and clinical manifestation in monkeys. Furthermore, the rRBD vaccine decreased viral load of lung, trachea and oropharyngeal swabs of monkeys. These data in NHP paves a way for further development of an effective human vaccine against MERS-CoV infection.

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1. Introduction

The Middle East respiratory syndrome coronavirus (MERS-CoV) is the only lineage C betacoronavirus known to infect humans (Annan et al., 2013; Anthony et al., 2013; van Boheemen et al., 2012). Similarly to severe acute respiratory syndrome coronavirus (SARS-CoV), MERS-CoV infection can result in acute respiratory distress syndrome and organ dysfunction, including progressive renal function impairment (Zaki et al., 2012). According to the World Health Organization, by the end of 12 August 2015, a total of 1401 cases had been laboratory confirmed, with at least 500 deaths following MERS-CoV infection. Among them, 186 MERS-CoV infection cases, including 36 deaths, had been reported by the Republic of Korea (http://www.who.int/csr/don/12-august-2015-mers-saudi-arabia/en/). These recent clustered cases firstly sprang up outside the Arabian Peninsula, indicating the potential human-to-human transmission of MERS-CoV. To date, no specific antiviral drug exists for MERS-CoV infection and supportive treatment is the mainstay of management (Zumla et al., 2015). Ribavirin and interferon alfa-2b exhibited potential in a rhesus macaque model (Falzarano et al., 2013a, b), but in a retrospective cohort study, ribavirin and interferon alfa-2a therapy was associated with significantly improved survival at 14 days, but not at 28 days in patients with severe MERS-CoV infection (Omran et al., 2014). Besides, specific peptide fusion inhibitors of MERS-CoV (Ji et al., 2014), convalescent sera from recovered patient and human monoclonal neutralising antibodies (Jiang et al., 2014; Tang et al., 2014; Ying et al., 2014) provided a novel approach to MERS-CoV treatment. However, more data are needed from animal studies and carefully done clinical studies (Zumla et al., 2015). Therefore, developing a prophylactic vaccine against MERS-CoV infection remains a priority (Papaneri et al., 2015).

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Considerable evidence has proved that recombinant receptor binding domain (rRBD)-based subunit vaccine is a promising candidate vaccine against the SARS-CoV infection. As rRBD of SARS-CoV Spike protein induced strong neutralisation antibody and long-term protective immunity in rabbits and mice and completely protected immunized mice from SARS-CoV infection (Zhu et al., 2013). Furthermore, high titres of neutralisation antibodies in non-human primates (NHP) were induced by vaccination with the rRBD of SARS-CoV (Wang et al., 2012). Experience using rRBD-based subunit vaccines against SARS could inform the design of a rRBD-based MERS vaccine. Several human neutralising antibodies targeting the RBD of the MERS-CoV spike protein, have been identified from the naïve-antibody library (Tang et al., 2014; Ying et al., 2014), suggesting that RBD contains epitopes that may mediate potent neutralising activity against MERS-CoV. Our group and others have confirmed rRBD protein induced strong neutralising activity against MERS-CoV infection in mice and rabbits (Du et al., 2013; Lan et al., 2014; Ma et al., 2014; Mou et al., 2013; Zhang et al., 2015). Although the rRBD subunit vaccine is a highly potent neutraliser of antibodies and T-cell immune responses, no formulation has been tested on a higher animal model with MERS-CoV challenge to verify its prophylactic efficacy (Gretebeck and Subbarao, 2015). Recently, MERS-CoV infection and disease animal models have been developed (Agrawal et al., 2015; de Wit et al., 2013a,b; Fälzrano et al., 2014; Munster et al., 2013; Pascal et al., 2015; Yao et al., 2013; Zhao et al., 2014), including a rhesus macaque model of naturally permissive MERS-CoV disease (de Wit et al., 2013a,b; Munster et al., 2013; Yao et al., 2013). We herein evaluate a rRBD subunit vaccine in a rhesus macaque model, to identify a prophylactic approach that could be used in humans to prevent MERS-CoV infection.

2. Methods

2.1. Ethics Statement

Animal studies were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the People’s Republic of China. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the Chinese Centre for Disease Control and Prevention. The approved registration number is 20140609015. To comply with 3R (reduction, replacement, refinement) animal experiment principles, a total of 9 rhesus macaques were used.

