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Systemic and mucosal immunity in mice elicited by a single immunization with human adenovirus type 5 or 41 vector-based vaccines carrying the spike protein of Middle East respiratory syndrome coronavirus

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Summary

An ideal vaccine against mucosal pathogens such as Middle East respiratory syndrome coronavirus (MERS-CoV) should confer sustained, protective immunity at both systemic and mucosal levels. Here, we evaluated the *in vivo* systemic and mucosal antigen-specific immune responses induced by a single intramuscular or intragastric administration of recombinant adenoviral type 5 (Ad5) or type 41 (Ad41) -based vaccines expressing the MERS-CoV spike (S) protein. Intragastric administration of either Ad5-S or Ad41-S induced antigen-specific IgG and neutralizing antibody in serum; however, antigen-specific T-cell responses were not detected. In contrast, after a single intramuscular dose of Ad5-S or Ad41-S, functional antigen-specific T-cell responses were elicited in the spleen and pulmonary lymphocytes of the mice, which persisted for several months. Both rAd-based vaccines administered intramuscularly induced systemic humoral immune responses (neutralizing IgG antibodies). Our results show that a single dose of Ad5-S- or Ad41-S-based vaccines represents an appealing strategy for the control of MERS-CoV infection and transmission.

Keywords: adenoviral vector; immunity; Middle East respiratory syndrome coronavirus; spike protein; vaccine.

Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV), a novel human coronavirus causing a severe acute respiratory syndrome (SARS)-like disease with a high case fatality rate, was first described in 2012.[1–3] As of 26 December 2014, a total of 941 laboratory-confirmed cases have been reported, resulting in at least 347 deaths.[4] However, despite this significant public health threat, no vaccine or effective treatment options exist to prevent a potential MERS-CoV pandemic.

Similarly to other coronaviruses, the spike (S) protein of MERS-CoV, a characteristic structural component of the virion membrane, forms a large protruding spike on the surface of the virus.[1,5] This protein plays an important role in both viral attachment and entry into the target cell.[6] In other coronaviruses, such as SARS-CoV, vaccination with recombinant S protein has been shown to induce potent neutralizing antibody responses capable of inhibiting infection.[7] Furthermore, inoculation with a modified vaccinia virus Ankara expressing the MERS-CoV S protein elicited high titres of S-specific neutralizing antibodies in mice,[8] highlighting the importance of this protein in viral propagation. Furthermore, S-driven lentiviral transduction was abrogated by serum from MERS-CoV-infected patients,[9] indicating that S-protein-specific neutralizing antibodies might contribute to the control of MERS-CoV infection.

Recombinant adenovirus (rAd) vectors have been widely used in studies of both gene therapy and vaccine delivery.[10] They represent attractive vectors for vaccine development because of their characteristics of growth to a high titre, manufacturability, and adequate space within the genome for gene insertion. Adenoviruses have been safely used as a vaccine for acute respiratory syndrome and are being tested as novel vaccine systems for numerous infectious

agents ranging from malaria to HIV-1.[10,11] Similar strategies based on serologically distinct adenoviruses could allow for successful repeat immunizations.[10–12]

There are more than 50 types of human adenoviruses, which are classified into seven distinct species (A–G), each with a different set of tissue-specific tropisms.[13] An example of these differences can be seen with adenoviruses Ad5 and Ad41; Ad5 is an airway pathogen shown to induce potent systemic responses, whereas Ad41 is a human serotype F virus that homes to the gastrointestinal tract,[14,15] which is resistant to inactivation by gastric acid, bile salt and proteases. Ad5 vectors have been tested for use in intramuscular (i.m.) immunizations and in DNA-rAd vaccine regimens in human clinical trials, providing an important proof of concept for adenovirus-based vaccine vectors.[9,16] Ad41-based vaccines have yet to be tested clinically; however, the natural tropism of this virus for the gastrointestinal tract may prove beneficial in terms of delivering antigen to an otherwise hostile gut environment.[14,15] This tropism may allow for the oral administration of vaccine vectors, as well as the enhanced induction of mucosal immunity,[17] thereby decreasing the likelihood of pathogen transmission via the mucosa. The recent development of a replication-deficient recombinant Ad41 (rAd41) vector expressing the HIV envelope protein (Env) strongly supports this hypothesis, as this construct has been shown to induce HIV Env-specific systemic and mucosal immune responses after prime-boost immunization.[18,19]

Most adenoviruses infect their host through the airway epithelium and replicate in the mucosal tissue of the respiratory tract.[9,13] Accordingly, these pathogens are capable of eliciting robust mucosal immune responses, making them an attractive antigen delivery system in the fight against other mucosal viruses, including MERS-CoV. Moreover, the S protein represents a promising target for preventive and therapeutic intervention against MERS-CoV because of its critical role in the early stages of viral infection.[7] Here, we evaluated the use of two adenovirus vectors for the development of recombinant vaccines against MERS-CoV. Replication-deficient Ad5 and Ad41 expressing the MERS-CoV S protein were used to immunize mice via different routes of delivery, and the humoral, cellular and mucosal immune responses of the animals were examined to assess the potential efficacy of the vaccines against MERS-CoV infection.

