Characterization and Demonstration of value of a Lethal Mouse Model of Middle East Respiratory Syndrome Coronavirus Infection and Disease

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Abstract

Characterized animal models are needed for studying pathogenesis of and evaluating medical countermeasures for the persisting Middle East Respiratory Syndrome-Coronavirus (MERS-CoV) infection. Here, we further characterized a lethal transgenic mouse model of MERS-CoV infection and disease that globally expresses hCD26/DPP4. The 50% infectious dose (ID$_{50}$) and lethal dose (LD$_{50}$) of virus were estimated to be <1 and 10 TCID$_{50}$ of MERS-CoV, respectively. Neutralizing antibody developed in surviving mice from the ID$_{50}$/LD$_{50}$ determinations and all were fully immune to challenge with 100 LD$_{50}$ of MERS-CoV. The tissue distribution and histopathology in mice challenged with a potential working dose of 10 LD$_{50}$ of MERS-CoV was subsequently evaluated. In contrast to the overwhelming infection in mice challenged with 10$^5$ LD$_{50}$ of MERS-CoV, we were only able to infrequently recover infectious virus from these mice although qRTPCR tests indicated early and persistent lung infection and delayed occurrence of brain infection. Persistent inflammatory infiltrates were seen in the lungs and brain stems at day 2 and day 6 after infection, respectively. While focal infiltrates were also noted in the liver, definite pathology was not seen in other tissues. Finally, using a receptor binding domain protein vaccine and a MERS-CoV fusion inhibitor, we demonstrated the value of this model for evaluating vaccines and antivirals against MERS. As outcomes of MERS-CoV infection in patients differ greatly, ranging from asymptomatic to overwhelming disease and death, having available both an infection and a lethal model makes this transgenic mouse model relevant for advancing MERS research.
Importance

Fully characterized animal models are essential for studying pathogenesis and for preclinical screening of vaccines and drugs against MERS-CoV infection and disease. When given a high-dose of MERS-CoV, our transgenic mice expressing hCD26/DPP4 viral receptor uniformly succumbed to death within 6 days, making it difficult to evaluate host responses to infection and disease. We further characterized this model by determining both the ID$_{50}$ and LD$_{50}$ doses of MERS-CoV in order to establish both an infection and a lethal model for MERS and followed this by investigating antibody responses and immunity of mice survived from MERS-CoV infection. Using the estimated LD$_{50}$ and ID$_{50}$ doses, we dissected the kinetics of viral tissue distribution and pathology in mice challenged with 10 LD$_{50}$ of virus, and utilized the model for preclinical evaluation of a vaccine and drug for MERS-CoV infection. This further characterized transgenic mouse model will be useful for advancing MERS research.
Severe acute respiratory syndrome (SARS)-coronavirus (CoV) emerged in Asia in 2002 and spread within months to other countries worldwide, including the United States and Canada, resulting in more than 8,000 cases of severe respiratory illness worldwide with a cases-mortality rate of ~10% before being brought under control using infection control measures (1). Ten years later (2012), another new CoV emerged in the Middle East as a cause of severe respiratory disease in humans, and was named Middle East Respiratory Syndrome (MERS)-CoV (2, 3).

Unlike the apparently high human-to-human transmissibility but short-lived SARS epidemic, MERS has continued to occur, especially in the Kingdom of Saudi Arabia, and recently appeared in the Republic of South Korea despite an apparent lower inter-human transmission rate than for SARS (4). As of July 3, 2015, more than 1,365 laboratory-confirmed cases of MERS-CoV disease, including at least 487 related deaths, have been identified globally (http://www.who.int/csr/don/03-july-2015-mers-korea/en/). No vaccines or antivirals known to be effective for control of MERS-CoV infection and disease in humans are currently available.

Animal models are needed for study of MERS-CoV infection and disease. Nonhuman primates (NHPs), such as rhesus macaques and marmosets, are naturally permissive to MERS-CoV infection and disease (5, 6) but they are expensive models of limited availability. Optimal development of knowledge and preventives and treatments for a new infectious disease of humans requires a small animal model to provide the numbers of animals needed for controlled and extensive studies of pathogenesis and immunity as well as for development of vaccines and antivirals. Mice are the most desirable small animal for this purpose because of availability and existence of a thorough knowledge base, particularly of genetics and immunology.

Unfortunately, the standard small animals, mice, hamsters and ferrets, all lack the functional
MERS-CoV receptor [human (h) CD26/DPP4] and are not susceptible to infection (7-9). Three humanized transgenic mouse models, each with strengths and weaknesses, have been reported, aiming to overcome the deficiency of small animal models that has impaired many aspects of MERS research (10-12). Of the three mouse models that have been described thus far, two are primarily lung infection models that develop a varying extent of lung pathology in response to $10^5$ to $10^6$ 50% tissue culture infectious doses (TCID$_{50}$) of MERS-CoV but lack morbidity (e.g., weight loss) and mortality; whereas, a transgenic mouse model globally expressing hCD26/DPP4, that was developed in our laboratory, exhibits an acute illness with profound weight loss ($\geq$ 20%), ruffled fur, hunching, squinting, decreased responsiveness to external stimuli, other clinical manifestations, and death within days after given an intranasal (i.n.) dose of $10^6$ TCID$_{50}$ of MERS-CoV.

