Elevated Human Dipeptidyl Peptidase 4 Expression Reduces the Susceptibility of hDPP4 Transgenic Mice to Middle East Respiratory Syndrome Coronavirus Infection and Disease

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Running title: Soluble human DPP4 and MERS-CoV infection

Main point of our study: We demonstrated that elevated levels of circulating soluble human (sh) DPP4 positively correlates with the resistance to MERS-CoV infection and identified trated a potential of recombinant shDPP4 as a treatment option for MERS.

Abstract

Background: The ongoing Middle East Respiratory Syndrome (MERS) Coronavirus (CoV) infections pose threats to public health worldwide, making an understanding of MERS pathogenesis and development of effective medical countermeasures (MCMs) urgent.

Methods: We used homozygous (+/+) and heterozygous (+/-) human dipeptidyl peptidase 4 (hDPP4) transgenic mice to study the effect of hDPP4 on MERS-CoV infection. Specifically, we determined values of 50% lethal dose (LD_{50}) of MERS-CoV for the two strains of mice, compared and correlated their levels of soluble (s) hDPP4 expression to susceptibility, and explored recombinant (r) shDPP4 as an effective MCM for MERS infection.

Results: $hDPP4^{+/+}$ mice were unexpectedly more resistant than $hDPP4^{+/-}$ mice to MERS-CoV infection, as judged by increased LD_{50}, reduced lung viral infection, attenuated morbidity and mortality, and reduced histopathology. Additionally, the resistance to MERS-CoV infection directly correlates with increased serum shDPP4 and serum virus neutralizing activity. Finally, administration of rshDPP4 led to reduced lung virus titer and histopathology.

Conclusions: Our studies suggest that the serum shDPP4 levels play a role in MERS pathogenesis and demonstrate a potential of rshDPP4 as a treatment option for MERS. Additionally, it offers a validated pair of Tg mouse for characterizing the effect of shDPP4 on MERS pathogenesis.

[Key words: Middle East respiratory syndrome-Coronavirus; MERS pathogenesis; human DPP4, transgenic mice; medical countermeasures for MERS]

Introduction

Middle East respiratory syndrome (MERS) is an emerging infectious disease caused by a coronavirus (MERS-CoV), first identified in Saudi Arabia in 2012, that has since spread to 27 mostly surrounding countries, resulting in more than 2,229 laboratory-confirmed cases of infection and 791 deaths (~ 36%), as of June, 2018 [1]. The pandemic potential of this infection calls for a better understanding of MERS pathogenesis and the development of effective medical countermeasures (MCMs) for humans.

Like other human CoVs, MERS-CoV uses an exo-aminopeptidase, human dipeptidyl peptidase 4 (DPP4), as the entry receptor for infection of permissive cells [2]. DPP4, also known as CD26, is involved in many physiological functions via its ubiquitous expression in a variety of tissues, its propensity to interact with adenosine deaminase (ADA) and other important regulatory molecules of the immune system, and its intrinsic proteolytic activity that cleaves many biologically active peptides or proteins that contain proline or alanine at the penultimate position [3,4]. Not only is DPP4 expressed as a type II transmembrane glycoprotein primarily on endothelial and epithelial cells, and subsets of immune cells, but it is also present in a functionally intact soluble form (sDPP4) in the circulation and other body fluids [3,4].

Since wild type mice are not susceptible to MERS-CoV, we established a heterozygous (+/-) transgenic (Tg) mouse model globally expressing hDPP4 for studies of MERS pathogenesis and development of MCMs against MERS-CoV infection [5,6]. To ensure a steady and cost-effective supply of the animals, a Tg mouse model with homozygous expression of hDPP4, designated hDPP4$^{+/+}$ Tg mice, was developed through mating of hDPP4$^{+/-}$ mice and used as

breeders for generating offspring of both genotypes of hDPP4 Tg mice. Since hDPP4 is the functional MERS-CoV receptor, the doubling of encoded hDPP4 gene in $hDPP4^{+/+}$ Tg mice could render them more susceptible than their $hDPP4^{+/-}$ counterparts to MERS-CoV infection and disease. To our surprise, we found that $hDPP4^{+/+}$ mice are more resistant than their $hDPP4^{+/-}$ counterparts to MERS-CoV infection. We subsequently found that an increased expression of functionally intact soluble (s) hDPP4 in the circulation of $hDPP4^{+/+}$ Tg mice, relative to that of $hDPP4^{+/-}$ mice, was associated with this increased resistance and might be, at least in part, accountable for the seemingly counterintuitive findings on susceptibility to MERS-CoV infection. This notion was supported by studies showing that elevated shDPP4 levels, brought about by administration of recombinant (r) shDPP4, resulted in increased resistance of recipient $hDPP4^{+/-}$ mice to MERS-CoV. Together, our results indicate that manipulation of shDPP4 might serve as a strategy for counteracting MERS-CoV infection and disease in humans.