Materials and methods

Mice

Six- to eight-week-old female BALB/c mice were purchased from the Animal Care Centre of the Chinese Academy of Medical Science (Beijing, China), and maintained under specific pathogen-free conditions. All experiments were carried out in strict compliance with the Guide for the Care and Use of Laboratory Animals of the People's Republic of China, and approved by the Committee on the Ethics of Animal Experiments of the Chinese Centre for Disease Control and Prevention.

Construction of adenovirus vectors

The MERS-CoV S protein gene sequence (GenBank number JX869059) was optimized for expression in adenovirus vectors using CODON OPTIMISATION software and synthesized chemically. The codon-optimized S gene (MERS-S) was cloned to generate pVRC–MERS-S; this was then digested with *Bgl*II, blunt-ended, and digested again with *Kpn*I to release the MERS-S fragment. The MERS-S fragment was purified from an agarose gel and inserted into the *Kpn*I/*Eco*RV sites of pShuttle–cytomegalovirus (CMV) to generate pSh5–MERS-S. The pSh5–MERS-S was linearized with *Pme*I, treated with calf intestinal alkaline phosphatase, and co-transformed with pAd-Easy-1 into *Escherichia coli* BJ5183 by electroporation to generate the rAd type 5 (HAdV-5) plasmid pAd5–MERS-S. Similarly, the MERS-S fragment was inserted into the *Kpn*I/*Eco*RV sites of pSh41–CMV to generate pSh41–MERS-S, after which it was linearized with *Pac*I, treated with calf intestinal alkaline phosphatase, and co-transformed with pAdbone41–ADP into *E. coli* BJ5183 to generate the recombinant HAdV-41 plasmid pAd41–MERS-S.

Rescue, amplification and purification of the rAd

The pAd5–MERS-S was linearized with *Pac*I then transfected into HEK293 cells using Lipofectamine 2000, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). The rescued Ad5-MERS-S virus was then further amplified in HEK293 cells. Similarly, pAd41–MERS-S was linearized with *Pme*I, and the recombinant Ad41-MERS-S virus was rescued and amplified in 293TE32 cells. The viruses were purified by two rounds of CsCl ultracentrifugation, dialysed against a buffer containing 150 mM NaCl, 1 mM $MgCl_2$, 5% glycerol and 10 mM Tris–HCl (pH 7·6), split into aliquots and preserved at -80°. The preparation of control viruses (Ad5-GFP and Ad41-GFP) expressing a green fluorescent protein (GFP) reporter gene is described elsewhere.[20]

Detection of MERS-S expression

HEK293 cells grown to the log phase were infected with Ad41-MERS-S or Ad5-MERS-S at a multiplicity of infection of 100 or 10 virus particles/cell. At 48 hr post-infection, total proteins were extracted and resolved by SDS–PAGE. The expression of MERS-S was analysed by Western blotting using polyclonal rabbit anti-emc (hCoV-EMC strain) antibodies (1 : 200). Proteins extracted from

HEK293 cells infected with GFP-carrying viruses were used as a negative control. For immunofluorescence assays, cells were fixed with cold methanol *in situ* and probed with polyclonal rabbit anti-emc (1 : 200) and FITC-conjugated goat anti-rabbit (1 : 200) antibodies.

Immunization and sample collection

The mice were randomly distributed to nine groups (n = 10 per group) and vaccinated with different recombinant viruses by different routes as described in Table 1. Half of the mice in each group were killed 4 weeks after immunization (short term), and their sera, spleens, lungs and intestines were harvested. The rest of the mice were killed 16 weeks after immunization (long term), and their organs and tissues were similarly collected. After washing the exterior of the lungs with PBS, lung lavage samples were collected by perfusing and draining the circulatory system with 1 ml of PBS. By removing the blood before lung lavage, any contamination of the lung washes with plasma antibodies was avoided. The small intestine and large intestine were collected in 2 ml of PBS, homogenized and centrifuged, with the resulting supernatant used for analysis. Notably, the serial dilution range of sampling for the SIgA detection is more than 200-fold using PBS containing 0·05% Tween 20 (PBST). The sera and other tissues were heat-inactivated at 56° for 30 min before the detection of MERS-CoV S protein receptor-binding domain (RBD)-specific and neutralizing antibodies.

ELISA

MERS-CoV S-RBD-specific IgG and IgA antibody responses were detected by ELISA. Briefly, serially diluted mouse sera were added to 96-well microtitre plates precoated with rRBD protein (100 ng/well).[21] The plates were incubated at 37° for 1 hr, and washed six times with PBST. Bound antibodies were reacted with horseradish peroxidase-conjugated anti-mouse IgG (1 : 5000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) or IgA (1 : 2000, Sigma-Aldrich, Madison, WI) at 37° for 1 hr. The samples were then washed six more times in PBST, after which 3,3′,5,5′-tetramethylbenzidine (Pierce, Rockford, IL) was added to the plates, and the reaction was stopped by adding 2 M H_2SO_4. The absorbance was read at 450 nm using an ELISA plate reader (Bio-Rad, Hercules, CA). The cut-off value was set to 2·1-fold above that of the negative control.