Although these globally expressing hCD26/DPP4 transgenic mice are highly permissive to MERS-CoV infection and disease, the acute onset of severe morbidity and mortality make it difficult to fully investigate the pathogenesis, host immune responses and immunity of the MERS-CoV infection and disease. To further develop this transgenic mouse model for MERS, we determined the 50% lethal dose (LD$_{50}$) and 50% infectious dose (ID$_{50}$) of MERS-CoV and described the tissue distribution of viral infection and histopathology in the hCD26/DPP4 transgene-positive (Tg$^+$) mice challenged with a much lower, potential working dose of MERS-CoV. Finally, we show that these transgenic mice can be used as a robust preclinical model for evaluating the efficacy of vaccines and antivirals against MERS.

**Material and Methods**
**Mice, virus, and cells.** Transgenic mice expressing hCD26/DPP4 were generated in-house in the barrier facility at the University of Texas Medical Branch as previously described (10). All animal studies were conducted strictly following an approved animal protocol and the guidelines and regulations of the National Institutes of Health and AAALAC. The EMC-2012 strain of MERS-CoV, provided by Heinz Feldmann (NIH, Hamilton, MT) and Ron A. Fouchier (Erasmus Medical Center, Rotterdam, Netherlands), was used throughout the study. Briefly, the MERS-CoV-EMC/2012 strain that we received was designated passage zero (P0) and further expanded with three passages in Vero E6 cells (American Type Culture Collection) for generating cell-free P1, P2, and P3 stocks; P3 was used as the working stock for experiments described in this study. The titers of individual stocks, determined by using Vero E6-based infectivity assays, were expressed as 50% tissue culture infectious doses (TCID₅₀)/ml. Aliquots of virus stock with an average of 10⁷ TCID₅₀/ml were stored at -80°C.

**Viral infections.** All of the *in vitro* and animal studies involving infectious MERS-CoV were conducted within approved bio-safety level 3 (BSL-3) and animal BSL-3 laboratories at the National Galveston Laboratory, strictly following approved notification of usage (NOU), animal protocols, and the guidelines and regulations of the National Institutes of Health and AAALAC. All of the designs and strategies involving intranasal challenge of Tg⁺ mice with live MERS-CoV were described in individual experiments.

**Virus isolations.** Collected tissue specimens of lungs, brain, heart, liver, kidney, spleen, and intestine, were weighed and homogenized in phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS) with a TissueLyser (Qiagen, Retsch, Haan, Germany), as described earlier.
(10). After clarification of the cellular and tissue debris by centrifugation, the resulting suspensions of infected tissues were titered in the standard Vero E6 cell-based infectivity assays for quantifying yields of infectious virus. The virus titers of individual samples were expressed as log_{10} TCID_{50} per gram (g) of tissue.

**RNA extraction and real time RT-PCR.** Tissues collected at indicated times were placed in individual vials containing RNA later solution (Qiagen), weighed, and stored at 4 °C until used for extracting total RNA. Briefly, tissues were homogenized in 1 ml of TriZol reagent (Life Technologies) with a TissueLyser. After clarifying by centrifugation at 12,000g for 5 min, the resulting suspensions were tested for total RNA, quantification of MERS-CoV-specific RNA that targeted the upstream E (upE) gene and mouse beta (β)-actin gene (internal control), as described previously (10). Briefly, 0.5 µg of RNA extracted from individual tissues was used in a one-step real-time RT-PCR with a set of primer/probes specific for upE gene of MERS-CoV, using the Superscript III One-step RT-PCR kit (Invitrogen) according to the manufacturer’s instructions. The primers and probes used for upE gene of MERS-CoV were as follows: forward, 5’GCC TCTACACGGGACCCATA-3’; reverse, 5’GCAACG CGC GAT TCA GTT-3’; and fluorescence probe, 5/-56-FAM/CTC TTC ACA TAA TCG CGA GCT CG/36-TAMSp/-3. The relative amount of targeted mRNA was obtained by normalizing with endogenous control gene (β-Actin) and expressed as fold change by the standard threshold cycle (ΔΔCT) method.

**Serological assays.** MERS-CoV-specific neutralizing antibody and S1 protein-specific IgG antibody responses were quantified by a classical infection reduction assay and a standard ELISA, respectively, as described previously (13, 14). For determining neutralizing antibody
titors, the standard Vero E6 cell-based micro-neutralization assay was used. Briefly, starting at 1:10 dilutions of 60 μl of serial 2-fold dilutions of heat-inactivated serum specimens obtained from surviving Tg+ mice at 21 dpi via retro-orbital bleeding were transferred into duplicate wells of 96-well plates containing 120 TCID<sub>50</sub> of MERS-CoV in 60 μl of M-2 medium/per well, giving a final volume of 120 μl/well. The antibody-virus mixtures were incubated at room temperature for one hour before transferring 100 μl of the mixtures (containing 100 TCID<sub>50</sub> of MERS-CoV) into confluent Vero E6 cell monolayers in 96-well plates. Six wells of Vero E6 cells cultured with equal volumes of M-2 medium with or without virus were included in these assays as positive and negative controls, respectively. When the wells of Vero E6 cells infected with virus alone developed advanced cytopathic effects (CPE), the neutralizing capacity of individual serum specimens was determined, based on the presence or absence of CPE. Reciprocals of the last dilutions of serum specimens capable of completely preventing the formation of CPE were used as the neutralizing antibody titer and expressed as neutralizing titer-100% (NT<sub>100</sub>.

