HHS Public Access

Author manuscript

*Sci Transl Med*. Author manuscript; available in PMC 2015 September 17.

Published in final edited form as:

*Sci Transl Med*. 2015 August 19; 7(301): 301ra132. doi:10.1126/scitranslmed.aac7462.

# A synthetic consensus anti–spike protein DNA vaccine induces protective immunity against Middle East respiratory syndrome coronavirus in nonhuman primates

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**Competing interests**: D.B.W. has grant funding, participates in industry collaborations, has received speaking honoraria, and fees for consulting. This service includes serving on scientific review committees and advisory boards. Remuneration includes direct payments and/or stock or stock options and in the interest of disclosure therefore he notes potential conflicts associated with this work with Inovio where he serves on the SAB, Merck, VGXI, OncoSec, Roche, Aldevron, and possibly others. Licensing of technology from his laboratory has created over 150 jobs in the biotech/pharma industry. The other authors declare no competing interests.

## Abstract

First identified in 2012, Middle East respiratory syndrome (MERS) is caused by an emerging human coronavirus, which is distinct from the severe acute respiratory syndrome coronavirus (SARS-CoV), and represents a novel member of the lineage C betacoronoviruses. Since its identification, MERS coronavirus (MERS-CoV) has been linked to more than 1372 infections manifesting with severe morbidity and, often, mortality (about 495 deaths) in the Arabian Peninsula, Europe, and, most recently, the United States. Human-to-human transmission has been documented, with nosocomial transmission appearing to be an important route of infection. The recent increase in cases of MERS in the Middle East coupled with the lack of approved antiviral therapies or vaccines to treat or prevent this infection are causes for concern. We report on the development of a synthetic DNA vaccine against MERS-CoV. An optimized DNA vaccine encoding the MERS spike protein induced potent cellular immunity and antigen-specific neutralizing antibodies in mice, macaques, and camels. Vaccinated rhesus macaques seroconverted rapidly and exhibited high levels of virus-neutralizing activity. Upon MERS viral challenge, all of the monkeys in the control-vaccinated group developed characteristic disease, including pneumonia. Vaccinated macaques were protected and failed to demonstrate any clinical or radiographic signs of pneumonia. These studies demonstrate that a consensus MERS spike protein synthetic DNA vaccine can induce protective responses against viral challenge, indicating that this strategy may have value as a possible vaccine modality against this emerging pathogen.

## INTRODUCTION

The Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified in 2012, with cases subsequently appearing and clustering predominantly in the Arabian Peninsula (1–4). More than 1300 cases have been reported and they are associated with a high rate of hospitalization and fatalities (about 40%). Accordingly, this emerging infection is of great public health concern (5, 6). This concern was further heightened by recent MERS cases reported in North America and Asia, as well as clear documentation of human-to-human spread (7). The virus's geographical distribution points to an intermittent transmission, and although the zoonotic reservoir remains to be conclusively identified, some indications suggest that bats and camels can function as the reservoir and/or intermediate/amplifying hosts for transmission to humans (2, 8, 9). In 2003, a similar outbreak of acute respiratory disease occurred caused by the related severe acute respiratory syndrome coronavirus (SARS-CoV) (10, 11). Similar to SARS-CoV, patients infected with MERS-CoV suffer from severe lower respiratory tract infections that are characterized by an acute fever, cough, and shortness of breath (12–16). MERS-CoV has been identified as a lineage C betacoronavirus that has segregated into more than two distinct clades (15, 17). A number of clusters have reported human-to-human transmission of the virus, which is a concern given the extent of global travel, as illustrated by the 2015 MERS outbreak in South Korea (6, 7, 18, 19).

Previous studies examining mechanisms of protection against SARS-CoV provide insight into vaccination strategies for pathogens such as MERS-CoV. Vaccination against SARS-CoV in animal studies illustrates that the coronavirus spike (S) protein is immunogenic, and that immunization of animals with S protein–based vaccines can induce neutralizing

antibodies (NAbs) (20) that are effective in preventing infection by homologous coronaviruses (21). Furthermore, patients infected with SARS naturally produce an antibody response against the S protein of SARS-CoV, and these antibodies are protective in passive transfer animal studies (7, 16, 22). However, in the case of MERS, the divergence of the virus and the current lack of a small animal challenge model provide major hurdles for vaccine design and study.

Here, we evaluated a synthetically designed consensus DNA vaccine developed through comparison of current database sequences focused on the MERS-CoV S glycoprotein. A consensus approach can, in principle, help to overcome some of the immune escape issues induced by variability of a pathogen, as we have previously described (23, 24). The synthetic, optimized, full-length consensus MERS vaccine induced strong $CD8^+$ and $CD4^+$ T cell immunity in small animals and rhesus macaques. Notably, the vaccine drives potent humoral immune responses in mice, camels, and nonhuman primates (NHPs), including NAbs that prevent infection. This vaccine was able to induce immune responses that protected rhesus macaques from clinical disease and its associated pathology.

## RESULTS

### Synthetic development of a MERS-CoV DNA vaccine

The consensus sequence for the MERS-CoV S protein vaccine was generated after analysis of the S protein genomic sequences, which were deposited in the GenBank-NCBI (National Center for Biotechnology Information) database. In previous reports, it was described that such consensus immunogens can induce broad cellular and humoral immune responses against diverse virus strains/isolates (24–27). Sequences from both clades A and B were included in the construct design. The MERS vaccine immunogen included several modifications to enhance in vivo expression, including the addition of a highly efficient immunoglobulin E (IgE) leader peptide sequence to facilitate expression and mRNA export. The insert was then subcloned into the pVax1 vector (Fig. 1A).

The MERS vaccine plasmid was transfected into 293T cells, and the expression of S protein was evaluated by Western blotting. Serum from MERS vaccine–immunized mice was used to detect the expression of S protein in the plasmid-transfected cell lysates. As expected, strong specific bands of MERS-CoV S protein (190 kD) were detected in MERS vaccine–transfected cells but not in lysates from cells transfected with the control vector (pVax1) (Fig. 1B).

In addition, the expression and localization of S protein expressed by the MERS vaccine were investigated using an immunofluorescence assay. The immunofluorescence assay with mouse anti–MERS vaccine serum revealed a strong signal in the cytoplasm in MERS vaccine–transfected cells (Fig. 1C). In contrast, the positive signal was not detected in cells transfected with pVax1 control vector. These results demonstrate the ability of the MERS vaccine to express strongly in mammalian cells and that antibodies induced by this construct can bind their target antigen.

## MERS vaccine induces potent antigen-specific cellular immune responses

The immunogenicity of the MERS vaccine was first investigated in mice. Female C57BL/6 mice ($n = 9$) were intramuscularly injected with 25 μg of either the MERS vaccine or the control pVax1 vector. Delivery of vaccines was facilitated by in vivo electroporation (EP), as previously described (24). Animals were vaccinated three times at 2-week intervals, and immune responses were measured 1 week after the third immunization.

Cell-mediated immunity was evaluated using a standard enzyme-linked immunospot (ELISpot) assay to monitor the ability of splenocytes from immunized mice to secrete interferon-γ (IFN-γ) after antigen-specific ex vivo restimulation with peptide pools encompassing the entire MERS S glycoprotein. As indicated in Fig. 2A, the MERS vaccine induced a strong cellular immune response [indicated by a high level of spot-forming units (SFU) per $10^6$ cells] in response to stimulation by multiple peptide pools. Peptides in pools 2 and 5 appeared immunodominant in this mouse haplotype.

On the basis of these T cell responses, a detailed mapping analysis using 31 matrix peptide pools spanning the entire MERS-CoV S protein was subsequently performed. After restimulation with peptide, a strong T cell response was detected against several regions on the S protein (Fig. 2B). There were 15 matrix pools demonstrating more than 100 spots per million cells, indicating that vaccination with the MERS vaccine elicited a broad cellular immune response. Using the matrix mapping method, we identified four peptides within the region from amino acids 301 to 334 that appeared to be the dominant epitopes (pools 18 to 21). In addition to this region, splenocytes from the immunized mice reacted to three other major regions spanning the peptide pools 4 to 6, 11 to 13, and 29 to 31. These pools include a predicted $CD8^+$ T lymphocyte immunodominant epitope at amino acids 307 to 321 (RKAWAAFYVYKLQPL).

## MERS vaccine generates highly polyfunctional T cell responses

To further determine the phenotype of the induced T cell response, polyfunctional T cell responses were analyzed. To accomplish this, polychromatic flow cytometry (28) was used to measure the production of IFN-γ, interleukin-2 (IL-2), and tumor necrosis factor–α (TNF-α) induced in an antigen-specific fashion in both $CD4^+$ and $CD8^+$ T cells. The flow cytometry profiles of MERS S–specific IFN-γ–, IL-2–, and TNF-α–secreting $CD4^+$ and $CD8^+$ T cells are shown in Fig. 2C. The magnitude of vaccine-induced $CD4^+$ and $CD8^+$ T cell responses after vaccination with the MERS vaccine was compared to those in animals with the control pVax1. Boolean gating was used to measure the ability of individual cells to produce multiple cytokines, that is, the polyfunctionality of the vaccine-induced $CD4^+$ and $CD8^+$ T cell response. Both the proportion of mono-, bi-, and trifunctional cells and the overall magnitude of the $CD4^+$ and $CD8^+$ T cell responses were superior in the MERS vaccine group. When the responses were then further divided into their seven possible functional combinations, it was observed that $CD8^+$ T cells in the vaccination group demonstrated a major increase in the number of $CD8^+$ T cells that produce IFN-γ and an increase in both $CD4^+$ and $CD8^+$ T cells that produce multiple cytokines.