Table 1. Vaccination groups and dosage

Group	Immunogen	Route	Dose (vp)*	No.
A (PBS)	PBS	i.m.		10
B (Ad41G i.g.)	HAd41V-GFP	i.g.	5×10^9	10
C (Ad41G i.m.)	HAd41V-GFP	i.m.	5×10^9	10
D (Ad5G i.g.)	HAd5V-GFP	i.g.	1×10^9	10
E (Ad5G i.m.)	HAd5V-GFP	i.m.	1×10^9	10
F (Ad41S i.g.)	HAd41V-MERS-S	i.g.	5×10^9	10
G (Ad41S i.m.)	HAd41V-MERS-S	i.m.	5×10^9	10
H (Ad5S i.g.)	HAd5V-MERS-S	i.g.	1×10^9	10
I (Ad5S i.g.)	HAd5V-MERS-S	i.m.	1×10^9	10

i.g., intragastric; i.m., intramuscular; vp., virus particles.
*The dosage of Ad41-based vaccine is fivefold that of Ad5-based vaccine for immunization.

Neutralization assay

The titres of neutralizing antibodies in the immunized mouse sera were determined using a previously reported MERS-CoV pseudovirus system.[22,23] Serum samples were serially diluted twofold in 96-well tissue culture plates and incubated at room temperature for 2 hr after the addition of MERS-CoV pseudovirus to each well. The resulting mixtures were then transferred to duplicate wells containing confluent Huh7 cells. After 72 hr of incubation, a luciferase assay was performed using an Ultra 384 luminometer (Tecan Group Ltd., Männedorf, Switzerland). All experiments were carried out in triplicate, with data expressed in relative luminescence units (RLU). Neutralizing antibody titres were expressed as the reciprocal of the highest serum dilution above the 90% relative inhibition rate, which was calculated as 100 × [1–(RLU in infected target cells in the presence of serum/RLU in infected control cells)].

Enzyme-linked immunospot assays

To evaluate antigen-specific T-cell responses in the immunized mice, an interferon-γ (IFN-γ) enzyme-linked immunospot assay was performed as described previously.[24] Briefly, 96-well plates were coated with 100 µl/well of 5 mg/ml anti-mouse IFN-γ antibodies (BD Pharmingen, San Jose, CA) under sterile conditions and incubated overnight at 4° in a humidified chamber. After washing with sterile PBST, the plates were blocked for 2 hr at room temperature. Freshly harvested mouse splenocytes (5×10^5 cells/well) and pulmonary lymphocytes (5×10^5 cells/well) were stimulated at 37° in 5% CO_2 with a synthesized 18-mer peptide library that overlapped with the MERS-CoV S-RBD by 10 amino acids. After adding a biotinylated detection antibody (BD Pharmingen) and streptavidin–horseradish peroxidase, blots were developed by the addition of 3-amino-9-ethycarbazole substrate solution. The plates were incubated in the dark for 5 min, after which IFN-γ spot-forming cells could be counted. Phorbol 12-myristate 13-acetate and ionomycin were added to the positive control group, whereas the negative control group received no stimuli.

The number of peptide-specific IFN-γ-secreting T cells was calculated by subtracting the negative control value from the spot-forming cell count.

Cytometric bead array

Cytometric bead array analysis was conducted to investigate the levels of T helper type 1 (Th1) and Th2 cytokine secretion[25] in mice 16 weeks after immunization. In brief, splenocytes (5×10^5 per well) of eight mice in each group were distributed in 96-well plates and stimulated with 4 mg/ml of pooled RBD peptide. Plates were incubated for 24 hr at 37° and supernatants were harvested. The concentrations of cytokines, including interleukin-2 (IL-2), IL-4, IL-6, IL-10, tumour necrosis factor-α (TNF-α), IL-17A and IFN-γ, were measured using a mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, San Jose, CA) and a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were analysed using the FCAP ARRAY software (Becton Dickinson).

Statistical analysis

Statistical analyses were performed by a one-way analysis of variance using GRAPHPAD PRISM 5 software. P values $\leq 0{\cdot}05$ were considered significant.

Results

Construction and identification of recombinant Ad41-MERS-S or Ad5-MERS-S

Adenovirus plasmids carrying human codon optimized S protein gene of MERS-CoV (Ad41-S or Ad5-S) were constructed by the homogeneous recombination of shuttle and backbone plasmids in *Escherichia coli* BJ5183. Adenovirus vectors were rescued, amplified and purified using traditional methods;[26,27] a schematic detailing the structure of these recombinant viruses is shown in Fig. 1(a). The expression of MERS-S in Ad41-MERS-S-infected or Ad5-MERS-S-infected cells was detected by Western blotting (Fig. 1b) and indirect immunofluorescence (Fig. 1c). Two prominent bands were detected by Western blotting. The higher band was thought to be full-length S protein (200 000–210 000 MW) from its glycosylation pattern, and the lower band (about 120 000 MW) was the S1 subunit of S cleavage by host cell protease. The size of indicated bands here is bigger than the predicted one based on its nucleotide sequence, but it was consistent with results shown in another previous report.[8] After verifying that both the rAd5 and rAd41 (fivefold dose of rAd5) vectors expressed the MERS-CoV S antigen at similar levels (Fig. 1b,c), the comparative immunogenicity of these vectors was analysed in mice using various regimens.