For quantifying the total MERS-CoV S1-specific IgG antibodies, 96-well ELISA plates were pre-coated with recombinant S1-His protein (1 μg/ml), as described previously (15, 16). After blocking with Tris-buffered saline (TBS) containing 10% FBS and 0.05% Tween 20 (TBS) for 1 hr at room temperature, 50 μl of serial 10-fold dilutions of mouse serum specimens, starting at dilutions of 1:100, were added to the plates (Corning, Cat. No. 3690), incubated for 1 hr at 37°C, and thoroughly washed with TBS before adding horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:4,000) (Southern Biotech, Cat. No.1030-05) for 1 hr at 37°C. For quantifying total specific IgG antibodies, the thoroughly washed plates were incubated in the dark with o-Phenylenediamine dihydrochloride (Sigma, Cat. No. P9187) for 15 min, the reactions were
stopped with 1N H$_2$SO$_4$, and evaluated in an ELISA plate reader (Molecular Device) for measuring the optical density (OD) at 450 nm. The highest dilutions of serum specimens with MERS-CoV S1-specific antibody with a mean OD reading greater than or equal to 2 standard deviation (SD) greater than the mean for specimens of naïve mice were used to define titers.

**Histopathology and IHC staining.** Tissue specimens harvested from animals at indicated times after infection were fixed in 10% buffered-formalin for 72 h, transferred to 70% ethanol, and paraffin-embedded for subsequent sectioning and processing for routine hematoxylin-eosin (H&E) staining for assessing pathological changes, as described previously (10). For testing for viral antigens in tissues of infected mice, the standard alkaline phosphatase-based colorimetric indirect IHC staining using a combination of a rabbit anti-MERS-CoV polyclonal antibody, provided by Dr. Heinz Feldmann, NIAID/NIH through Dr. Thomas Ksiazek at UTMB, and a biotinylated swine anti-rabbit immunoglobulin (Dako, Cat. No. E0353) were employed as we previously described (10, 17). Irrelevant rabbit antibodies were also included in this IHC staining as negative controls. Nuclei were counterstained with Mayer’s hematoxylin (Fisher Scientific) before subjecting to microscopic examination.

**Vaccine and Antiviral Evaluations.** Groups of age-matched Tg$^+$ mice were immunized intramuscularly (i.m.) twice, three-weeks apart, with S377-588-Fc (10 µg in 50 µl of PBS) formulated with an equal amount of MF59 adjuvant (AddaVax™, Cat. No. vac-adx-10, InvivoGen) or MF59 alone, designated S377-588-Fc/MF59 and PBS/MF59, respectively. Sera of immunized mice post second immunization were subjected to serological assays for quantifying neutralizing and MERS-CoV S1 protein-specific IgG antibodies. Immunized mice were
subsequently challenged (i.n.) at day 10 post second immunization with $10^3$ TCID$_{50}$ of MERS-CoV in a volume of 60 µl. Three mice in each group were sacrificed at 3 dpi for quantifying infectious virus and viral RNA expression, whereas the remaining five in each group were monitored daily for morbidity (weight loss) and mortality.

Both the preventive and therapeutic efficacy of a recently proven effective fusion inhibitor peptide, HR2M6 (18), were evaluated. For measuring the prophylactic potential, groups of Tg$^+$ mice were treated (i.n.) with 200 µg of HR2M6 in 50 µl of PBS/per mouse or PBS alone at 1 and/or 4 hrs prior to challenge (i.n.) with 100 TCID$_{50}$ of MERS-CoV in 60 µl. For assessing the therapeutic effect, groups of Tg$^+$ mice previously infected (i.n.) with 100 TCID$_{50}$ of MERS-CoV were treated with 50 µl of PBS or 200 µg of HR2M6 in 50 µl PBS at 1, 12, and 24 hrs after infection and then once daily to day 7 p.i. Three mice in each group were sacrificed at 2 dpi for assessing yields of infectious virus or viral RNA in lungs, whereas the remaining five animals in each group were monitored daily for morbidity and mortality.

Statistical Analysis

Neutralizing antibody titers and virus titers were averaged for each group of mice. Comparisons were conducted using Students $t$ test and 1-way analysis of variance as indicated.

Results

Determination of LD$_{50}$ and ID$_{50}$, immune responses, and immunity of hCD26/DPP4 transgenic mice to MERS-CoV infection.

To determine the LD$_{50}$ and ID$_{50}$, we initially administered (i.n.) serial 10-fold decreasing doses of MERS-CoV from $10^6$ to $10^1$ TCID$_{50}$ in a volume of 60 µl, to groups of four or eight
naïve Tg^+ mice and monitored them daily for clinical manifestations (weight loss) and mortality for at least 21 dpi. We found that all mice receiving virus dosages of 10^2 to 10^6 TCID_{50} of MERS-CoV succumbed to the infection (100% mortality) with day of death later with reducing dosage (Table 1). Weight loss was extreme (≥20%) for dosages of 10^3 and higher; all mice given a dose of 10^2 died but weight loss was 8% or less (data not shown). Only 5 of 8 mice given 10^1 died; deaths were between days 8 to 13 p.i. and weight loss was only 4% (Experiment 1, Table 1). All of the surviving mice continued to appear well up to 21 dpi when the experiment was terminated.