## MERS vaccine induces binding and NAb responses in mice

The induction of functional humoral immune responses in mice by vaccination with the MERS vaccine was evaluated. Serum samples were obtained before and after DNA immunization. The anti–S protein humoral immune responses were analyzed for binding to recombinant S antigen as well as for functional NAb responses (29, 30). As indicated in Fig. 3A, immunized with the MERS vaccine animals produced a robust S protein–specific antibody response compared to the control animals (pVax1) as measured by enzyme-linked immunosorbent assay (ELISA). Endpoint titers of S protein–specific antibodies in mice immunized with the MERS vaccine also increased after each immunization (Fig. 3B). The antibodies generated by immunized with the MERS vaccine mice also bound to recombinant S protein in a Western blot assay (Fig. 3C). The neutralizing activity in sera from mice immunized with the MERS vaccine was assessed via a viral neutralization assay, which used a clade A strain of MERS-CoV, designated EMC/2012. As indicated in Fig. 3D, immunization with the MERS vaccine induced NAb titers that were higher than those in sera from mice immunized with the control vector (pVax1) alone.

Conventional neutralization assays as well as various infection assays using live MERS-CoV can logistically and technically be cumbersome and require biosafety level 3 facilities. This, in turn, creates challenges for conducting immunopathogenesis and functionality studies. Therefore, a pseudovirus neutralization assay was used. Several such assays have been recently reported (31, 32). This assay is very sensitive and quantitative and can be conducted using biosafety level 2 facilities and methods (33). MERS-CoV pseudoviral particles were produced by cotransfection of 293T cells with plasmids encoding the MERS S protein and an HIV-1 luciferase reporter plasmid, which does not express a native envelope. A panel of DNA plasmids (England/2/2013, Al-Hasa_1_2013, HUK1, and NL63) were synthesized as described previously (34) and were used in this study. Pseudovirus expressing vesicular stomatitis virus glycoprotein G (VSV-G) was included as a positive control, and pseudovirus without any envelope protein was used as a negative control. Furthermore, the HIV-1 core antigen p24 can be quantified by ELISA, allowing for standardization during the pseudoviral infection. Specifically, sera were evaluated for neutralizing activity against different S proteins with the MERS pseudovirus–based inhibition assay. As indicated in Fig. 3E, antisera from immunized with the MERS vaccine mice ($n = 4$) efficiently inhibited infection of Vero cells by the pseudoviruses tested. However, England/2/2013 and Al-Hasa_1_2013 MERS coronaviruses appear to be closer than HKU1 and NL63, which are related coronaviruses, on the basis of the neutralization pattern observed. These data support the relevance of the MERS vaccine–induced humoral responses.

## MERS vaccine induces binding and NAbs in camels

Three dromedary camels were immunized three times at 4-week intervals with the MERS vaccine delivered with EP, and the humoral immune response was examined by Western blot as well as viral neutralization assay (Fig. 4A). S protein–specific antibodies were detected by Western blot in sera from all three immunized camels at week 11 (3 weeks after the third immunization), whereas no specific antibody response was detected in sera samples from week 0 (prebleed) in any animal (Fig. 4B). Robust NAb titers were also detected in

two of three immunized animals after vaccination (Fig. 4C). These data show that synthetic MERS vaccine is capable of inducing S protein–specific binding and NAbs in camels, a natural host to the MERS virus.

## MERS vaccine induces high antigen-specific cellular immune responses in rhesus macaques

The immunogenicity and efficacy of the MERS vaccine against a virulent MERS-CoV challenge were assessed in rhesus macaques. Rhesus macaques were vaccinated with EP-enhanced delivery three times at 3-week intervals with the MERS vaccine, as described in Materials and Methods. A low dose (0.5 mg per immunization) and a high dose (2 mg per immunization) were used to determine the optimal dose in rhesus macaques. Figure 5A provides details of the MERS vaccine immunization protocol, along with the time points for immunological evaluation and viral challenge. To determine the impact of the novel MERS vaccine on cellular immune responses, an IFN-γ ELISpot was used to measure the T cell response in the blood of vaccinated animals. After three immunizations, the number of MERS S protein–specific cells present in the blood of the low-dose group ranged between 500 and 1100 SFU per million peripheral blood mononuclear cells (PBMCs), whereas in the high-dose group, responses ranged between 500 and 1500 SFU per million PBMCs (Fig. 5B).

To gain further insight into the responses of the MERS vaccine–specific $CD4^+$ and $CD8^+$ T cells, we also measured the polyfunctionality of these populations on the basis of the expression of IFN-γ, TNF-α, and IL-2 after peptide stimulation (Fig. 5C). Both the low- and high-dose groups produced $CD4^+$ and $CD8^+$ T cells secreting IFN-γ, TNF-α, and, to a lesser extent, IL-2. The high-dose group produced significantly higher percentages of $CD4^+$ and $CD8^+$ T cells secreting IFN-γ, IL-2, and TNF-α. Thus, it was concluded that the MERS vaccine can induce substantial T cell responses in immunized rhesus macaques.

## MERS vaccine induces binding and NAb responses in rhesus macaques

To assess the humoral immune response in rhesus macaques after MERS vaccine immunization, we measured MERS-CoV S protein specific IgG in serum obtained from vaccinated animals at various time points throughout the immunization schedule. First, an ELISA using full-length MERS-CoV S protein as the immobilized antigen was performed. The binding ELISA results are shown in Fig. 6A. All prevaccination (day 0) sera were negative for MERS S protein–specific antibodies. A robust increase in endpoint antibody titers of >10,000 was observed in both the low- and high-dose groups. No statistically significant difference was noted between the two vaccine doses; however, all four rhesus macaques in the high-dose group seroconverted after a single immunization, whereas the low-dose group took two immunizations to see complete serocon-version. To verify that the immune sera reacted with recombinant MERS S protein, we performed a Western blot analysis and compared the ability of a commercially available monoclonal antibody and pooled sera collected from the rhesus macaques after the final immunization to bind to recombinant S protein (Fig. 6B). The result confirms the ELISA data that the MERS vaccine is able to induce antibodies that are specific for the MERS S target protein. To determine the level of NAb present in the seraof MERS vaccine–immunized rhesus macaques, we

performed a MERS-CoV neutralization assay using sera collected 2 weeks after the final immunization. Rhesus macaques immunized with both low and high doses of MERS vaccine displayed elevated neutralization titers against live MERS-CoV strain EMC/2012 (Fig. 6C). MERS-CoV genomes are phylogenetically classified into multiple clades (3, 17). To determine whether MERS vaccine immunization would induce cross-clade neutralizing activity in NHP, MERS pseudoviruses expressing S protein from different isolates from multiple clades were studied using two macaques from each dose group. Both the low- and high-dose animal sera contained antibodies capable of blocking entry of the pseudoviruses as measured by a decrease in luciferase activity compared to pseudovirus alone (Fig. 6D). All four NHP immune sera could neutralize all five pseudoviral S antigens with subtle differences. HKU1 and NL63, which are MERS-related coronaviruses, exhibited weaker neutralization. Cross-neutralization against various coronaviruses has been reported, and of particular relevance, cross-neutralization against MERS-CoV by SARS immune sera has been described (35, 36). Previous studies have also reported that pseudotype neutralization assays appear more sensitive than traditional viral neutralization assays (37). Immune responses in both assays therefore require more study but likely provide important information.

## MERS vaccine protects rhesus macaques from MERS viral challenge

The immunogenicity and protective efficacy of the MERS vaccine were evaluated in a MERS-CoV rhesus macaque challenge model, as described previously (38). The eight MERS vaccine– and four pVax1 control–immunized rhesus macaques were inoculated with $7 \times 10^6$ tissue culture infectious dose ($TCID_{50}$) of MERS-CoV clinical isolate EMC/2012 via combined intratracheal, intranasal, oral, and ocular routes 4 weeks after the final immunization and were monitored for signs of pneumonia (39, 40). Animals underwent dorsoventral and lateral x-ray during examinations on days 0, 1, 3, 5, and 6 after infection. On day 3 after infection, all four animals vaccinated with the pVax1 control showed signs of diffuse interstitial infiltration in both caudal lobes, occasionally extending to the middle lobe as well (Table 1). By day 5 after infection, control animals showed increased respiration, and radiographic changes of varying severity had progressed to serious diffuse interstitial infiltration in the caudal lobes consistent with a viral pneumonia. Upon necropsy on day 6 after infection, gross pathological lesions consistent with previous studies (38–40) were observed encompassing about 10% (range, 1 to 37% of a lobe) of the total lung. Lesions were characterized as multifocal, mild to marked, interstitial pneumonia frequently centered on terminal bronchioles (Fig. 7A). The pneumonia was characterized by thickening of alveolar septae by edema fluid and fibrin and small to moderate numbers of macrophages and fewer neutrophils (39). The alveoli contained moderate numbers of pulmonary macrophages and neutrophils. In regions with moderate to marked changes, there was abundant alveolar edema and fibrin with multifocal formation of hyaline membranes, as well as abundant type II pneumocyte hyperplasia. There were also perivascular infiltrates of inflammatory cells multifocally within and adjacent to affected areas of the lung (Fig. 7B). In contrast, six of the eight MERS vaccine–immunized animals failed to demonstrate radiographic evidence of infiltration at any time point, whereas the other two animals (high dose) showed evidence of minor infiltration that resolved by day 5 after infection. MERS vaccine–immunized animals did not have increased respiration, and at necropsy, no gross

lesions were noted in these animals. There were no histologic differences between the high- and low-dose vaccine groups. All eight animals in these groups were essentially normal. Rare, small foci of interstitial pneumonia that were characterized by mild thickening of the alveolar interstitium with small numbers of lymphocytes and macrophages were observed (Fig. 7B). Very small numbers of these inflammatory cells are present in adjacent alveolar spaces.