Methods:

Human DPP4 transgenic (hDPP4 Tg) mice

hDPP4$^{+/-}$ transgenic mice were established, as previously reported [5,6]. hDPP4$^{+/+}$ breeder mice were derived by mating two parental hDPP4$^{+/-}$ mice. The homozygosity was determined by quantitative PCR analysis of tail DNA (data not shown) and verified by their subsequent mating with wild-type (wt) mice. Only those mice uniformly yielding heterozygous offspring were selected as hDPP4$^{+/+}$ breeders. Inter-breeding between hDPP4$^{+/+}$ mice produced additional hDPP4$^{+/+}$ mice, whereas backcrossing them to *wt* mice generated hDPP$^{+/-}$ mice.

Viral infection, isolation, and titration

All of the *in vitro* and animal studies involving infectious MERS CoV were conducted at the biosafety level 3 (BSL3) laboratory and animal BSL3 facilities at the Galveston National Laboratory in accordance with approved protocols and the guidelines and regulations of the NIH and AAALAC. Detailed methodologies for viral infection, isolation from infected lungs and brain, and determination of infectious viral loads have been established and routinely used in our laboratory [5,6]. The original stock of MERS-CoV EMC-2012 strain, a gift of Heinz Feldmann (NIH, Hamilton, MT) and Ron A. Fouchier (Erasmus Medical Center, Rotterdam, Netherlands), was expanded in Vero E6 cells three times consecutively. Passage 3 containing a titer of ~5 x 10^6 $TCID_{50}$/ml of infectious virus was used throughout the study.

50% lethal dose (LD_{50}) determination

LD_{50} values for hDPP4$^{+/+}$ and hDPP4$^{+/-}$ mice was determined by using traditional virus dilution assays and the Reed-Muench method as we previously described [6]. Briefly, groups of four young (6-8 weeks) or old (7-10 months) hDPP4$^{+/+}$ and hDPP4$^{+/-}$ mice were inoculated, via intranasal (i.n.) route, with dosages of EMC-2012 MERS-CoV in 10-fold decrements from 10^2 to 10^{-1} $TCID_{50}$ in a volume of 60 µl. Mice were monitored daily for clinical manifestations (weight loss) and mortality for at least 21 dpi. LD_{50} values for each strain of mice were estimated based on the ratio of the surviving mice to the total inoculated mice as previously described [6]. Those surviving for more than 21 days were also evaluated for specific antibody responses to MERS-CoV receptor binding domain (RBD) protein by ELISA [6]. Only those showing specific antibody to RBD were considered as "MERS-CoV-infected".

Quantification of circulating soluble human DPP4 in Tg mouse sera

To quantify the circulating shDPP4 in the sera of naïve DPP4$^{+/+}$, DPP4$^{+/-}$, and DPP4$^{-/-}$ mice, a commercial ELISA-based assay was used, following the manufacturer's instructions (eBioscience catalog # BMS235). Absorbance at 450 nm in 96-well plates was read in an ELISA plate reader (Molecular Device).

Serological and micro-neutralization assays

ELISA- and Vero E6 cell-based micro-neutralization assays, previously described [7], were used to determine the titers of MERS-CoV RBD-specific serum IgG and neutralizing antibodies in hDPP4 Tg mice in response to MERS-CoV infection..

Binding specificity and anti-MERS-CoV activity of rshDPP4 in tissue cultures

Purified insect cell-derived human DPP4 ectodomain (residues 39–766; GenBank accession no. NP_001926.2) containing an N-terminal human CD5 signal peptide and a C-terminal His6 tag, as we previously described and characterized [7,8], was prepared and used for treatment studies. Testing binding specificity of the rshDPP4 to the receptor-binding domain (RBD) proteins of MERS-CoV and SARS-CoV, both RBDs are generous gifts of Drs. Du and Jiang at New York Blood Center, NY, was determined in ELISA-based assays [7,9]. For determining the capacity of rshDPP4 to inhibit MERS-CoV infection in vitro we initially used our standard micro-neutralization procedure with cytopathic effect (CPE) inhibition as the endpoint. These assays revealed a dose-dependent reduction of cytopathic effects (CPE) at 72 hrs, ranging from less than 5% for 100, 50, and 25 μg/ml and gradually increased to ~30% for 12.5 μg/ml of rshDPP4. In addition to standard micro-neutralization assay with a CPE endpoint, we measured the antiviral effect of rshDPP4 using virus yield of each rshDPP4 dilution from 100 to 0.8 μg/ml, expressed as $\log_{10}$ $TCID_{50}$/ml.