To further assess the LD_{50} and ID_{50} of the MERS-CoV stock, we challenged (i.n.) another four groups of four Tg^+ mice with 2-fold decrements of MERS-CoV, starting from 10 TCID_{50}; dosages were 10, 5, 2.5 and 1.25 TCID_{50} of virus. Mice were followed daily for morbidity (weight loss) and mortality for at least 3 weeks. Although none of the infected mice exhibited any significant weight loss (data not shown), we noted a death at days 9 and 10 in mice infected with 10 TCID_{50} and a single death in mice infected with 5 or 1.25 TCID_{50}; whereas, all mice challenged with 2.5 TCID_{50} of MERS-CoV survived without clinical illness (Table 1, Experiment 2). From the data in Table 1, we estimated the LD_{50} and ID_{50} of MERS-CoV for this transgenic mouse model to be 10 and <1 TCID_{50}, respectively, further emphasizing the extreme susceptibility of hCD25/DPP4 transgenic mice to MERS-CoV infection and disease.

All but one mouse that survived challenge with the low-doses of virus had developed serum neutralizing antibodies and MERS-CoV S1 protein-specific IgG antibodies by 21 days following infection, with NT_{100} and ELISA titers of 1:10 to 1:20 and 1:400 to 1:800, respectively (Table 2). We subsequently challenged (i.n.) these low-dose challenge survivors, along with two naïve Tg^+ mice, with 10^3 TCID_{50} (100 LD_{50}) of MERS-CoV at 35 dpi to determine if they had
developed immunity to a lethal infection dose. While both naive mice simultaneously challenged
lost more than 20% body weight and succumbed to infection within 10 dpi, all mice that had
survived the prior low dose challenge, including the one that failed to exhibit a serum antibody
response, were immune to a subsequent lethal challenge, surviving without significant weight
loss for more than 3 weeks after re-challenge. The rechallenged mouse without serum antibody
in the standardized tests did exhibit evidence of neutralizing and ELISA antibody for lower end
point criteria. Thus, these results indicate that previous infection with a non-lethal dose of
MERS-CoV was sufficient to induce immune responses that fully protect Tg^+ mice against lethal
infection.

Kinetics and tissue distribution of viral infection in hCD26/DPP4 Tg^+ mice challenged with 10
LD50 of MERS-CoV

We have shown that Tg^+ mice challenged (i.n.) with 10^6 TCID50 of MERS-CoV suffered
profound weight loss of ≥ 20% with 100% death within 6 days after infection. While infectious
virus could be readily recovered from the lungs and brains and development of a progressive
pneumonia, as evidenced by extensive infiltration of inflammatory cells, was seen, no
histopathological lesion was identified in brains of infected mice (10). For determining the tissue
distribution of viral infection and histopathology over time with a potential working dose of
virus, eighteen age-matched (10-14 weeks old) Tg^+ mice were challenged (i.n.) with 10^2 TCID50
(10 LD50) of MERS-CoV. The initial plan was to sacrifice three mice at 2, 4, 6, 8, 10 and 12 dpi
for assessing viral infection in the lungs, brain, heart, liver, kidney, spleen, and intestine by
quantifying infectious virus and viral RNA expression using Vero E6 cell-based infectivity and
qRT-PCR assays, respectively. Standard immunohistochemistry (IHC) with a rabbit anti-MERS-CoV hyperimmune serum was also performed for detecting viral antigens in tissues.

In contrast to the acute onset of extensive weight loss and mortality seen in Tg\(^+\) mice infected with a high-dose of MERS-CoV, those challenged with a dose of 10 LD\(_{50}\) (10\(^2\) TCID\(_{50}\)) only exhibited a maximum of 8% weight loss before dying. We were able to collect tissues from all three animals at two day intervals up to 8 dpi but from only a single mouse at day 10 and none at day 12 p.i. due to infection-associated deaths. Unlike the consistent recovery of \(\geq 10^7\) TCID\(_{50}/g\) of infectious virus from the lungs of mice inoculated with 10\(^5\) LD\(_{50}\) (10\(^6\) TCID\(_{50}\)), we detected a much lower titer of virus from the lung \((\sim 10^{4.6} \text{ TCID}_{50}/g)\) of only a single mouse at days 2 and 4 p.i. (Figure 1A). Also, we only detected approximately 10\(^{4.2}\) TCID\(_{50}/g\) of infectious virus from the brain of a single mouse at 8 dpi (Figure 1B). Additionally, IHC staining with specific rabbit antibodies failed to reveal the expression of viral antigens in paraffin-embedded lung and brain tissues, including those positive for infectious virus (data not shown). All tests for infectious virus from other tissues over time, including liver, spleen, kidneys, and intestines, were negative (data not shown).

Although infectious virus could only be sporadically detected, results of qRT-PCR analyses targeting the upstream E gene of MERS-CoV clearly indicated a consistent expression of viral RNA, especially in lungs and brains (Figure 1C). All lung specimens collected over time were positive for viral RNA, with the highest level detected on day 4 p.i.. In contrast, viral RNA was undetectable in brains until day 6; however, expression increased thereafter, reaching the highest level at day 10 p.i.. Although attempts to isolate virus from the GI tract were unsuccessful, viral RNA expression was detected at day 6 and increased thereafter, reaching a level equivalent to 10\(^{3.4}\) TCID\(_{50}/g\) at day 10 p.i. (Figure 1C), an increasing trend also observed in
Tg+ mice challenged earlier with a high-dose of virus (10). Viral RNA was detectable in all other tissues over time but at low levels. Taken together, these results indicate that, despite differences in kinetics and intensities of viral infection, lung, brain and, possibly GI tracts appear to be the major tissues supporting MERS-CoV infection in Tg+ mice.