Antigen-specific humoral IgG and neutralizing antibody production induced by a single vaccination with Ad5-MERS-S or Ad41-MERS-S

To evaluate the ability of Ad5-MERS-S and Ad41-MERS-S to induce a systemic humoral immune response via intragastric (i.g.) or i.m. injection, we collected mouse sera at different time-points (4 and 16 weeks post-vaccination)and screened for humoral IgG against MERS-CoV S-RBD using ELISA. Notably, the dosage of Ad41-based vaccines is fivefold that of the Ad5-based vaccines in this study (Table 1) as the expression level of S antigen of rAd5-based vaccine *in vitro* was comparable with that of a fivefold dose of rAd41-based vaccine.

Both the Ad5-MERS-S and Ad41-MERS-S viruses induced strong RBD-specific IgG antibody responses 4 weeks (short term) after vaccination (Fig. 2a). The MERS-CoV S-RBD-specific IgG responses induced by Ad5-MERS-S were significantly higher in mice immunized by i.m. injection when compared with mice receiving either Ad5-MERS-S or Ad41-MERS-S via the i.g. route ($P < 0{\cdot}05$). No significant difference was seen in the IgG titres of mice vaccinated intramuscularly with Ad41-MERS-S and Ad5-MERS-S ($P > 0{\cdot}05$).

Next, we assessed the capacity of Ad41-MERS-S and Ad5-MERS-S to induce long-term antibody responses in mice vaccinated with i.g. or i.m. injection. As expected, MERS-S-specific IgG could still be detected 16 weeks after vaccination, with the exception of the Ad41-MERS-S i.g. vaccination group (Fig. 2b). The RBD-specific IgG titres induced in the Ad41-MERS-S i.m. and the Ad5-MERS-S i.g. and i.m. vaccination groups were significant; however, the differences among these three groups were not significant ($P > 0{\cdot}05$).

To investigate the effect of vaccination route on the ability of Ad-MERS-S to induce neutralizing antibodies, the neutralization capacity of mouse sera collected from each of the four treatment groups was analysed using a pseudovirus-based inhibition assay. By 4 weeks post-vaccination, both the i.g. and i.m. vaccination groups exhibited strong neutralizing antibody responses against MERS-CoV. Interestingly, no difference in neutralizing titre was evident between the i.g. and i.m. Ad41-MERS-S vaccination groups ($P > 0{\cdot}05$; Fig. 3a). Intramuscular vaccination with Ad5-MERS-S induced significantly higher levels of neutralizing antibodies than did i.m. vaccination with Ad41-MERS-S ($P < 0{\cdot}01$); no differences were seen between the i.g. vaccination with Ad5-MERS-S and Ad41-MERS-S groups. Both the i.g. and i.m. Ad-GFP control groups induced only background levels of neutralizing antibodies.

To assess the ability of the recombinant viruses to induce long-term neutralizing antibodies, mice were killed 16 weeks after vaccination and analysed as above. In agreement with IgG results, neutralizing antibody could