Administration of rshDPP4 to mice before and after challenge with MERS-CoV

The effect of rshDPP4 for inhibiting MERS-CoV infection in Tg mice was determined using $hDPP4^{+/-}$ mice in two pilot studies with two different batches of rshDPP4 showing similar, but not identical, binding capacity to MERS-CoV RBD and *in vitro* neutralizing activity. Briefly, groups of $hDPP4^{+/-}$ mice (N=3 per group) were treated twice with either 100 μg, 400 μg of rshDPP4 or PBS (as control) via the intra-peritoneal (i.p.) route 2-hr before (-2) and 24-hr after

(+24) infection (i.n.) with 10^3 $TCID_{50}$ of MERS-CoV. Mice were sacrificed at 3 dpi for assessing infectious viral loads and histopathology in the lungs.

Histopathology

Inflated lung specimens and brain tissues were fixed in 10% neutral buffered formalin for 48 hours before paraffin-embedding and processing for routine hematoxylin and eosin stain (H&E) for assessing the histopathology, as we previously described [5,6] .

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Neutralizing antibody titers and virus titers were averaged for each group of mice and compared using *Students' t*-test, one-way ANOVA, or others as indicated.

Results:

hDPP4$^{+/+}$ mice are more resistant than DPP4$^{+/-}$ mice to MERS-CoV infection and disease

For the initial comparison of the susceptibility of hDPP4$^{+/+}$ and hDPP4$^{+/-}$ mice to MERS-CoV, we determined the LD_{50} values for mice 7 to 10 months of age, as we previously described [6]. Since hDPP4 is the functional receptor of MERS-CoV, we anticipated that DPP4$^{+/+}$ mice might be more, or at least equally, permissive as DPP4$^{+/-}$ mice to MERS-CoV infection. To our surprise, we found that hDPP4$^{+/+}$ mice were more resistant than hDPP4$^{+/-}$ mice as indicated by LD_{50} values of 4.3 and 32.4 $TCID_{50}$ of MERS-CoV for hDPP4$^{+/-}$ and hDPP4$^{+/+}$ mice, respectively. To confirm this seemingly counterintuitive finding and rule out any potential effect of age and gender, we repeated the study using age- (6-8-weeks old) and sex-matched Tg mice of both genotypes. Shown in *Figure 1A* is a representative of two independently performed experiments that confirmed the LD_{50} difference; values for the hDPP4$^{+/+}$ and hDPP4$^{+/-}$ mice were 70.0 and 7.7 $TCID_{50}$ of MERS-CoV, respectively, indicating that hDPP4$^{+/+}$ mice are more resistant than their age- and sex-matched hDPP4+/- counterparts to MERS-CoV infection.

Using sera collected 21 dpi from each strain of Tg mice that survived the lower challenge dosages, we quantified MERS-CoV RBD-specific IgG antibodies by ELISA. We found that infection had occurred in Tg mice of both strains. Infection rates for those given 100 $TCID_{50}$ were similar (1/1 for DPP4$^{+/-}$ mice and 2/2 for DPP4$^{+/+}$ mice) and 10 $TCID_{50}$ (2/2 for each strain) but were greater for DPP4$^{+/-}$ mice (3/3) than DPP4$^{+/+}$ mice (1/4) for 1 $TCID_{50}$. The difference in infection rates is consistent with the increased resistance of DPP4$^{+/+}$ mice described earlier.