Histopathology of hCD26/DPP4 transgenic mice infected with 10 LD50 of MERS-CoV

In contrast to the profound gross lesions solely detected in the lungs of animals challenged with 10^6 TCID50 of MERS-CoV, no gross organ pathology was noted in the lungs, brains, and other organs of animals sacrificed at 2 day intervals for virological and histological evaluations. However, microscopic lesions were noted on different days after infection in lungs, brains, and, to a lesser extent, in livers, but not in other tissues examined, including spleens, kidneys, and small intestines. As shown in Figure 2, lung histopathology of infected mice primarily consisted of mild and multifocal perivascular, peribronchial, and interstitial infiltrations with mononuclear cells on days 2 and 4 post-infection (p.i.). The intensity of these pulmonary infiltrates was slightly increased in 2 of 3 animals and moderately increased in one animal at day 6, and reached the maximum in all three animals sacrificed at day 8 p.i. A decreasing trend of the pulmonary inflammatory response was seen in the sole survivor at day 10 p.i., suggesting some resolution was underway.

Unlike the earlier high-dose viral challenge (10^6 TCID50/mouse) in which an inconsistent mild perivascular effect was the only pathological change seen in infected brains (10), mice infected with 10^2 TCID50/mouse (10 LD50) developed progressive inflammatory responses. As shown in Figure 3, no abnormalities could be detected in brain stem tissues on either days 2 or 4 p.i. However, pathological changes consisting of perivascular cuffing, microglia activation, and
apoptotic bodies that likely represent neuronal death were noted in brain stem tissues from 6 to 10 dpi. While no intracerebral pathology was seen in brain tissues, a mild meningitis was noted in cerebral tissues from 8-10 dpi.

Focal mononuclear infiltrations were noted in liver specimens collected on 6-10 dpi, but not 2 and 4 dpi (data not shown); however, we did not detect definite pathology in kidney, small intestine, and spleen specimens.

*HCD26/DPP4 transgenic mice as a robust preclinical model for development of vaccines and treatment*

Having further characterized this transgenic mouse lineage with regard to the LD$_{50}$ and ID$_{50}$ (Tables 1 &2), we explored whether it can be used as a small and economical animal model for development of vaccines and treatments for MERS-CoV infection and disease. Since MERS-CoV receptor binding domain (RBD)-based subunit vaccine (S377-588-Fc) and fusion inhibitor peptide (HR2M6) have been demonstrated to be preventive and therapeutic candidates for MERS (16, 19, 20), we evaluated their efficacy in our transgenic mice against MERS-CoV infection.

For evaluating the efficacy of S377-588-Fc as a subunit vaccine, we first determined its immunogenicity in Tg$^+$ mice by measuring serum neutralizing antibody responses. Specifically, two groups of Tg$^+$ mice (eight animals in each group) were immunized (i.m.) twice at a 3-week interval with S377-588-Fc plus MF59 adjuvant (S377-588-Fc/MF59) or PBS/MF59 (as control). Sera of vaccinated mice were collected at day 10 after the second immunization for assessing immunogenicity in neutralizing antibody tests. As shown in Figure 4A-D, consistent with the absence of any detectable neutralizing antibody response (< 1:10), control mice given PBS/MF59 exhibited $10^{4.9}$ TCID$_{50}$/g of MERS-CoV in lung tissues 2 days after challenge i.n.
with 100 LD$_{50}$ (10$^3$ TCID$_{50}$) of MERS CoV and profound weight loss (≥20%) with 100% mortality by 8 dpi. In contrast, those vaccinated with S377-588-Fc/MF59 elicited an average serum neutralizing antibody (NT$_{100}$) titer of ~1:800. Although none of the three vaccinated and challenged mice tested at day 2 p.i. had infectious virus in lung specimens, the remaining five vaccinated and challenged mice exhibited mild weight loss and a single death occurred at 10 dpi; the remaining four animals recovered from the mild morbidity and survived until the experiment was terminated at day 21 p.i. (data not shown).

