1   **Acute respiratory infection in human dipeptidyl peptidase 4-transgenic**

2   **mice infected with Middle East respiratory syndrome coronavirus**

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17   Running head: A transgenic mouse model of MERS-CoV

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24 **ABSTRACT**

25   Middle East respiratory syndrome coronavirus (MERS-CoV) infection can manifest

26 as a mild illness, acute respiratory distress, organ failure, or death. Several animal models

27 have been established to study disease pathogenesis and to develop vaccines and

28 therapeutic agents. Here, we developed transgenic (Tg) mice on a C57BL/6 background;

29 these mice expressed human CD26/dipeptidyl peptidase 4 (hDPP4), a functional receptor

30 for MERS-CoV, under the control of an endogenous hDPP4 promoter. We then

31 characterized this mouse model of MERS-CoV. The expression profile of hDPP4 in these

32 mice was almost equivalent to that in human tissues, including kidney and lung; however,

33 hDPP4 was overexpressed in murine CD3-positive cells within peripheral blood and

34 lymphoid tissues. Intranasal inoculation of young and adult Tg mice with MERS-CoV led

35 to infection of the lower respiratory tract and pathological evidence of acute multifocal

36 interstitial pneumonia within 7 days, with only transient loss of body weight. However, the

37 immunopathology in young and adult Tg mice was different. On Day 5 or 7

38 post-inoculation, lungs of adult Tg mice contained higher levels of pro-inflammatory

39 cytokines and chemokines associated with migration of macrophages. These results suggest

40 that the immunopathology of MERS infection in the Tg mouse is age-dependent. The

41 mouse model described herein will increase our understanding of disease pathogenesis and

42 host mediators that protect against MERS-CoV infection.

43

44   **IMPORTANCE**

45   Middle East respiratory syndrome coronavirus (MERS-CoV) infections are endemic

46   in the Middle East and a threat to public health worldwide. Rodents are not susceptible to

47   the virus because they do not express functional receptors; therefore, we generated a new

48   animal model of MERS-CoV infection based on transgenic mice expressing human

49   (h)DPP4. The pattern of hDPP4 expression in this model was similar to that in human

50   tissues (except lymphoid tissue). In addition, MERS-CoV was limited to the respiratory

51   tract. Here, we focused on host factors involved in immunopathology in MERS-CoV

52   infection and clarified differences in antiviral immune responses between young and adult

53   transgenic mice. This new small animal model could contribute to more in-depth study of

54   the pathology of MERS-CoV infection and aid development of suitable treatments.

55

56   **INTRODUCTION**

57   Middle East respiratory syndrome coronavirus (MERS-CoV) was originally

58   isolated as a novel coronavirus from a fatal case of acute respiratory distress syndrome

59   and renal failure in 2012 (1). A human receptor for the virus, called human

60   CD26/dipeptidyl peptidase 4 (hDPP4), was identified subsequently (2). Many

61   epidemiological and virological investigations have been undertaken since then; however,

62   information about the pathogenesis of MERS-CoV is limited. In addition, because

63   MERS-CoV is endemic in the Middle East, the development of effective prophylactic

64   and therapeutic treatment strategies remains a high priority. Therefore, appropriate

65   animal models are needed to better understand the pathogenesis of MERS-CoV and

66   facilitate development of effective vaccines and drugs. Some research groups

67   experimentally infected nonhuman primates and small experimental animals with

68   MERS-CoV (3-7). Rhesus macaques appear to develop a transient lower respiratory tract

69   infection after a combination of intratracheal, ocular, oral, and intranasal inoculation with

70   MERS-CoV (3), whereas the common marmoset develops progressive and severe

71   pneumonia, which can be lethal (4). However, animal models based on nonhuman

72   primates present both ethical and economic problems. Thus, establishing a small animal

73   model of MERS-CoV infection is desirable. Unfortunately, MERS-CoV does not infect

74   or replicate in small rodents such as Syrian hamsters (8), mice (9), or rats (10) because

75   they lack a functional MERS-CoV receptor. Zhao *et al.* described lung infection in a

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76   mouse model transduced with an adenovirus expressing hDPP4 (11); thus a transgenic

77   (Tg) mouse carrying hDPP4 should be suitable for MERS-CoV studies (5). Some

78   research groups developed Tg mice overexpressing the hDPP4 receptor under the control

79   of CAG or cytokeratin 18 promoters (5-7). These mice developed severe lung disease,

80   along with infection of the brain. Autopsy data are available from only one MERS

81   patient; therefore, it is unclear whether MERS-CoV causes a systemic infection, although

82   there is no evidence that MERS-CoV infects the human brain. Other studies describe

83   development of a hDPP4 knock-in mouse (12-14). Although the tissue distribution and

84   expression levels of hDPP4 in these models are largely equivalent to those of DPP4 in

85   wild-type mice, the phenotype that determines MERS-CoV susceptibility varies from

86   model to model. The hDPP4 knock-in mouse model described by Coleman *et al.* (12)

87   succumbed to infection with wild-type MERS-CoV. By contrast, model mice described

88   by Cockrell *et al.* (14) and Li *et al.* (13) are susceptible to infection by serially passaged

89   MERS-CoV, which induces severe lung pathology and diffuse alveolar damage (DAD).

90   These mice would be good models for studying pathogenesis of MERS. Here, we

91   developed a new Tg mouse model expressing hDPP4 under the control of its endogenous

92   promoter to better mimic physiological expression of hDPP4. These Tg mice were then

93   backcrossed onto Th1-prone C57BL/6 mice. After evaluating susceptibility to

94   MERS-CoV infection, we investigated age-dependent differences in disease

95   pathogenesis; because older age is one of the common factors related to MERS severity,

96   and mortality (15-20). Both young and adult Tg mice infected with MERS-CoV showed

97   transient weight loss along with moderate pneumonia and MERS-CoV replication in the

98   lung; however, they did not recapitulate the severe disease and lethal infection seen in

99   humans. Young and adult Tg mice infected with MERS-CoV did, however, show

100   differing immunopathology. Adult Tg mice showed higher levels of pro-inflammatory

101   cytokine- and chemokine-mediated macrophage infiltration of the lungs than young Tg

102   mice.   Taken together, these results suggest that age affects the immunopathology of

103   MERS-CoV infection in Tg mice.   The data suggest that other factors are required to

104   recapitulate severe human disease in these Tg mice; however, this mouse model will be

105   useful for identifying host mediators that protect against MERS-CoV infection. This

106   animal model will provide new insight into factors that cause severe MERS-CoV

107   infection.

108

109   **RESULTS**

110   **Expression of hDPP4 in Tg mouse tissues**

111   To generate Tg mice showing tissue- or cell type-specific hDPP4 expression

112   mimicking that in humans, we first looked at research involving Tg mice harboring human

113   enterovirus receptors (such as the human poliovirus receptor) and SCARB2 receptor-driven

114   endogenous promoters (21, 22). Promoter sequences, which normally include a

115   transcriptional start site, are usually isolated from the upstream regions of endogenous

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116   mammalian genes (23). Therefore, we used a bacterial artificial chromosome (BAC) clone

117   (RP11-345J9) containing the complete hDPP4 gene and an endogenous promotor to

118   generate Tg mice harboring hDPP4 (Fig. 1A). To screen the generated Tg mice, we

119   confirmed presence of the transgene by PCR genotyping using two primers sets specific for

120   hDPP4 (Table 1; exon 3 and exon 10). hDPP4 is a protease expressed on the surface of cells

121   in various organs, including T cells (24, 25). The enzyme is expressed by approximately

122   60% of resting T cells isolated from blood (26). Since handling of peripheral blood in a

123   laboratory is relatively simple, we conducted flow cytometry analysis using a

124   FITC-conjugated anti-human CD26/hDPP4 monoclonal antibody that does no react with

125   murine DPP4 to detect expression of hDPP4 in mice. CD3-positive lymphocytes from 2/15

126   tested pups were positive for hDPP4. These mice were then crossed with C57BL/6 mice to

127   establish two independent Tg lines (Tg1 and Tg2), which were maintained as hemizygotes

128   carrying the hDPP4 gene. The Tg animals were born at the expected Mendel's ratio and

129   were outwardly indistinguishable from control littermates. Because CD3-positive

130   lymphocytes from peripheral blood of line Tg2 showed higher hDPP4 expression that those

131   from line Tg1 (Fig. 1B), Tg2 was used for further analyses. PCR genotyping using primer

132   sets specific for hDPP4 revealed that the complete hDPP4 gene had integrated into the

133   genome of Tg2 mice (Fig. 1C, Table 1).