During experiments, all procedures were performed under ethyl ether anaesthesia, and every effort was made to minimise suffering. Following inoculation with MERS-CoV, all experiments were conducted within an animal biosafety level 3 (ABSL-3) facilities.

2.2. Virus and Cell Culture

MERS-CoV strain (hCoV-EMC/2012) was kindly provided by Professor Fouchier (Erasmus Medical Centre, Netherlands). MERS-CoV seed stocks were propagated in Vero cells (ATCC Number: CCL-81) without mycoplasma contamination maintained in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% foetal bovine serum (FBS), 100 international units (IU)/mL penicillin, and 100 μg/mL streptomycin, cultured at 37 °C in 5% CO2. Seed stocks were diluted to the desired titre and used to determine the MERS-CoV 50% tissue culture infection dose (TCID50), and for neutralising antibody and virus inoculation assays.

2.3. Vaccine Formulation

MERS-CoV rRBD protein, containing a 240-amino-acid fragment spanning residues 367–606, was prepared using a baculovirus expression vector system and purified through a HisTrap HP and Superdex 200 column (GE Healthcare, Bucks, UK) as described previously (Lan et al., 2014; Lu et al., 2013). The rRBD protein was admixed with aluminium hydroxide (alum) adjuvant 1 day before immunisation.

2.4. Animal Immunisation and MERS-CoV Challenge

Nine monkeys were randomly assigned to high-dose (H), low-dose (L), and mock (M) groups (Table 1). High-dose animals were primed with 200 μg rRBD and boosted with 100-μg rRBD admixed with 1 mg of alum adjuvant. The three low-dose animals were primed with 50 μg rRBD and boosted with 25 μg rRBD admixed with 1 mg of alum adjuvant; control animals were immunised with PBS with 1 mg of alum adjuvant. Animals received their second immunisation 8 weeks subsequent to their first. To assess long-term immunological responses, the interval between the second and third immunisations was 17 weeks. Each vaccine formulation was administered intramuscularly (i.m.). Two weeks after each immunisation, animals were bled periodically to obtain serum and peripheral blood mononuclear cells (PBMCs) for immunomonitoring analysis.

One week after the final immunisation, animals were transferred to an ABSL-3 laboratory. Following an adaptive phase of 1 week, nine immunised monkeys were anaesthetised and inoculated intratracheally with MERS-CoV of the hCoV-EMC strain at a dosage of $6.5 \times 10^7$ TCID50, diluted in 1-mL DMEM. After the challenge with MERS-CoV, monkeys were observed twice daily, with detailed recording of clinical signs and symptoms, morbidity and mortality, including the nature, onset, severity and duration of all gross or visible changes. Chest X-rays were performed 1 day pre- and 1 and 3 days post-inoculation (dpi) with MERS-CoV. Nasal, oropharyngeal and rectal swabs were collected at the same time points. Three days after challenge with MERS-CoV, monkeys were euthanized and tissue specimens, including lungs, trachea, spleen and kidney, were collected for various pathological, virological, and immunological tests. The schematic of the vaccination schedule and biological specimen collection timeline are displayed in Fig. 1.

2.5. IgG Antibody Analyses Using Enzyme-linked Immunosorbent Assay (ELISA)

Animals were bled periodically throughout the study (Fig. 1) to derive serum for antibody detection. Endpoint anti-RBD IgG antibody titres were determined by ELISA as described previously (Lan et al., 2014).

2.6. Pseudovirus Neutralisation Assay

A MERS-CoV pseudovirus system was conducted within biosafety level-2 facilities as reported previously (Lan et al., 2014). Relative light units were determined immediately using a Gaomax lumimeter (Promega). All experiments were performed in triplicate. The pseudovirus inhibition (PI) rate was calculated as follows:

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\text{Relative luciferase units of mock sera} = \frac{\text{Relative luciferase units of immune serum for a given dilution}}{\text{Relative luciferase units of mock sera}}
\]

2.7. Neutralising Antibody to hCoV-EMC

The virus neutralisation assay was conducted on Vero Cells. Briefly, sera were diluted twofold and mixed with 50 μL of $2 \times 10^5$ TCID50/mL virus, incubated at $37^\circ\text{C}$ for 1 h in quadruplicate. Thereafter, 100-μL virus-serum mixture was added to Vero cells previously seeded at $1.5 \times 10^5$/well. Inoculated plates were incubated in a CO2 incubator at $37^\circ\text{C}$ for 3 days, following which the cytopathic effects (CPEs) of the virus were observed microscopically at $40 \times$ magnification. All tests were repeated twice independently.
Additionally, nasal, oral and rectal swabs were collected at trachea, spleen and kidney specimens of challenged monkeys were acquired at 3 dpi for detection.

