ChAdOx1 and MVA based vaccine candidates against MERS-CoV elicit neutralising antibodies and cellular immune responses in mice

Naif Khalaf Alharbi [a,b,*], Eriko Padron-Regalado [a], Craig P. Thompson [a,c], Alexandra Kupke [d,e], Daniel Wells [a], Megan A. Sloan [a], Keith Grehan [f], Nigel Temperton [f], Teresa Lambe [a], George Warimwe [a], Stephan Becker [d,e], Adrian V.S. Hill [a], Sarah C. Gilbert [a]

[a] The Jenner Institute, University of Oxford, Oxford OX3 7DQ, UK
[b] King Abdullah International Medical Research Center, Riyadh, Saudi Arabia
[c] Department of Zoology, University of Oxford, Oxford, UK
[d] Institute of Virology, Philipps University of Marburg, Marburg, Germany
[e] German Center for Infection Research, TTU Emerging Infections, Germany
[f] Viral Pseudotype Unit, School of Pharmacy, University of Kent, Chatham Maritime, Kent ME4 4TB, UK

ABSTRACT

The Middle East respiratory syndrome coronavirus (MERS-CoV) has infected more than 1900 humans, since 2012. The syndrome ranges from asymptomatic and mild cases to severe pneumonia and death. The virus is believed to be circulating in dromedary camels without notable symptoms since the 1980s. Therefore, dromedary camels are considered the only animal source of infection. Neither antiviral drugs nor vaccines are approved for veterinary or medical use despite active research on this area. Here, we developed four vaccine candidates against MERS-CoV based on ChAdOx1 and MVA viral vectors, two candidates per vector. All vaccines contained the full-length spike gene of MERS-CoV; ChAdOx1 MERS vaccines were produced with or without the leader sequence of the human tissue plasminogen activator gene (tPA) where MVA MERS vaccines were produced with tPA, but either the mH5 or F11 promoter driving expression of the spike gene. All vaccine candidates were evaluated in a mouse model in prime only or prime-boost regimens. ChAdOx1 MERS with tPA induced higher neutralising antibodies than ChAdOx1 MERS without tPA. A single dose of ChAdOx1 MERS with tPA elicited cellular immune responses as well as neutralising antibodies that were boosted to a significantly higher level by MVA MERS. The humoral immunogenicity of a single dose of ChAdOx1 MERS with tPA was equivalent to two doses of MVA MERS (also with tPA). MVA MERS with mH5 or F11 promoter induced similar antibody levels; however, F11 promoter enhanced the cellular immunogenicity of MVA MERS to significantly higher magnitudes. In conclusion, our study showed that MERS-CoV vaccine candidates could be optimized by utilising different viral vectors, various genetic designs of the vectors, or different regimens to increase immunogenicity. ChAdOx1 and MVA vectored vaccines have been safely evaluated in camels and humans and these MERS vaccine candidates should now be tested in camels and in clinical trials.

1. Introduction

Middle East respiratory syndrome (MERS) is caused by a novel betacoronavirus (MERS-CoV) that was isolated in late 2012 in Saudi Arabia [1]. The syndrome (MERS) is described as a viral infection that causes fever, cough, and/or shortness of breath and to a lesser extent gastrointestinal symptoms such as diarrhea [2]. Severe disease from MERS-CoV infection can cause respiratory failure and organ failure, and cases can be fatal, especially in patients with co-morbidities such as diabetes and cardiac complications. However, the infection can be asymptomatic or mild in many cases [3–7]. MERS-CoV has spread to 27 countries and infected more than 1900 humans with a mortality rate of 40% [2]. Dromedary camels, especially juveniles, contract the infection and shed the virus, without notable symptoms of disease; this is now known to have been occurring since the early 1980s [8–13]. The mechanism of camel to human transmission is still not clear, but several primary cases have been associated with camel contact, which is considered an important risk factor [14–16]. Therefore, camels

are being considered an intermediate host and one of the sources of MERS-CoV infection [8–13]. Other livestock animals such as sheep, goats, cows, chicken, and horses have proved seronegative in many studies [17–20]. Further, these animals did not productively contract MERS-CoV when they were inoculated experimentally [21,22]. Therefore, to date, dromedary camels are the only confirmed animal reservoir. There is currently no approved vaccine against MERS-CoV for camels or humans despite active vaccine research and development. A number of vaccine candidates have been developed using various platforms and regimens and have been tested in several animal models [23]. Viral vectors are potent platform technologies that have been utilised to develop vaccines against malaria, tuberculosis, influenza, HIV, HCV, Ebola, and many viral pathogens. These vectors include adenoviruses, poxviruses, yellow fever viruses, and alphaviruses [24,25], and they are preferred for their ability to induce cellular immune responses in addition to humoral immunity. Here, we report development of MERS-CoV vaccine candidates that are based on two different viral vectors: Chimpanzee Adenovirus, Oxford University #1 (ChAdOx1) [26] and Modified Vaccinia virus Ankara (MVA) [27,28]. Each viral vector was developed by generating two alternative versions, resulting in four vaccine candidates that all encode the same complete MERS-CoV spike gene (S). The two ChAdOx1 based vaccines were produced with or without the signal peptide of the human tissue plasminogen activator gene (tPA) at the N terminus. Previous studies have shown that encoding tPA upstream of recombinant antigens enhanced immunogencity, although results differed depending on the antigens employed. The tPA encoded upstream of influenza A virus nucleoprotein, in a DNA vector, enhanced both cellular and humoral immune responses in mice [29,30], whereas the same leader sequence resulted in increased humoral sequences but decreased cellular responses to HIV Gag [30]. The two MVA based vaccines were produced with either the mH5 or F11 poxviral promoter driving antigen expression, both including the tPA sequence at the N terminus of MERS-CoV Spike protein. Previously, we reported the ability of the strong early F11 promoter to enhance cellular immunogenicity of vaccine antigen candidates for malaria and influenza, as compared to utilising p7.5 or mH5 early/late promoters which resulted in a lower level of gene expression immediately after virus infection of target cells, but higher levels at a later stage [31]. Here, we continue to assess the F11 promoter in enhancing cellular immunogenicity, and to investigate its ability to impact on humoral immune responses. The four vaccine candidates were evaluated in a number of different regimens in mouse models that showed a single dose of ChAdOx1 MERS inducing higher cellular and humoral immunogenicity than a single dose of MVA MERS, or equivalent to two doses of MVA MERS. ChAdOx1 based vaccines have been tested in different animal models, including camels [32], and in human clinical trials and proved safe and immunogenic [33]. Therefore, based on our data, ChAdOx1 MERS can be readily developed for use as a MERS vaccine in humans. Furthermore, utilising ChAdOx1 MERS for camel vaccination can serve the one-health approach whereby blocking MERS-CoV transmission in camels is expected to prevent human infections.