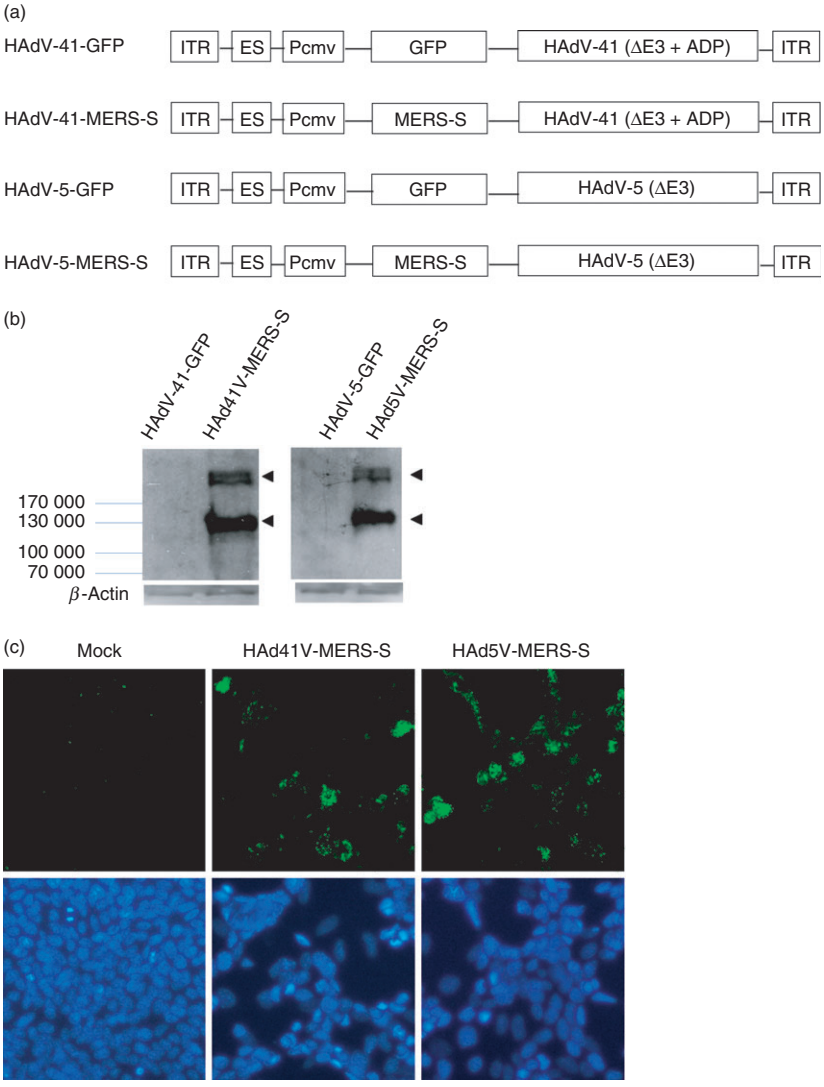

Figure 1. Construction and identification of recombinant adenovirus type 41–Middle East respiratory syndrome coronavirus spike protein (Ad41-MERS-S) or Ad5-MERS-S. (a) Schematic of recombinant Ad41-MERS-S and Ad5-MERS-S. E1 region-deleted Ad41 or Ad5 vectors were constructed, with target genes (GFP or MERS-S) inserted into the deleted E1 region. A cytomegalovirus promoter and simian virus 40 polyA tail were used to control target gene expression. (b) Detection of MERS-S by Western blotting. Exponentially growing HEK293 cells were infected by Ad41-MERS-S or Ad5-MERS-S for 48 hr. Total proteins were then extracted and resolved by SDS–PAGE. MERS-S expression was confirmed by Western blotting using polyclonal rabbit anti-emc antibodies; proteins from GFP-containing viruses were used as a negative control. (c) Detection of MERS-S by immunofluorescence. HEK293 cells were infected as described in (b). The cells were then fixed with cold methanol *in situ* and probed with polyclonal rabbit anti-emc antibodies and FITC-conjugated goat anti-rabbit antibodies. Uninfected HEK293 cells were used as a negative control.

still be detected 16 weeks after vaccination, with the exception of the Ad41-MERS-S i.g. vaccination group (Fig. 3b). Neutralizing antibody titres induced in the Ad41-MERS-S i.m. and the Ad5-MERS-S i.g. and i.m. vaccination groups were significantly higher than in either the Ad5-G or Ad41-G vaccination group .

To assess the ability of the Ad5-MERS-S and Ad41-MERS-S viruses to induce local mucosal immune responses via different vaccination routes, mucosal IgA responses were analysed in the sera, lung lavage fluid and intestines of vaccinated mice by ELISA. Our results indicated that both Ad5-MERS-S and Ad41-MERS-S vaccination only induced lower but detectable MERS-S-specific IgA responses among some of the tissues and organs examined (data not shown). Overall, the IgA detected here may have been underestimated because of the limited amount of fluid that can be collected at each sampling and the dilution factor (more than 200-fold dilution at this study) and optimization of the assay would have been warranted.

Intramuscular vaccination with Ad-MERS-S induces robust cellular immune responses in spleen and lung of mice

We evaluated the effects of i.g. or i.m. vaccination on cellular immunity by counting IFN-γ-producing T cells in the splenocytes of immunized mice. At 4 weeks post-immunization, i.m. Ad5-MERS-S and i.m. Ad41-MERS-S vaccination induced significant IFN-γ-secreting T-cell responses (Fig. 4a); and the responses were at similar levels between these two test groups. The response was substantially stronger in mice vaccinated intramuscularly compared with those vaccinated intragastrically for either Ad5-MERS-S or Ad41-MERS-S ($P < 0{\cdot}01$), and no difference was seen between groups administered with either vaccine viruses (Ad5-MERS-S or Ad41-MERS-S). Similar results were seen in the long-term observation (16 weeks, Fig. 4b). As expected, no antigen-specific cellular immune responses were detected in the spleen and lungs of mice immunized with control vectors. These results suggest