Table 1
Rhesus macaque vaccination group.

| Group          | Code | Gender | Age | Vaccine | Immunisation (i.m.) |
|----------------|------|--------|-----|---------|---------------------|
|                |      |        |     |         | 1st       | 2nd       | 3rd       | MERS-CoV (TCID50) |
| M (mock)       | 6#   | M      | 6   | PBS     | 100 μL | 100 μL | 100 μL | 6.5 × 10^7 |
| 4#            | M    | 7      | 5   |         |         |         |         |             |
| 12#           | F    | 5      |     |         |         |         |         |             |
| H (high-dose)  | B#   | M      | 6   | rRBD    | 200 μg | 100 μg | 100 μg | 6.5 × 10^7 |
| F#            | F    | 5      |     |         |         |         |         |             |
| G#            | F    | 7      |     |         |         |         |         |             |
| L (low-dose)   | A#   | M      | 5   | rRBD    | 50 μg  | 25 μg  | 25 μg  | 6.5 × 10^7 |
| D#            | M    | 7      |     |         |         |         |         |             |
| H#            | F    | 7      |     |         |         |         |         |             |

2.8. Enzyme-linked Immunospot Assay (ELISPOT)

To evaluate the antigen-specific T-cell response induced by rRBD protein in immunised monkeys, an IFN-γ ELISPOT assay was performed as described previously (Wen et al., 2013). After PBMCs were isolated from EDTA-treated blood, IFN-γ ELISPOT assay was carried out according to the manufacturer’s instructions. Briefly, 2.5 × 10^5 cells per well were stimulated with pools of 15-mer peptides overlapping by 11 amino acids spanning the RBD protein of MERS-CoV at a final concentration of 4 μg/ml into a PVDF membrane-bottomed plate pre-coated with anti-monkey IFN-γ capture antibody. A set of negative control wells comprising media only were included on each plate. After stimulation for 20–24 h, IFN-γ-spot-forming cells were detected by staining membranes with the detection antibody (7-B6-1-biotin) followed by streptavidin-horseradish peroxidase and development with TMB (3, 3′, 5, 5′-tetramethylbenzidine) substrate solution. Results are presented as spot-forming cells per 10^6 PBMCs. Values were reported as the mean of the test wells minus the mean of all negative-control wells.

2.9. RNA Extraction and Quantification Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from individual samples using the RNeasy Mini Kit (Qiagen) and reverse transcription reactions were performed using the Superscript III First Strand Synthesis Kit (Invitrogen), as described previously (Yao et al., 2013), to derive complementary DNA (cDNA). All cDNA samples were stored at −20 °C until use in the qRT-PCR, targeted to upE as described previously (Corman et al., 2012).

2.10. Viral Isolation From Tissue and Swab Samples

Even though the qRT-PCR is considered as a highly sensitive assay for detection the viral nucleic acid, an assay of viral isolation is absolute necessity to detect the real viral replication. To this end, all tissues were homogenised to a final 10% suspension in DMEM and clarified by low-speed centrifugation at 4500 g for 30 min at 4 °C. Swab samples were immersed in 1-mL DMEM, vortexed, and clarified by low-speed centrifugation at 5000 g for 10 min at 4 °C. Virus titres were determined in Vero cell monolayers grown in 96-well plates as described previously (Yao et al., 2013). In brief, 1.5 × 10^4/well of Vero cells were seeded in a 96-well plate and incubated overnight at 37 °C in a CO2 incubator. One-hundred microliters of 10-fold serially diluted suspension was then added to each well in quadruplicate. The virus was allowed to adsorb to the cells at 37 °C for 1 h. After adsorption, viral inocula were removed, and 100 μL of DMEM (2% FBS) was added to each well. Plates were incubated in a CO2 incubator at 37 °C for 3 days, following which CPEs were observed microscopically at 40× magnification. The virus titres expressed as the TCID50 of each specimen were calculated by the Reed–Muench method.