2. Materials and methods

2.1. Transgene and shuttle vector cloning

The spike (S) gene of MERS-CoV camel isolate (Genbank accession number: KJ650098.1) was synthesised by GeneArt Gene Synthesis (Thermo Fisher Scientific). The S transgene was then cloned into four shuttle plasmid vectors following In-Fusion cloning (Clontech). Two plasmids contained the S transgene within the E1 homologous region of ChAdOx1, driven by the human cytomegalovirus major immediate early promoter (IE CMV) that includes intron A. One of the ChAdOx1 shuttle plasmids was designed to include the tPA signal sequence upstream of the transgene sequence while the second plasmid did not contain the tPA. The ChAdOx1 shuttle plasmids contained the S transgene within Gateway® recombination cassettes. To construct MVA MERS, one of the shuttle plasmids for MVA was designed to have the upstream and downstream (flanks) of the F11L ORF as homologous sequence arms. Inserting the S transgene within these arms enabled the utilisation of the endogenous F11 promoter, which is part of the right homologous arm, while deleting the native F11L ORF. This resulted in the shuttle vector for generation of F11-MVA MERS (F11 shuttle vector). The mH5 promoter sequence was subcloned upstream of the S transgene; and this mH5-S transgene was then subcloned into the F11 shuttle vector. This resulted in the shuttle vector for generation of mH5-MVA MERS (F11/mH5 shuttle vector). mH5-MVA MERS contained the mH5 promoter at the F11L locus, however, the endogenous F11 promoter is intact and located upstream of the mH5 promoter. The endogenous F11 promoter could not be replaced with the mH5 since it is part of the essential upstream ORF.

2.2. Immunostaining for transgene expression

The ChAdOx1 shuttle plasmid, described above, was used to validate the expression of MERS-CoV spike protein in vitro. An African green monkey kidney cell line (Vero cells) was seeded into 6-well plate to 80% confluence. Then the plasmid DNA was transfected into Vero cells using Lipofectamine® 2000 (Thermo Fisher Scientific) following manufacturer's instruction. Twenty-four hours after transfection, cells were fixed, permeabilised, and immunostained using a rabbit polyclonal anti-MERS-CoV spike antibody, following standard protocols. DAPI stain was used to label nuclei.

2.3. Construction of recombinant ChAdOx1 and MVA encoding MERS-CoV S antigens

The ChAdOx1 MERS vaccines were prepared by Gateway® recombination between the ChAdOx1 destination DNA BAC vector (described in [26]) and entry plasmids containing the coding sequence for MERS-CoV spike gene (ChAdOx1 shuttle vectors explained above), according to standard protocols. ChAdOx1 MERS genomes were then derived in HEK293A cell lines (Invitrogen, Cat. R705-07), the resultant viruses were purified by CsCl gradient ultracentrifugation as previously described [34]. The titres were determined on HEK293A cells using anti-hexon immunostaining assay based on the QuickTiter™ Adenovirus Titer Immunoassay kit (Cell Biolabs Inc). For MVA MERS vaccines chicken embryo fibroblast cells (CEFs) were infected with MVA parental virus that encodes dsRed marker instead of the native F11L ORF and transfected with MVA shuttle plasmids containing MERS-CoV spike gene (explained above) to allow recombination with the MVA genome and deletion of dsRed marker whilst keeping the F11 promoter sequence. Recombinant MVA expressing MERS-CoV S protein was purified by plaque-picking and fluorescent selection using the sorting function of CyCLONE robotic module of a MoFlo Flow cytometer (Dako Cytomation, Denmark) as previously described [31]. F11-MVA MERS and mH5-MVA MERS were confirmed to lack the native F11L ORF (and the dsRed marker), and contain MERS-CoV S by PCR (identity and purity PCR screening). The sequence of the S transgene amplified from these vaccines was confirmed. The recombinant viruses (vaccines) were amplified in 1500 cm^2 monolayers of CEFs cells, partially purified over sucrose cushions and titrated in CEFs cells according to standard practice, and purity and identity were again verified by PCR.

2.4. Mouse immunogenicity

Female BALB/c mice (Harlan, UK) aged 6–8 weeks were immunised intramuscularly (i.m.) in the upper leg (total volume 50 μL) with a total of 10^8 IU of ChAdOx1 MERS with or without tPA or with a total of 10^6 pfu of either F11-MVA MERS or mH5-MVA MERS. For induction of short-term anaesthesia, animals were anaesthetised using vaporised IsoFloH. In prime only regimens, mice were vaccinated with ChAdOx1 with blood samples taken at 14 days post immunisation (d.p.i) or 28 d.p.i. for serum isolation; and spleens were collected at 28 d.p.i. In heterologous prime-boost regimens, mice were vaccinated with ChAdOx1 MERS and boosted with MVA MERS at 28 d.p.i; mice were bled at 28 d.p.i. (post-prime) or 42 d.p.i (14 days post-boost) for serum isolation, and spleens were collected at 42 d.p.i. In homologous regimens, mice were vaccinated with MVA MERS and boosted with MVA MERS at 21 d.p.i; mice were bled on 21 d.p.i. (post-prime) or 42 d.p.i (post-boost) for serum isolation and spleens were collected at 42 d.p.i.

2.5. ELISpot, ICS, and flow cytometry

Splenocytes were harvested for analysis by IFN-γ ELISpot or intracellular cytokine staining (ICS) and flow cytometry as previously described [35,36], using re-stimulation with 2 μg/mL S291 MERS-CoV S-specific peptide (VYDTIKYYSIIPHSI); for vaccine cellular immunogenicity [37]); or 1 μg/mL E3 and F2(G) MVA vector-specific peptides [38] (for anti-MVA immune responses). In the absence of peptide re-stimulation, the frequency of IFN-γ$^+$ cells, which was typically 0.1% by flow cytometry or less than 50 SFC by ELISpot, was subtracted from tested re-stimulated samples.

2.6. ELISA

2 μg/mL with capturing antigen (S1 recombinant protein from MyBioSource, CA, USA) were used to coat ELISA plates, and standard endpoint ELISA protocol was followed, as previously described [39]. Sera were prepared in a 10-fold serial dilution in PBS/T and then 50 μL were plated in duplicate wells. Serum from a naïve BALB/c mouse was included as a negative control. Goat anti-mouse total IgG conjugated to alkaline phosphatase (Sigma) and PNPP tablet (20 mg p-nitrophenylphosphate, SIGMA) substrate were used in the assay.

2.7. Merspp neutralisation assay

MERS pseudotyped viral particles (MERSpp) were produced and titrated using Huh7.5 cell line as described previously [40]. For the MERSpp neutralisation assay, serum samples were serially diluted in 96-well white plates (Nunc). A standard concentration of the MERSpp were added to the wells and plates were incubated for 1 h at 37 °C. After incubation, Huh7.5 cells (10,000 cells per well) were added to the plate in duplicates. Following 48 h incubation, cells were lysed and luciferase activity was measured. IC90 neutralisation titres were calculated for each mouse serum sample using GraphPad Prism.

2.8. Virus neutralisation assay

Induction of virus-neutralising antibodies was confirmed according to previously published protocols [37,41]. Briefly, mouse serum samples were tested for their capacity to neutralise MERS-CoV (EMC isolate) infections in vitro with 100 50% tissue culture infective doses ($TCID_{50}$) in Huh-7 cells. Sera of non-immunised mice served as negative control.

2.9. Statistical analysis

GraphPad Prism (GraphPad software) was used for statistical analysis and to plot data.

2.10. Ethics statement

All animal procedures were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act (ASPA) for the project licenses 30/2414 or 30/2889 and were approved by the University of Oxford Animal Care and Ethical Review Committee. All mice were housed for at least 7 days for settlement prior to any procedure in the University animal facility, Oxford, UK under Specific Pathogen Free (SPF) conditions.