134   To examine hDPP4 expression in human and Tg2 tissues, we first performed

135   Western blot analysis with a goat anti-CD26/hDPP4 polyclonal antibody (AF1180; R&D

136   Systems), which detected hDPP4 but cross-reacted weakly with mouse DPP4. Bands of

137   about 110 kDa (hDPP4) were detected in all tested human tissues (liver, spleen, kidney,

138   heart, lung, stomach, small intestine, large intestine, pancreas, brain, spinal cord, and

139   skeletal muscle), except brain (Fig. 2A). All of the tissues from hDPP4 Tg mice expressed

140   hDPP4, including liver, spleen, kidney, heart, lung, stomach, small intestine, large intestine,

141   pancreas, brain, spinal cord, and skeletal muscle (Fig. 2A). These results suggest that the

142   human transgene was expressed in the majority of organs/tissues in Tg2 mice.

143   To further determine hDPP4 distribution in tissues, immunohistochemistry (IHC)

144   was performed (Fig. 2B). IHC using a goat anti-CD26/hDPP4 antibody detected hDPP4

145   antigens in pneumocytes in the lung, in bile capillaries in the liver, in renal tubular

146   epithelium, on the surface of epithelial cells lining the small intestine, in pancreatic islets,

147   in lymphocytes in the lymph nodes, and in several types of endothelial cell and serous

148   membranes (Fig. 2B, left column). While hDPP4 expression was undetectable in brain

149   tissue by Western blot analysis, IHC revealed that endothelial cells lining blood vessels and

150   leptomeninges of the human brain were positive for hDPP4, although neurons and glia were

151   negative. In Tg2 mice, pneumocytes and bronchial epithelial cells in the lungs, bile

152   capillaries in the liver, the renal tubular epithelium, and the surface of epithelial cells in the

153   small intestine were positive for hDPP4 (Fig. 2B, middle column). In addition, several

154   types of endothelial cells and serous membranes in all tested tissues, including the central

155   nervous system, from Tg2 mice were positive for hDPP4. Notably, most lymphocytes in

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156   the T cell zones of the spleen and lymph nodes from Tg2 mice were positive for hDPP4.

157   Staining of tissues from non-Tg mice was very weak or absent (except for the small

158   intestine) (Fig. 2B, right column). These data suggest that the pattern of hDPP4 expression

159   in Tg2 mice is similar to that in humans (except for pancreas and lymphoid tissues).

160   Expression of hDPP4 was higher in lymphocytes from Tg2 mice than in those from

161   humans (Fig. 1C and Fig. 2B). Therefore, we investigated the immune response profile in

162   Tg2 mice. To assess innate immune responses in the lungs of Tg2, non-Tg, and C57BL/6

163   mice, all animals received intranasal administration of PBS with or without poly(I:C), a

164   synthetic analog of double-strand RNA (Fig. 3). There was no statistically significant

165   difference in cytokine expression between Tg2, non-Tg, and C57BL/6 mice at 24 h after

166   inoculation with poly(I:C) or PBS (Fig. 3). However, when we set expression levels after

167   PBS treatment as 1, we noted that expression of MIP-1α, GM-CSF, IL-1β, IL-12, and IL-2

168   in poly(I:C)-treated Tg2 mice was 0.8–2-fold higher than in the other two strains. These

169   results suggest that hDPP4 expression in mice does not have a marked effect on basal

170   innate immune responses in the three mouse strains; however, Tg2 show slightly stronger

171   or earlier innate immune responses than C57BL/6 mice and non-Tg mice. Thus, when we

172   investigated immune responses in this animal model we made comparisons between

173   MERS-CoV-infected and non-infected Tg mice.

174

175   **Susceptibility of hDPP4-Tg mice to MERS-CoV infection**

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176   In this experiment, C57BL/6 mice were used instead of non-Tg mice, because we

177   were unable to prepare a sufficient number of non-Tg mice from littermate mice for this

178   experiment. After intranasal inoculation of 10-week-old Tg2 and C57BL/6 mice with 10^5

179   TCID$_{50}$ (50% of the tissue culture infectious dose) of MERS-CoV, neither group was

180   lethargic; however, Tg2 mice showed mild but transient weight loss from Days 6 to 7

181   post-inoculation (p.i.) (Tg2 mice, n = 8 [four females and four males]; and C57BL/6 mice,

182   n = 10 [five female and five male]; Fig. 4A). Tg2 mice showed seroconversion at 35 days

183   p.i., whereas C57BL/6 mice did not (Tg2 mice, n = 5 [three females and two males]; and

184   C57BL/6 mice, n = 6 [three females and three males]; Fig. 4B), suggesting that Tg2 mice

185   are susceptible to infection by MERS-CoV.

186   We next examined viral replication kinetics and sites of viral replication in

187   10-week-old Tg2 and C57BL/6 mice (n = 3–4 [two females and 1–2 males per time point]).

188   Tg2 mice and C57BL/6 mice were inoculated intranasally with 10^5 TCID$_{50}$ of MERS-CoV,

189   and tissue specimens (the maxilla [including the nostril], nasal wash fluid, lung, and lung

190   wash fluid) were collected at 6 h p.i. and on Days 1, 3, 5, and 7 p.i. The nasal wash fluid

191   from three out of four Tg2 mice contained barely detectable levels of infectious virus at

192   Days 1 and 5 p.i. (Fig. 4C). Supernatants from maxilla tissue homogenates (20%) from Tg2

193   mice contained $10^{2.8}$ TCID$_{50}$/g at Day 1 p.i., although the titer fell by 5 days p.i. The viral

194   titers in lung wash fluid and supernatants of lung tissue homogenates (20%) from Tg2 mice

195   contained $10^{2.3}$ TCID$_{50}$/ml and $10^{4.6}$ TCID$_{50}$/g, respectively, at 1 day p.i.; these values were

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196 significantly higher than those at 6 h p.i. (P < 0.05 and P < 0.01, respectively; one-way

197 ANOVA). The virus titers in the lungs were detectable up until 5 days p.i. Virus was

198 undetectable in the respiratory tract at 7 days p.i. Although IHC revealed that various

199 organs from Tg2 mice were positive for the hDPP4 antigen (Fig. 2B), no infectious virus

200 was detected in the liver, spleen, kidney, heart, intestines, and brain up to 7 days p.i. (Table

201 2). By contrast, virus was detected in the respiratory tract of C57BL/6 mice at 6 h p.i. only.

202 These observations suggest that MERS-CoV infects and replicates mainly in the lower

203 respiratory tract of Tg2 mice and is eliminated within 7 days of infection.

204   Several research groups have developed Tg mouse models of MERS-CoV infection;

205 however, in these models, viral replication and MERS-CoV RNA were detected in the

206 brain (5-7, 27). Thus, we next measured the amount of viral RNA in the brain of Tg2 mice

207 at 6 h and at 1, 3, 5, and 7 days p.i. by real-time RT-PCR using primers specific for

208 MERS-CoV; however, no MERS-CoV RNA was detected in the brain of Tg2 mice.

209 Furthermore, another study showed that experimental infection of common marmosets with

210 MERS-CoV resulted in viremia (4). Quantitative examination of viral RNA levels in Tg2

211 mice revealed very low copy numbers of viral RNA in the blood at 3 and 5 days p.i., while

212 sera from Tg2 mice were negative for the virus (Table 2). These results suggest that,

213 although intranasal inoculation of Tg2 mice with MERS-CoV causes no neuroinvasion, it

214 may induce viremia.