2.11. Histopathology and Immunohistochemistry

Necropsy was performed on all animals according to the standard protocol. Lung, trachea, spleen and kidney tissue samples were collected for histopathological and immunohistochemical examination. Procedures were performed using fixed 10% neutral buffered formalin, embedded in paraffin, sectioned at 4-μm thickness, and stained with haematoxylin and eosin (HE) prior to examination by light microscopy. For immunohistochemistry analysis, staining methods included antigen retrieval with citrate buffer. A rabbit-serum-derived polyclonal antibody against nucleoprotein (Sino Biological Inc., cat: 100213-RP02) at 1:1000 dilution, was then incubated with the sections. The secondary antibody used was goat anti-rabbit (ZSGB-Bio, cat: pv-9001) at 1:2000. Finally, results were evaluated using light microscopy. To acquire a subject result, different and multiple sites of the lung, trachea, spleen and kidney tissue samples were collected for the histopathology and immunohistochemistry analysis. Finally, quantification of 4–5 slides per animal was used to assess the effect and impact of the immunized animals.

Fig. 1. Schematic diagram of the rRBD vaccination and MERS-CoV challenge schedule. Monkeys were immunised three times intramuscularly (i.m.), at intervals of 8 or 17 weeks, and inoculated intratracheally with hCoV-EMC at a dosage of 6.5 × 10^7 TCID50 (red arrow). Monkeys were bled periodically at 2, 10, and 25 weeks (−14 days), and at −1 day, 1 day and 3 days. Additionally, nasal, oral and rectal swabs were collected at −1 day, 1 day and 3 days. Chest X-rays were performed 1 day before inoculation, and at 1 and 3 dpi. Following euthanasia, lung, trachea, spleen and kidney specimens of challenged monkeys were acquired at 3 dpi for detection.
2.12. Statistical Analysis

Statistical analyses were conducted using one-way ANOVA with Bonferroni post-test via the SPSS for Windows software package (ver. 17, SPSS Inc., Chicago, IL, USA). Unpaired two-tailed Student’s t test was used to compare means between different groups. A value of \( p < 0.05 \) was taken to indicate statistical significance. Results are expressed as mean ± SD. All figures were rendered using the Prism 5 software package (GraphPad Software, CA, USA).

3. Results

3.1. rRBD Vaccination Induced Sustained and Robust Immunity in the Rhesus Macaque

To assess the humoral immune response to the rRBD vaccine in rhesus macaques, total anti-MERS-CoV RBD IgG antibody titres were determined by ELISA. Low titres of IgG in serum samples before prime vaccination were detected, possibly due to non-specific responses. IgG titres in the high-dose group increased significantly (\( p < 0.05 \)) after the first vaccination (Fig. 2a), and achieved the highest titre of RBD-specific IgG 2 weeks after the second immunisation. Sera IgG titre in the low-dose group increased (\( p < 0.05 \)) only after the second immunisation, to almost the same level as that of the high-dose group. RBD-specific IgG in the sera of immunised rhesus macaques was preserved for at least 17 weeks without obvious decline. In addition, IgG titre increased rapidly after a subsequent boost. Increased IgG titre was particularly marked in the low-dose group.

Circulating neutralising antibody titres were monitored simultaneously in a pseudovirus system. Neutralisation antibodies were detected only after the second vaccination. When the pseudovirus inhibition rate was at 50% (\( PI_{50} \)), the neutralisation antibody titres of sera 2 weeks after the second vaccination were 1:800 and 1:1600, in the low- and high-dose groups, respectively (Fig. 2b). Non-specific neutralisation antibody titres in the mock group were at 1:300. Detection of neutralisation antibodies in rRBD immunised monkeys persisted for at least 17 weeks. Neutralisation antibody titres (\( PI_{50} \)) of the sera were at 1:400 and 1:700 in the low- and high-dose groups, respectively (Fig. 2c). Encouragingly, following the next boost, immunisation neutralisation antibody titres (\( PI_{50} \)) were as high as 1:800 and 1:1200.
Neutrality antibody titres in sera following rRBD vaccination were also detected in Vero cells by the hCoV-EMC strain of MERS-CoV. Neutralisation antibody titres in the sera of rRBD immunised monkeys at week 10 were at 1:269 and 1:363 in the low- and high-dose groups, respectively (Fig. 2e). After 17 weeks, neutralisation antibody titres decreased slightly. Following a subsequent immunisation boost, neutralisation antibodies 1 day pre-MERS-CoV challenge were almost as high as at week 10. At all detection time points, non-specific neutralisation antibody titres in the mock group were lower or equal to 1:10.