3. Results

3.1. Construction and antigen expression of MERS-CoV vaccine candidates

The spike gene from a camel isolate (Camel/Qatar_2_2014 MERS-CoV isolate, GenBank accession number KJ650098.1) was cloned into four shuttle vectors that facilitate homologous recombination with the genome of ChAdOx1 or MVA. Four recombinant viral vectors, two ChAdOx1 and two MVA, were derived as described in the materials and methods. ChAdOx1 based vaccine candidates were generated with or without the signal peptide of the human tissue plasminogen activator gene (tPA). The spike transgene expression in ChAdOx1 MERS vaccine candidates is under the control of the human cytomegalovirus major immediate early promoter (CMV IE) that includes intron A. In MVA MERS vaccine candidates, the tPA was also inserted upstream of the spike transgene, which was under the control of either the ectopic mH5 promoter or the endogenous F11 promoter (Fig. 1A). All of our MERS-CoV vaccine candidates contain the same codon-optimized spike transgene. The expression of the newly synthesized transgene was first tested by transfection of an African green monkey kidney cell line (Vero cells) with the adenovirus shuttle vector, and immunofluorescence staining of the transfected cells (Fig. 1B and 1C). This was performed to confirm the expression of the codon optimized spike transgene in mammalian cells. The level of transgene expression from the four vaccine candidates was not evaluated in vitro. We have previously reported that differences in MVA promoter activity detectable in vitro does not correlate with in vivo immunogenicity [31], and that only in vivo expression correlates with the in vivo immunogenicity.

3.2. Humoral immunogenicity of ChAdOx1 based MERS-CoV vaccine candidates

To evaluate humoral immune responses to ChAdOx1 MERS with or without tPA, BALB/c mice were vaccinated with 1×10^8 IU of ChAdOx1 intramuscularly. Serum samples from 14 and 28 d.p.i. were collected and evaluated by ELISA. Both vaccine candidates induced a high level of S1-specific antibodies (mean endpoint titre (Log_{10}) = 4.8 with tPA, 4.7 without tPA), unlike the control vaccine, ChAdOx1 encoding enhanced green fluorescent protein (ChAdOx1-eGFP, mean endpoint titre (Log_{10}) = 1). These antibody levels were similar between the two candidates (with or without tPA) at day 14. However, at 28 d.p.i. ChAdOx1 MERS with tPA induced significantly higher S1-specific antibodies than ChAdOx1 MERS without tPA (mean endpoint titre (Log_{10}) = 5.13 with tPA, 4.6 without tPA, Fig. 2A). Serum samples from day 28 were selected for MERSpp neutralisation assay. Serum antibodies induced by ChAdOx1 MERS with tPA showed significantly higher neutralisation activity than
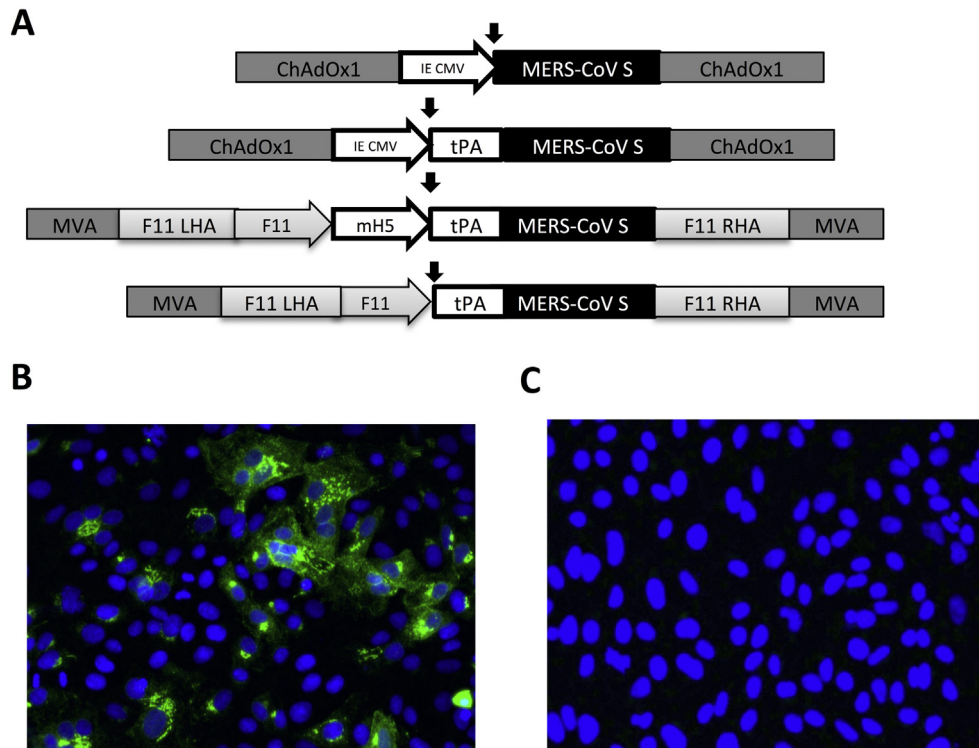
Fig. 1. Construction of MERS-CoV vaccine candidates. (A) Schematic representation of ChAdOx1 and MVA based vaccines, each encodes the same MERS-CoV spike gene (Genbank accession number: KJ650098.1). The S gene was inserted into the E1 region of ChAdOx1 genome or into the *F11L* locus of MVA genome (see Section 2). tPA: Human tissue plasminogen activator (tPA) signal peptide sequence. IE CMV: The human cytomegalovirus major immediate early promoter. mH5 and F11: Poxviral promoters. LHA: left homology arm sequence. RHA: right homology arm sequence. (B) The expression of spike transgene, cloned into a plasmid vector, was confirmed by transfection into an African green monkey kidney cell line (Vero cells) and immunostaining. (C) Untransfected cells control. Green colour represents detection of the spike protein. Blue colour represents nuclei by staining nucleic acid with DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

without tPA (mean titre IC_{90} (Log_{10}) = 2.8 with tPA, 2.2 without tPA; Fig. 2B). In order to confirm that the psuedotyped virus neutralisation assay was producing biologically relevant results, serum samples from mice immunised with ChAdOx1 MERS with tPA were also tested in a neutralisation assay utilising wildtype MERS virus. This assay confirmed the neutralisation activity of mouse antibodies (nAb) with a median of 360 VNT (Virus Neutralization Test antibody titre; Fig. 2C). We therefore continued to evaluate ChAdOx1 MERS with tPA in addition to generating MVA MERS vaccine candidates with tPA.

3.3. Cellular immunogenicity of ChAdOx1 based MERS-CoV vaccine candidates

Having established the utility of tPA in ChAdOx1 MERS vaccines (referred to as ChAdOx1 MERS in the rest of this report) at increasing humoral responses, spleens were collected at 28 d.p.i. from immunised BALB/c mice. Splenocytes were processed to evaluate cellular immune responses to ChAdOx1 MERS in ELISpot and Intracellular cytokine staining (ICS). Peptide S291, described by others [37], was used to re-stimulate the cells in both assays and ELISpot data showed a high level of IFN-γ secreting splenocytes (Median = 1300 $SFU/10^6$ splenocytes; Fig. 3A). ICS data confirmed the IFN-γ secreting $CD8^+$ splenocytes also secreted TNF-α and IL-17 (Fig. 3B).