215   Several animal studies have identified MERS-CoV RNA in the lymphoid organs of

216   infected animals (3, 4, 28), but no infectious virus was detected in the spleen from Tg2

217   mice up to 7 days p.i. (Table 2). To investigate whether lymphoid organs in Tg2 mice are

218   susceptible to MERS-CoV infection, we harvested splenocytes from Tg2 and C57BL/6

219   mice and estimated infectivity and MERS-CoV RNA levels (Fig. 4D). Although the

220   amount of infectious MERS-CoV in splenocytes was below the detection limit, viral RNA

221   was detected in splenocytes from Tg2 mice (peaking at 2 days p.i.). Thus, lymphoid organs

222   of Tg2 mice were as susceptible to MERS-CoV infection as those reported in other animal

223   studies, although lymphoid organs were not a major site of MERS-CoV replication in Tg2

224   mice after intranasal inoculation.

225

226   **Acute inflammatory changes in the lungs of hDPP4-Tg mice after MERS-CoV**

227   **inoculation**

228   MERS-CoV infection of the nasal cavity, lungs, brain, spinal cord, liver, spleen,

229   kidney, heart, and gastrointestinal tract of Tg2 mice was examined histopathologically on

230   Days 1, 3, 5, 7, 14, and 35 p.i (n = 3 [one or two females and one or two males per time

231   point]). Histopathological investigations revealed that Tg2 mice showed progressive

232   pulmonary inflammation associated with acute virus infection, from which they recovered

233   (Fig. 5). On Day 1 p.i., there was no obvious infiltration of the lungs in Tg2 mice; however,

234   mild cellular degeneration and viral antigen-positive cells were seen in the bronchioles and

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235   a few alveolar areas (Fig. 5A–C). Double immunofluorescence staining revealed that

236   MERS-CoV antigen co-localized with hDPP4-positive bronchioles and alveolar cells on

237   Day 1 p.i. (Fig. 6). On Days 3 and 5 p.i., inflammatory reactions, including partial and/or

238   mild perivascular and peribronchiolar infiltration by mononuclear cells and eosinophils in

239   response to viral antigens, were observed in alveolar areas of lung tissue from Tg2 mice

240   (Fig. 5D–I). From Day 3 p.i. onwards, the alveolar area was the main site of inflammatory

241   responses to viral replication (Fig. 5F, I, L). On Day 7 p.i., the point at which Tg2 mice

242   showed weight loss, there was evidence of severe lung inflammation, including

243   perivascular and alveolar septal thickening, caused by infiltrating mononuclear cells; some

244   alveoli were positive for viral antigens (Fig. 5K). At Day 14 p.i., focal cellular infiltration

245   was still evident in the peribronchioles and alveolar septa, although viral antigens and

246   inflammatory responses had cleared from the lungs by Day 35 p.i. (Fig. 5M–P). There was

247   no evidence of diffuse alveolar damage in the lungs up to Day 35 p.i. These findings

248   suggest that progressive immunopathology occurred uniformly throughout the lungs, but

249   resolved within 14 days after infection. Neither histopathology nor IHC detected

250   inflammatory infiltration or viral antigens in the nasal cavity through 35 days p.i. In

251   addition, there was no inflammation or viral antigen expression in the brain through 35

252   days p.i. (Fig. 7). By contrast, C57BL/6 mice showed no histopathological changes or viral

253   antigen in any organ, including the lung. These results indicate that Tg2 mice suffer acute

254   pneumonia after infection of the lungs with MERS-CoV, which is related to expression of

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255 hDPP4 in the bronchiolar epithelium and pneumocytes. Splenocytes from Tg2 mice were

256 susceptible to *ex vivo* infection with MERS-CoV (Fig. 4D); however, the spleen and other

257 lymphoid tissues did not harbor viral antigens. Furthermore, MERS-CoV induced T cell

258 apoptosis upon infection *in vitro* (28), whereas immunohistochemical staining detected no

259 evidence of apoptosis in lymphoid tissues, including spleen and lymph nodes, of

260 MERS-CoV-infected Tg2 mice. Similar to the other mouse models in which mouse DPP4

261 was replaced with hDPP4 (12), MERS-CoV replication and pathology were localized in the

262 lungs of Tg2 mice.

263

264 **Differences in the immunopathology of MERS-CoV infection between young and**

265 **adult hDPP4-Tg mice**

266   According to an epidemiological study (29), age (> 45 years) is considered to be one

267 of the risk factors for MERS-CoV infection in humans. Therefore, we infected 25-week-old

268 mice with MERS-CoV. Tg2 mice showed significant weight loss from Days 7 and 8 p.i,

269 before recovering by Day 14 p.i. (n = 6 [one female and five male Tg2 mice] and n = 7

270 [three female and four male non-Tg mice]; Fig. 8A). However, 25-week-old Tg2 mice

271 showed no obvious clinical signs (such as respiratory illness and mortality). The

272 25-week-old Tg2 mice had seroconverted by 35 days p.i., whereas the C57BL/6 mice had

273 not (Fig. 8B). Next, we examined viral replication kinetics and sites of viral replication in

274 25-week-old Tg2 and non-Tg mice (n = 4 [two females and two males per time point]). The

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275 nasal wash fluid from one out of four Tg2 mice contained barely detectable levels of

276 infectious virus at 1, 3, and 5 days p.i. (Fig. 8C). Supernatants from maxilla tissue

277 homogenates (20%) from Tg2 mice contained $10^{1.7}$ and $10^{2.0}$ TCID$_{50}$/g at 1 and 3 days p.i.,

278 respectively, although the titer was undetectable at 5 days p.i. The viral titers in lung wash

279 fluid from Tg2 mice were detectable up until 3 days p.i., while the supernatants from lung

280 tissue homogenates (20%) from Tg2 mice showed a high viral load from 1 to 5 days p.i.;

281 infectious virus was detectable up until 7 days p.i. Viral loads in the respiratory tract

282 peaked at 3 days p.i.. Although no virus was detectable in the respiratory tract of

283 10-week-old Tg mice at 7 days p.i., infectious virus was detected in the lungs of

284 25-week-old Tg mice up until 5 days p.i. In addition, infectious virus was detected in the

285 lungs of one of four 25-week-old Tg mice ($10^{2.5}$ TCID$_{50}$/g) even at 7 days p.i. We also

286 found that viral titers in the nasal wash fluid, maxilla (including nostril), lung wash fluid,

287 and lungs of 25-week-old Tg2 mice on Day 3 p.i. ($10^{2.6}$/ml, $10^{3.3}$/ml, $10^{2.3}$/ml, and $10^{4.9}$/ml,

288 respectively) were slightly higher than those in 10-week-old Tg2 mice ($10^{1.6}$/ml, $10^{2.9}$/ml,

289 $10^{1.8}$/ml, and $10^{4.5}$/ml, respectively) (P = 0.04, Student's *t* test with Welch's correction).

290   Histopathological analysis revealed that 25-week-old Tg2 mice showed delayed and

291 prolonged inflammatory responses in the lung compared with those in 10-week-old Tg2

292 mice (Fig. 9). Interestingly, viral antigen-positive cells were seen in the alveolar area on

293 Day 1 p.i., and then in the bronchi on Day 3 p.i., along with a sparse cellular infiltrate (Fig.

294 9A–F). Cellular infiltration (which included mononuclear cells and polynuclear cells) was

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295   observed in the alveoli from 5 days p.i.; this expanded on Day 7 p.i. (Fig. 9G–L). Focal cell

296   infiltration was seen in the alveoli on Day 14 p.i., and lymphoid cell aggregates were seen

297   around bronchioles and blood vessels on Day 35 p.i. (Fig. 9M–R).