To assess the T-cell immune response, rhesus macaque PBMCs derived at different times following rRBD immunisation were stimulated with overlapping synthetic peptides of RBD; ELISpot analyses were then conducted. Immunisation with rRBD proteins induced antigen specific IFN-γ-secreting T-cells. In addition, high-dose rRBD induced significantly higher levels of IFN-γ-secreting T-cells (Fig. 2f).

3.2. rRBD Vaccination Alleviated Pneumonia in the Rhesus Macaque Upon Challenge With MERS-CoV

Following MERS-CoV challenge, all monkeys were observed twice daily, with detailed recording of clinical signs, symptoms, morbidity, and mortality, including the nature, onset, severity, and duration of all gross or visible changes. After challenge, all monkeys, including rRBD-immunised animals, drastically decreased their water intake. No infected monkeys exhibited excessive weight loss or died during the experiment.

To monitor signs of pneumonia in MERS-CoV challenged animals, X-ray examination was performed 1 day pre-, 1 and 3 days after MERS-CoV inoculation. Radiography revealed that all monkeys were negative for pneumonia before MERS-CoV challenge (−1 day). In mock group animals, local interstitial inflammation was detected at 1 dpi, with pneumonia diffused to adjacent pulmonary lobes at 3 dpi. Pneumonia generated in the high-dose group was delayed to 3 dpi, with inflammation confined to a single lobe (Fig. 3a). Although pneumonia was detected in low-dose monkeys at 1 dpi, infiltration and interstitial inflammation was less severe compared with the mock group.

The gross pathology of challenged animals was investigated and necropsies were performed at 3 dpi following euthanasia. The lungs of mock group animals were congested with palpable nodules (Fig. 3b). Dark red discoloration and multifocal consolidation was consistent with the interstitial pneumonia indicated by X-ray examination. In contrast, haemorrhage and necrosis shown by black circle in the rRBD-groups were smaller in both high and low dose immunization (Fig. 3b).

To investigate pneumonia following MERS-CoV infection, histopathological and immunohistochemical analyses were conducted to elucidate the effect of rRBD vaccination. Control monkey lung tissues exhibited moderate lesions (Fig. 4a) with abundant alveolar oedema, fibrin formation in the hyaline member and a small-to-moderate degree of macrophage fibrous infiltration (Fig. 4b). In contrast, a mild-to-moderate sized lesion was observed in the lung tissue of rRBD-immunised monkeys (Fig. 4c–f). Infiltration of inflammatory cells was also detected in the tracheas of mock group monkeys (Fig. 4g). Although inflammatory cell infiltration and impaired epithelium in the tunica mucosa bronchiorum were also found in the rRBD vaccination group (Fig. 4h–i), all of the lesion were alleviated compared to the control group. Glomerular capsule expansion was observed in the kidneys of all challenge monkeys (data not shown), but this was considered an incidental, clinically non-significant finding. No specific pathological changes were observed in the spleens of immunised monkeys (data not shown). Viral antigen, using a polyclonal antibody against nucleoprotein, was found by IHC in the lungs (Fig. 5a–c) and tracheas (Fig. 5d–f) of all monkeys. No specific antigen was detected in the kidneys of challenged monkeys (data not shown).
3.3. rRBD Vaccination Decreased Viral Load in the Rhesus Macaque Upon Challenge

qRT-PCR, using upE gene primers specific for MERS-CoV, was conducted to determine viral loads in serum, swabs and tissues, including lung, trachea and kidney. Viral RNA was detected in throat, nasal and rectal swabs (Fig. 6a) at 1 dpi and 3 dpi. Similarly, viral RNA in lung and tracheal tissue homogenate were detected at 3 dpi (Fig. 6b). Compared with control monkeys, the rRBD vaccine decreased \((p < 0.05)\) viral loads in the oronasopharynx swab, lungs and trachea. No viral RNA was detected in the kidney or serum of challenged monkeys.