3.4. Immunogenicity of heterologous ChAdOx1 and MVA vaccination against MERS-CoV

To evaluate humoral immune responses to heterologous prime-boost vaccination, BALB/c mice were immunised with ChAdOx1

MERS vaccine and boosted with one of two different MVA MERS vaccine candidates four weeks later. The MVA based candidates differ in the promoters that controls the transgene expression: F11-MVA MERS utilises the endogenous strong early F11 promoter and mH5-MVA MERS utilises the ectopic early/late mH5 promoter. Serum samples from 28 d.p.i. (post-prime) or 42 d.p.i. (post-boost) were collected and evaluated by ELISA and MERSpp neutralisation assay. At 28 d.p.i. ChAdOx1 MERS induced similar levels of S1-specific antibodies and nAb as observed previously (Fig. 4A and B). At 42 d.p.i. S1-specific antibodies were boosted to a higher level (mean endpoint titre (Log_{10}) = 5 by ChAdOx1 MERS boosted to 5.8 by mH5-MVA MERS or 5.9 by F11-MVA MERS); Fig. 4A) with nAb also enhanced to a statistically significant level (mean titre IC_{90} (Log_{10}) = 2.87 by ChAdOx1 MERS boosted to 3.3 by mH5-MVA MERS or 3.5 by F11-MVA MERS; Fig. 4B). There was no difference in antibody levels induced using either the F11 or mH5 promoter in the MVA.

At 42 d.p.i. splenocytes were also processed to evaluate cellular immune responses to ChAdOx1 MERS MVA MERS prime-boost vaccination in ELISpot and ICS as shown in Fig. 3. The T cell responses to MERS S were boosted by the MVA vaccinations; in the ICS experiments, F11-MVA and mH5-MVA boosted the percentage of IFN-γ$^+$ splenic $CD8^+$ T cells to 7.3 and 5.2% respectively (Fig. 4D) whereas the percentage was 2.5% after ChAdOx1 MERS prime in Fig. 3B. The percentage of TNF-α$^+$ splenic $CD8^+$ T cells were also increased by MVA boost (comparing Fig. 3B and 4D). Utilising the F11 promoter resulted in a trend towards greater cell-mediated immunogenicity (Fig. 4C and D). Splenocytes were also re-stimulated with MVA backbone-specific E3 and F(G)2 peptides and evaluated in ICS. Both MVA based vaccines induced similar responses to E3 or to F(G)2 pep-
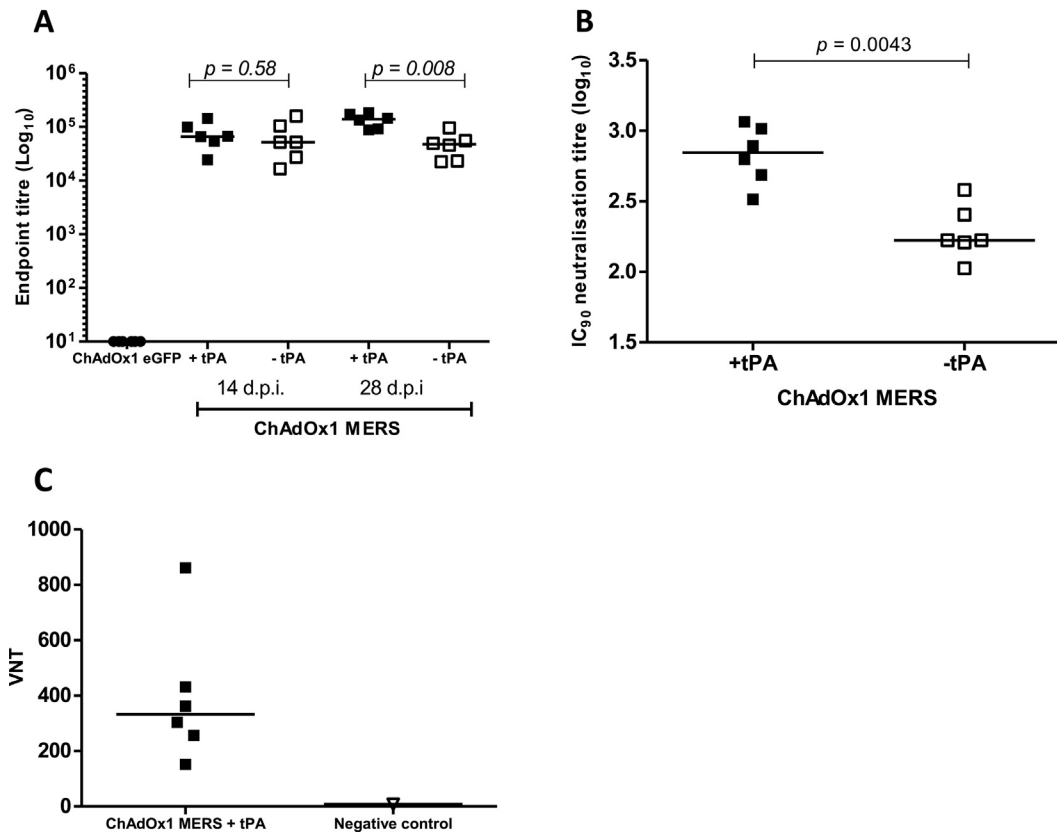
Fig. 2. Antibody responses to ChAdOx1 MERS vaccine candidates. BALB/c mice (n = 6) were immunised intramuscularly with 1×10^8 IU ChAdOx1 MERS that either encodes or lacks tPA signal peptide upstream of the antigen sequence. A control group of mice were immunised with ChAdOx1 expressing eGFP instead of MERS-CoV S gene (given at 1×10^8 IU intramuscularly). Serum samples were collected at 14 and 28 days post immunisation (d.p.i.). S1-binding antibodies were measured by ELISA (A) and neutralisation activity of the antibodies were confirmed by MERS-CoV pseudotyped viral particles (MERSpp) neutralisation assay (B) or neutralisation assay (C). Individual data points (representing individual mice) are shown with line as the median. Data are representative of two independent experiments. Statistical significance by Kruskal–Wallis test is shown.
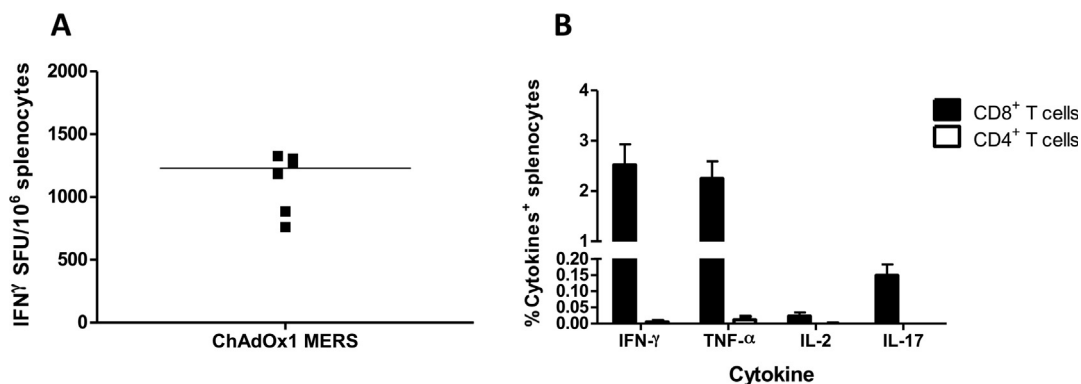

Fig. 3. Cellular immune responses to ChAdOx1 MERS vaccine candidate. BALB/c mice (n = 6) were immunised with intramuscularly with 1×10^8 IU ChAdOx1 MERS that encodes tPA signal peptide upstream of the antigen sequence. Twenty-eight days post-immunisation, IFN-γ *ex vivo* ELISpot (A) or Intracellular Cytokine Staining (ICS (B)), were performed to determine the percentage of splenic IFN-γ secreting CD4⁺ and CD8⁺ after *in vitro* re-stimulation with a MERS-CoV S-specific peptide. Individual data points (representing individual mice) are shown with line as the median (A) or error bars as the SD (B). Data are representative of two independent experiments.

tides, 2 weeks after MVA vaccination (Fig. 4E and F). This similarity confirmed the efficiency of vaccine titration, vaccination, and sample processing because responses to each of those peptides are not expected to be different unless there is variation in the doses administered or sample preparation. Overall, MVA MERS vaccines were able to boost the humoral and cellular immune responses to ChAdOx1 MERS prime vaccination. There was no difference between the F11 and mH5 promoter in the resulting antibody titres after ChAdOx1 prime/MVA boost, but there was a trend towards increased cellular immunogenicity when the F11 promoter was used.