298   Next, we compared inflammation of the alveoli in 10-week-old Tg mice and

299   25-week-old Tg mice on Day 7 p.i. Ionized calcium binding adaptor molecule 1 (Iba-1) is

300   expressed specifically by monocytes/macrophages and is upregulated when these cells are

301   activated. The predominant inflammatory infiltrate within the lungs of both 10-week-old

302   and 25-week-old Tg mice comprised Iba-1-positive large cells and CD3-positive

303   mononuclear cells (Fig. 10). Phagocytic vacuoles were prominent in large Iba-1 positive

304   cells from 25-week-old Tg mice.

305

306   **Expression of pro-inflammatory cytokines and chemokines in hDPP4-Tg mice after**

307   **MERS-CoV infection**

308   Dysregulated cytokine and chemokine expression is observed in

309   MERS-CoV-infected patients (30). Therefore, we measured the levels of 20 cytokines and

310   chemokines in lung samples from both 10-week-old and 25-week-old Tg2 mice inoculated

311   with either MERS-CoV or minimal essential medium (MEM). Measurements were made at

312   6 h, and at 1, 3, 5, and 7 days, p.i. (n = 3–4 [two females and 1–2 males per time point], Fig.

313   11A; and n = 4 [two females and two males per time point], Fig. 11B). On Day 3 p.i., we

314   observed early expression of gamma IFN-induced protein 10 (IP-10) in the lungs of both

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315   10- and 25-week-old Tg2 mice, which was significantly higher than that in control mice;

316   this increase lasted through Day 7. This was followed by a transient increase in expression

317   of interleukin (IL)-6, IL-13, and monocyte chemotactic protein-1 (MCP-1) in lungs from

318   both 10- and 25-week-old Tg2 mice at Day 5 p.i. High levels of macrophage inflammatory

319   protein 1 alpha (MIP-1α) and monokine induced by gamma IFN (MIG) were detected in

320   the lungs of both groups of Tg2 mice from Days 3 or 5 to 7 p.i., whereas IL-12 levels

321   increased at Day 7 p.i. IFN-γ production in the lungs of 10-week-old Tg2 mice peaked

322   significantly on Day 5 p.i. while high values were seen in both infected and non-infected

323   25-week-old mice during the observation period. By contrast, expression of IP-10, IL-12,

324   and IL-1β in 25-week-old Tg mice was higher than that in 10-week-old Tg2 mice.

325   Interestingly, IL-1α and IL-17 were detected only in 25-week-old Tg2 mice infected with

326   MERS-CoV. IL-1α, a potent pro-inflammatory cytokine associated with inflammation and

327   fever, was detected from 3 days p.i. and remained significantly elevated until 7 days p.i.;

328   IL-17 (a pro-inflammatory cytokine that recruits monocytes and neutrophils) was detected

329   from 3 days p.i., peaking at 5 days p.i. before falling at 7 days p.i. These results indicate

330   that MERS-CoV infection induces production of acute inflammatory chemokines and

331   cytokines in the lungs. In addition, aging causes more severe immunopathology; this means

332   that young and adult Tg mice show differing pathology in the lung after MERS-CoV

333   infection.

334   Furthermore, we measured expression of mRNA encoding interferon (IFN)-α4 and

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335 IFN-β (type 1 IFN with antiviral activity) in the lungs of 10- and 25-week-old mice at 6 h

336 and at 1, 3, 5, and 7 days p.i. by real-time reverse transcription RT-PCR (31). We did not

337 find any evidence of IFN-α4 or IFN-β in the lungs from 10-week-old Tg2 mice at early

338 times post-infection; however, we observed a transient increase in IFN-α4 expression in the

339 lungs of two out of four Tg2 mice at 5 days p.i.; this fell by 7 days p.i. (Fig. 11C). Day 5 p.i.

340 was the time point at which the amount of virus in the lungs of Tg2 mice began to fall. No

341 IFN-β mRNA was detectable in lung samples from either group. On the other hand, the

342 lungs of 25-week-old Tg2 mice showed a transient increase in IFN-α4 and IFN-β mRNA

343 expression at 3 days p.i. (Fig. 11C). In addition, IFN-α4 and IFN-β mRNA expression was

344 higher than that in 10-week-old Tg2 mice. Taken together, these results indicate that both

345 type 1 and type 2 IFN contribute to the immunopathology of the lungs of 25-week-old Tg2

346 mice infected with MERS-CoV.

347

348 **DISCUSSION**

349   Here, we describe a new hDPP4-Tg mouse expressing the human gene under the

350 control of an endogenous human promoter. This mouse model shows a pattern of hDPP4

351 expression that closely mimics that in human tissues and is similar to that in other recent

352 models (5-7). This mouse model also shows susceptibility to infection by MERS-CoV; this

353 mimics the non-lethal observations in other mouse models (13, 14). After intranasal

354 inoculation with a human isolate of MERS-CoV, the Tg mice developed acute and mild

19

355   interstitial pneumonia; however, the infection was non-lethal and so did not mimic severe

356   cases seen in human MERS-CoV patients. MERS-CoV infection can cause severe illness,

357   resulting in acute respiratory distress syndrome, although a large number of MERS-CoV

358   infections follow a mild or asymptomatic course in healthy individuals (32-35). Thus, this

359   Tg mouse model reflects the natural course of a mild MERS-CoV infection.

360   The majority of severe MERS-CoV cases occur in middle-aged and older males (36,

361   37). Therefore, we infected Tg2 mice aged 25 weeks with MERS-CoV. The mice showed

362   prominent pro-inflammatory responses and prolonged pulmonary inflammation when

363   compared with Tg2 mice aged 10 weeks. However, none of the infected Tg2 mice had a

364   severe outcome, such as respiratory distress, that led to death. Epidemiologically, patients

365   with diabetes, kidney failure, or chronic lung disease, all of which might weaken the

366   immune system, tend to have a poor outcome after infection by MERS-CoV

367   (http://www.who.int/csr/don/23-september-2015-mers-kuwait/en/). Thus, it is presumed

368   that a combination of older age and underlying disease might also increase mortality in this

369   animal model.

370   This hDPP4-Tg mouse model, which lacks the clinical signs and mortality

371   associated with severe MERS-CoV infection, is likely to be less advantageous than other

372   lethal mouse models with respect to development of novel vaccines or antiviral agents

373   (12-14). When we asked why the Tg2 mice showed non-lethal responses to infection, we

374   could not ignore the fact that virus levels in lungs were lower than those reported for other

20

375   MERS mouse models (5, 12, 13, 38, 39). One reason for this is that the transgene used in

376   this study is a hemizygote, meaning that the copy number or expression level may be lower

377   than that in mice homozygous for hDPP4. In addition, the DPP4 protein is active as a dimer

378   (40), but the Tg2 mice harbor both murine and hDPP4. We presume that the viral yield in

379   the lungs of Tg2 mice was low because of heterodimers formed by hDPP4 and murine

380   DPP4. To address this, we constructed structural models of homo- and heterodimers

381   comprising human and mouse DPP4. Notably, the estimated interaction energy of the DPP4

382   heterodimers was greater than that of murine and hDPP4 homodimers (-358.2, -347.6, and

383   -344.6 kcal/mol, respectively). These results suggest that DPP4 heterodimers are as stable

384   as DPP4 homodimers. Cockrell *et al*. reported that mouse DPP4 does not support

385   MERS-CoV entry (38). Thus, the presence of stable DPP4 heterodimers may be a reason

386   for the lower levels of infection in our mouse model. Further study is necessary to clarify

387   this.

388   Some research groups generated a mouse-adapted MERS-CoV for use in

389   severe/lethal MERS-CoV infection mouse models (13, 14). This may be one way to

390   establish severe MERS infection in our Tg2 mice.