The efficacy of the HR2M6 virus fusion inhibitor was also evaluated in Tg$^+$ mice. We initially tested it as prophylaxis by intranasal administration of a single dose of 200 µg HR2M6 or PBS alone at 1 and 4 hrs before challenging with 10 LD$_{50}$ (100 TCID$_{50}$) of MERS-CoV. Titers of infectious virus and viral RNA in lungs of three animals sacrificed at 2 dpi were determined by Vero E6-based infectivity assays and qRT-PCR, respectively. Although no infectious virus could be recovered from challenged mice regardless of whether treated with HR2M6 or not, lung viral RNA titers were significantly reduced from 3.7 of PBS-treated mice to 1.2 and 1.4 log$_{10}$ TCID$_{50}$ eq./g in those pretreated with HR2M6 at 1 and 4 hrs, respectively (Figure 5A). All of the remaining five and four out of five mice pretreated with HR2M6 at 1 and 4 hrs, respectively, were protected from death, whereas four out of five PBS-treated mice succumbed to the infection (Figure 5B). To evaluate the therapeutic efficacy of HR2M6, Tg$^+$ mice previously infected with 10 LD$_{50}$ (10$^2$ TCID$_{50}$) of MERS-CoV were administered (i.n.) either PBS or 200 µg of HR2M6 at 1, 12, and 24 hrs, and then daily after infection until 7 dpi. Three mice were sacrificed at 2 dpi for quantifying viral RNA, whereas the remaining five animals were monitored for morbidity and mortality. In contrast to the earlier report, we did not see any therapeutic benefit of HR2M6 as neither the viral load tests nor the mortality rate were significantly reduced (Figure 5C-D) (21,
Results obtained from additional Tg+ mice treated with this fusion inhibitor prior to and/or post exposure to different doses of MERS-CoV consistently indicated that HR2M6 was effective as a prophylactic, but not as a therapeutic agent against MERS-CoV infection and disease in the Tg+ mouse (data not shown).

**Discussion**

Using a cytomegalovirus promoter in a manner previously successful for developing transgenic mouse models of SARS-CoV infection and disease, we identified candidate MERS-CoV susceptible transgenic mouse lineages (10). One lineage was selected and further evaluated. A 10^6 TCID₅₀ (Vero cell cultures) intranasal dose of MERS-CoV strain EMC/2012 induced a severe pneumonia leading to death in 4 to 6 days. Lung virus was highest on day 2 post challenge and dissemination then ensued to many other organs including the brain (10). Based on RT-PCR assays, virus titer was highest in lung on day 2 and brain on day 4 post challenge. Both extensive gross and microscopic lung pathology developed. Of interest is that lung histopathology was major on day 4 but brain had minor to no pathology despite detection of high titers of virus and viral antigens in neurons and glial cells. The extensive infection and disease with MERS-CoV in this transgenic mouse model was similar to that reported in marmosets challenged with MERS-CoV (6). A concern was that the challenge to our transgenic mice and that given to marmosets might represent an overwhelming dose in very susceptible animals that caused a very severe acute lung infection with dissemination to numerous organs. Although currently available clinical information is inadequate to exclude dissemination of MERS-CoV as a component of MERS-CoV pneumonia in humans, MERS is considered to be a respiratory infection and disease (23, 24).
To clarify the role of challenge dosage in our transgenic mouse model and to provide guidance for study of MERS-CoV infection and disease as well as for evaluation of vaccines and antivirals, we proceeded to conduct infectivity assays in the transgenic mice. These studies yielded an estimated ID$_{50}$ of <1 TCID$_{50}$ (Vero cell cultures) and a LD$_{50}$ of 10 TCID$_{50}$ (Table 1). Thus, the initial challenge study with a challenge dose of $10^6$ TCID$_{50}$ represented a challenge with more than 1 million ID$_{50}$ and 100,000 LD$_{50}$ of virus. This might be designated as an “overwhelming” dosage and suggests that this may have also been true for the marmoset study (6).

As indicated earlier, two other MERS-CoV mouse infection models with some associated disease have been reported (11, 12). The approaches used to provide the human DPP4 receptor were transduction with an adenovirus type 5 vector (Ad5), gene replacement with a commercial procedure (VelociGene) and our transgenic method. While challenge dosages for the 3 models (including our model) in the published data were similar, results of challenge differed considerably. Most striking is that the Ad5 and VelociGene models induced lung infections with some histopathology but little to no clinical disease and no mortality. In contrast, our transgenic model also induced lung infection at about the same level but with severe gross and microscopic pathology, virus dissemination to other organs, including brain, and severe clinical disease preceding death in 4 to 6 days. Tests for dissemination were apparently not done for the Ad5 model and were limited to brain on days 2 and 4 (reported negative) for the VelociGene model. In the present study, using a lower challenge dosage (10 LD$_{50}$), transgenic mice still exhibited dissemination of virus, including to brain, but this was first detected later (day 6 p.i.). Clinical disease occurred but was milder than seen earlier; however, mortality was still 100% although occurring later (days 6 to 12). Mortality was not reported at these later times for the Ad5 model.
but the VelociGene model was apparently not followed beyond day 4. In summary, all 3 models appear suitable for studies with desired endpoints of lung virus yield and some lung histopathology. The transgenic models add virus dissemination, severe gross lung pathology and histopathology, severe clinical disease and mortality as potential endpoints for study. Whether either of the other 2 models would have exhibited the more substantial endpoints with higher challenge dosages, more extensive testing or longer follow is unknown.

On the basis of available data, it seems reasonable to suggest that virus dissemination and infection of other organs may occur during MERS-CoV infections, particularly in those with severe disease. Virus has been detected in blood and urine of a MERS case (25). Moreover, the receptor for MERS-CoV, CD26/DPP4 (9), is ubiquitous in human tissues, and, presumably, in primates and humanized mice; included are demonstrated presence in lung, kidney, GI tract, brain and most (if not all) other organs (26). Given access to the organ, virus infection may occur and yield virus and local abnormalities.