3.5. Immunogenicity of homologous MVA vaccination against MERS-CoV

To evaluate humoral immune responses to a homologous MVA MERS prime-boost vaccination, two groups of BALB/c mice were immunised with F11-MVA MERS or mH5-MVA MERS and boosted with the same vaccine after three weeks. Serum samples from 21 d.p.i. (post-prime) or 42 d.p.i. (post-boost) were collected and evaluated in ELISA and MERSpp neutralisation assays. At 21 d.p.i. F11-MVA MERS and mH5-MVA induced similar levels of S1-specific
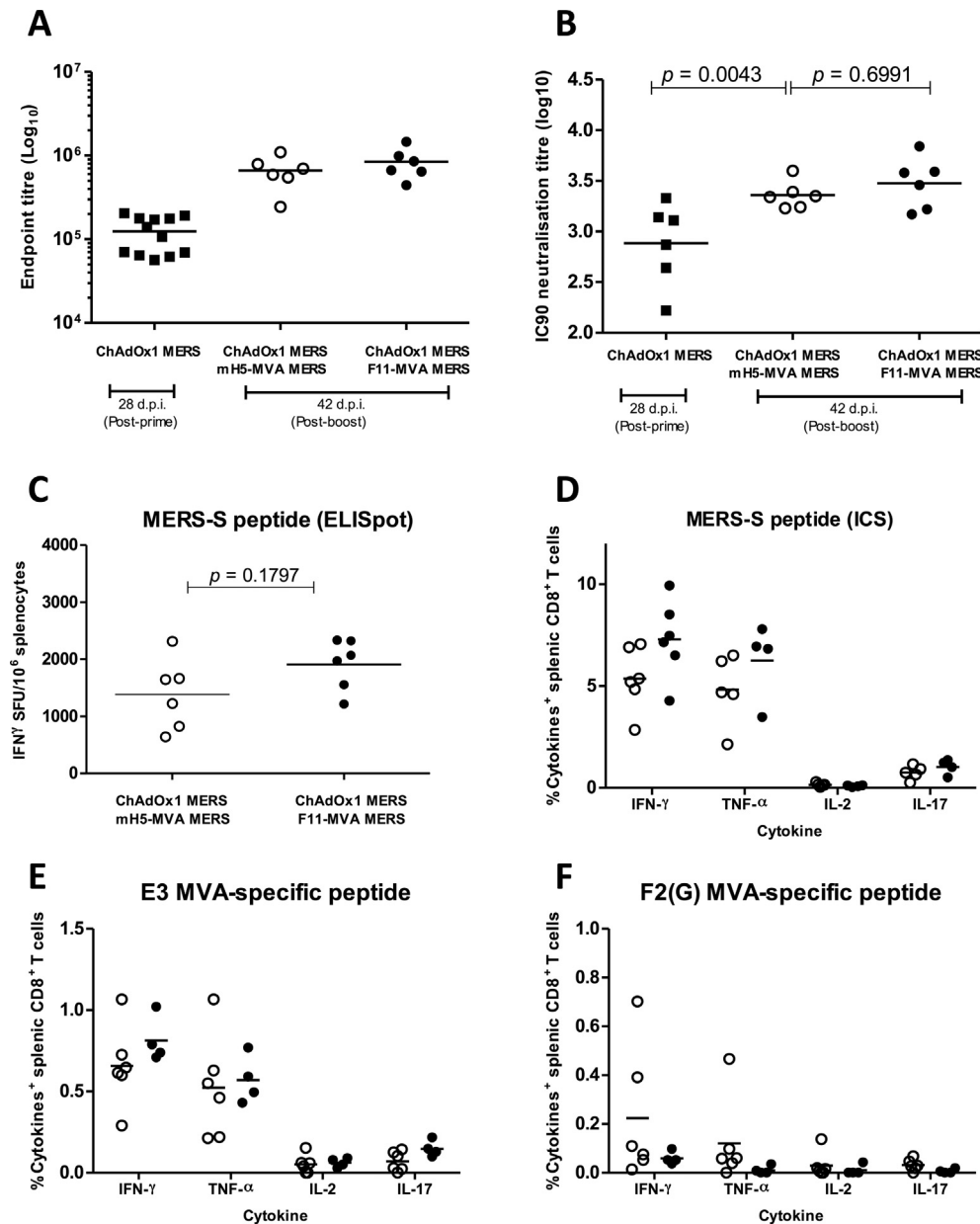
Fig. 4. Humoral and cellular immunogenicity of heterologous ChAdOx1 MERS and MVA MERS vaccination. BALB/c mice (n = 6) were immunised intramuscularly with 1×10^8 IU ChAdOx1 MERS that encodes tPA signal peptide upstream of the antigen sequence. At 28 d.p.i. mice were boosted intramuscularly with 1×10^6 pfu MVA MERS. MVA MERS vaccine candidates utilise either the mH5 or F11 promoter for transgene expression. Serum samples were collected at 28 (post-prime) and 42 (post-boost) d.p.i. S1-binding antibodies were measured by ELISA (A) and neutralisation activity of serum antibodies at 42 d.p.i. were confirmed by MERSpp neutralisation assay (B). At 42 d.p.i, IFN-γ *ex vivo* ELISpot (C) or Intracellular Cytokine Staining (ICS (D)) were performed to determine the percentage of CD8$^+$ IFN-γ$^+$ splenocytes after *in vitro* re-stimulation with a MERS-CoV S-specific peptide. ICS of splenocytes re-stimulated with MVA-specific peptides (F(G)2 and E3) was also performed (E and F). Individual data points (representing individual mice) are shown with line as the median. Data are representative of two independent experiments. Statistical significance by Kruskal–Wallis test is shown. Symbols are closed squares (■) for ChAdOx1 prime responses, open circles (○) for mH5-MVA boost responses, and closed circles (●) for F11-MVA boost responses.

antibodies (mean endpoint titre (Log$_{10}$) = 3.2 and 2.8 respectively; Fig. 5A). At 42 d.p.i S1-specific antibody levels had increased to 4.7 and 4.8 respectively (Fig. 5A). The titres of nAb (MERS pp assay) were also similar for both vaccines (mean titre IC$_{90}$ (Log$_{10}$) = 2.71 (F11-MVA MERS) and 2.76 respectively; Fig. 5B). Utilising different promoters in MVA vectors did not result in differences in the induced antibody levels. However, at 42 d.p.i. IFN-γ secreting splenocytes induced by F11-MVA MERS were statistically significantly higher than those of mH5-MVA MERS ((Median = 525 and 249 SFU/10^6 splenocytes, respectively, Fig. 5C). Both MVA vaccines induced similar vector-specific immune responses as expected (Fig. 5D and E).