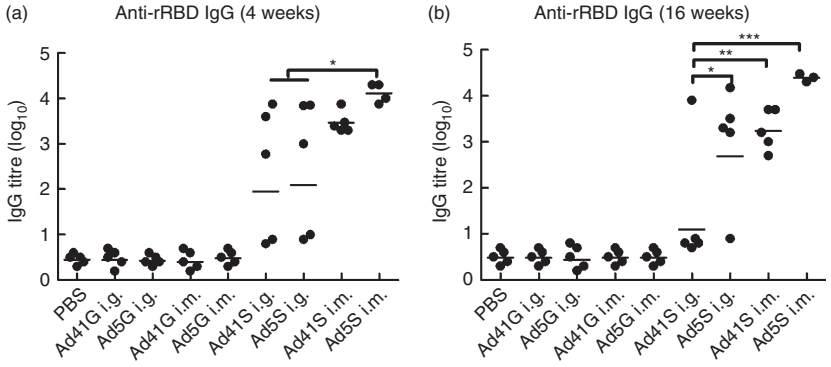

Figure 2. Adenovirus- and Middle East respiratory syndrome coronavirus spike protein (MERS-S)-specific IgG responses in the immunized mice. BALB/c mice ($n = 10$ per group) were immunized with different immunogens or by different routes of exposure, as described in Table 1. Four weeks post-immunization (short term), half of the mice in each group were killed; various tissues and organs were then analysed for antibody responses against adenovirus or MERS-S by ELISA. The remaining mice were killed 16 weeks post-immunization (long term) and analysed as for the short-term group. (a) short-term anti-recombinant receptor-binding domain (rRBD) IgG in serum; (b) long-term anti-rRBD IgG in serum. Statistically significant differences are indicated as follows: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

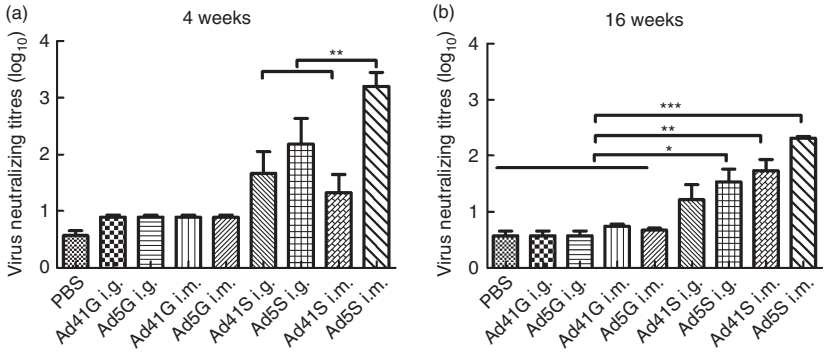

Figure 3. Neutralization antibody responses in immunized mice. Sera were collected 4 (a) and 16 weeks (b) post-vaccination, heat-inactivated, and examined for neutralizing antibodies using the Middle East respiratory syndrome coronavirus (MERS-CoV) pseudovirus system. Virus neutralizing titres were defined as the dilution at which the relative inhibition rate was 90%. The data are presented as means ± standard error of the mean (SEM). Statistically significant differences are indicated as follows: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, and $***P < 0{\cdot}001$.

that Ad41-MERS-S and Ad5-MERS-S can induce the cellular immune response, and the response is relatively stronger when i.m. administration is employed.

A sustained high frequency of S-specific Th1 cytokine-producing T cells was induced with either Ad41-MERS-S or Ad5-MERS-S via i.m. route

The cytokine profiles of spleen cells from immunized mice at 16 weeks were analysed after stimulation with RBD-specific peptides. During cytometric bead array analysis, splenocytes from mice immunized with either Ad41-MERS-S or Ad5-MERS-S via an i.m. route produced IFN-γ (Fig. 5a), IL-2 (Fig. 5b), IL-10 (Fig. 5c) and TNF-α (Fig. 5d), which were at much higher levels than those produced via the i.g. route. Cytokines at background level were seen in mock-infected or control vector-infected groups. Splenocytes from mice immunized with Ad5-MERS-S (i.m.) induced a significantly higher level ($P < 0{\cdot}01$) of IFN-γ (Fig. 5a), IL-2 (Fig. 5b), IL-10 (Fig. 5c) and TNF-α (Fig. 5d) compared with Ad41-MERS-S (i.m.) group. Interleukin-4, IL-6, and IL-17A were detected in none of the groups (data not shown). These data indicated that a higher and sustained frequency of S-specific, Th1 cytokine-producing T cells were induced when Ad41-MERS-S or Ad5-MERS-S were vaccinated via an i.m. route.

Discussion

As a first step toward developing a new vaccine to prevent MERS-CoV infection and transmission, the present study explored the immunogenicity of rAd5-MERS-S and rAd41-MERS-S in mice following a single i.m. or i.g. injection. Our data suggest that single administration with Ad5-S or Ad41-S by i.m. or i.g. injection induced antigen-specific systemic (IgG production, neutralizing antibody responses, and potent cytokine secretion by