391   While the Tg2 mice expressed hDPP4 protein in the liver, spleen, kidney, heart,

392   lung, gastrointestinal tract, pancreas, and brain, viral infection and replication were limited

393   (mainly) to the lower respiratory tract, with little upper respiratory tract involvement, after

394   intranasal inoculation of MERS-CoV. Tg2 mice developed interstitial pneumonia, and

21

395   MERS-CoV antigens were detected in the lungs. Virus yields in the lung were up to

396   100-fold higher than those in the upper respiratory tract. Most MERS patients exhibit a

397   severe lower respiratory tract infection, with little involvement of the upper respiratory tract

398   (36). This suggests that MERS-CoV infection in Tg2 mice mimics mild infection in

399   humans.

400   *In vitro* analysis of MERS-CoV suggests that the virus also infects human T cells

401   and macrophages (28, 41, 42). We detected MERS-CoV RNA in serum and spleen cells

402   from Tg2 mice. These data are similar to those generated from another Tg mouse model in

403   which mouse DPP4 was replaced with hDPP4 under control of the endogenous mouse

404   DPP4 promoter (12). MERS-CoV infection of T cells might affect immunopathology or

405   induction of apoptosis in Tg mice, but we found no clear evidence of this. Although Tg2

406   mice showed systemic viremia, infection of organs (except lung) did not lead to secondary

407   complications. The disease phenotype (including clinical symptoms, viral titer in the lung,

408   and acute pneumonia) appeared to be driven by infiltration by macrophages and

409   lymphocytes; this is similar to the phenotype observed in another Tg mouse model

410   harboring hDPP4 under the control of the endogenous mouse DPP4 promoter (12).

411   The Tg2 mice described herein did not show any brain or renal lesions after

412   MERS-CoV infection. Other Tg mouse models in which hDPP4 is expressed under a strong

413   ubiquitous promoter show high levels of viral RNA and inflammation in the lungs, which

414   are accompanied by brain lesions (5, 7, 11). A fatal case of human MERS-CoV infection

415 published by Ng *et al.* showed no sign of MERS-CoV infection in the brain (43). To date,

416 no reports suggest that MERS-CoV shows tropism for brain tissue. The primary target of

417 MERS-CoV is the lower respiratory tract; however, patients with MERS often show signs

418 of acute kidney failure (1, 43). In addition, MERS-CoV was identified in the urine of

419 MERS patients (44, 45). Data from the first autopsy case did find pathological signs in the

420 patient's kidneys, although IHC revealed no evidence of MERS-CoV replication in the

421 kidneys (43). In addition, acute renal failure in this patient was not caused by MERS-CoV

422 directly; rather, it was caused by hypotension (43) and/or acute respiratory distress

423 syndrome (46).

424   Histopathological analysis identified CD3-positive T cells and Iba-1-positive

425 macrophages in the lungs of Tg2 mice on Day 7 p.i., which correlated with expression of

426 inflammatory cytokines and inflammatory infiltrates in the lung. Tg2 mice aged 10 and 25

427 weeks showed increased expression of cytokines and chemokines associated with migration

428 of T cells and activation of macrophages, including IP-10, IL-6, IL-13, MCP-1, IFN-γ,

429 MIP-1α, MIG, and IL-12, in the lungs at Days 5 and/or 7 p.i. This result is the same as that

430 observed in a hDPP4 knock-in mouse model reported by Coleman *et al.* (12). In this hDPP4

431 knock-in mouse model, CD8$^+$ T cells and macrophages affected the course of

432 MERS-CoV-induced disease (12). In addition, Tg2 mice expressed mRNA encoding the

433 type 1 IFN, IFN-α4, during the early phase of the MERS-CoV infection. Importantly, the

434 pathogenic and immune response data from the knock-in mouse model and our own model

23

435　　are similar. Thus, an acute inflammatory reaction (including production of type 1 and type

436　　2 IFNs) and infiltration by macrophages might clear the virus from the lung, thereby

437　　preventing progression to MERS. Interestingly, we detected IL-1α and IL-17 in the lungs of

438　　25-week-old Tg2 mice, but not those of 10-week-old Tg2 mice, after MERS-CoV infection.

439　　Both IL-1α and IL-17 are pro-inflammatory cytokines that attract monocytes and

440　　neutrophils; therefore, they may exacerbate immunopathology after infection. These

441　　findings support the notion that the severity of MERS-CoV infection is age-dependent. Age

442　　is one of the most common factors related to severity and mortality of MERS infection;

443　　however, the underlying pathology is unclear (47).

444　　　　Many studies have examined immune responses of MERS patients (30, 48-52).

445　　Indeed, elevated serum levels of IL-6, IL-12, IP-10, and IFN-γ are observed in patients

446　　during the early period after severe infection (48-51). In addition, a prominent

447　　pro-inflammatory Th1 and Th17 response, including production of IFN-γ, TNF-α, IL-15,

448　　and IL-17, is seen in patients during the acute phase of MERS-CoV infection (52). By

449　　contrast, we found that administration of poly(I:C) to Tg2 mice induced a mild increase (or

450　　earlier induction) in innate immune responses when compared with those in C57BL/6 mice

451　　and non-Tg mice. This suggests that overexpression of hDPP4 might influence immune

452　　responses in Tg2 mice. Thus, we must exercise caution when assessing the relationship

453　　between immunopathology and outcome in patients and animal models of MERS-CoV;

454   however, pro-inflammatory responses seem to contribute to immunopathology during the

455   acute phase of MERS-CoV infection, both in patients and mouse models.

456   In summary, we generated a hDPP4-Tg mouse model showing mild respiratory

457   infection by MERS-CoV. While this Tg mouse has limitations as a model for human

458   MERS (i.e., lower virus titer in the lungs and mild disease), the immunopathology seems to

459   resemble a mild and early stage of infection in humans. Even though it has limitations, this

460   Tg mouse model will increase our understanding of the mechanisms underlying

461   MERS-CoV infection. Indeed, we recently used this mouse model to confirm a role for

462   transmembrane protease serine type2 (TMPRSS2) during MERS-CoV infection (Naoko

463   Iwata-Yoshikawa *et al.*, submitted). This animal model may provide new insight into

464   disease pathogenesis and guide development of therapeutic interventions that mitigate

465   MERS.

466

467   **MATERIALS AND METHODS**

468   **Ethics statements.** Experiments using recombinant DNA and pathogens were approved by

469   the Committee for Experiments using Recombinant DNA and Pathogens at the National

470   Institute of Infectious Diseases, Tokyo, Japan. All animal experiments were approved by

471   the Animal Care and Use Committee of the National Institute of Infectious Diseases, and

472   the NCGM Research Institute, and were conducted in accordance with institutional

473   Guidelines for the Care and Use of Animals. All animals were housed in a Japan Health

25

474   Sciences Foundation-certified facility. All human samples used in this study were obtained

475   from US Biomax, Inc., GeneTex, Inc., Alpha Diagnostics International, or Protein

476   Biotechnologies. The protocols were approved by the Health Insurance Portability and

477   Accountability Act (HIPAA) or Institutional Review Board (IRB).

478

479   **Cells and viruses.** MERS-CoV, HCoV-EMC 2012 strain, was kindly provided by Dr. Bart

480   Haagmans and Dr. Ron Fochier (Erasmus Medical Center, Rotterdam, the Netherlands) and

481   was used throughout the study. Vero E6 cells, purchased from the American Type Cell

482   Collection (Manassas, VA), were cultured in Eagle's MEM containing 5% fetal bovine

483   serum (FBS), 50 IU/ml penicillin G, and 50 μg/ml streptomycin (5% FBS-MEM). Stocks of

484   MERS-CoV were propagated and titrated on Vero E6 cells and cryopreserved at -80°C.

485   Viral infectivity titers are expressed as the $TCID_{50}$/ml on Vero E6 cells and were calculated

486   according to the Behrens-Kärber method. Work with infectious MERS-CoV was performed

487   under biosafety level 3 conditions.