To confirm that the MERS vaccine–immunized animals were protected from MERS-CoV infection after challenge, we measured viral loads by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in tissues that were collected at necropsy. Using this very sensitive assay, we measured viral RNA in the infected rhesus lung tissues. In all of the lung tissues analyzed, the viral loads were lower in specimens from the vaccinated animals compared to the pVax1 control–vaccinated animals (Fig. 7C). With the combined values from all the entire lung specimens from each animal, the mean viral load in the vaccinated animals (both low- and high-dose groups) was significantly lower than that in the control pVax1–immunized animals ($P = 0.0254$ and 0.0274, respectively) (Fig. 7D). There was not a statistically significant difference in the viral loads between the low- and the highdose vaccinated macaques. In summary, animals immunized with the MERS vaccine exhibited protection from symptoms of MERS disease after viral challenge with the MERS-CoV. These data provide compelling evidence that this consensus MERS vaccine can provide protection from disease in a relevant NHP animal model.

## DISCUSSION

The recent identification and rapid spread of MERS-CoV coupled with its high associated morbidity and mortality illustrate that the infection is an emergent global health issue (2, 7, 41–43). Clinically, MERS-CoV presents as an acute lower respiratory tract infection that can cause severe pneumonia, particularly in elderly and immunocompromised populations. Additionally, the identified spread of the infection from human to human illustrates that the MERS-CoV pathogen presents a significant public health and epidemiological concern. Although much remains to be understood about the spread of MERS-CoV, it is likely that camels represent a potentially important intermediate/amplifying host reservoir as well as a mode of transmission to humans (19, 42, 44).

Accordingly, the development of an efficacious vaccine against MERS-CoV is an important goal (2, 18). New approaches involving a combination of animal and human health measures to limit the zoonotic spread of MERS-CoV are important. Such a strategy would benefit from having new tools to limit infection in camels and humans, including an efficacious vaccination approach targeting both populations. MERS-CoV has demonstrated a propensity to mutate with the subsequent generation of antigenic diversity (45), an observation that could be problematic for the development and utility of single strain– derived vaccines. Currently, two clades have been identified that account for the observed genetic diversity (19). This viral variability suggested to our group that a consensus-based vaccine against the MERS-CoV S protein might provide effective protection across both clades (7, 18, 46). The MERS S protein is a class I membrane fusion protein that represents the major envelope protein on the surface of CoVs. The S protein binds to the MERS-CoV receptor dipeptidyl

peptidase 4 (DPP4, also called CD26) as a method for entry into the target cell (47). It encodes the determinants for both host range and cell tropism (46, 47). Viral binding spares the hydrolase domain on DPP4, thus rendering drugs against this target ineffective for treatment. However, antibodies targeting the S protein are effective at blocking entry of MERS-CoV as measured by in vitro laboratory assays. Our group has previously reported that focused consensus sequences can provide long-lasting immune responses against divergent viruses within several infectious disease models, including influenza A, hepatitis B, Ebola, Chikungunya virus, and human papillomavirus (23–27, 48). Thus, an immunogen based on a consensus sequence of the MERS S glycoprotein covering both of the known clades was developed as a first approach to vaccine development.

A synthetic DNA plasmid–based vaccine containing a full-length consensus MERS S protein sequence was constructed. A strong T cell response was elicited by the MERS vaccine in mice and NHPs as measured by an IFN-γ ELISpot assay. Furthermore, intracellular cytokine staining demonstrated the polyfunctionality of both the $CD4^+$ and $CD8^+$ T cell compartments in both animal models. A robust humoral immune response was also generated in MERS vaccine–immunized mice, camels, and NHPs.

Strong NAb responses were also detected in mice, camels, and NHP immune sera in a live MERS-CoV neutralization assay. To determine the ability of the same immune sera to neutralize with some diversity including MERS-CoV, we took advantage of a pseudovirus-based neutralization assay. In mouse and NHP models, MERS vaccine–induced antibodies were able to prevent entry of MERS-CoV pseudoviral particles into target cells (Figs. 3E and 6D). These findings were supported using a traditional MERS viral neutralization assay with a prototypic clade A infectious virus (EMC/2012) and the pseudovirus neutralization assay with S proteins. The pseudotype assay also allowed us to test additional related CoVs where we also observed neutralization but at a somewhat lower titer in this assay.

However, there are limitations in interpreting data from the pseudotype assay. The pseudotype assay and the traditional NAb assay have been previously reported to give similar data, but both assays provide somewhat unique views of neutralization (18, 30, 35–37, 49). Pseudotype assays, likely because of their increased sensitivity, may provide information that is not easily observed in other viral neutralization assays. For example, broadly neutralizing anti-hemagglutinin (HA) stem antibodies have been reported using pseudotype assay formats; however, this same activity is not observed in influenza HA-inhibition (HAI) neutralization formats (50). Additional studies on CoV neutralization phenotypes in these and perhaps additional assays are important to provide additional color around this issue. However, it is important to note that neutralization of MERS-CoV by sera from SARS infection, a divergent CoV infection, has recently been reported (35, 36), supporting that cross-neutralization appears to be detectable, at least in specific assays, and that more work is needed to understand cross-neutralization for this emerging viral family.

The immune sera data from vaccinated mice, camels, and NHPs all support that the vaccine presented here induced humoral responses of relevance to vaccine development against MERS-CoV. In rhesus macaques, the synthetic consensus DNA vaccine MERS vaccine delivered with EP produced a balanced cellular and humoral response, including the

induction of strong cytotoxic T lymphocyte responses as well as potent NAbs. These antibody responses appeared as soon as after a single immunization. The vaccine was protective against MERS-CoV challenge. The rhesus macaques from both the low- and high-dose vaccinated groups displayed mostly normal clinical parameters, showing no breathing irregularities and only limited evidence of infiltration by x-ray analysis. Additionally, vaccination reduced viral RNA copy number by several logs. Upon necropsy, there were essentially no signs of infection and an absence of gross lesions. Notably, protection was achievedina short 6-week period. This rapid induction of protective immune responses could be imperative in an outbreak situation, and additional studies to improve these results with more rapid protocols are of interest. In addition, although there were some differences in vaccine-induced responses between the low-dose and the high-dose regimens, these differences did not seem to affect the challenge outcome because protection was similar in the two groups.

Together, these studies support the robustness of the consensus DNA vaccine approach for the development of a potential protective vaccine against MERS-CoV. The data emphasize the significant contribution of NAbs to abrogate MERS-CoV infection. These findings are of value in understanding the role of the S glycoprotein in MERS-CoV infection and in vaccine development as well as for the design and development of vaccines against related emerging pathogens.

## MATERIALS AND METHODS

### Cells and MERS vaccine construction

Human embryonic kidney (HEK) 293T cells [American Type Culture Collection (ATCC) #CRL-N268] and Vero-E6 cells (ATCC #CRL-1586) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (51). The MERS vaccine plasmid DNA construct encodes a consensus S glycoprotein developed by comparing the sequences of current MERS-CoV S protein sequences. In addition, a panel of DNA plasmids encoding S glycoproteins from England/2/2013 (GenBank: KM015348.1), Al-Hasa_1_2013 (AGN70962.1), HKU1 (AGW27872.1), and NL63 (AFD98834.1) strains were also synthesized for subsequent evaluation. An Ig heavy chain ε-1 signal peptide was fused to the N terminus of each sequence, replacing the N-terminal methionine, to facilitate expression. The vaccine insert was genetically optimized for improved expression, including codon and RNA optimization, among other proprietary modifications that enhance protein expression (51, 52). The optimized gene was then subcloned into a modified pVax1 mammalian expression vector under the control of the cytomegalovirus immediate-early promoter (GenScript). The MERS S glycoprotein–expressing DNA vaccine is referred to as MERS vaccine and the control plasmid backbone as pVax1.

### MERS vaccine expression

For in vitro expression studies, transfections were performed using the TurboFectin 8.0 reagent, following the manufacturer's protocols (OriGene). Briefly, cells were grown to 80% confluence in a 35-mm dish and transfected with 1, 2.5, or 5 μg of MERS vaccine. The cells were harvested 2 days after transfection, washed twice with phosphate-buffered saline

(PBS), and lysed with cell lysis buffer (Cell Signaling Technology). Western blot analysis was used to verify the expression of the S protein from 25 μg of harvested cell lysate, as described previously (51). Sera from MERS vaccine–immunized mice were used at a dilution of 1:100 as a primary antibody. Blots were stripped and reprobed with anti–β-actin antibody as a loading control.

For the immunofluorescence assay, Vero cells were grown on coverslips and transfected with 5 μg of MERS vaccine. Two days after transfection, the cells were fixed with ice-cold acetone for 5 min. Nonspecific binding was then blocked with 5% skim milk in PBS at 37°C for 30 min. The slides were then washed in PBS for 5 min and subsequently incubated with sera from immunized mice at a 1:100 dilution for 1 hour. Slides were washed as described above and incubated with goat anti-mouse IgG-AF488 (Invitrogen) at 37°C for 30 min. After washing, DAPI was used to stain the nuclei of all cells. Coverslips were mounted with ProLong Gold antifade reagent (Invitrogen), and the slides were observed under a confocal microscope (LSM710; Carl Zeiss). The resulting images were analyzed using Zen software (Carl Zeiss) (51).