4. Discussion

Vaccines against MERS-CoV have been developed and tested in a number of animal models (including non-human primates [42–44] and camels [45]) as well as in human clinical trials [46]. All vaccine candidates focused on the spike antigen because it contains the receptor-binding domain used for cell entry by the virus, against which neutralising antibodies may be induced, and it is conserved. Therefore, the improvement of MERS-CoV vaccines focuses on platform and vaccination regimens rather than antigen selection and optimisation. Here, we focused on using the same antigen (transgene) to develop a vaccine against MERS-CoV, and
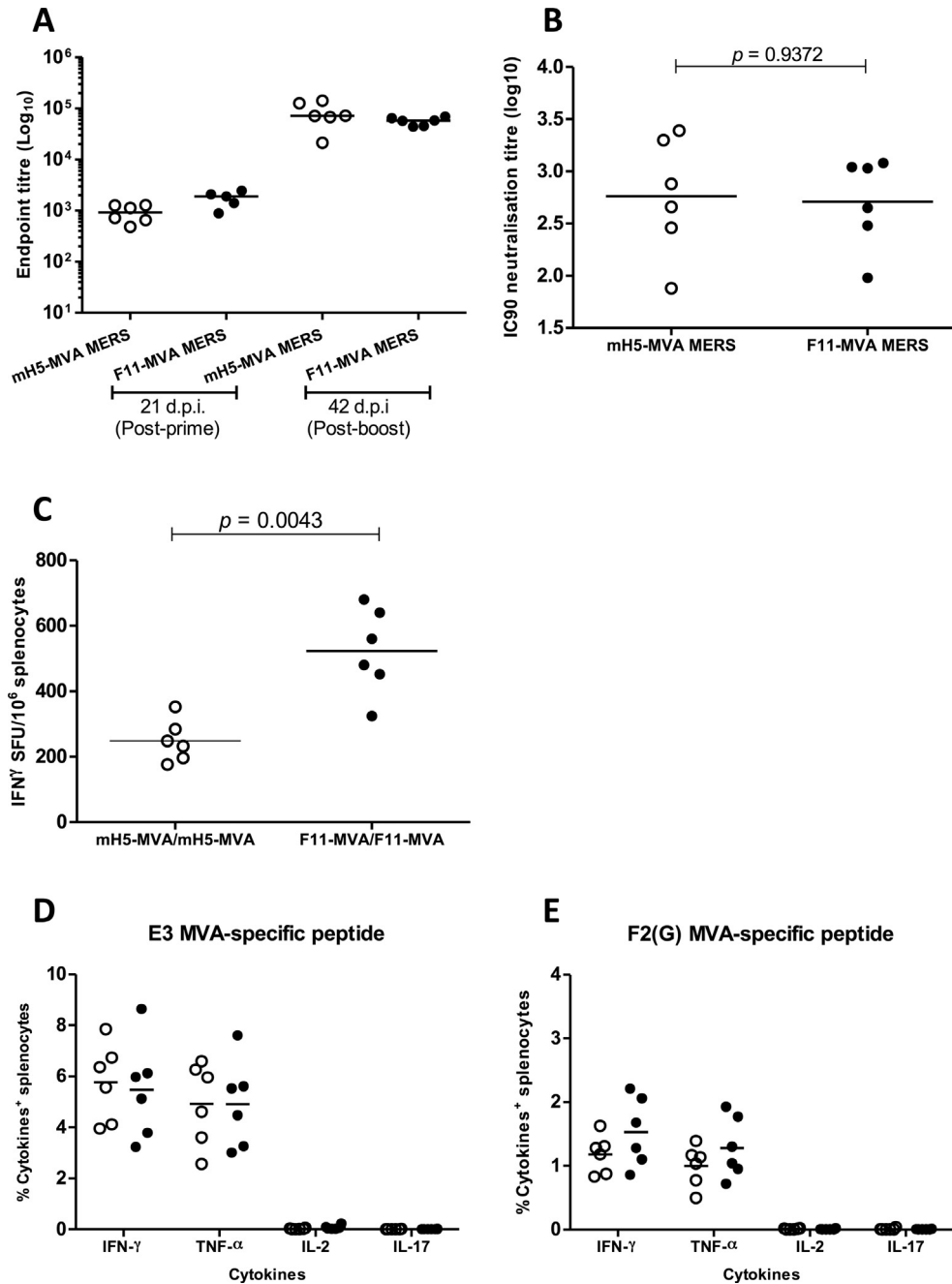
Fig. 5. Humoral and cellular immunogenicity of homologous MVA MERS vaccination. BALB/c mice (n = 6) were immunised intramuscularly with 1×10^6 pfu MVA MERS, in a homologous prime-boost vaccination with three-weeks interval between vaccination. MVA MERS vaccine candidates utilise either the mH5 or F11 promoter for transgene expression. Serum samples were collected at 21 (post-prime) and 42 (post-boost) d.p.i. S1-binding antibodies were measured by ELISA (A) and neutralisation activity of serum antibodies at 42 d.p.i. were confirmed by MERSpp neutralisation assay (B). At 42 d.p.i splenocytes were processed and re-stimulated with a MERS-CoV S-specific peptide (CD8+ T cell specific) for IFN-γ *ex vivo* ELISpot (C). ICS of splenocytes re-stimulated with MVA-specific peptides (F(G)2 and E3) was also performed (D and E) as was performed in Fig. 4. Individual data points (representing individual mice) are shown with line as the median. Data are representative of two independent experiments. Statistical significance by Kruskal–Wallis test is shown. Symbols are open circles (○) for mH5-MVA and closed circles (●) for F11-MVA.

to assess different vectors, different versions of each vector, and different vaccination regimens. We generated a number of MERS-CoV vaccine candidates based on the same codon optimized spike transgene and ensured its expression *in vitro* before we evaluated the humoral and cellular immunogenicity in a pre-clinical BALB/c mouse model. ChAdOx1 based vaccine candidates were produced with or without tPA. The tPA signal peptide was predicted to enhance the humoral immunogenicity of encoded vaccine antigens, based on previous reports [29]. Our data supported this hypothesis and showed a significant increase in the S1-specific

antibody levels at 28 d.p.i. The level of neutralising antibodies was also increased when tPA was utilised. However, ChAdOx1 MERS without tPA was still a potent vaccine candidate, inducing a high level of both S1-specific binding antibodies and MERS-CoV neutralising antibodies. Neutralisation activity of mouse serum antibodies was assayed by using MERS-CoV pseudotyped viral particles (MERSpp), an approach used by a number of researchers for other human pathogens such as HIV, Influenza, and HCV to overcome the necessity of handling BSL-3 viruses [40]. Additionally, we confirmed the ability of serum samples from vaccinated mice

to neutralise live MERS virus. We therefore selected ChAdOx1 MERS with tPA (simply referred to ChAdOx1 MERS) for further evaluation.

ChAdOx1 MERS also induced cellular responses for MERS S, with polyfunctional CD8+ T cells detected in the spleen of immunised mice. This supports the potency of the ChAdOx1 viral vector in inducing T cellular immunity, observed previously in animal models [26,32,47] as well as in humans [33]. Following ChAdOx1 prime/MVA boost, MVA significantly boosted the neutralising antibody titres to higher levels. No difference in humoral immunity was found when either the F11 or mH5 promoter was used. Regarding the promoter effect on MVA cellular immunogenicity, we have previously reported that utilising the F11 promoter enhanced malaria and influenza antigens in MVA [31]. Here, we again report that F11-MVA MERS induced higher T cell responses than mH5-MVA MERS in a homologous prime-boost MVA MERS vaccination.