Initial reports of SARS emphasized the lung disease, its severity, and problems in management (1, 27-29). Gastrointestinal infection and disease were reported commonly in early reports as for MERS, but disease in other organs was not. However, subsequent reports of autopsies on SARS related deaths noted dissemination and a high frequency of CNS disease, particularly of neurons (28, 30, 31). It seems possible that an encephalopathy/encephalitis-type of abnormality might have been missed in patients with severe lung disease. In this regard, it is of interest that the reports of infection and disease for the mouse-adapted MA-15 strain of SARS-CoV and the ACE receptor transgenic mouse models that were capable of causing severe disease and death in infected mice exhibited SARS-CoV dissemination and presence of virus in the brain (32-34). Thus, SARS-CoV appears to have a capacity for dissemination with infection...
and disease in other organs, including brain. In a report of three severe cases of CNS disease in association with MERS, the authors suggested CNS disease might be missed among cases of severe disease cared for in intensive care units with use of sedation and sometimes neuromuscular blockade in the care of patients (35). These findings for SARS-CoV and MERS-CoV infections and diseases in humans suggest a need for caution in drawing conclusions about patterns of human infection and disease until a complete set of data are available. Similarly, data on animal model infections suggest conclusions about properties of a model should await a full characterization of the course of infection and disease in the model.

Although further refinement of our transgenic mouse model is desirable, a major value of a small animal model of an infection and disease of humans is for preclinical evaluations of infection and vaccine-induced immunity and of antimicrobials for prevention and treatment. For a test of our model, we conducted a pilot study of immunity induced in surviving mice in the ID_{50}/LD_{50} determinations and a preliminary test of a candidate vaccine and antiviral for MERS-CoV. Mice surviving infection had developed serum neutralizing antibody and all were completely immune to challenge with 100 LD_{50} of MERS-CoV (Tables 1&2). Similarly, a receptor binding domain protein vaccine, S377-588-Fc, induced serum neutralizing antibody to MERS-CoV and vaccinated animals were significantly protected to challenge with 100 LD_{50} of virus (Figure 4). Finally, although no benefit was seen with post challenge treatment in our test, we verified a previous report that intranasal administration of a MERS-CoV fusion inhibitor peptide, HR2M6, before virus challenge prevented disease and death from challenge (Figure 5, (18). Thus, the utility of our MERS-CoV model for studies of immunity and for development of vaccines and antivirals has been demonstrated.
Although we have not yet developed a model of infection not leading to death, the ID$_{50}$ data available for our virus and test system assure an effort would be successful. A variation in severity and pattern of infection and disease in a MERS-CoV model is potentially important as human infection and disease apparently spans a spectrum from infection with little or no disease to overwhelming disease and death (24, 36). Currently available data indicate that our transgenic mouse model can completely span this spectrum of infection and disease. To have available both an infection and a lethal model of MERS-CoV infection is highly desirable.

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References


Figure 1. Kinetics and tissue distribution of MERS-CoV infection in hCD26/DPP4 transgenic mice. Eighteen hCD26/DPP4 transgene-positive (Tg⁺) mice were challenged i.n. with 10 LD50 (10² TCID₅₀) of MERS-CoV/mouse in 50 µl. Three animals were euthanized at 2 day intervals starting from 2 dpi for assessing the magnitudes of viral infection in tissues by Vero E6-based infectivity and qRT-PCR targeting MERS-CoV-specific E gene. (A) Low levels of virus were recovered from infected lung homogenates of a single mouse (out of three) at 2 and 4 dpi. Dotted lines represent “Limit of Detection” (B) A barely detectable level of virus was recovered from brain homogenates of one mouse (out of three) at 8 dpi. Dotted lines represent “Limit of Detection” (C) Kinetics of viral loads in various tissue homogenates harvested at indicated dpi, as assessed by RNA levels of upstream E gene-specific viral RNA expression in tissues harvested at indicated times post infection are shown. Data are presented as Mean ± standard error (SE); error bars indicate standard error.

Figure 2. Low and high power photomicrographs of lungs of hCD26/DPP4 Tg⁺ mice challenged with 10 LD₅₀ of MERS-CoV. H&E stained paraffin-embedded sections of lung specimens collected from Tg⁺ mice at indicated days after infection were evaluated for pathology as briefly described in Materials and Methods. Multifocal perivascular and peribronchiolar infiltrates predominately comprised of mononuclear cells were detected at 2 and 4 dpi. The inflammatory responses gradually increased and extended to bronchi and alveolar interstitium through 6-8 dpi. Some resolution was noted at 10 dpi (single surviving mouse) but discrete perivasculitis remained detectable.
Figure 3. High power photomicrographs of brain stem and cortex of hCD26/DPP4 Tg+ mice challenged with 10 LD₅₀ of MERS-CoV. Brain tissues obtained from the same infected mice described in Figure 2 were processed for assessing histopathology. No pathological lesions were seen at 2 and 4 dpi. However, pathological changes, including perivascular cuffing, apoptotic bodies, and activated microglia, were seen in the brainstem on days 6 to 10 pi. No intracerebral pathology was seen but mononuclear cell collections in cortical meninges were seen on days 8 and 10 p.i.