Virus titres of various tissues and swabs of immunised animals were determined by end-point titration in Vero cells. Viral loads in the lungs and trachea of control monkeys were as high as 50 and 100 TCID\(_{50}\)/mL, respectively (Table 2), but rRBD-immunised monkeys could not isolate MERS-CoV from the lungs. Although MERS-CoV could be isolated from two of the six rRBD-immunised monkeys, virus titres were lower vs. the mock group.

4. Discussion

The MERS virus, which emerged in 2012, is now considered a threat to global public health. Developing an effective vaccine is important to control MERS-CoV infection. To date, virus-vector vaccines, such as recombinant modified vaccinia virus Ankara (MVA), which expresses full-length MERS-CoV spike protein (MVA-MERS-S), and recombinant adenoviral vectors encoding the full-length MERS-CoV S protein (Ad5.MERS-S) and the S1 extracellular domain of S protein (Ad5.MERS-S1), have been developed and tested for their ability to induce virus-neutralising antibodies in mice (Kim et al., 2014; Song et al., 2013; Volz et al., 2015). Besides, the rRBD subunit vaccine confers a highly potent neutralising antibody and T-cell immune response in mice (Zhang et al., 2014). However, few studies have been assessed in response to MERS-CoV challenge to verify their efficacy, due to a lack of animal models. For example, mice (Coleman et al., 2014; Scobey et al., 2013), hamsters (de Wit et al., 2013a,b) and ferrets (Raj et al., 2014) had not been proved to infect with MERS-CoV naturally. Recently, a small animal model of MERS-CoV infection was developed by transducing mice with an adenovirus vector expressing human DPP4 (Zhao et al., 2014). However, MERS-CoV infection in this model is highly dependent on the transduction of cells and the level of DPP4 expression from the adenovirus vector, and therefore does not necessarily reflect the natural disease process (Falzarano et al., 2014). More recently, a transgenic mouse model of MERS-CoV has been published (Agrawal et al., 2015). However, in this model, all cells of the mouse express human DPP4. This kind of non-physiological expression patterns resulted in extensive brain infection of MERS-CoV and rapidly succumbs to infection in mice. Moreover, a novel humanized mouse model of MERS-CoV has been published (Agrawal et al., 2015). Encouragingly, rhesus macaque models are naturally permissive to MERS-CoV disease, and more closely mimic the disease course in human patients (de Wit et al., 2013a,b; Munster et al., 2013; Yao et al., 2013). Furthermore, the effects of...
interferon-α2b and ribavirin treatment have been evaluated in these models (Falzarano et al., 2013a,b). Therefore, we herein assessed rRBD vaccine efficacy in a NHP model.

Similar as the rRBD protein of SARS-CoV which induced high titres of protective anti-RBD antibody responses in NHPs (Wang et al., 2012), the rRBD vaccination of MERS-CoV induced effective IgG and neutralisation antibodies in our rhesus macaque model. Besides, the induced IgG and neutralisation antibodies maintained for at least 17 weeks without obvious attenuation. Following a subsequent boost, antibody titres reached almost their previous peak level. There was no evident difference for induction of the humoral immunity between high and low dose groups. However, high-dose vaccination induced a stronger T-cell response than the low-dose vaccination. Following MERS-CoV challenge, rhesus macaques exhibited a transient lower respiratory tract infection, in accordance with previous reports (de Wit et al., 2013a,b; Munster et al., 2013; Yao et al., 2013). Although infection was also detected in rRBD-immunised animals, clinical signs were alleviated. Pathological changes in the lungs and tracheas of rRBD-immunised animals indicated reduced, but not fully absent lesions. Using qRT-PCR assay, viral loads in the lungs, trachea and oropharyngeal swabs of the rRBD vaccination groups were decreased. Furthermore, rRBD-immunised monkeys could not isolate MERS-CoV from the lungs while viral loads in the lungs and trachea of mock monkeys were as high as 50 and 100 TCID₅₀/mL, respectively. Consistent with the results of immunity elicited by high and low

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**Fig. 5.** IHC staining of immunised rhesus macaque lung and trachea 3 days after MERS-CoV infection by a polyclonal antibody against nucleoprotein. a–c. IHC analysis of lung tissue. d–f. IHC staining of trachea tissue. Black arrows indicate the distribution of viral antigen. M, H and L represent mock, high- and low-dose groups, respectively.