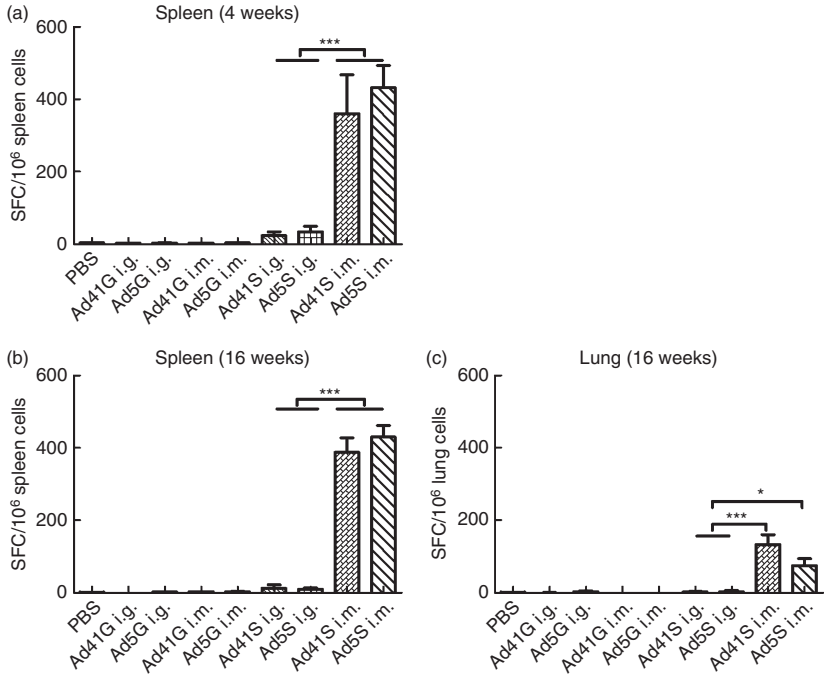

Figure 4. ELISPOT analysis of interferon-γ (IFN-γ) secretion by splenocytes and pulmonary lymphocytes. Lymphocytes were isolated 4 and 16 weeks after immunization. The data are expressed as spot-forming cells (SFCs) responding to receptor-binding domain (RBD)-specific peptides and presented as means $\pm$ standard error of the mean (SEM). Statistically significant differences are indicated as follows: $*P < 0{\cdot}05$ and $***P < 0{\cdot}001$. Lymphocytes were isolated from the spleens (a) of the 4-week post-immunization mice, and from the spleens (b) and lungs (c) of the 16-week post-immunization mice.

spleen-resident lymphocytes) and mucosal (pulmonary lymphocyte IFN-γ secretion) immunity in mice. Furthermore, a higher dose of rAd41-S induced humoral and cellular immune responses, similar to rAd5-S, following an i.m. challenge, suggesting that rAd41 is a good substitute for rAd5 in the induction of systemic immunity. However, antigen-specific immunity in sera and mucosal sites were dramatically higher following the i.m. vaccination with Ad5-S than other groups.

Novel vectors that possess natural mucosal tropism may have advantages over some of the more common vectors in terms of administration, safety and vaccine potency.[18,19,28–30] The gut tropism of Ad41 indicates that the rAd41 vector may be a more efficient inducer of mucosal immune responses when administered via an oral or i.g. route.[14,18,30] Here, a single i.g. dose of rAd41-MERS-S induced significant humoral immunity in serum. However, no significant antigen-specific T-cell responses were detected in the spleens and pulmonary lymphocytes of mice immunized intragastrically with Ad5-MERS-S or Ad41-MERS-S, in contrast to studies suggesting that mucosal administration is advantageous for inducing T-lymphocyte responses at mucosal surfaces.[19] Additional studies will be necessary to fully define these differences and their underlying mechanisms, and they may improve our ability to design vaccines and regimens that target polyfunctional systemic and mucosal immunity to particular infectious agents.

Mucosal immunity is considered important for protection against many infectious diseases,[17] as mucosal surfaces are the first line of defence against the majority of pathogens, including MERS-CoV. Although recent progress has been made, robust stimulation of mucosal immunity remains difficult. Several approaches have been used to elicit mucosal immune responses using rAd5-based vaccines.[18,19,31] Intranasal immunization with a rAd5 vaccine generated stronger IgA responses in systemic and mucosal compartments than i.m. immunization in mice; however, safety concerns may limit the use of the approach in humans.[28] In addition, oral immunization is the most convenient way to deliver mucosal vaccines. Heterologous rAd41 oral or ileal priming followed by an i.m. rAd5 boost elicits enhanced intestinal mucosal cellular immunity.[18] In this study, we found that antigen-specific IgG and neutralizing antibodies were induced by the i.g. administration of rAd5-based or rAd41-based vaccines expressing the S protein of MERS-CoV. This immunization strategy is associated with increased safety and applicability relative to an oral challenge, making it an excellent choice for future vaccine development efforts. Furthermore, as increasing evidence supports a role for dromedary camels as a reservoir of MERS-CoV,[32] our vaccination strategy may hold promise for the development of a preventive vaccine that targets the animal reservoir; and this may be an effective approach to eliminate the transmission of MERS-CoV to humans.