488

489   **Virus isolation and titration.** Lung wash fluids and liver, kidney, heart, spleen, intestine,

490   and brain tissue samples from Tg2, non-Tg, and C57BL/6 mice were collected at the time

491   of postmortem examination and stored at -80°C. Tissue homogenates (20% [w/v]) were

492   prepared in 2% FBS-MEM, and samples were inoculated onto Vero E6 cell cultures, which

493   were then examined for cytopathic effects (CPE) for 5 days. Blind-passage was performed

26

494 after freezing and thawing cells from the first- or second-round passages. If

495 MERS-CoV-specific CPE were not observed in the first-, second- or third-round cultures,

496 the samples were deemed negative for infectious virus. Viral infectivity titers were

497 determined in Vero E6 cell cultures using the micro-titration assay described above.

498

499 **MERS-CoV neutralizing assay.** Blood was obtained from each mouse and allowed to clot.

500 Sera were then obtained by centrifugation and inactivated by incubation at 56°C for 30 min.

501 One hundred $TCID_{50}$ aliquots of MERS-CoV were incubated for 1 h in the presence or

502 absence of mouse serum (serially diluted 2-fold) and then added to confluent Vero E6 cell

503 cultures in 96-well microtiter plates. The presence of a viral CPE was determined on Day 5,

504 and the titers of neutralizing antibody were determined as the reciprocal of the highest

505 dilution at which no CPE was observed. The lowest and highest serum dilutions tested were

506 1:2 and 1:512, respectively.

507

508 **Generation of hDPP4-Tg mice.** To generate Tg mice expressing hDPP4, a BAC vector

509 carrying the hDPP4 gene (RP11-345J9) was purchased from Advanced GenoTechs Co.,

510 Japan. The BAC DNA was purified using a Large-Construct Kit (Qiagen) according to the

511 manufacturer's instructions and suspended in TE buffer (10 mM Tris·HCl and 0.1 mM

512 EDTA, pH 7.5) at a concentration of 4 ng/μl. Tg mice were generated using standard

513 procedures (23). The purified BAC clones were microinjected into the pronuclei of

27

514   fertilized eggs from BDF1×C57BL/6NCr mice (SLC Inc., Hamamatsu, Japan) and then

515   transplanted into pseudopregnant ICR mice (SLC Inc.). Expression of the transgene was

516   assessed by FACS analysis as described below. The Tg mice were then backcrossed onto

517   C57BL/6NCr for five generations. After weaning, the mice were tested for Tg integration

518   by PCR and FACS analysis. Briefly, genomic DNA isolated from ear punch tissues was

519   subjected to PCR using hDPP4-specific primers (Table 2) and lymphocytes were isolated

520   from blood taken from the tail vein. Lymphocytes were screened for hDPP4 protein

521   expression by flow cytometry analysis. Tg mice and their non-Tg littermates were used for

522   the MERS-CoV infection study.

523

524   **Flow cytometry analysis.** Blood was collected from the retro-orbital venous plexus under

525   isoflurane anesthesia using heparinized capillary tubes. The samples were then treated with

526   Red Blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO) to remove erythroid cells. For

527   immunofluorescence staining, cells were re-suspended in staining buffer (PBS containing

528   2.5% FBS, 0.5 mM EDTA, 0.05% NaN$_3$) and Fc-receptors were blocked by incubation for

529   20 min on ice with an unlabeled anti-mouse CD16/CD32 monoclonal antibody (clone:

530   2.4G2; Bay bioscience Co., Ltd., Kobe, Japan). After washing, the cells were stained for 30

531   min with a FITC-labeled anti-hDPP4 antibody (clone: BA5b) or with a control antibody

532   against mouse IgG2a (clone: MOPC-173; BioLegend, San Diego, CA) and an APC-labeled

533   anti-human CD3 antibody (clone: 145-2C11; BioLegend). Cells were then washed and

28

534   analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Data were

535   processed using Cell Quest software (BD Biosciences).

536

537   **Western blot analysis.** To examine expression of DPP4 in human tissues, human tissue

538   lysates prepared from the liver, spleen, kidney, heart, lung, stomach, small intestine, large

539   intestine, pancreas, and brain were purchased from Alpha Diagnostics International (San

540   Antonio, TX). Human skeletal muscle lysates were purchased from Protein Biotechnologies

541   (Ramona, CA) and used under IRB-approval protocols. To prepare protein samples from

542   the Tg and non-Tg mouse organs, tissues were homogenized in 0.5 ml of RIPA buffer (50

543   mM Tris/HCl [pH 7.5], 150 mM NaCl, 1% [v/v] Nonidet P40 [NP40], 0.5% Sodium

544   deoxycholate, 0.1% SDS) containing a protease inhibitor mixture (Complete Mini; Roche

545   Diagnostics, Basel, Switzerland) and the protein concentrations were measured using a

546   Pierce™ BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA).

547   Homogenates (equivalent to 5 μg of protein) were subjected to SDS/PAGE on 4–12%

548   Bis-Tris Protein Gels (Thermo Fisher Scientific), followed by transfer to a PVDF

549   membrane (Millipore Corp., Billerica, MA). After blocking, the membranes were incubated

550   for 1 h with a goat anti-hDPP4 antibody (0.1 μg/ml, Cat# AF1180; R&D Systems, Inc.,

551   Minneapolis, MN) or a rabbit anti-β-actin antibody (1 μg/ml, Cat# ab8227; Abcam,

552   Cambridge, UK), followed by incubation with a donkey anti-goat HRP-conjugated

553   antibody (Abcam) and an anti-rabbit HRP-conjugated antibody (Abcam). The bands were

554   detected by an Immobilon Western Chemiluminescent HRP Substrate (Millipore) and an

555   LAS-3000 apparatus (FUJIFILM, Tokyo, Japan).

556

557   **Intranasal administration of poly(I:C).** Tg2, non-Tg, and C57BL/6 mice (10 weeks old)

558   were anesthetized and received 20 μg of poly(I:C) (Invitrogen, San Diego, CA) in 20 μl of

559   saline intranasally. The dose of poly(I:C) was determined from previous studies (53).

560   Control mice received saline alone. To evaluate production of cytokines and chemokines in

561   the lung, mice were sacrificed at 1day post-administration (n = 4 per group [two females

562   and two males]) and lungs were collected. Inflammatory cytokine profiles in 20% (w/v)

563   lung homogenates were detected using a commercial Mouse Cytokine 20-Plex antibody

564   bead kit (Thermo Fisher Scientific), as described by the manufacturer.

565

566   **Inoculation of mice with MERS-CoV.** Tg2 and non-Tg mice (9–10 weeks or 25 weeks

567   old), and Tg2-BALB mice (12–22 weeks old), were anesthetized and inoculated

568   intranasally with 1×10^5 TCID$_{50}$ (30 μl) of MERS-CoV. Body weight was measured daily

569   for 14 days (n = 4–8 per group), and animals were sacrificed at 6 h and at 1, 3, 5, 7, 14, and

570   35 days p.i. to analyze virus replication, hematological parameters, cytokine expression,

571   and disease pathology (n = 3–6 per group). Clinical signs were observed up until 14 days

572   p.i. All mock-infected mice were inoculated with 2% FBS-MEM and used as controls for

573   all analyses involving mice aged 9–10 weeks.

574

575 **Histopathology and IHC.** Formalin-fixed paraffin-embedded normal human tissue

576 sections were purchased from separate sources: liver, spleen, lung, trachea, small intestine,

577 colon, pancreas, cerebrum, cerebellum, and skeletal muscle were obtained from US Biomax,

578 Inc. (Rockville, MD), whereas kidney, heart, spinal cord, stomach, and lymph node were

579 obtained from GeneTex, Inc. (Irvine, CA). These human tissues were collected under

580 HIPAA-approved protocols. To obtain animal tissues, mice were anesthetized and perfused

581 with 2 ml of 10% phosphate-buffered formalin and the lungs, liver, spleen, kidney, heart,

582 gastrointestinal tract, salivary glands, and brain tissue were harvested and fixed. Fixed

583 tissues were routinely embedded in paraffin, sectioned, and stained with hematoxylin and

584 eosin. For IHC, antigen retrieval of formalin-fixed mouse tissue sections was performed by

585 autoclaving at 121°C for 10 min in retrieval solution at pH 6.0 (Nichirei, Tokyo, Japan).