All of our vaccine candidates induced humoral (with nAb) and cellular immune (with polyfunctional CD8+ T cell) responses against MERS-CoV spike antigen. Modest effects on immunogenicity of different versions of the vaccines were noted, with the use of the tPA leader sequence in ChAdOx1, and the use of the F11 promoter in MVA producing small increases in immunogenicity compared to no leader sequence, or the mH5 promoter. The protective level of either antibodies or cellular immunity required to counter MERS-CoV infection in humans or in animal models is not yet defined, despite some efforts [48–51]. The ideal vaccine would provide rapid onset of immunity and complete protective efficacy after a single dose, with a long duration of immunity. Complete protective efficacy of one dose of ChAdOx1 expressing the external glycoprotein of Rift Valley Fever Virus has been demonstrated in multiple species and it is already known that ChAdOx1 RVF is highly immunogenic in camels [32]. To date, the only vaccine against MERS to be tested in camels is an MVA vectored vaccine [41] which was protective in hDPP4 transgenic mice immunised with a homologous prime/boost regimen [37] but in camels required two doses given both intranasally and intramuscularly to provide partial protection and reduction of virus shedding [45]. Here we find that a single dose of ChAdOx1 MERS is as immunogenic as two doses of MVA MERS, suggesting that this regimen should be tested for protective efficacy in camels. However if this is not completely protective, administration of MVA MERS as a heterologous boost should be considered next. In our hands one dose of MVA resulted in an endpoint titre of 3 logs, two doses of MVA produced 4.7 logs, one dose of ChAdOx1 produced 5 logs, and ChAdOx1/MVA prime boost produced 5.9 logs. If a single dose of ChAdOx1 MERs is not protective and a two dose regimen is required, ChAdOx1/MVA would be more likely to provide complete protection than MVA/MVA.

ChAdOx1 MERS should now be evaluated for immunogenicity and efficacy in larger animal species, including both camels and humans.

Conflict of interest

References

[1] Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. New Engl J Med 2012;367(19):1814–20.

[2] World Health Organisation. Middle East respiratory syndrome coronavirus (MERS-CoV) 2016. Available from: http://www.who.int/emergencies/mers-cov/en/ [accessed 20.02.2017].

[3] Lessler J, Salje H, Van Kerkhove MD, Ferguson NM, Cauchemez S, Rodriquez-Barraquer I, et al. Estimating the severity and subclinical burden of Middle East respiratory syndrome coronavirus infection in the Kingdom of Saudi Arabia. Am J Epidemiol 2016;183(7):657–63.

[4] Al Hammadi ZM, Chu DK, Eltahir YM, Al Hosani F, Al Mulla M, Tarnini W, et al. Asymptomatic MERS-CoV infection in humans possibly linked to infected dromedaries imported from Oman to United Arab Emirates, May 2015. Emerg Infect Dis 2015;21(12):2197–200.

[5] Oboho IK, Tomczyk SM, Al-Asmari AM, Banjar AA, Al-Mugti H, Aloraini MS, et al. 2014 MERS-CoV outbreak in Jeddah–a link to health care facilities. N Engl J Med 2015;372(9):846–54.

[6] Who Mers-Cov Research G. State of knowledge and data gaps of Middle East respiratory syndrome coronavirus (MERS-CoV) in humans. PLoS Curr 2013;5.

[7] Memish ZA, Zumla AI, Assiri A. Middle East respiratory syndrome coronavirus infections in health care workers. New Engl J Med 2013;369(9):884–6.

[8] Muller MA, Corman VM, Jores J, Meyer B, Younan M, Liljander A, et al. MERS coronavirus neutralizing antibodies in camels, Eastern Africa, 1983–1997. Emerg Infect Dis 2014;20(12):2093–5.

[9] Perera RA, Wang P, Gomaa MR, El-Shesheny R, Kandeil A, Bagato O, et al. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin 2013;18(36): pii=20574.

[10] Hemida MG, Perera RA, Al Jassim RA, Kayali G, Siu LY, Wang P, et al. Seroepidemiology of Middle East respiratory syndrome (MERS) coronavirus in Saudi Arabia (1993) and Australia (2014) and characterisation of assay specificity. Euro Surveillance: Bull Eur sur les maladies transmissibles = Eur Commun Dis Bull 2014;19(23).

[11] Gutierrez C, Tejedor-Junco MT, Gonzalez M, Lattwein E, Renneker S. Presence of antibodies but no evidence for circulation of MERS-CoV in dromedaries on the Canary Islands, 2015. Euro Surveillance: Bull Eur sur les maladies transmissibles = Eur Commun Dis Bull 2015;20(37).

[12] Ali MA, Shehata MM, Gomaa MR, Kandeil A, El-Shesheny R, Kayed AS, et al. Systematic, active surveillance for Middle East respiratory syndrome coronavirus in camels in Egypt. Emerg Microbes Infect 2017;6(1):e1.

[13] van Doremalen N, Hijazeen ZS, Holloway P, Al Omari B, McDowell C, Adney D, et al. High prevalence of Middle East respiratory coronavirus in young dromedary camels in Jordan. Vector Borne Zoonotic Dis 2017;17(2):155–9.

[14] Alraddadi BM, Watson JT, Almarashi A, Abedi GR, Turkistani A, Sadran M, et al. Risk factors for primary Middle East respiratory syndrome coronavirus illness in humans, Saudi Arabia, 2014. Emerg Infect Dis 2016;22(1):49–55.

[15] Gossner C, Danielson N, Gervelmeyer A, Berthe F, Faye B, Kaasik Aaslav K, et al. Human-dromedary camel interactions and the risk of acquiring zoonotic Middle East respiratory syndrome coronavirus infection. Zoonoses Public Health 2016;63(1):1–9.

[16] Funk AL, Goutard FL, Miguel E, Bourgarel M, Chevalier V, Faye B, et al. MERS-CoV at the animal-human interface: inputs on exposure pathways from an expert-opinion elicitation. Front Veterin Sci 2016;3:88.

[17] Reusken CB, Ababneh M, Raj VS, Meyer B, Eljarah A, Abutarbush S, et al. Middle East Respiratory Syndrome coronavirus (MERS-CoV) serology in major livestock species in an affected region in Jordan, June to September 2013. Euro Surveillance: Bull Eur sur les maladies transmissibles = Eur Commun Dis Bull 2013;18(50):20662.

[18] Hemida MG, Perera RA, Wang P, Alhammadi MA, Siu LY, Li M, et al. Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013. Euro Surveillance: Bull Eur sur les maladies transmissibles = Eur Commun Dis Bull 2013;18(50):20659.

[19] Alagaili AN, Briese T, Mishra N, Kapoor V, Sameroff SC, Burbelo PD, et al. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. mBio 2014;5(2):e00884–e914.

[20] Reusken CB, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, et al. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. Lancet Infect Dis 2013;13(10):859–66.

[21] Vergara-Alert J, van den Brand JM, Widagdo W, Mt Munoz, Raj S, Schipper D, et al. Livestock susceptibility to Infection with Middle East respiratory syndrome coronavirus. Emerg Infect Dis 2017;23(2):232–40.