Virus-specific CD8 T cells are required for pathogen clearance following primary respiratory CoV infection.[33] One recent report also showed that virus-specific CD8 T cells by a prime-boost immunization protect susceptible 8- to 10-month-old mice from lethal SARS-CoV challenge. It is widely accepted that systemic immunization with protein- or peptide-based vaccine induces only weak or short-term cellular immune responses. Compared with

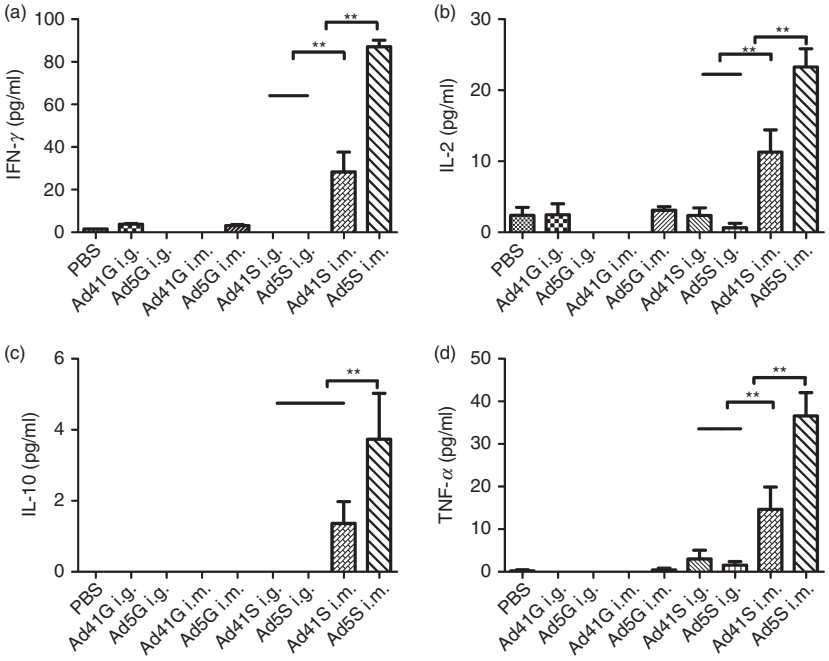

Figure 5. Cytometric bead assay to determine the *in vitro* cytokine production of splenocytes from immunized mice 16 weeks after immunization. (a) Interferon-γ (IFN-γ), (b) interleukin-2 (IL-2), (c) IL-10 and (d) tumour necrosis factor-α (TNF-α). Statistical significance was defined by $^{**}P < 0{\cdot}01$. Each group contained six mice.

rRBD subunit immunization studies, we reported here that a sustained mucosal as well as systemic cellular immune response was induced in mice by single administration with Ad5-S or Ad41-S via the i.m. route (Fig. 4 and 5). Together, these studies demonstrate the possibility of generating sustained mucosal and systemic T-cell-mediated immunity using rAd5- or rAd41-based MERS-CoV vaccines; however, further optimization will be necessary before advancing into human clinical trials.

Previous reports showed that the route of vaccination critically impacts not only the magnitude but also the phenotype and trafficking of antigen-specific immunity in mice.[19] Despite the many attractive features of mucosal vaccination, it has often proven difficult in practice to stimulate strong immune responses and protection by mucosal administration of antigens. Oral or i.g. immunization with rAd5 or rAd41 vectors targeted the digestive epithelium, where the induction of mucosal and systemic immunity is well documented.[14,18,19,30] Other studies have suggested that mucosal routing of vaccine vectors may be used to optimize mucosal and cellular immunity.[33–36] In this study, we found that the route of rAd vector delivery critically impacted both systemic and mucosal immunity. In particular, i.m. rAd immunization consistently generated high-level T-lymphocyte responses among the systemic and mucosal compartments. In contrast, i.g. immunization generated no detectable T lymphocytes in both serum and mucosal sites. Our finding suggests that a single i.m. dose of Ad5-S or Ad41-S induced strong, persistent and complementary systemic and mucosal immunity, and represents an appealing strategy for the control of MERS-CoV transmission. Continued development of appropriate formulations such as enteric coatings or nanoparticles might allow these vectors to be more amenable to clinical applications.[37,38]

Overall, we examined the effects of administration route on immune responses using a single dose of Ad5 or Ad41 vectors as the vaccination agent in this study. During the preparation of this manuscript, a different group reported that antibody responses against the S protein were sufficient for neutralizing MERS-CoV *in vitro*.[39] These responses could be induced using an i.m. vaccination with rAd5-based MERS-CoV candidate vaccines followed by a secondary intranasal boost 3 weeks later. Our results indicated that a single i.m. or i.g. administration of Ad5-S or Ad41-S induced S-antigen-specific systemic and mucosal immunity. Furthermore, the most significant and sustained neutralizing antibody production as well as cell-mediated immunity could be elicited by rAd5-based vaccine via the intramuscular route. Because no full-length S protein was available on hand, all evolution of the antibody response (IgG and IgA) against S was limited to RBD-specific antibodies. We understand that other S-protein-specific antibodies may play a role in the neutralization and protection of the infection, although the majority of RBD-specific antibodies are neutralizing. In addition, the protocol of IgA assay for evaluation of mucosal immunity in this study needs to be further optimized. Although further study will be necessary to evaluate the protective efficacy of rAd5-MERS-S or rAd41-MERS-S vectors in humans and dromedary camels, our results strongly support a role for rAd5- or rAd41-encoding MERS-S antigens as a safe and effective strategy for MERS-CoV prevention.

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Disclosures

The authors declare no commercial or financial conflict of interest.

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