## Mice and immunization protocols

Female C57BL/6 mice (6 to 8 weeks old; Jackson Laboratories) were divided into three experimental groups. All animals were housed in a temperature-controlled, light-cycled facility in accordance with the guidelines of the National Institutes of Health (NIH) and the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Immunizations consisted of 25 μg of DNA in a total volume of 25 μl of water delivered into the tibialis anterior muscle with in vivo minimally invasive EP delivery. The protocols for the use of EP have been previously described in detail (24). Mice were immunized three times at 2-week intervals and sacrificed 1 week after final immunization. Blood was collected after each immunization, and sera were isolated for analysis of humoral immune responses (51).

Single-cell suspensions of splenocytes were prepared from all mice. Briefly, spleens from mice were collected individually in 10 ml of RPMI 1640 supplemented with 10% FBS (R10), then processed with a Stomacher 80 paddle blender (A.J. Seward and Co. Ltd.) for 60 s on high speed. Processed spleen samples were filtered through 45-μm nylon filters and then centrifuged at 800g for 10 min at room temperature. Cell pellets were resuspended in 5 ml of ACK (ammonium-chloride-potassium) lysis buffer (Life Technologies) for 5 min at room temperature, and PBS was then added to stop the reaction. Samples were again centrifuged at 800*g* for 10 min at room temperature. Cell pellets were resuspended in R10 at a concentration of $1 \times 10^7$ cells/ml and then passed through a 45-μm nylon filter before use in ELISpot assay and flow cytometric analysis (51).

## Immunization of camels with MERS vaccine

Three female adult dromedary camels (*Camelus dromedarius*) were housed at a private farm, and all treatments and sample collections were done under the supervision of a licensed veterinarian. The animals were healthy and were maintained under standard feeding and housing conditions. The camels received three intramuscular immunizations with the

MERS vaccine at 4-week intervals. All immunizations were formulated in sterile water and delivered with EP using the CELLECTRA constant current device (Inovio Pharmaceuticals Inc.). Blood was collected immediately before the first immunization (week 0) and 3 weeks after the last immunization (week 11), and sera were isolated to evaluate the humoral immune response.

### IFN-γ ELISpot analysis

Antigen-specific T cell responses were determined using IFN-γ ELISpot analysis. Briefly, for mouse samples, 96-well polyvinylidene difluoride plates (Millipore) were coated with purified anti-mouse IFN-γ capture antibody and incubated for 24 hours at 4°C (R&D Systems). The following day, the plates were washed and blocked for 2 hours with 1% bovine serum albumin and 5% sucrose. Two hundred thousand splenocytes were added to each well and stimulated overnight at 37°C in 5% $CO_2$ with R10 (negative control), concanavalin A (3 μg/ml; positive control), or specific peptide antigens (5 μg/ml; GenScript). Peptide pools consisted of 15-mer peptides overlapping by 11 amino acids and spanned the entire S protein (GenScript). After 24 hours of stimulation, the plates were washed and incubated for 24 hours at 4°C with biotinylated anti-mouse IFN-γ antibodies (R&D Systems). The plates were washed, streptavidin-alkaline phosphatase (R&D Systems) was added to each well, and the plates were incubated for 2 hours at room temperature. The plates were washed, 5-bromo-4-chloro-3′-indolyl phosphate *p*-toluidine salt and nitro blue tetrazolium chloride (R&D Systems) were added to each well, and the plates were incubated until spots appeared. The plates were then rinsed with distilled water and dried at room temperature overnight. Spots were counted by an automated ELISpot reader (Cellular Technology Ltd.). For NHP samples, the ELISpot$^{PRO}$ for Monkey IFN-γ kit (MABTECH) was used as directed by the manufacturer. Two hundred thousand PBMCs were stimulated with peptide pools, and plates were washed and spots were developed and counted as described above (51).

### Flow cytometry and intracellular cytokine staining assay

Splenocytes were added to a 96-well plate ($2 \times 10^6$ per well) and were stimulated with MERS S protein peptides for 5 to 6 hours at 37°C/5% $CO_2$ in the presence of Protein Transport Inhibitor Cocktail (brefeldin A and monensin) (eBioscience) according to the manufacturer's instructions. The Cell Stimulation Cocktail (phorbol 12- myristate 13-acetate, ionomycin, brefeldin A, and monensin) (eBioscience) was used as a positive control and the R10 medium as a negative control. All cells were then stained for surface and intracellular proteins. Briefly, the cells were washed in FACS buffer (PBS containing 0.1% sodium azide and 1% FBS) before surface staining with fluorochrome-conjugated antibodies. The cells were washed with FACS buffer and then fixed and permeabilized using the BD Cytofix/Cytoperm according to the manufacturer's protocol, followed by intracellular staining. For mice, the following antibodies were used for surface staining: LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen), CD19 (V450, clone 1D3; BD Biosciences), CD4 (FITC, clone RM4-5; eBioscience), CD8 [APC (allophycocyanin)-Cy7, clone 53-6.7; BD Biosciences], and CD44 (AF700, clone IM7; BioLegend). For mouse intracellular staining, the following antibodies were used CD3 [PerCP (peridinin chlorophyll protein)-Cy5.5, clone 145-2C11; BioLegend], IFN-γ (APC, clone XMG1.2; BioLegend),

TNF-α [phycoerythrin (PE), clone MP6-XT22; eBioscience], and IL-2 (PE-Cy7, clone JES6-SH4; eBioscience). For the rhesus macaque vaccination and viral challenge studies, the following antibodies were used for surface staining: CD4 (AF700, clone OKT4; BioLegend), CD8 (PE, clone SK1; BD Biosciences), CD16 [Pacific blue (PB), clone 3G8; BD Biosciences), CD14 (PB, clone MϕP9; BD Biosciences), and CD19 (PB, clone HIB19; BioLegend). For NHP intracellular staining, the following antibodies were used: CD3 (APC-Cy7, clone SP34-2; BD Biosciences), IFN-γ (APC, clone B27; BioLegend), TNF-α (PE-Cy7, clone MAb11; BioLegend), and IL-2 (FITC, clone MQ1-17H12; BioLegend). All data were collected using an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star) and SPICE (Simplified Presentation of Incredibly Complex Evaluations) version 5 (NIH). Boolean gating was performed using FlowJo software to examine the polyfunctionality of the T cells from vaccinated animals (28).

### Antigen-specific ELISA assay

An ELISA was used as previously described to determine antigen-specific antibody levels present in sera (25). Briefly, purified recombinant human betacoronavirus S protein 2c EMC/2012 (clade A) (5 μg/ml; Sino Biologicals) was used to coat 96-well microtiter plates (Nalge Nunc International) at 4°C overnight. After blocking with 10% FBS in PBS, the plates were washed five times with 0.05% PBST (Tween 20 in PBS). Serum samples from immunized mice and NHPs were serially diluted in 1% FBS and 0.05% PBST, added to the plates, and incubated for 1 hour at 37°C. The plates were again washed five times in 0.05% PBST and then incubated with horseradish peroxidase (HRP)–conjugated anti-mouse IgG for the mouse sera or anti-human IgG for the NHP sera (Sigma-Aldrich) for 1 hour at room temperature. After washing five times in 0.05% PBST, the bound antibody was detected by adding SIGMAFAST OPD (*o*-phenylenediamine dihydrochloride) tablets according to the manufacturer's instructions (Sigma-Aldrich). The reaction was terminated after 15 min with the addition of 1 M $H_2SO_4$. The plates were then read at 450 nm on a GloMax 96 Microplate Luminometer (Promega). All samples were plated in duplicate. Endpoint titers were determined using the method described by Frey *et al*. (53).

### Antigen-specific antibody detection by Western blot

Tris-acetate NuPAGE gels (Life Technologies) were loaded with 100 ng of recombinant full-length MERS S protein (Sino Biologicals) or 100 ng of recombinant gp120-pTRJO4551 (Immune Tech) as a negative control. Gels were run at 150 V for 1 hour in tris-acetate buffer. The protein was transferred onto nitrocellulose membranes using the iBlot 2 Gel Transfer Device (Life Technologies). The membranes were blocked in Odyssey blocking buffer (LI-COR) for 1 hour at room temperature. Sera were diluted 1:250 in 0.5× Odyssey blocking buffer with 0.1% Tween 20 (Bio-Rad) and incubated with the membranes overnight at 4°C. A commercial mouse anti-MERS S antibody was used as a positive control (Sino Biologicals). The membranes were washed and then incubated with the appropriate secondary antibody [goat anti-human IRDye680RD for NHP samples (LI-COR), goat anti-mouse IRDye800CW for mouse samples and the positive control antibody (LI-COR), or rabbit anti-camel IgG-HRP for camel samples (ABclonal)] for 1 hour at room temperature. After washing, the membranes were imaged on the Odyssey infrared imager (LI-COR) or developed using the Amersham ECL Prime Western Blotting Detection

Reagent (GE Healthcare) and Amersham Hyperfilm ECL high-performance chemiluminescence film (GE Healthcare).

### Viral neutralization assay

The 50% $TCID_{50}$ was calculated and a standard concentration of virus (that is, 100 $TCID_{50}$) was used for the neutralization test throughout the study. Briefly, the heat-inactivated mouse/NHP serum was serially diluted in 50 μl of DMEM and incubated for 1 hour with 50 μl of DMEM containing 100 infectious MERS-CoV EMC/2012 particles per well at 37°C. The virus-serum mixture was then added to a monolayer of Vero cells (10,000 cells per well, plated 24 hours earlier) in a 96-well flat-bottomed plate and incubated for 1 hour at 37°C. Then, 100 μl of DMEM supplemented with 4% FBS was added to each well, and the samples were incubated for 2 days at 37°C in a 5% $CO_2$ incubator. The titer of NAb for each sample is reported as the reciprocal of the highest dilution with which less than 50% of the cells show cytopathic effects. All samples were run in duplicate. The percent neutralization was calculated as follows: percent neutralization = 1 − PFU (plaque-forming units) of serum of interest (each concentration)/mean PFU of negative control (all concentrations).