To further verify the difference in susceptibility to MERS-CoV infection, we infected (i.n.) hDPP4$^{+/+}$ (N=9) and hDPP4$^{+/-}$ (N=11) Tg mice with an equal dose of MERS-CoV (10^3 $TCID_{50}$/per mouse) and monitored them daily for morbidity (weight loss) and mortality. Three mice of each strain, unless indicated otherwise, were euthanized at 3, 5, and 7 dpi for assessing infectious viral titers and the histopathology of lungs and brains. In contrast to DPP4$^{+/-}$ mice that exhibited marked weight loss, starting at 3-4 dpi and two deaths at 6 dpi (data not shown), infected hDPP4$^{+/+}$ mice exhibited minimal weight changes and uniformly survived through 7 dpi when the experiment was terminated (*Figure 1B*). When the viral loads were measured at 3 dpi, we readily recovered infectious virus from the lungs, but not the brains, of all three hDPP4$^{+/-}$ mice examined, but from the lung of only one of three hDPP4$^{+/+}$ mice. Although we usually recover virus from lungs of some mice, efforts to recover infectious virus from both lung and brain specimens of both strains of Tg mice at 5 dpi were unsuccessful (data not shown); however, we were able to retrieve infectious virus from the brain (but not lungs) of the sole hDPP4$^{+/-}$ survivor and from all three hDPP4$^{+/+}$ mice that survived to 7 dpi. The virus titer in the brain was $10^{6.2}$/per gram for that single hDPP4$^{+/-}$ mouse, a titer significantly higher than the average of $10^{3.7}$/per gram for three hDPP4$^{+/+}$ mice (*Figure 1C*). This ability to recover infectious virus from the lungs approximately 2-3 days earlier than from the brains is consistent with the pattern, kinetics, and tissue distribution of MERS-CoV infection in DPP4 Tg mice we have previously reported [6]. We also compared the histopathology of lungs and brains, two of the prime targets of MERS-CoV infection of hDPP4 Tg mice [5]. Although infected DPP4$^{+/-}$ mice elicited mild-to-moderate histopathological changes within the lungs at 3 dpi after a dose of 10^3 $TCID_{50}$ of MERS-CoV infection as in our earlier study [6], infected DPP4$^{+/+}$ mice exhibited

reduced or no lung histopathology (data not shown). Brain histopathology at 7 dpi for the sole hDPP4$^{+/-}$ survivor had infiltrations of mononuclear cells in the meninges (*Figure 1D:c, arrows*), perivascular cuffing (*Figure 1D:a&c*), microglial nodules (*Figure 1D:c, circle*), microhemorrhage (*Figure 1D:a, asterisks*) and cell death at the junctions of gray and white matter (*Figure 1D:a*) but not in DPP4$^{+/+}$ mice. Collectively, the significant differences between these two strains of mice in their LD_{50} values, seroconversion rates, viral loads, weight loss, and histopathology support the notion that DPP4$^{+/+}$ mice are more resistant than DPP4$^{+/-}$ mice to MERS-CoV infection.

hDPP4$^{+/+}$ mice exhibit significantly higher levels of soluble hDPP4 in sera than those of hDPP4$^{+/-}$ mice.

Since functionally active soluble (s) DPP4 exists in the circulation and other body fluids of humans [10,11], we explored whether the levels shDPP4 expression in the sera of these two strains of hDPP4 Tg mice could be different, thereby contributing to their difference in susceptibility to MERS-CoV infection. Using a commercial ELISA-based analysis (eBioscience), we were unable to detect shDPP4 in age- and sex-matched hDPP4-negative (hDPP4$^{-/-}$) littermates. However, as shown in *Figure 2A*, a representative of two independently performed studies, an average of 8.1 ± 0.2 μg/ml (Mean ± SD) and 6.2 ± 0.4 μg/ml of shDPP4 was detected for hDPP4$^{+/+}$ (N=16) and DPP4$^{+/-}$ mice (N=10), respectively. As shDPP4 retains its binding specificity to MERS-CoV RBD protein [7], the significantly different expression of shDPP4 in the sera of these two strains of Tg mice ($P < 0.001$) prompted us to examine if

elevated shDPP4 expression might relate to the higher resistance of $DPP4^{+/+}$ to MERS-CoV infection by possibly acting like a decoy that binds MERS-CoV RBD and prevents virus infection. Using micro-neutralization tests, we noted that the 50% neutralization titers (NT_{50}), expressed as the geometric mean titers (GMT), were 3.9 ± 0.3 (Mean ± SE, P = 0.026) , 3.1 ± 0.1, and 3.0 for $hDPP4^{+/+}$, $hDPP4^{+/-}$, and $hDPP4^{-/-}$ mice, respectively (*Figure 2B*).

