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Middle East Respiratory Syndrome Coronavirus Spike Protein Delivered by Modified Vaccinia Virus Ankara Efficiently Induces Virus-Neutralizing Antibodies

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Middle East respiratory syndrome coronavirus (MERS-CoV) has recently emerged as a causative agent of severe respiratory disease in humans. Here, we constructed recombinant modified vaccinia virus Ankara (MVA) expressing full-length MERS-CoV spike (S) protein (MVA-MERS-S). The genetic stability and growth characteristics of MVA-MERS-S make it a suitable candidate vaccine for clinical testing. Vaccinated mice produced high levels of serum antibodies neutralizing MERS-CoV. Thus, MVA-MERS-S may serve for further development of an emergency vaccine against MERS-CoV.

Middle East respiratory syndrome coronavirus (MERS-CoV), a novel infectious agent causing severe respiratory disease and death in humans, was first described in 2012 (1–3). To date, a total of 108 cases of infection with MERS-CoV have been confirmed, including 50 deaths (http://www.who.int/csr/don/2013_08_30/en/index.html). Most infections were geographically linked to the Middle East, i.e., Jordan, Saudi Arabia, Qatar, and United Arab Emirates, but cases also occurred in the United Kingdom, Germany, France, and Italy. The epidemiology of MERS-CoV infection remains unclear. The virus is suspected to persist in animal reservoirs and cause zoonotic infections in humans (4, 5). The MERS-CoV spike (S) protein, a characteristic structural component of the virion membrane, forms large protruding spikes on the surface of the virus; its S1 domain mediates binding to dipeptidyl peptidase 4, which serves as the host cell receptor of MERS-CoV (6). Importantly, the S protein is considered a key component of vaccines against coronavirus infection, including severe acute respiratory syndrome (SARS) (7, 8).

Modified vaccinia virus Ankara (MVA), a highly attenuated strain of vaccinia virus originating from growth selection on chicken embryo fibroblasts (CEF), shows a characteristic replication defect in mammalian cells (9, 10, 11). At present, MVA serves as one of the most advanced recombinant poxvirus vectors in preclinical research and human clinical trials for developing new vaccines against infectious disease and cancer (12, 13, 14).

Here, we show that the full-length S protein of MERS-CoV, expressed by MVA, is produced as an ~210-kDa N-glycosylated protein that is specifically recognized by antibodies in Western blot analysis. Further studies suggest cleavage of the mature full-length S glycoprotein into an amino-terminal domain (S1) and an ~85-kDa carboxy-terminal domain (S2) that is putatively anchored to the membrane. When tested as a vaccine in mice, recombinant MVA expressing the S protein induced high levels of circulating antibodies that neutralize MERS-CoV in tissue culture infections.

Construction and characterization of recombinant MVA. cDNA containing the entire gene sequence encoding MERS-CoV S (GenBank accession no. JX869059) was obtained by DNA synthesis (Invitrogen Life Technology, Regensburg, Germany) and modified by introducing silent mutations that remove three termination signals (TTTTTNT) for vaccinia virus transcription (MERS-S). Furthermore, we generated a second version containing a tag sequence encoding nine amino acids (YPYDVPDYA) from influenza virus hemagglutinin (HA tag) attached at the C terminus of S (MERS-S_{HA}). MERS-S and MERS-S_{HA} were cloned under the transcriptional control of the vaccinia virus early/late promoter PmH5 (15) and introduced by homologous recombination into an existing deletion site (deletion III) in the MVA genome (Fig. 1A).

MVA expressing MERS-S or MERS-S_{HA} (MVA-MERS-S or MVA-MERS-S_{HA}, respectively) was obtained using standard methods to generate recombinant MVA vaccines suitable for clinical testing, as described previously (13). Briefly, transient coproduction of the fluorescent marker protein mCherry (under the control of the vaccinia virus late promoter P11 [16]) was used to isolate clonal recombinant viruses by screening for fluorescent cell foci during repetitive plaque purification. At this stage, immunostaining of infected cell cultures with anti-HA tag monoclonal or polyclonal antibodies from MERS-CoV-infected macaques suggested synthesis of the recombinant S_{HA} and S proteins in CEF and Vero cells (ATCC CCL-81) (Fig. 2). MVA-MERS-S and MVA-MERS-S_{HA} were genetically stable and replicated efficiently in CEF but not in human HeLa or HaCat cells (Fig. 1B and C). The latter findings confirmed that the recombinant viruses could be handled under biosafety level 1 conditions.