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**Fig. 6.** MERS-CoV loads in the swabs and tissues of immunised rhesus macaques following MERS-CoV infection detected by Real-time-PCR. a. Viral loads in swab samples at 1 dpi and 3 dpi. b. The viral loads in lung and trachea tissue 3 days after MERS-CoV infection. All of the detections were replicated for three times. M, H and L represent mock, high- and low-dose groups, respectively. *p < 0.05.
dose of rRBD vaccine, the protection against MERS-CoV infection in monkey could be contributed to the humoral immune especially the neutralising antibodies rather than the cellular mediated immunity.

Although rRBD vaccination alleviated pneumonia and decreased viral load in the rhesus macaque upon challenge with MERS-CoV in this study, it did not prevent the infection of MERS-CoV completely. The data should be interpreted with some cautions. First, the rhesus macaque models to mimic MERS-CoV infection had inevitable limitations. In both the present and previous reports, MERS-CoV challenge in the rhesus macaque models resulted in transient infection of the lower respiratory tract (de Wit et al., 2013a,b; Munster et al.; Yao et al., 2013) and the more severe or even lethal disease course frequently associated with human cases was not observed herein (van den Brand et al., 2015). Therefore, the prophylactic effects of the rRBD vaccine were not fully demonstrated in this model. Second, MERS-CoV challenge in the trachea bypasses the natural entry sites, such as nasal mucosa and laryngeal areas of the pharynx which show as crucial regions of mucosal immunity (Sato and Kiyono, 2012), while, the bypassed mucosal immunity is the first defence against lots of infectious diseases, including MERS-CoV (Neutra and K. P., 2006). Third, the challenge dose of 6.5 × 107 TCID50 was markedly greater than typical MERS-CoV exposure in humans. More recently, a progressive and lethal pneumonia was observed in a MERS-CoV challenged common marmoset model (Annan, A., Baldwin, H.J., Corman, VM, Klose, SM, Dvoua, M, Nikrumah, E.E, Badu, E.K., Anit, P., Agbenyea, O., Meyer, B., Oppong, S., Sarkodie, Y.A., Kalko, E.K.V., Uima, P.H.C., Godlewska, E.V., Reusken, C., Seebens, A., Glozo-Rausch, F., Vallo, P., Tschapka, M., Drosten, C., Drexler, J.F., 2013. Human betacoronavirus 2c EMC/2012-related viruses in bats, Ghana and Europe. Emerg. Infect. Dis. 19 (3), 456–459. Anthony, S.J., Ojeda-Flores, R., Rico-Chavez, O., Navarrete-Macias, I., Zambrana-Torrelio, C.M., Rostal, M.K., Epstein, J.H., Tippis, T., Liang, E., Song, M., Sotomayor-Bonilla, J., Aguirre, A.A., Avila-Flores, R., Medellin, R.A., Rolde, T., Sanz, G., Diazak, P., Lipkin, W.J., 2013. Coronavirus in bats from Mexico. J. Gen. Virol. 94 (Pt. 5), 1028–1038. Coleman, C.M., Matthews, K.L., Gicquelco, L., Biermark, M.B., 2014. Wild-type and innate immune-deficient mice are not susceptible to the Middle East respiratory syndrome coronavirus. J. Gen. Virol. 95 (Pt. 2), 408–412. Corman, V.M., Muller, M.A., Costabel, U., Timm, J., Binger, T., Meyer, B., Krehmer, P., Lattwein, E., Eschbach-Bludau, M., Nitsche, A., Bleicker, T., Landt, O., Schweiger, B., Drexler, J.F., Osterhaus, A.D., Haagmans, B.L., Dittmer, U., Bonif, F., Wolff, T., Drosten, C., 2012. Asays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro Surveill. 17 (49).

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