Administration of recombinant soluble hDPP4 (rshDPP4) significantly inhibits MERS-CoV infection in $hDPP4^{+/-}$ mice

Since the significantly higher expression of shDPP4 with better neutralizing activity might contribute to the increased resistance of $hDPP4^{+/+}$ mice to MERS-CoV infection, we explored whether an increased shDPP4 expression might increase resistance of naïve $hDPP4^{+/-}$ mice to MERS-CoV infection. Using our limited amount of insect cell-derived rshDPP4, known to specifically bind to RBD of MERS-CoV but not SARS-CoV, and to neutralize MERS-CoV *in vitro* in a dose-dependent manner (*Figure 3A, B*), we administered 100 μg of rshDPP4, via the intra-peritoneal route, into each of three $DPP4^{+/-}$ mice 2 hrs before (- 2) and 24 hrs after (+ 24) infection with 10^3 $TCID_{50}$ of MERS-CoV. The effect of the rshDPP4 against MERS-CoV infection was assessed at 3 dpi by using the titers of infectious virus within the lungs as the end point for this pilot study. While all of three PBS-treated mice exhibited moderate titers of live virus, we were unable to recover infectious virus from any of three rshDPP4-treated mice (*Table 1, Experiment-1*). Encouraged by the preliminary data, we generated another batch of rshDPP4 shown to inhibit MERS-CoV infection in a dose-dependent manner (*Figure 3B*) to repeat the experiment. We gave groups of three $DPP4^{+/-}$ mice either 100 or 400 μg of rshDPP4, or PBS (as

control) 2 hrs before and 24 hrs after virus infection as before. To determine if administration of rshDPP4 would increase the circulating levels of shDPP4, serum levels of shDPP4 in mice prior to and 2-hr after the first administration and before challenge with MERS-CoV were measured. We found that titers at 0 and 2hrs of Tg mice given 100 μg (6.6 ± 0.4 versus 6.6 ± 0.9 μg/ml), were similar to those of PBS-treated mice (7.5 ± 0.8 versus 7.3 ± 1.0 μg/ml). However, mice treated with 400 μg of rshDSPP4 showed significantly increased titers 2hrs after treatment [6.6 ± 0.6 to 13.7 ± 0.8 μg/ml (P = 0.002, *t*-test)]. These results suggest that an increased serum level of shDPP4 can be achieved by administration of rshDPP4 in a dose-dependent manner.

In addition to the viral loads within the lungs at 3 dpi, the pulmonary histopathology was examined for investigating the effect of rshDPP4 on MERS-CoV infection. Unlike the first study in which treatment with two-doses of 100 μg (at -2 and +24 hrs) of rshDPP4 fully protected against MERS-CoV infection, we were able to recover reduced titers of infectious virus from each of three mice given 100 μg of the second batch of rshDPP4 when compared to those of PBS-treated controls (*P = 0.077*, *t*-test). However, as shown in *Table 1*, *Experiment-2*, the titers of infectious virus in mice treated with 400 μg of rshDPP4 were significantly reduced from an average of 4.3 ± 0.3 (Mean ± SE) in control mice to 2.6 ± 0.1 $TCID_{50}$/g (*P* = 0.011, *t*-test). This finding is consistent with the reduced potency of batch 2 of rshDPP4 as shown in *Figure 3B*. Yet, the histopathology in mice treated with the high-dose of rshDPP4 (i.e., 400 μg) was reduced as well when compared to that of the PBS controls (data not shown*)*.

Discussion

In this study we found that hDPP4$^{+/+}$ mice were more resistant than hDPP4$^{+/-}$ mice to MERS-CoV infection, as evidenced by ~10-fold increases of LD_{50}, reduced infectious viral yields and seroconversion rates, as well as less weight loss and lower mortality than their age- and sex-matched hDPP4$^{+/-}$ counterparts (*Figure 1*). We also found that hDPP4$^{+/+}$ mice had significantly higher levels of shDPP4 in their circulation than did hDPP4$^{+/-}$ mice (*Figure 2*). Moreover, these higher serum levels of shDPP4 exhibited higher titers of neutralizing activity against MERS-CoV in Vero E6 cell-based assays. Finally, we showed that administration with functionally active rhsDPP4 proteins (*Figure 3*) enabled hDPP4$^{+/-}$ mice to better resist MERS-CoV infection in a dose-dependent manner, (*Table 1*), a finding in accordance with increased levels of shDPP4 in their circulation. Taken together, these results support the notion that increasing the levels of shDPP4 is a potential option for counteracting MERS-CoV infection and disease of humans.