Characterization of MERS-CoV S produced by recombinant MVA. We specifically detected a protein with an estimated molecular mass of about 200 kDa in lysates from MVA-MERS-S- and MVA-MERS-S_{HA}-infected Vero cells by using sera from MERS-

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BioNTech-Pfizer Exhibit 1109
BioNTech SE and Pfizer Inc. v. ModernaTX, Inc. IPR2023-01358
Page 1 of 5

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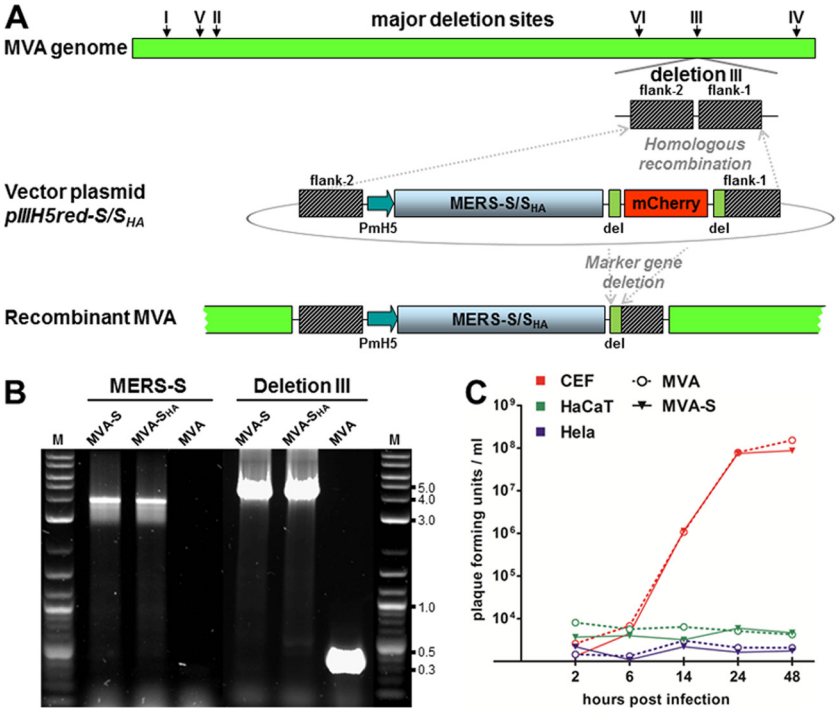

FIG 1 Generating and characterizing recombinant MVA. (A) Schematic diagram of the MVA genome and the locations of major deletion sites I to IV, with deletion III being the site used to insert the MERS-CoV S gene sequences. Flank-1 and flank-2 refer to MVA DNA sequences adjacent to deletion site III which were originally prepared by PCR and cloned into MVA transfer plasmids targeting deletion site III for insertion of recombinant genes. In MVA vector plasmids pIIIH5red-S and -S_{HA}, the S coding gene sequences (MERS-S/S_{HA}) are placed under transcriptional control of the vaccinia virus promoter PmH5 and introduced by homologous recombination between the flanking sequences in the vector and the MVA genome. MVA-MERS-S and MVA-MERS-S_{HA} were isolated in plaque passages by screening for transient coexpression of the fluorescent marker gene mCherry under transcriptional control of the vaccinia virus late promoter P11. Repetitive sequences (del) are designed to remove the mCherry marker by intragenomic homologous recombination (marker gene deletion). (B) Genetic integrity and genetic stability of MVA-MERS-S and MERS-S_{HA}. PCR analysis of genomic viral DNA using oligonucleotide primers to confirm the identity (MERS-S) and proper insertion (deletion III) of S gene sequences. (C) Multiple-step growth analysis of recombinant MVA-MERS-S. Recombinant MVA (MVA-S) and wild-type MVA (MVA) can be efficiently amplified in CEF (multiplicity of infection [MOI], 0.1) but fail to productively grow in HeLa and HaCat human cell lines.