[22] Adney DR, Brown VR, Porter SM, Bielefeldt-Ohmann H, Hartwig AE, Bowen RA. Inoculation of goats, sheep, and horses with MERS-CoV does not result in productive viral shedding. Viruses 2016;8(8).

[23] Alharbi NK. Vaccines against Middle East respiratory syndrome coronavirus for humans and camels. Rev Med Virol 2016.

[24] Draper SJ, Heeney JL. Viruses as vaccine vectors for infectious diseases and cancer. Nat Rev Microbiol 2010;8(1):62–73.

[25] Tao D, Barba-Spaeth G, Rai U, Nussenzweig V, Rice CM, Nussenzweig RS. Yellow fever 17D as a vaccine vector for microbial CTL epitopes: protection in a rodent malaria model. J Exp Med 2005;201(2):201–9.

[26] Dicks MD, Spencer AJ, Edwards NJ, Wadell G, Bojang K, Gilbert SC, et al. A novel chimpanzee adenovirus vector with low human seroprevalence: improved systems for vector derivation and comparative immunogenicity. PloS one 2012;7(7):e40385.

[27] Gomez CE, Perdiguero B, Garcia-Arriaza J, Esteban M. Clinical applications of attenuated MVA poxvirus strain. Exp Rev Vacc 2013;12(12):1395–416.

[28] Gilbert SC. Clinical development of modified vaccinia virus Ankara vaccines. Vaccine 2013;31(39):4241–6.

[29] Luo M, Tao P, Li J, Zhou S, Guo D, Pan Z. Immunization with plasmid DNA encoding influenza A virus nucleoprotein fused to a tissue plasminogen activator signal sequence elicits strong immune responses and protection against H5N1 challenge in mice. J Virol Meth 2008;154(1–2):121–7.

[30] Wallace A, West K, Rothman AL, Ennis FA, Lu S, Wang S. Post-translational intracellular trafficking determines the type of immune response elicited by DNA vaccines expressing Gag antigen of Human Immunodeficiency Virus Type 1 (HIV-1). Hum Vaccin Immunother 2013;9(10):2095–102.

[31] Alharbi NK, Spencer AJ, Salman AM, Tully CM, Chinnakannan SK, Lambe T, et al. Enhancing cellular immunogenicity of MVA-vectored vaccines by utilizing the F11L endogenous promoter. Vaccine. 2016;34(1):49–55.

[32] Warimwe GM, Gesharisha J, Carr BV, Otieno S, Otingah K, Wright D, et al. Chimpanzee adenovirus vaccine provides multispecies protection against rift valley fever. Sci Rep 2016;6:20617.

[33] Antrobus RD, Coughlan L, Berthoud TK, Dicks MD, Hill AV, Lambe T, et al. Clinical assessment of a novel recombinant simian adenovirus ChAdOx1 as a vectored vaccine expressing conserved Influenza A antigens. Mol Ther: J Am Soc Gene Ther 2014;22(3):668–74.

[34] Cottingham MG, Carroll F, Morris SJ, Turner AV, Vaughan AM, Kapulu MC, et al. Preventing spontaneous genetic rearrangements in the transgene cassettes of adenovirus vectors. Biotechnol Bioeng 2012;109(3):719–28.

[35] Sridhar S, Reyes-Sandoval A, Draper SJ, Moore AC, Gilbert SC, Gao GP, et al. Single-dose protection against Plasmodium berghei by a simian adenovirus vector using a human cytomegalovirus promoter containing intron A. J Virol 2008;82(8):3822–33.

[36] Cottingham MG, Andersen RF, Spencer AJ, Saurya S, Furze J, Hill AV, et al. Recombination-mediated genetic engineering of a bacterial artificial chromosome clone of modified vaccinia virus Ankara (MVA). PloS one 2008;3(2):e1638.

[37] Volz A, Kupke A, Song F, Jany S, Fux R, Shams-Eldin H, et al. Protective efficacy of recombinant Modified Vaccinia virus Ankara (MVA) delivering Middle East Respiratory Syndrome coronavirus spike glycoprotein. J Virol 2015.

[38] Tscharke DC, Woo WP, Sakala IG, Sidney J, Sette A, Moss DJ, et al. Poxvirus CD8 + T-cell determinants and cross-reactivity in BALB/c mice. J Virol 2006;80 (13):6318–23.

[39] Draper SJ, Moore AC, Goodman AL, Long CA, Holder AA, Gilbert SC, et al. Effective induction of high-titer antibodies by viral vector vaccines. Nat Med 2008;14(8):819–21.

[40] Grehan K, Ferrara F, Temperton N. An optimised method for the production of MERS-CoV spike expressing viral pseudotypes. MethodsX. 2015;2:379–84.

[41] Song F, Fux R, Provacia LB, Volz A, Eickmann M, Becker S, et al. Middle East respiratory syndrome coronavirus spike protein delivered by modified vaccinia virus Ankara efficiently induces virus-neutralizing antibodies. J Virol 2013;87(21):11950–4.

[42] Muthumani K, Falzarano D, Reuschel EL, Tingey C, Flingai S, Villarreal DO, et al. A synthetic consensus anti-spike protein DNA vaccine induces protective immunity against Middle East respiratory syndrome coronavirus in nonhuman primates. Sci Transl Med 2015;7(301):301ra132.

[43] Lan J, Yao Y, Deng Y, Chen H, Lu G, Wang W, et al. Recombinant receptor binding domain protein induces partial protective immunity in rhesus macaques against Middle East respiratory syndrome coronavirus challenge. EBioMedicine 2015;2(10):1438–46.

[44] Wang L, Shi W, Joyce MG, Modjarrad K, Zhang Y, Leung K, et al. Evaluation of candidate vaccine approaches for MERS-CoV. Nat Commun 2015;6:7712.

[45] Haagmans BL, van den Brand JM, Raj VS, Volz A, Wohlsein P, Smits SL, et al. An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels. Science 2016;351(6268):77–81.

[46] Pellerin C. Army Scientists Begin First MERS Vaccine Clinical Trial. DoD News, Defense Media Activity; 2016.

[47] Warimwe GM, Lorenzo G, Lopez-Gil E, Reyes-Sandoval A, Cottingham MG, Spencer AJ, et al. Immunogenicity and efficacy of a chimpanzee adenovirus-vectored Rift Valley fever vaccine in mice. Virol J 2013;10:349.

[48] Wang W, Wang H, Deng Y, Song T, Lan J, Wu G, et al. Characterization of anti-MERS-CoV antibodies against various recombinant structural antigens of MERS-CoV in an imported case in China. Emerg Microb Infect 2016;5(11): e113.

[49] Min CK, Cheon S, Ha NY, Sohn KM, Kim Y, Aigerim A, et al. Comparative and kinetic analysis of viral shedding and immunological responses in MERS patients representing a broad spectrum of disease severity. Sci Rep 2016;6:25359.

[50] Park WB, Perera RA, Choe PG, Lau EH, Choi SJ, Chun JY, et al. Kinetics of Serologic Responses to MERS Coronavirus Infection in Humans, South Korea. . Emerg Infect Dis 2015;21(12):2186–9.

[51] Corman VM, Albarrak AM, Omrani AS, Albarrak MM, Farah ME, Almasri M, et al. Viral shedding and antibody response in 37 patients with Middle East respiratory syndrome coronavirus infection. Clin Infect Dis 2016;62 (4):477–83.