DPP4, ubiquitously expressed on many types of cells and tissues, has been well-characterized as critically involved in regulating many important physiological functions, in part, through its intrinsic enzymatic activity and propensity to interact with other key regulatory molecules of the immune system [3,12]. As the functional receptor that mediates entry of MERS-CoV to permissive host cells, the membrane-associated hDPP4 plays a pivotal role in MERS-CoV infection and disease. However, specific role(s) that shDPP4 might have in MERS pathogenesis remain much less understood. While the levels of sDPP4 vary significantly, even among healthy individuals, it has been shown that the intensities of sDPP4 expression in the circulation, along with its intrinsic enzymatic activity, could be a factor in dictating the severity of many human

diseases, including malignancies, autoimmune and inflammatory diseases, diabetes mellitus and other metabolic syndromes, and chronic infectious disease such as AIDS and Hepatitis C [4,10,11,13,14]. It has been recently reported that serum levels of sDPP4 expression in confirmed MERS patients were significantly reduced when compared to those of healthy individuals [15]; however, the suggestion that these reduced levels could serve as biomarkers for susceptibility requires knowledge regarding the levels in MERS cases before onset of infection and disease. In addition, further studies of the therapeutic value of shDPP4 as either a significant resistance factor or a potential countermeasure for MERS-CoV in humans is warranted. Of note, soluble form of the viral receptors for several other viruses, including those caused by SARS-CoV, rhinovirus, and HIV, has been proposed as potentially effective antiviral therapeutics [16–18].

We showed in this study that sera derived from naive hDPP4 Tg mice of either strain, especially hDPP4$^{+/+}$, possess detectable neutralizing antibody-like activity against MERS-CoV (*Figure 2B*). Whether the significantly higher shDPP4 expression of hDPP4$^{+/+}$ mice could be solely accountable for its greater resistance to MERS-CoV infection through functioning as receptor decoys, seems unlikely, since it took at least 12.5 μg of rshDPP4, a level higher that the ~ 8 μg/ml in sera of hDPP4$^{+/+}$ mice, to significantly inhibit MERS-CoV infection in Vero E6 cells (*Figure 3B*). Additional studies are needed to better understand the shDPP4-related protective mechanisms against MERS-CoV, especially those of the immune system. However, the validated direct correlation between the level of shDPP4 and the susceptibility to MERS-CoV infection, as shown in this study, may provide a possible genetic basis for the observed wide-

spectrum of diseases, ranging from asymptomatic, mild-to-moderate, to severe infection and death in MERS patients [19,20]

With the limited supplies of rshDPP4, we have shown in two independently performed "proof-of-principle" studies that administration of exogenous rshDPP4 might be a treatment option for MERS-CoV infection (*Table 1*). Additional studies are required to determine if increasing shDPP4 levels by rshDPP4 treatment could be a useful treatment option for human MERS. The study presented in this report demonstrates the usefulness of this homozygous and heterozygous pair of hDPP4 Tg mice to fully explore the interactions between hDPP4 and MERS-CoV infection and disease, studies that could lead to identification of new and novel molecular and cellular targets for MCMs for MERS-CoV infection and disease in humans.

Footnote:

Conflict of Interest:

No reported conflict interests from all of the authors

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Presentations:

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Figure legends:

Figure 1. Homozygous hDPP4 (hDPP4$^{+/+}$) transgenic (Tg) mice are more resistant than heterozygous hDPP4 (hDPP4$^{+/-}$) Tg mice to Middle East respiratory syndrome-associated Coronavirus (MERS-CoV) infection and disease. A: The 50% lethal dose (LD_{50}) values of age-and-sex-matched hDPP4$^{+/+}$ and hDPP4$^{+/-}$ mice. Data shown are represetative of two independently performed studies. B: Kinetics of weight loss of hDPP4$^{+/+}$ (n=9) and hDPP4$^{+/-}$ (n=11) mice in response to infection with 10^3 $TCID_{50}$ of MERS-CoV. C: Titers of infectious virus recovered from MERS-CoV (10^3 $TCID_{50}$)-infected hDPP4$^{+/+}$ and hDPP4$^{+/-}$ mice at 3 (lungs) and 7 (brains) dpi. ** $P < 0.01$ (*t*-test, lung titers at 3 dpi), D: Histopathological changes were detectable in MERS-CoV (10^3 $TCID_{50}$)-infected hDPP4$^{+/-}$, but not hDPP4$^{+/+}$, mice at 7 dpi, and consisted of infiltration of mononuclear cells within the meninges (*c, arrows*), perivascular cuffing (*a&c*), microglial nodule (*c, circle*), microhemorrhage (*a, asterisks*) and cell death at the junctions of gray-white matter (*a*).