586 hDPP4 and MERS-CoV antigens were detected using a standard immunoperoxidase

587 method and a goat anti-hDPP4 antibody (R&D Systems), a rabbit anti-MERS-CoV

588 nucleocapsid antibody (Cat# 40068-RP01; Sino Biological Inc., Beijing, China).

589 For double staining of CD3 (T cells) and Iba-1 (macrophages) antigen, we used a rabbit

590 anti-human CD3 antibody (Cat# 790-4341; Ventana Medical System Inc., Tucson, AZ) and

591 a rabbit anti-human Iba-1 antibody (Cat# 019-19741; Wako Pure Chemical Industries, Ltd.,

592 Osaka, Japan). DAB (Sigma-Aldrich Co., MO, USA) and the Vina Green Chromogen Kit

593 (Biocare Medical, CA, USA) were used as chromogens for HRP visualization. Following

31

594   the first staining of CD3 using the polymer-based detection system with DAB, denaturing

595   was performed by hydrolytic autoclaving in citrate buffer (pH 6.0) for 10 min at 121°C.

596   The second staining was performed for Iba-1 with Vina Green. Nuclei were counterstained

597   with hematoxylin for 10 sec. To detect apoptosis, TUNEL labeling was performed using the

598   In Situ Cell Death Detection Kit (Roche).

599

600   **Quantitative real-time RT-PCR.** To measure the levels of type I IFN mRNA expression

601   and the number of viral genome copies, RNA was extracted from 20% (w/v) lung and brain

602   tissue homogenates and from the blood of Tg2 and non-Tg mice infected with MERS-CoV

603   using RNeasy Mini kits (Qiagen, Hilden, Germany), according to the manufacturer's

604   instructions. mRNA encoding IFN-α, IFN-β, and the E gene of MERS-CoV was examined

605   by real-time RT-PCR using an ABI Prism 7900HT Fast real-time PCR system (Applied

606   Biosystems, Foster City, CA). The TaqMan probes and primers, and the reaction conditions,

607   have been described previously (31). Expression of each gene was normalized to that of

608   β-actin.

609

610   **Detection of inflammatory cytokines and chemokines.** Cytokines and chemokines in

611   mouse lung homogenates (10% w/v) were measured using a commercial Mouse Cytokine

612   20-Plex antibody bead kit (Thermo Fisher Scientific). A panel of inflammatory cytokines

613   and chemokines (basic fibroblast growth factor [bFGF], granulocyte-macrophage,

32

614   colony-stimulating factor [GM-CSF], IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10,

615   IL-12p40/p70, IL-13, IL-17, IP-10, keratinocyte chemoattractant [KC], MCP-1, MIG,

616   MIP-1α, TNF-α, and vascular endothelial growth factor) was measured according to the

617   manufacturer's protocols.

618

619   **Isolation of splenocytes and infection with MERS-CoV.** Spleens were removed

620   aseptically from Tg2 and C57BL/6 mice (n = 3 each), dissociated in RPMI-1640 medium,

621   and pressed gently through a 40 µm nylon mesh filter. The cell suspension was centrifuged

622   at 400 g for 10 min, and red blood cells were lysed with blood cell lysis buffer (final

623   concentration: 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; pH 7.3) at room

624   temperature for 5 min. The cells were washed twice with RPMI-1640 medium and

625   centrifuged at 1000 g for 10 min. hDPP4-expressing $CD3^+$ T cells within the splenocyte

626   population were detected by flow cytometry analysis. The percentage of $CD3^+$ T cells was

627   39.11±3.8%, and that of hDPP4-expressing $CD3^+$ T cells was 26.46±1.9%. The cells were

628   re-suspended in the medium and infected with MERS-CoV (MOI of 1). Viral replication

629   was determined after 1 and 2 days of culture. Viral infectivity titers were measured in Vero

630   E6 cell cultures using a micro-titration assay. To detect the MERS-CoV genome in

631   splenocytes, RNA from splenocytes infected with MERS-CoV was extracted at 1 and 2

632   days p.i. and subjected to quantitative real-time RT-PCR (10).

633

634 **Molecular modeling of DPP4 homo- and heterodimers.** DPP4 dimer models were

635 constructed using the Molecular Operating Environment (MOE) (Chemical Computing

636 Group Inc., Montreal, Quebec, Canada) based on the crystal structure of hDPP4 at a

637 resolution of 2.55 Å (PDB code: 2ONC). Stereochemical quality was assessed using the

638 Ramachandran Plot and Atom Clashes applications in MOE. Interaction energy, which is

639 an indicator of the affinity of the dimer, was calculated using the Potential Energy

640 application in MOE.

641

642 **Statistical analysis.** Data are expressed as the mean and standard error of the mean.

643 Statistical analyses were performed using Graph Pad Prism 5 software (GraphPad Software

644 Inc., La Jolla, CA). Intergroup comparisons were performed using one-way, two-way

645 ANOVA, or Student's t test with Welch's correction. A P-value < 0.05 was considered

646 statistically significant.

647

648 **FUNDING INFORMATION**

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656

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REFERENCES

1. **Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA.** 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. The New England journal of medicine **367:**1814-1820.

2. **Raj VS, Mou H, Smits SL, Dekkers DH, Muller MA, Dijkman R, Muth D, Demmers JA, Zaki A, Fouchier RA, Thiel V, Drosten C, Rottier PJ, Osterhaus AD, Bosch BJ, Haagmans BL.** 2013. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature **495:**251-254.

3. **de Wit E, Rasmussen AL, Falzarano D, Bushmaker T, Feldmann F, Brining DL, Fischer ER, Martellaro C, Okumura A, Chang J, Scott D, Benecke AG, Katze MG, Feldmann H, Munster VJ.** 2013. Middle East respiratory syndrome coronavirus (MERS-CoV) causes transient lower respiratory tract infection in rhesus macaques. Proceedings of the National Academy of Sciences of the United States of America **110:**16598-16603.

4. **Falzarano D, de Wit E, Feldmann F, Rasmussen AL, Okumura A, Peng X, Thomas MJ, van Doremalen N, Haddock E, Nagy L, LaCasse R, Liu T, Zhu J, McLellan JS, Scott DP, Katze MG, Feldmann H, Munster VJ.** 2014. Infection with MERS-CoV causes lethal pneumonia in the common marmoset. PLoS pathogens **10:**e1004250.

5. **Agrawal AS, Garron T, Tao X, Peng BH, Wakamiya M, Chan TS, Couch RB, Tseng CT.** 2015. Generation of a transgenic mouse model of Middle East respiratory syndrome coronavirus infection and disease. Journal of virology **89:**3659-3670.

6. **Zhao G, Jiang Y, Qiu H, Gao T, Zeng Y, Guo Y, Yu H, Li J, Kou Z, Du L, Tan W, Jiang S, Sun S, Zhou Y.** 2015. Multi-Organ Damage in Human Dipeptidyl Peptidase 4 Transgenic Mice Infected with Middle East Respiratory Syndrome-Coronavirus. PloS one **10:**e0145561.

7. **Li K, Wohlford-Lenane C, Perlman S, Zhao J, Jewell AK, Reznikov LR,**

693   **Gibson-Corley KN, Meyerholz DK, McCray PB, Jr.** 2016. Middle East
694   Respiratory Syndrome Coronavirus Causes Multiple Organ Damage and
695   Lethal Disease in Mice Transgenic for Human Dipeptidyl Peptidase 4.
696   The Journal of infectious diseases **213:**712-722.