### Preparation of MERS-CoV S pseudoviruses

S protein pseudovirus was prepared similarly to previously described methods using an HIV-1 genome expressing a luciferase reporter in HEK 293T cells. Specifically, $2 \times 10^6$ HEK 293T cells were seeded in 10-cm tissue culture plates and transfected using the TurboFectin 8.0 reagent (OriGene) at ~80% confluency. To produce S pseudoparticles, 10 μg of pNL4-3.Luc.$R^-$.$E^-$ (NIH AIDS Reagent Program) and 10 μg of S construct (MERS vaccine, England/2/2013, Al-hasa_1_2013, HKU1, NL63, or VSV-G as a positive control) were cotransfected into the cells. After 12 hours, the transfection medium was removed and replaced with fresh medium. The cells were incubated for 48 hours at 37°C. The pseudovirus-containing medium was collected, and HIV-p24 viral protein was quantified. The pseudoviruses were stored at −80°C (51).

### Pseudovirus neutralization assay

For the pseudovirus neutralization assay, S pseudovirus (25 ng of p24 protein) was preincubated with serially diluted pooled mouse or NHP serum (1:100 dilution) for 30 min at 37°C. After incubation, the mixture was added to the target cells. Virus infectivity was determined 48 hours later by measuring the amount of luciferase activity expressed in infected cells. One hundred microliters of cell lysate was mixed with 100 μl of luciferase substrate, and luciferase activity [designated relative luminescence units (RLU)] was measured in a GloMax 96 Microplate Luminometer (Promega).

### NHP immunization with MERS vaccine followed by viral challenge

Three groups of four healthy rhesus macaques (*Macaca mulatta*) ($n = 4$) received three immunizations (prime vaccination plus two boosters) administered 3 weeks apart (weeks 0, 3, and 6). The animals were randomly assigned to groups in a nonblinded manner. Group 1 received 0.5 mg of MERS vaccine per immunization (low dose), group 2 received 2 mg of MERS vaccine per immunization (high dose), and group 3 received 2 mg of empty vector

per immunization (pVax1). The animals were anesthetized intramuscularly with ketamine hydrochloride (10 to 30 mg/kg). The vaccine was administered intramuscularly in each thigh (one injection site per thigh per vaccination), and immediately after the injections of the experimental or control plasmids, EP (three pulses at 0.5 A constant current with 52 ms pulse length with 1 s between pulses) was applied. The dose and the immunization regimen of the DNA vaccine used in these studies were previously determined to be optimal in rhesus macaques (25). Blood was collected one week after each immunization to analyze serum antibody levels and to test for the presence of NAbs in addition to monitoring systemic T cell responses. For challenge, the animals were inoculated with $7 \times 10^6$ $TCID_{50}$ of MERS-CoV (EMC/2012) by combined intratracheal, intranasal, oral, and ocular routes as previously established (38–40). After challenge, the animals were monitored three times daily through clinical scoring and/or examinations (1, 3, 5, and 6 dpi) as described previously (39). Clinical examinations included radiography, body temperature, blood pressure, heart rate, respiration rate, pulse oximetry, venous bleeding, and collection of swabs from nasal and oral mucosa. On 6 dpi, all animals were necropsied, and respiratory tract tissues were collected for virological and histopathological analysis. The tissues were placed in cassettes and fixed in 10% neutral buffered formalin for 7 days. The tissues were subsequently processed with a Sakura VIP 5 Tissue-Tek, on a 12-hour automated schedule, using a graded series of ethanol, xylene, and Paraplast X-tra. Embedded tissues were sectioned at 5 μm and dried overnight at 42°C before staining. The tissue sections were stained with hematoxylin and eosin.

### Animal ethics statement for the rhesus macaques studies

This study was carried out in strict accordance with the recommendations described in the *Guide for the Care and Use of Laboratory Animals* of the NIH, the Office of Animal Welfare, and the U.S. Department of Agriculture. All animal immunization work was approved by the Bioqual Animal Care and Use Committee (IACUC), and the challenge studies were approved by the IACUC at Rocky Mountain Laboratories (RML). Both facilities are accredited by the American Association for Accreditation of Laboratory Animal Care. All procedures were carried out under ketamine anesthesia by trained personnel under the supervision of veterinary staff, and all efforts were made to ameliorate the welfare of the animals and to minimize animal suffering in accordance with the "Weatherall report for the use of non-human primates" recommendations. The animals were housed in adjoining individual primate cages allowing social interactions, under controlled conditions of humidity, temperature, and light (12-hour light/12-hour dark cycles). Food and water were available ad libitum. The animals were monitored twice daily (before and after challenge) and fed commercial monkey chow, treats, and fruits twice daily by trained personnel. Early endpoint criteria, as specified by the RML IACUC-approved score parameters, were used to determine when the animals should be humanely euthanized. The work with infectious MERS-CoV (EMC/2012) was approved under biosafety level 3 conditions by the Institutional Biosafety Committee (IBC) at RML. Sample inactivation was performed according to standard operating procedures approved by the IBC for removal of specimens from high containment.

### Statistical analysis

The mouse experiments evaluating immune responses were repeated two times. All data are presented as mean ± SEM. Prism version 5.0 (GraphPad Software Inc.) was used to perform unpaired *t* tests on data obtained from animal studies and various immune assays. *P* values < 0.05 were considered significant.

## Acknowledgments

We thank D. Long, R. Rosenke, T. Thomas, P. Hanley, and the animal caretakers of the Rocky Mountain Veterinary Branch for assistance with the rhesus macaque vaccination and viral challenge studies. E. Ramsay from University of Tennessee College of Veterinary Medicine for guidance with animal studies. We also thank Penn Center for AIDS Research and Abramson Cancer Center core facilities for their support.

**Funding:** This work was partially funded by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases of NIH and R01-AI092843 to D.B.W. D.B.W. and K.M. also note funding by Inovio Pharmaceuticals Inc.

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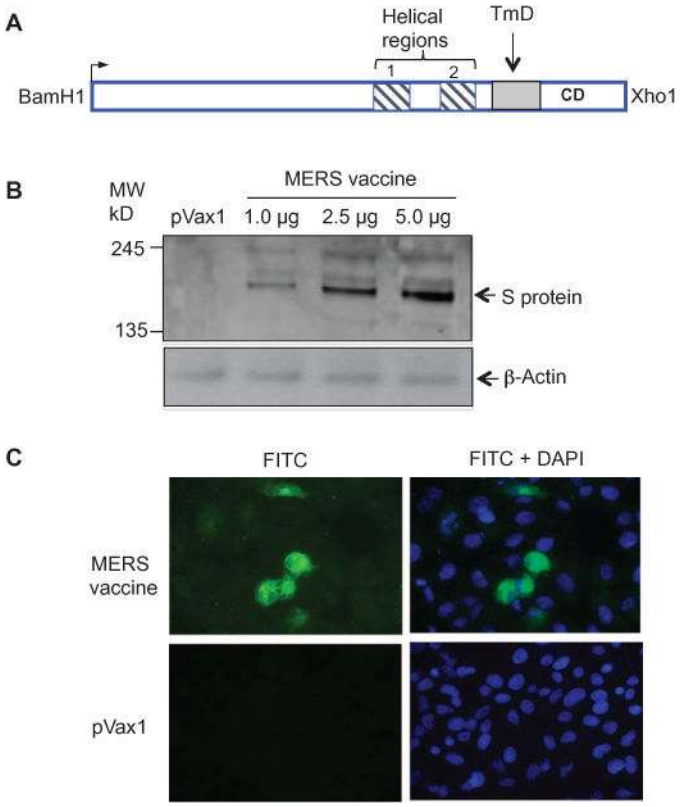


**Fig. 1. Construction and characterization of the MERS vaccine plasmid construct**

(**A**) Schematic diagram of MERS S protein gene inserts used to generate the codon-optimized DNA vaccines, designated as MERS vaccine. Different S protein domains (TmD, transmembrane domains; CD, cytoplasmic domain) are indicated. (**B**) Expression of the MERS S protein detected by SDS–polyacrylamide gel electrophoresis and Western blot. The expression of S protein from the indicated amount of MERS vaccine in 293T cells was analyzed. The arrows indicate theSprotein and β-actin control. (**C**) Immunofluorescence assay of Vero cells transfected with the MERS vaccine. S protein expression is indicated by Alexa Fluor 488 (AF488) staining, and 4′,6-diamidino-2-phenylindole (DAPI) staining shows cell nuclei. MW, molecular weight; FITC, fluorescein isothiocyanate.