Figure 2. Sera of naïve hDPP4$^{+/+}$ mice contain significantly higher levels of soluble (s) hDPP4 that exhibit higher levels of neutralizing antibody-like activity than those of naïve hDPP4$^{+/-}$ mice. A: Groups of at least 10 age-and-sex-matched hDPP4$^{+/+}$, hDPP4$^{+/-}$, and their transgene-negative (hDPP4$^{-/-}$) littermates were subjected to retro-orbital bleeding for assessing the contents of shDPP4, using commercially available ELISA kits (eBioscience) that quantify specific hDPP4 (hDPP4$^{+/+}$ vs hDPP4 $^{+/-}$, $P < 0.001$ *t*-test). B: Sera obtained from naïve hDPP4$^{+/+}$ and hDPP4$^{+/-}$ mice (N=10 of each) and hDPP4$^{-/-}$ mice (N=6) were subjected to the standard Vero

E6-based micro-neutralization tests to determine their potential to neutralize MERS-CoV. Data are presented as the geometric mean neutralization titers (log_2) of 50% neutralization titer (NT_{50}). A 1-10 dilution of sera is ~3.4 log_2, *: P = 0.026 (t-test and Mann-Whitney Rank Sum Test, when compared to those of $hDPP4^{-/-}$ mice). Dotted line: Limit of detection.

Figure 3. Recombinant soluble human DPP4 (rshDPP4) proteins derived from insect cells specifically bind to RBD protein of MERS-CoV, but not SARS-CoV, and are capable of neutralizing MERS-CoV in a dose-dependent manner. A: Binding specificity of rshDPP4 to MERS-CoV RBD. Briefly, ELISA 96-well plates, pre-coated with RBD protein of MERS-CoV or SARS-CoV, were used to determine the binding specificity of rshDPP4 by using the standard ELISA-based assays. The absorbance was measured at a wavelength of 450 nm. B: The dose-dependent neutralizing activities of two different batches of rshDPP4 (rshDPP4-1 and -2) against MERS-CoV. The modified Vero E6-based micro-neutralization test (virus yield) was used to quantify the neutralizing antibody-like capacity of rshDPP4, as described earlier [21] and in the Methods.

Table 1. Effect of administration of recombinant soluble human DPP4 (rshDPP4) on MERS-CoV infection within the lungs of infected DPP4$^{+/-}$ mice[§]

Treatments	Experiment-1	Experiment-2
	Lung viral titers ($TCID_{50}$/ml)	
PBS	2.7	3.7
	3.6	4.5
	3.4	4.8
	3.2 ± 0.3[a]	4.3 ± 0.3[a]
rshDPP4 (100 µg)	≤ 2.4[b]	2.8
	≤ 2.4	3.7
	≤ 2.4	3.6
	≤ 2.4 [a,c] (P = 0.038)	3.4 ± 0.3[a] (P = 0.077)
rshDPP4 (400 µg)	NT[d]	≤ 2.4[b]
		2.7
		2.8
		2.6 ± 0.1[e] (P = 0.011)

§: Mice (N=3 each group) were given either 100 µl of PBS or PBS containing 100 or 400 µg of rshDPP4/per mouse by the intra-peritoneal route 2-hr

before and 24-hr after intranasal challenge with 100 LD_{50} (~ 10^3 $TCID_{50}$) of MERS-CoV. Lung infectious viral titers were quantified at 3 dpi using a

standard Vero E6 cell-based infectivity assay. a: Mean ± SE; b: None detected (Limit of detection is 2.5 $\log_{10}$ $TCID_{50}$/g); c: P = 0.038 (t-test) using

2.4 as the value for non-detectable samples; d: Not tested; e: P = 0.011, One Way ANOVA