CoV-infected macaques in Western blots (Fig. 3A, upper panel). Further immunoblot analysis with monoclonal anti-HA tag antibody (Roche, Penzberg, Germany) confirmed the production of a protein doublet of 200 to 210 kDa in cells infected with MVA-MERS-S_{HA} as well as a second protein of about 85 kDa (Fig. 3A, lower panel).

The molecular masses of the 200- to 210-kDa polypeptides detected by SDS-PAGE were significantly higher than the 149 kDa predicted for MERS-CoV S protein based on its nucleotide sequence. NetNGlyc server analysis suggested at least 17 likely N-glycosylation sites [Asn-X-(Ser/Thr)] for co- and posttranslational modification. Therefore, we investigated the glycosylation pattern of the expressed S_{HA} protein by using treatment with peptide-N-glycosidase F (PNGase F) or endoglycosidase H (endo H) (both from New England BioLabs), followed by Western blotting (Fig. 3B). S_{HA} produced in MVA-MERS-S_{HA}-infected Vero cells was treated with PNGase F, which removes all N-linked oligosaccharide chains from glycoproteins. This treatment reduced the ~210-kDa protein doublet to a sharp protein band of 150 kDa, a value closely matching the predicted mass of unmodified MERS-CoV S protein.

Similarly, PNGase F treatment converted the 85-kDa protein fragment to a polypeptide with a molecular mass of 55 kDa. Endo H cleaves N-linked high-mannose oligosaccharides synthesized

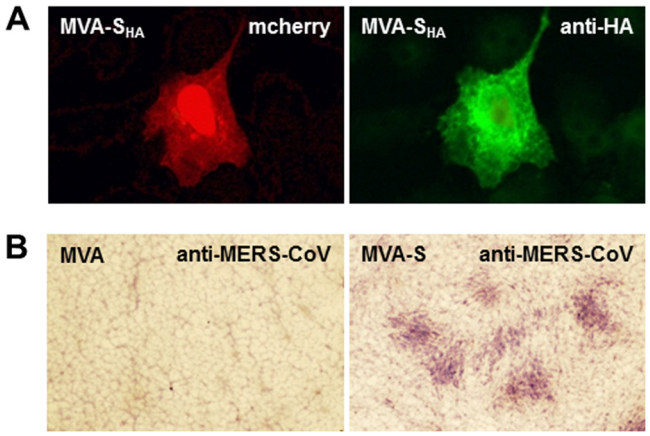

FIG 2 Immunostaining of S proteins in recombinant MVA-infected cells. (A) Transient expression of the marker protein mCherry served to localize single virus-infected cells (left panel). Monoclonal antibody directed against the HA tag (anti-HA) (right panel) reveals the presence of S_{HA} in Vero cells infected (MOI, 0.1) with MVA-MERS-S_{HA} (MVA-S_{HA}). (B) Polyclonal antibodies from a MERS-CoV-infected cynomolgous macaque (anti-MERS-CoV) detected S-producing cell foci in CEF infected with MVA-MERS-S (MVA-S; MOI, 0.1) but no foci when CEF were infected with nonrecombinant MVA (MVA).

BioNTech-Pfizer Exhibit 1109
BioNTech SE and Pfizer Inc. v. ModernaTX, Inc. IPR2023-01358
Page 2 of 5

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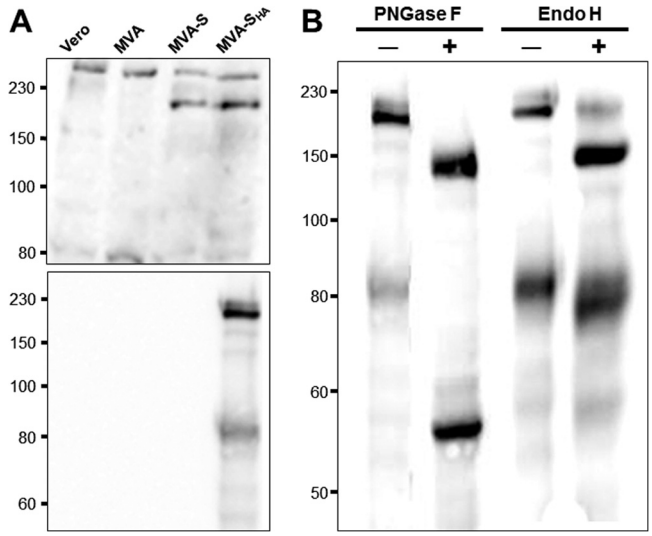