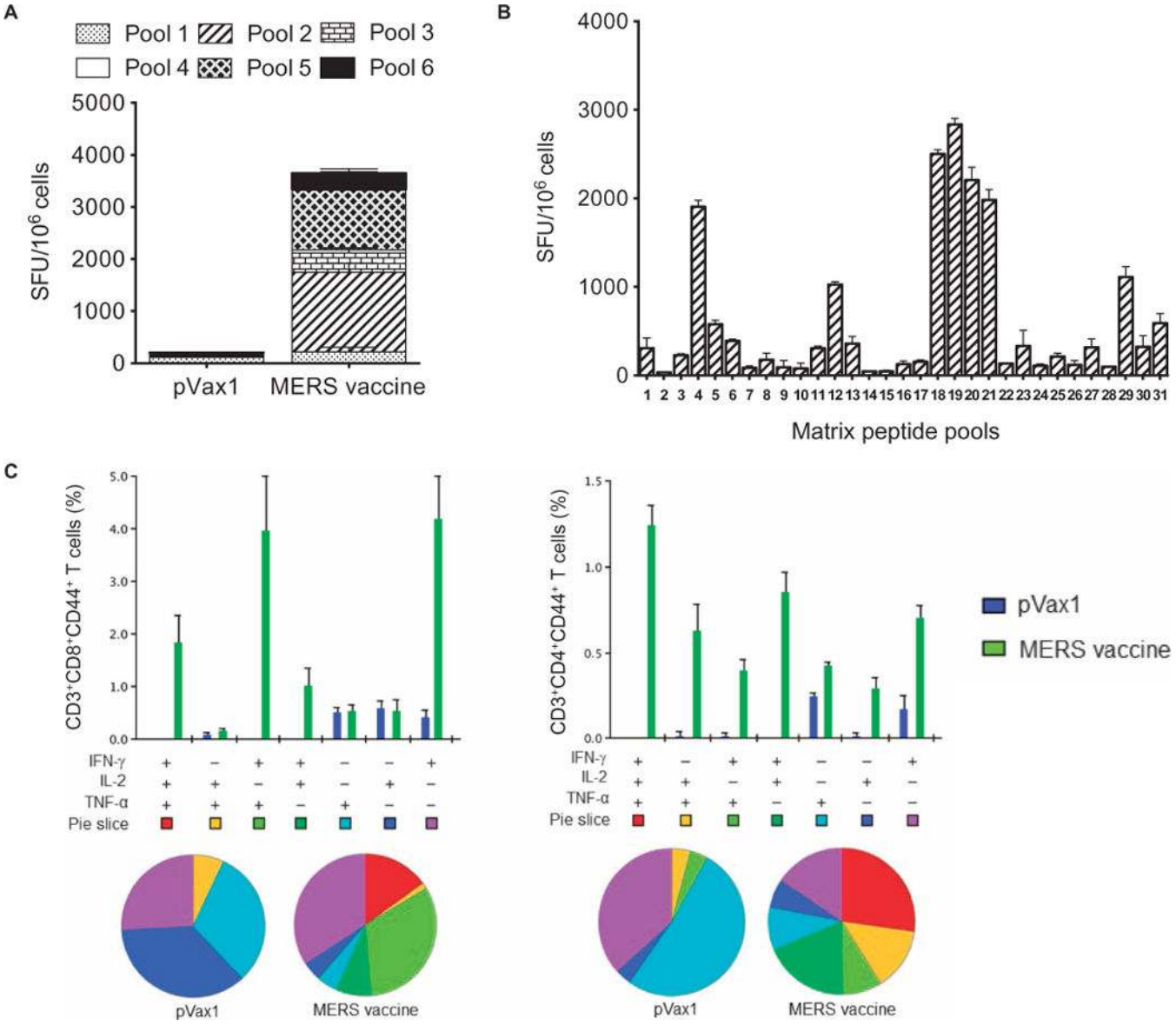


**Fig. 2. Functional profile of cellular immune responses elicited by MERS vaccine in mice**

(**A**) The S protein-specific cellular immune response in mice 1 week after the final immunization with the MERS vaccine. IFN-γ responses were assessed by ELISpot assays using six peptide pools encompassing the entire S protein. Values (that is, SFU per $10^6$ cells) represent mean responses in each group ($n = 3$) ± SEM. (**B**) Characterization of MERS-CoV S protein-specific dominant epitopes in C57BL/6 mice. IFN-γ responses were assessed by ELISpot assays with matrix pools of peptides, indicating the presence of immunodominant epitopes. Values represent mean responses in each group ($n = 3$) ± SEM. Similar results were obtained in two separate experiments. (**C**) The functional profile of $CD4^+$ and $CD8^+$ T cell responses elicited by MERS vaccine. Mouse splenocytes ($n = 3$) were isolated 1 week after the final DNA immunization and were stimulated with pooled MERS S protein peptides ex vivo. Cells were stained for intracellular production of IFN-γ, TNF-α, and IL-2, and then analyzed by fluorescence-activated cell sorting (FACS). The bar

graph shows subpopulations of mono-, double-, and triple-positive $CD4^+$ and $CD8^+$ T cells releasing the cytokines IFN-γ, TNF-α, and IL-2. The pie charts show the proportion of each cytokine subpopulation. Values represent mean responses in each group ($n = 3$) ± SEM.

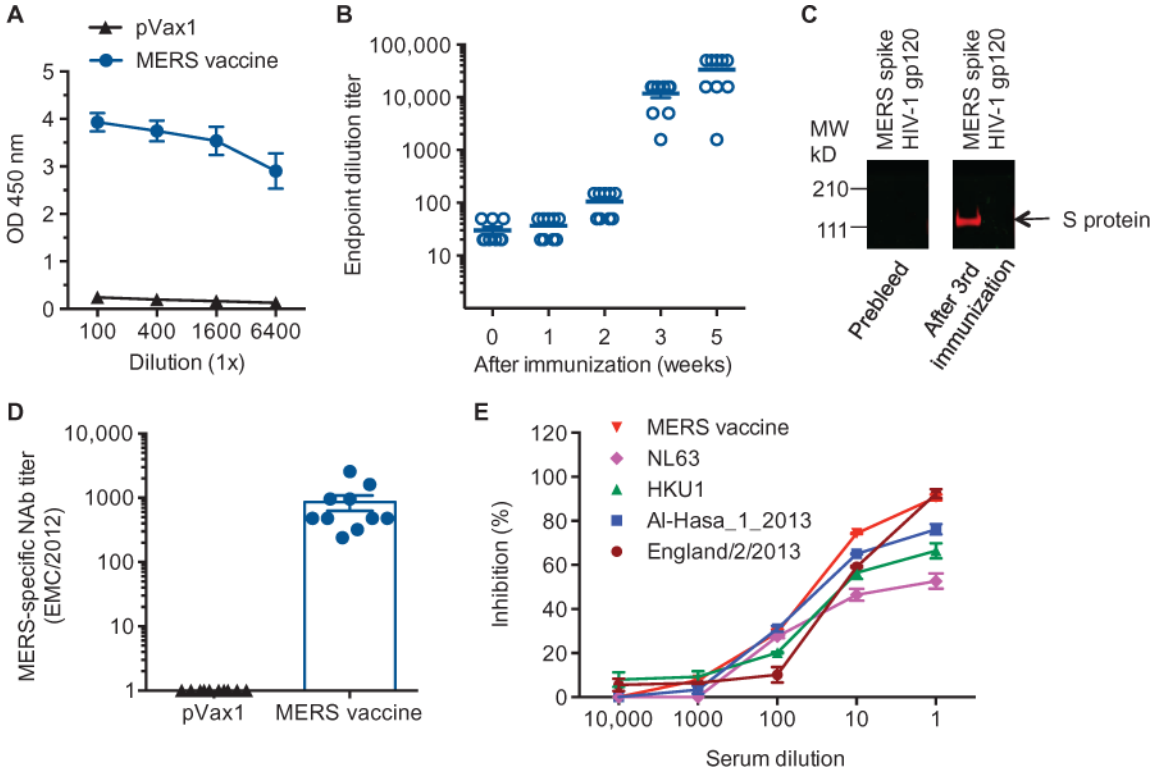


**Fig. 3. Humoral immune responses elicited by MERS vaccine in mice**

(**A**) Serum IgG responses specific for MERS S protein. Serum from individual mice (1 week after the third immunization) was serially diluted, and anti-MERS S protein–specific total IgG was measured by ELISA. Values represent mean responses in each group ($n = 9$) ± SEM. (**B**) Endpoint binding titers for the MERS vaccine– immunized mouse sera were calculated at the indicated time points. Values for individual mice are shown ($n = 9$) and lines represent the geometric mean ± SEM. (**C**) Western blot analysis of the presence of IgG specific for recombinant full-length MERS S protein (or recombinant HIV gp120 as a negative control) in immune sera. Pooled sera were used as the primary antibody at a 1:250 dilution. (**D**) NAb responses detected by the viral infection assay in sera collected 1 week after the final immunization. NAb titers are presented as the sera dilution that mediates 50% inhibition ($IC_{50}$) of virus infection of the target cells. Values of individual mice are shown ($n = 9$) and lines indicate the mean of each group ± SEM. (**E**) Neutralization with MERS and related CoV pseudoviruses by MERS vaccine–immunized mouse sera. Serially diluted pooled sera from four mice 1 week after the third immunization were analyzed in duplicate. These assays were performed twice for consistency, with one of these shown. VSV-G–pseudotyped virus was used as the control for neutralization specificity. OD, optical density.