Figure 4. Immunization of hCD26/DDp4 Tg+ mice with a receptor binding domain (RBD) and challenge with 100 LD₅₀ of MERS-CoV. Two groups of Tg+ mice, eight animals in each group, were immunized (twice, three-weeks apart) with MF59-adjuvanted RBD fragment fused with Fc or MF59/PBS alone. Resulting neutralizing antibody titers were determined prior to viral challenge (A). Lung viral loads of three animals were determined at day 4 after infection with 100 LD₅₀ (10³ TCID₅₀) of MERS-CoV by qRT-PCR targeting the upstream E gene, and were expressed as log₁₀ TCID₅₀ equivalents/per gram (B). The remaining five mice in each group were monitored daily for weight loss (C) and survivor rates (D). Error bars indicate standard error. *** P < 0.001, Students t test between control and test groups.

Figure 5. Prophylactic and therapeutic evaluations of the HR2M6 fusion inhibitor against MERS-CoV infection and disease in hCD26/DPP4 transgenic mice. For evaluating the prophylactic efficacy, groups of Tg+ mice, eight in each group, were given a single dose of either HR2M6 (200 μg in 60 μl) or PBS (control) at 1 or 4 hrs before viral challenge. Mice were challenged i.n. with 10 LD₅₀ (10² TCID₅₀) of MERS-CoV in 60 μl. Lung viral loads were
determined in three infected mice per group on day 3 p.i. by qRT-PCR targeting the upstream E
653 gene, and expressed as log_{10} TCID_{50} equivalents per gram (A). Error bars indicate standard error.
654 Survivor rates of the remaining five animals in each group were assessed daily (B). For assessing
655 the therapeutic efficacy, two groups of Tg\(^*\) mice, eight animals/group, were treated i.n. with
656 HR2M6 (200 \(\mu\)g in 60 \(\mu\)l) or PBS at 1, 12, and 24 hrs and then daily for 7 days after infection,
657 viral challenge was with 10LD_{50} (10\(^2\) TCID_{50}); viral load was assessed on day 2 p.i. (C) and
658 survivor rates for 12 days (D). ** \(P < 0.01\), 1-way analysis of variance (ANOVA) compared with
659 control group.
Brainstem | Meninges

4 dpi

6 dpi

8 dpi

10 dpi

.LEFT: apoptotic body | .RIGHT: activated microglia | .UP: perivascular cuffing
Table 1. Determining the 50% lethal dose (LD$_{50}$) and infectious dose (ID$_{50}$) of MERS-CoV in hCD26/DPP4 transgenic mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Challenge Dose (TCID$_{50}$/mouse)</th>
<th>No. Deaths/No. Challenged (%)</th>
<th>Day of death post challenge</th>
<th>No. infected/No. tested (%)$^a$</th>
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<tbody>
<tr>
<td>1</td>
<td>$10^6$</td>
<td>8/8 (100)</td>
<td>4-6</td>
<td>NA$^b$</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>4/4 (100)</td>
<td>5-7</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>4/4 (100)</td>
<td>5-8</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$10^3$</td>
<td>4/4 (100)</td>
<td>6-10</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$10^2$</td>
<td>8/8 (100)</td>
<td>6-12</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$10^1$</td>
<td>5/8 (62.5)</td>
<td>8-13</td>
<td>NA$^c$</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>2/4 (50)</td>
<td>9,10</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1/4 (25)</td>
<td>9</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0/4 (0)</td>
<td>NA$^a$</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>1/4 (25)</td>
<td>10</td>
<td>3/3 (100)</td>
</tr>
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</table>

$^a$ Infection determined by serum antibody response in neutralization a/o ELISA tests.

$^b$ Not applicable (NA)

$^c$ Not determined (ND)

NOTE: Estimated LD$_{50}$ and ID$_{50}$ are 10 and < 1 TCID$_{50}$, respectively.
Table 2. Serum antibody titers to MERS-CoV in survivals of initial challenge and their response to re-challenge

<table>
<thead>
<tr>
<th>Initial challenge dose (TCID\textsubscript{50})</th>
<th>Number of survivors</th>
<th>Neutralizing antibody\textsuperscript{b}</th>
<th>ELISA IgG antibody\textsuperscript{c}</th>
<th>Death or wt loss on rechallenge\textsuperscript{d}</th>
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<tbody>
<tr>
<td>10</td>
<td>2</td>
<td>&lt; 10, 10</td>
<td>800,800</td>
<td>0/2</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>10, &lt; 10, 20</td>
<td>800,400,800</td>
<td>0/3</td>
</tr>
<tr>
<td>2.5</td>
<td>4</td>
<td>20, 20, &lt; 10, 20</td>
<td>400,400,&lt;100</td>
<td>0/4</td>
</tr>
<tr>
<td>1.25</td>
<td>3</td>
<td>&lt; 10, 10, &lt; 10</td>
<td>400,400,400</td>
<td>0/3</td>
</tr>
</tbody>
</table>

a: Antibody responses were determined at day 21 p.i.
b: The highest dilution of sera that completely inhibited CPE formation in 100% of infected Vero E6 cultures (NT\textsubscript{100})
c: The highest dilution of sera with MERS-CoV S1-specific antibody with a mean optical density (OD) ≥ 2 standard deviation (SD) greater than the mean for naïve mice
d: Re-challenged with 100 LD\textsubscript{50} (10\textsuperscript{3} TCID\textsubscript{50}) of MERS-CoV at day 35 after the initial infection. Two out of two simultaneously challenged naive Tg\textsuperscript{+} mice exhibited severe weight loss (>20%) and death occurred within 10 days p.i.