FIG 3 Synthesis of full-length S glycoprotein in recombinant MVA-infected cells. (A) Western blot analysis of cell lysates from MVA-MERS-S (MVA-S)- or MVA-MERS-S_{HA} (MVA-S_{HA})-infected Vero cells 24 h postinfection. Polypeptides were analyzed by SDS-PAGE and immunoblotting using serum from a MERS-CoV-infected macaque (1:1,000; upper panel) or monoclonal rat anti-HA tag antibody (1:50; lower panel). Lysates from uninfected (Vero) or wild-type MVA-infected (MVA) cells served as controls. (B) Western blot analysis of MVA expressed S_{HA} following treatment with glycosidases. Vero cells were infected with MVA-MERS-S_{HA} for 24 h. Cell lysates were incubated with (+) or without (−) glycosidase PNGase F or endo H and analyzed by SDS-PAGE and immunoblotting with monoclonal rat anti-HA tag antibody (1:50). Numbers on the left indicate molecular masses of marker proteins in kilodaltons.

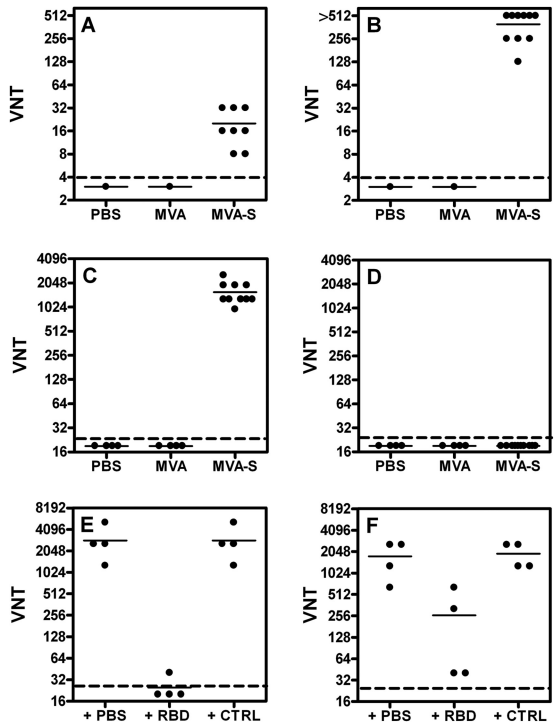

FIG 4 Antibody responses after intramuscular immunization with recombinant MVA-MERS-S. BALB/c mice (n = 10) were vaccinated twice within a 21-day interval with 10^8 PFU MVA-MERS-S (MVA-S). Groups of mice (n = 4) vaccinated with wild-type MVA (MVA) or saline (PBS) served as controls. MERS-CoV virus-neutralizing titers (VNT) were determined 20 days after primary immunization (A) and 10 days after the second immunization (B and C) using Huh7 (A and B) and Vero cells (C). Sera obtained after the second boost were also tested against SARS-CoV on Vero cells (D). Serum from a rabbit (17) immunized with the MERS-CoV receptor binding domain (RBD) (E) or sera from mice obtained after the second MVA-S boost (F) were preincubated with PBS, the RBD, or a SARS-CoV-derived control protein (CTRL) at 5 μg/ml for 1 h before incubation with MERS-CoV, followed 1 h later by inoculation on Vero cells.

in the endoplasmic reticulum but not those matured to more complex oligosaccharides in the Golgi apparatus. Western blot analysis of S_{HA} digested with endo H revealed a band of 150-kDa proteins, indicating complete hydrolysis of N-linked oligosaccharides. An additional subpopulation of S_{HA} migrated with the original size of about 210 kDa, indicating resistance to endo H treatment. Of note, the 85-kDa protein subpopulation remained largely unaffected by endo H digestion.