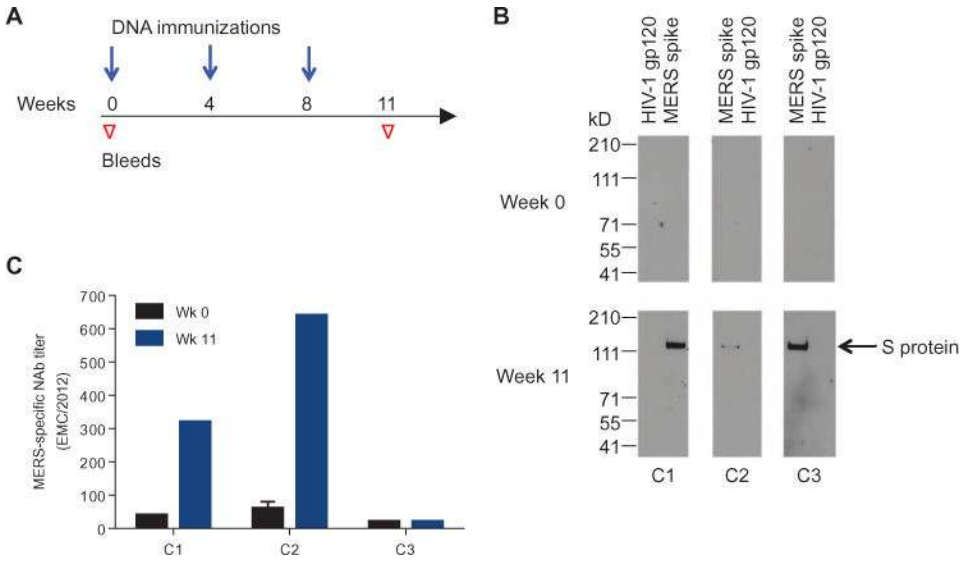


**Fig. 4. Humoral immune responses elicited by MERS vaccine in camels**

(**A**) Three dromedary camels were immunized three times at 4-week intervals with the MERS vaccine delivered by EP. Blood was taken at week 0 (prebleed) and week 11 (3 weeks after the third immunization), and sera were isolated for the assessment of the humoral immune response. (**B**) Western blot analysis of the presence of IgG specific for recombinant full-length MERS S protein (or recombinant HIV gp120 as a negative control) in immune sera. Sera from individual animals were used as the primary antibody at a 1:250 dilution. (**C**) NAb responses detected by the viral neutralization assay in sera collected 3 weeks after the final immunization. NAb titers are presented as the sera dilution that mediates $IC_{50}$ of virus infection of the target cells. Each sample was run in duplicate. The data shown are the mean titers for each animal ± SEM.

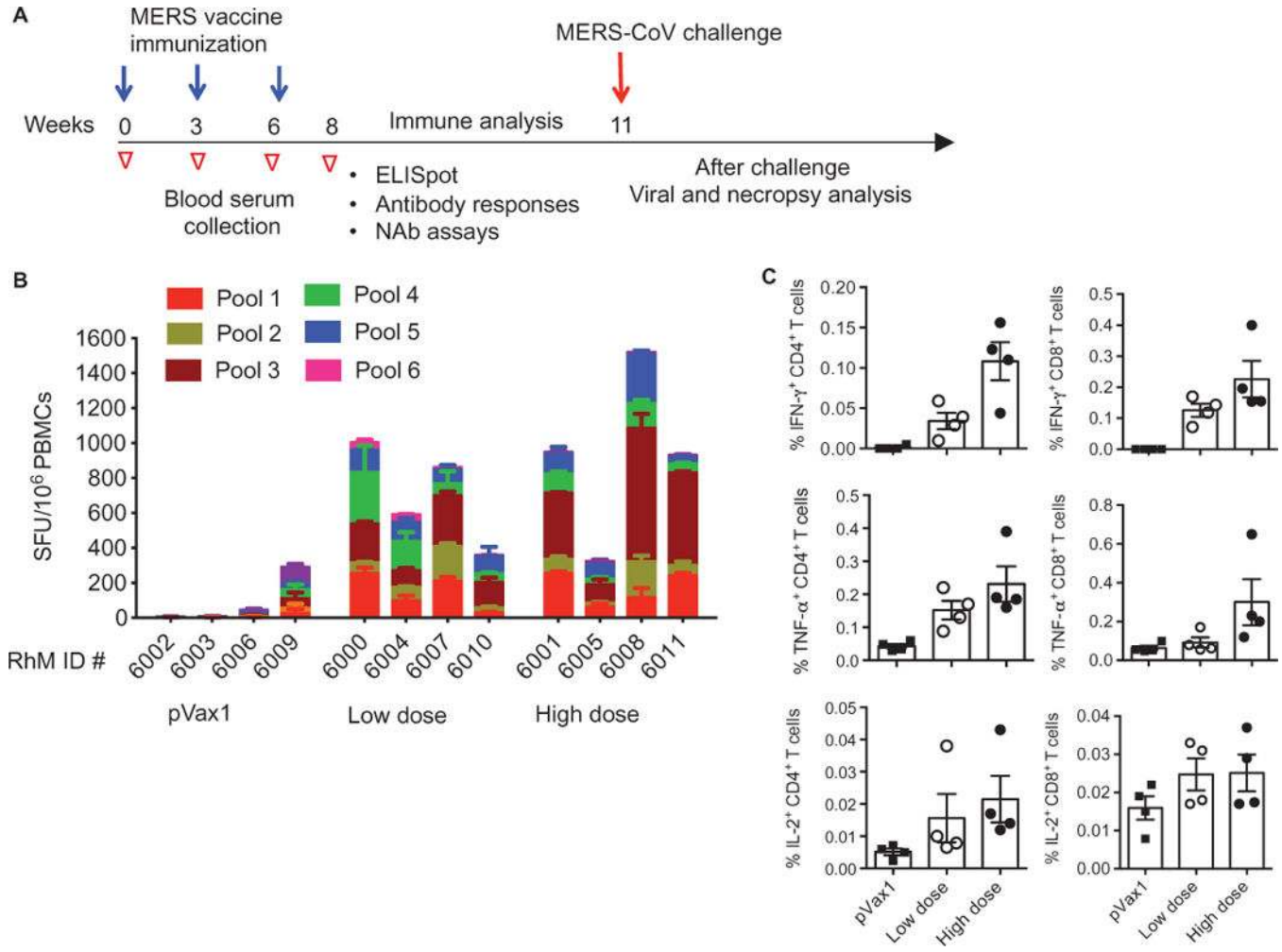


**Fig. 5. Potent T cell responses elicited by MERS vaccine in rhesus macaques**

(**A**) Time course of MERS vaccine immunization, viral challenge, and immune analysis. (**B**) The S protein–specific cellular immune response in PBMCs isolated from NHP 2 weeks after the final immunization with MERS vaccine. IFN-γ responses were assessed by ELISpot assays using six peptide pools encompassing the entire S protein. Values represent mean responses in each group ($n = 4$) ± SEM. (**C**) The functional profile of $CD4^+$ and $CD8^+$ T cell responses elicited by low and high dose MERS vaccine. PBMCs ($n = 4$) were isolated 2 weeks after the final MERS vaccine immunization and were stimulated with pooled MERS S protein peptides ex vivo. Cells were stained for intracellular production of IFN-γ, TNF-α, and IL-2. The bar graph shows the mean total percentage ± SEM of $CD4^+$ and $CD8^+$ T cells in the blood expressing the indicated cytokine. RhM, rhesus macaque.