Figure 1

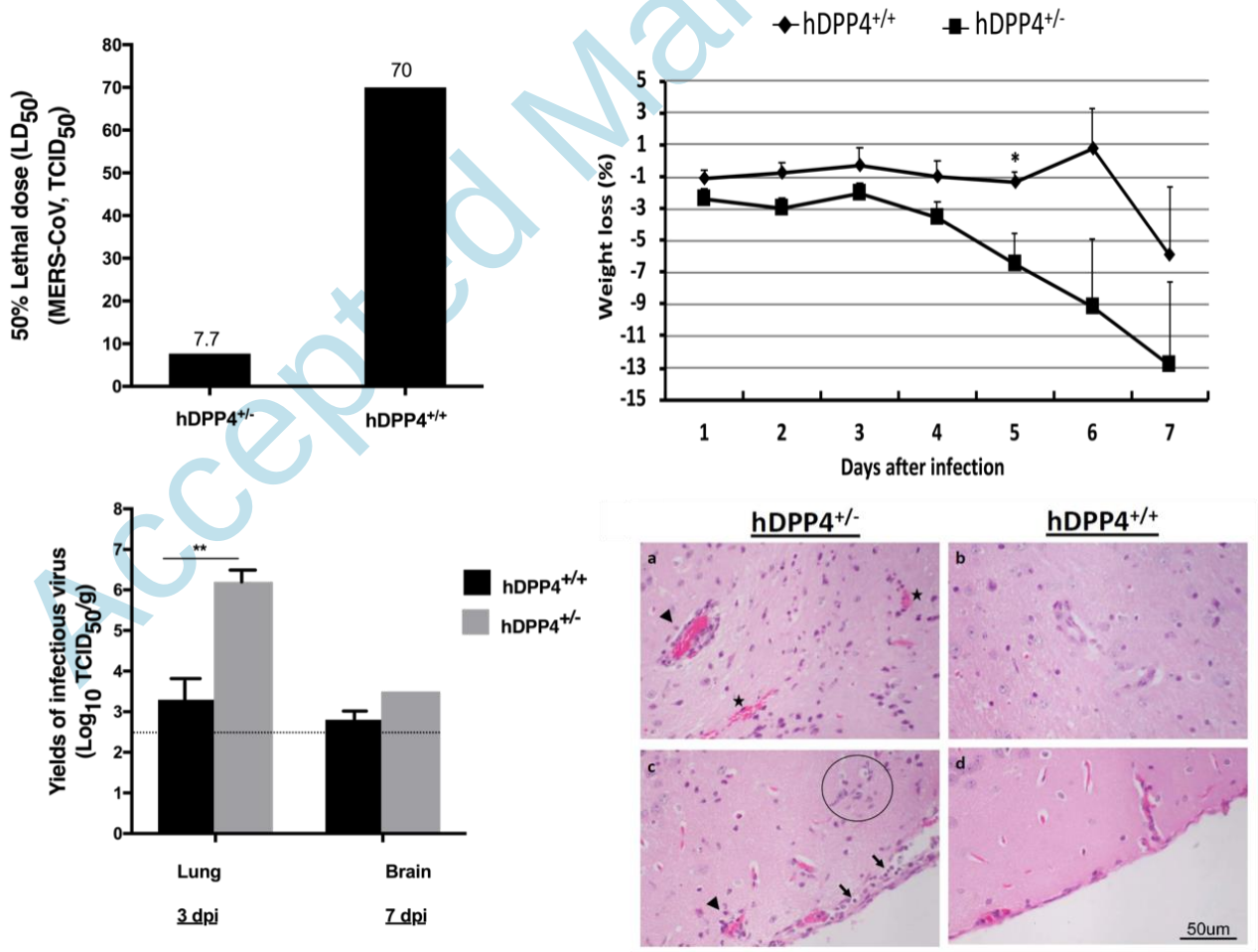

Figure 2

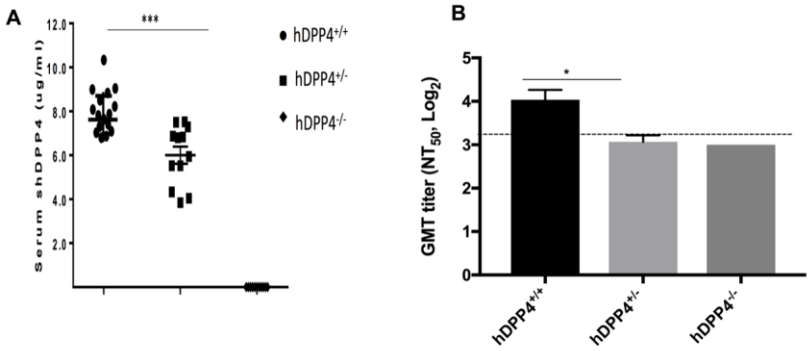

Figure 3