Immunogenicity of MVA-MERS-S in mice. BALB/c mice were vaccinated intramuscularly with 10^8 PFU MVA-MERS-S at 0 and 3 weeks. Twenty days after the first immunization and 10 days after the second immunization, serum samples were tested for their capacity to neutralize MERS-CoV (EMC isolate) in tissue culture infections by using 200 50% tissue culture infective doses ($TCID_{50}$) in Vero cells or 100 $TCID_{50}$ in Huh-7 cells. Even a single application of MVA-MERS-S induced low levels of virus-neutralizing antibodies in all eight animals tested (Fig. 4A). After booster immunization, all vaccinated animals produced high levels of circulating antibodies that neutralized MERS-CoV (Fig. 4B and C). In contrast, neutralizing antibodies were not detected in serum samples from control animals inoculated with nonrecombinant MVA or saline (phosphate-buffered saline [PBS]). The specificity of the induced MVA-MERS-S antibodies for MERS-CoV was confirmed by the absence of detectable neutralization against SARS coronavirus (SARS-CoV) (strain HKU39849) (Fig. 4D). In addition, the MERS-CoV-neutralizing activity of these serum samples could be only partly blocked by preincubation with proteins that encompass the receptor binding domain (RBD) (Fig. 4E and F). This last observation is consistent with the fact that besides the RBD, other parts of the spike protein are able to induce neutralizing antibodies (17).

Conclusions. Our objective was to use MVA vectors compatible with clinical evaluation to express mostly native S antigen to induce antibodies that would neutralize MERS-CoV. The S gene of MERS-CoV expressed by recombinant MVA produced a glycoprotein migrating at a molecular mass of about 210 kDa. Glycosidase treatment to remove all N-linked carbohydrates resulted in a polypeptide of 150 kDa, closely corresponding to the molecular mass predicted from the S gene nucleotide sequence. A subpopulation of protein apparently acquired endo H resistance, indicating trafficking of S to the Golgi apparatus, which correlates with observations made for SARS-CoV S protein (18, 19, 20).

In addition, we obtained evidence for putative S1 and S2 cleavage of full-length S as predicted recently (6, 21). The HA tag fused to the C terminus of S_{HA} enabled us to detect two glycoprotein subpopulations, full-length S (~210 kDa) and subdomain S2 (~85 kDa), by anti-HA Western blot analysis (Fig. 3). We considered furin cleavage sites because spike proteins of some betacoronaviruses and all gammacoronaviruses are typically activated by intracellular furin-dependent cleavage (22, 23; for a review, see

BioNTech-Pfizer Exhibit 1109
BioNTech SE and Pfizer Inc. v. ModernaTX, Inc. IPR2023-01358
Page 3 of 5

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references 24 and 25); the ProP1.0 server indicated three possible furin cleavage sites at amino acid positions 751, 887, and 1113 of MERS-CoV S (data not shown). We favor putative cleavage at amino acid 887 since this cleavage would produce a predicted S2 subdomain of 54.5 kDa for the nonglycosylated protein matching our Western blot data (Fig. 3). Moreover, prominent endo H resistance of the 85-kDa S2 subdomain suggests that S cleavage occurs predominantly during or after passage through the Golgi apparatus.

Since biochemical characterization of the MVA-expressed S suggested synthesis of a mature and properly folded spike antigen, we investigated whether MVA-MERS-S would elicit virus-neutralizing antibodies. Indeed, mice immunized with MVA-MERS-S via an intramuscular route developed circulating antibodies that neutralized MERS-CoV infections in highly permissive tissue cultures. Interestingly, compared to results of previous studies eliciting neutralizing antibodies to SARS-CoV (18), MVA-MERS-S induced relatively high levels of antibodies that efficiently block MERS-CoV infection. This observation may be explained by MERS-CoV-specific differences in receptor usage and entry mechanisms, as discussed previously (18). Previous work with SARS-CoV showed that S-specific neutralizing antibodies correlated with the protective capacity of vaccination in various animal models (18, 26, 27; for a review, see reference 28).

Future studies will be necessary to monitor for S antibody-dependent enhancement of MERS-CoV infections, as previously discussed for SARS-CoV and feline coronavirus infections (28, 29, 30). Furthermore, the safety and protective capacity of MVA-MERS-S immunization should be tested in animal models that reproduce MERS-CoV infections in humans. However, the current absence of suitable preclinical models recommends the development of an MVA vaccine delivering MERS-CoV S.

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BioNTech-Pfizer Exhibit 1109
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Page 4 of 5

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BioNTech-Pfizer Exhibit 1109
BioNTech SE and Pfizer Inc. v. ModernaTX, Inc. IPR2023-01358
Page 5 of 5

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