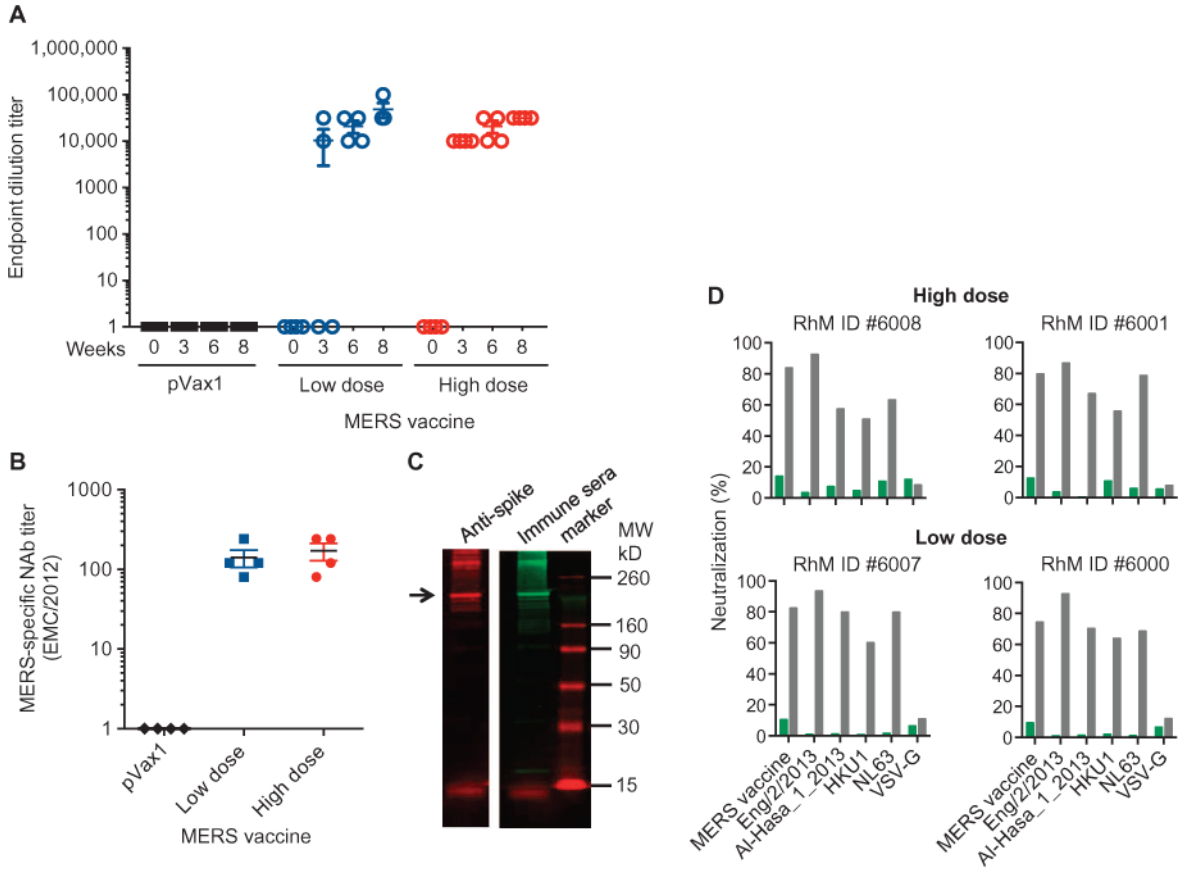


**Fig. 6. Humoral immune responses elicited by MERS vaccine in rhesus macaques**

(**A**) Endpoint antibody titers were determined for all rhesus macaques before and after each immunization with MERS vaccine. Values for individual NHP are shown ($n = 4$) and lines represent the group mean ± SEM. (**B**) NAb responses detected by the viral infection assay in sera collected 2 weeks after the final immunization. NAb titers are presented as the sera dilution that mediates $IC_{50}$ of virus infection of the target cells. Values of individual NHP are shown ($n = 4$) and lines indicate the mean of each group ± SEM. (C) Western blot analysis of the presence of IgG specific for recombinant full-length MERS S protein in immune sera. Pooled immune sera were used as the primary antibody at a 1:250 dilution. (**D**) Percent neutralization of S protein–pseudotyped viruses by sera from MERS vaccine–immunized NHP. The values are expressed as percent neutralization of the average of duplicate wells. The assay was performed two times. Gray bar represents immunized sera, and green bar represents prebleed sera. VSV-G pseudotyped virus was used as the control for neutralization specificity.

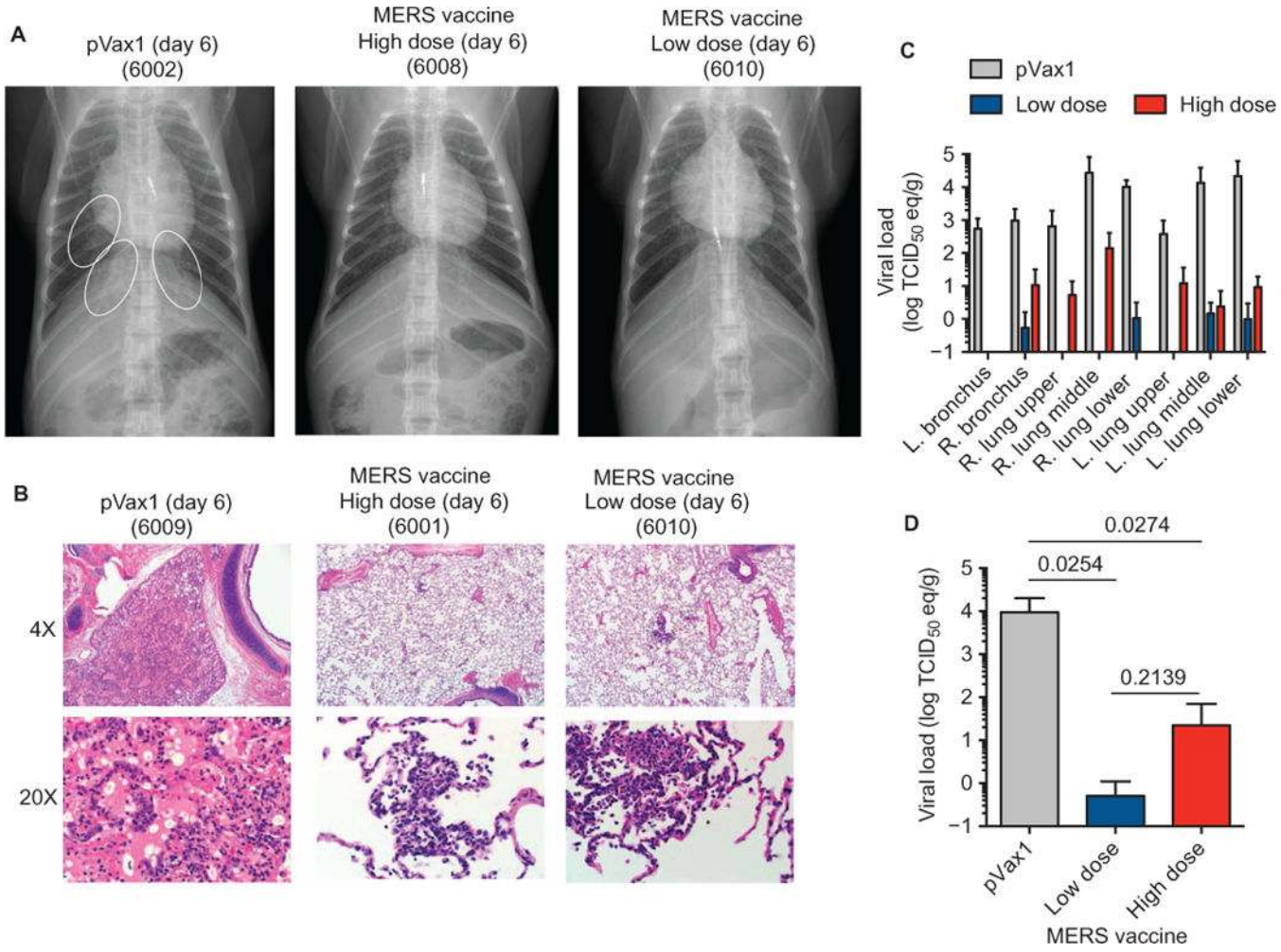


**Fig. 7. Protection from live MERS-CoV viral challenge by MERS vaccine in rhesus macaques: Evaluation of clinical signs and viral loads**

(**A**) Radiographic changes. Ventrodorsal thoracic x-rays from pVax1- and MERS vaccine–immunized rhesus macaques imaged on day 6 after MERS-CoV infection. Infiltration is highlighted by the white circles. (**B**) Histology of lung sections. Lung from a pVax1-vaccinated animal (4× and 20×) indicating coalescing subacute bronchointerstitial pneumonia with abundant alveolar edema and fibrin and type II pneumocyte hyperplasia. Lungs from rhesus macaques immunized with high or low doses of the MERS vaccine demonstrating minimal focal interstitial pneumonia with mild subacute perivasculitis and minimal focal interstitial pneumonia. Histology pictures are all taken of tissue from the left middle lobe. (**C**) Viremia in the indicated tissues from rhesus macaques immunized with MERS vaccine and challenged with MERS-CoV ($n = 4$ per group). RNA was extracted from control and vaccinated NHPs, and viral load was determined as $TCID_{50}$ equivalents (TCID eq/g) by qRT-PCR. $TCID_{50}$ eq/g were extrapolated from standard curves generated by adding dilutions of RNA extracted from a MERS-CoV EMC/2012 stock with known virus titer in parallel to each run. All values are mean ± SEM. (**D**) Cumulative viremia in all tissues from rhesus macaques in each vaccination group ($n = 4$ per group). All values are

mean ± SEM. *P* values determined by an unpaired *t* test are indicated as comparison between different groups.

*Sci Transl Med*. Author manuscript; available in PMC 2015 September 17.

**Table 1**

**Radiographic findings in lungs of rhesus macaques inoculated with MERS-CoV between 1 and 6 days postinfection (dpi)**

Images and clinical observations were made on days 1, 3, 5, and 6.

| Group | Animal | Day 1 | Day 3 | Day 5 | Day 6 |
|---|---|---|---|---|---|
| pVax1 | (6002) | Interstitial infiltration present in both caudal lobes | Diffuse interstitial infiltration present in both caudal lobes; bronchial pattern present in right middle lobe | Diffuse interstitial infiltration present in both caudal lobes; bronchial pattern present in right middle lobe | Serious diffuse interstitial infiltration present in both caudal lobes; bronchial pattern present in right middle lobe |
| | (6003) | Interstitial infiltration present in both caudal lobes | Diffuse interstitial infiltration present in both caudal lobes | Diffuse interstitial infiltration present in both caudal lobes and right middle lobe | Diffuse interstitial infiltration present in both caudal lobes and right middle lobe |
| | (6006) | Normal | Interstitial infiltration present in both caudal lobes | Interstitial infiltration present in both caudal lobes; small mass in right caudal lobe; bronchial pattern present in both caudal lobes | Interstitial infiltration present in both caudal lobes; small mass in right caudal lobe; bronchial pattern present in both caudal lobes |
| | (6009) | Interstitial infiltration present in both caudal lobes; air bronchograms observed in right middle lobe | Interstitial infiltration present in both caudal lobes; air bronchograms observed in left caudal, right caudal, and middle lobes | Interstitial infiltration present in both caudal lobes; air bronchograms observed in left caudal, right caudal, and middle lobes | Interstitial infiltration present in both caudal lobes; air bronchograms observed in left caudal, right caudal, and middle lobes |
| MERS vaccine (high dose) | (6001) | Normal | Normal | Normal | Normal |
| | (6005) | Interstitial infiltration present in both caudal and middle lobes | Interstitial infiltration present in both caudal and middle lobes | Normal | Normal |
| | (6008) | Normal | Normal | Normal | Normal |
| | (6011) | Interstitial infiltration present in both caudal and middle lobes | Interstitial infiltration present in both caudal and middle lobes; air bronchograms observed in both caudal and middle lobes | Normal | Normal |
| MERS vaccine (low dose) | (6000) | Normal | Normal | Normal | Normal |
| | (6004) | Normal | Normal | Normal | Normal |
| | (6007) | Normal | Normal | Normal | Normal |
| | (6010) | Normal | Normal | Normal | Normal |