697 8.   **de Wit E, Prescott J, Baseler L, Bushmaker T, Thomas T, Lackemeyer**
698   **MG, Martellaro C, Milne-Price S, Haddock E, Haagmans BL, Feldmann**
699   **H, Munster VJ.** 2013. The Middle East respiratory syndrome coronavirus
700   (MERS-CoV) does not replicate in Syrian hamsters. PloS one **8:**e69127.

701 9.   **Coleman CM, Matthews KL, Goicochea L, Frieman MB.** 2013. Wild type
702   and innate immune deficient mice are not susceptible to the Middle East
703   Respiratory Syndrome Coronavirus. The Journal of general virology.

704 10.   **Iwata-Yoshikawa N, Fukushi S, Fukuma A, Suzuki T, Takeda M, Tashiro**
705   **M, Hasegawa H, Nagata N.** 2016. No susceptibility of neonatal and adult
706   rats against the Middle East respiratory syndrome coronavirus.
707   Japanese journal of infectious diseases.

708 11.   **Zhao J, Li K, Wohlford-Lenane C, Agnihothram SS, Fett C, Zhao J, Gale**
709   **MJ, Jr., Baric RS, Enjuanes L, Gallagher T, McCray PB, Jr., Perlman S.**
710   2014. Rapid generation of a mouse model for Middle East respiratory
711   syndrome. Proceedings of the National Academy of Sciences of the United
712   States of America **111:**4970-4975.

713 12.   **Coleman CM, Sisk JM, Halasz G, Zhong J, Beck SE, Matthews KL,**
714   **Venkataraman T, Rajagopalan S, Kyratsous CA, Frieman MB.** 2017.
715   CD8+ T Cells and Macrophages Regulate Pathogenesis in a Mouse Model
716   of Middle East Respiratory Syndrome. Journal of virology **91**.

717 13.   **Li K, Wohlford-Lenane CL, Channappanavar R, Park JE, Earnest JT,**
718   **Bair TB, Bates AM, Brogden KA, Flaherty HA, Gallagher T, Meyerholz**
719   **DK, Perlman S, McCray PB, Jr.** 2017. Mouse-adapted MERS coronavirus
720   causes lethal lung disease in human DPP4 knockin mice. Proceedings of
721   the National Academy of Sciences of the United States of America
722   **114:**E3119-E3128.

723 14. **Cockrell AS, Yount BL, Scobey T, Jensen K, Douglas M, Beall A, Tang XC,**
724   **Marasco WA, Heise MT, Baric RS.** 2016. A mouse model for MERS
725   coronavirus-induced acute respiratory distress syndrome. Nature
726   microbiology **2:**16226.
727 15. **Korea Centers for Disease Control and Prevention.** 2015. Middle East
728   Respiratory Syndrome Coronavirus Outbreak in the Republic of Korea,
729   2015. Osong public health and research perspectives **6:**269-278.
730 16. **Saad M, Omrani AS, Baig K, Bahloul A, Elzein F, Matin MA, Selim MA,**
731   **Al Mutairi M, Al Nakhli D, Al Aidaroos AY, Al Sherbeeni N, Al-Khashan**
732   **HI, Memish ZA, Albarrak AM.** 2014. Clinical aspects and outcomes of 70
733   patients with Middle East respiratory syndrome coronavirus infection: a
734   single-center experience in Saudi Arabia. International journal of
735   infectious diseases : IJID : official publication of the International Society
736   for Infectious Diseases **29:**301-306.
737 17. **Ahmed AE.** 2017. The predictors of 3- and 30-day mortality in 660
738   MERS-CoV patients. BMC infectious diseases **17:**615.
739 18. **Alsahafi AJ, Cheng AC.** 2016. The epidemiology of Middle East
740   respiratory syndrome coronavirus in the Kingdom of Saudi Arabia,
741   2012-2015. International journal of infectious diseases : IJID : official
742   publication of the International Society for Infectious Diseases **45:**1-4.
743 19. **Majumder MS, Kluberg SA, Mekaru SR, Brownstein JS.** 2015. Mortality
744   Risk Factors for Middle East Respiratory Syndrome Outbreak, South
745   Korea, 2015. Emerging infectious diseases **21:**2088-2090.
746 20. **Choi WS, Kang CI, Kim Y, Choi JP, Joh JS, Shin HS, Kim G, Peck KR,**
747   **Chung DR, Kim HO, Song SH, Kim YR, Sohn KM, Jung Y, Bang JH, Kim**
748   **NJ, Lee KS, Jeong HW, Rhee JY, Kim ES, Woo H, Oh WS, Huh K, Lee YH,**
749   **Song JY, Lee J, Lee CS, Kim BN, Choi YH, Jeong SJ, Lee JS, Yoon JH, Wi**
750   **YM, Joung MK, Park SY, Lee SH, Jung SI, Kim SW, Lee JH, Lee H, Ki**
751   **HK, Kim YS.** 2016. Clinical Presentation and Outcomes of Middle East
752   Respiratory Syndrome in the Republic of Korea. Infection &

38

753   chemotherapy **48:**118-126.

754 21.   **Koike S, Taya C, Kurata T, Abe S, Ise I, Yonekawa H, Nomoto A.** 1991.
755   Transgenic mice susceptible to poliovirus. Proceedings of the National
756   Academy of Sciences of the United States of America **88:**951-955.

757 22.   **Fujii K, Nagata N, Sato Y, Ong KC, Wong KT, Yamayoshi S, Shimanuki**
758   **M, Shitara H, Taya C, Koike S.** 2013. Transgenic mouse model for the
759   study of enterovirus 71 neuropathogenesis. Proceedings of the National
760   Academy of Sciences of the United States of America **110:**14753-14758.

761 23.   **Haruyama N, Cho A, Kulkarni AB.** 2009. Overview: engineering
762   transgenic constructs and mice. Current protocols in cell biology /
763   editorial board, Juan S. Bonifacino ... [et al.] **Chapter 19:**Unit 19 10.

764 24.   **Geppert TD, Davis LS, Gur H, Wacholtz MC, Lipsky PE.** 1990. Accessory
765   cell signals involved in T-cell activation. Immunological reviews **117:**5-66.

766 25.   **Fleischer B.** 1994. CD26: a surface protease involved in T-cell activation.
767   Immunology today **15:**180-184.

768 26.   **Hegen M, Niedobitek G, Klein CE, Stein H, Fleischer B.** 1990. The T cell
769   triggering molecule Tp103 is associated with dipeptidyl aminopeptidase
770   IV activity. J Immunol **144:**2908-2914.

771 27.   **Fan C, Wu X, Liu Q, Li Q, Liu S, Lu J, Yang Y, Cao Y, Huang W, Liang C,**
772   **Ying T, Jiang S, Wang Y.** 2018. A Human DPP4-Knockin Mouse's
773   Susceptibility to Infection by Authentic and Pseudotyped MERS-CoV.
774   Viruses **10**.

775 28.   **Chu H, Zhou J, Wong BH, Li C, Chan JF, Cheng ZS, Yang D, Wang D, Lee**
776   **AC, Li C, Yeung ML, Cai JP, Chan IH, Ho WK, To KK, Zheng BJ, Yao Y,**
777   **Qin C, Yuen KY.** 2016. Middle East Respiratory Syndrome Coronavirus
778   Efficiently Infects Human Primary T Lymphocytes and Activates the
779   Extrinsic and Intrinsic Apoptosis Pathways. The Journal of infectious
780   diseases **213:**904-914.

781 29.   **Alghamdi IG, Hussain, II, Almalki SS, Alghamdi MS, Alghamdi MM,**
782   **El-Sheemy MA.** 2014. The pattern of Middle East respiratory syndrome

783   coronavirus in Saudi Arabia: a descriptive epidemiological analysis of
784   data from the Saudi Ministry of Health. International journal of general
785   medicine **7:**417-423.

786 30. **van den Brand JM, Smits SL, Haagmans BL.** 2015. Pathogenesis of
787   Middle East respiratory syndrome coronavirus. The Journal of pathology
788   **235:**175-184.

789 31. **Iwata-Yoshikawa N, Uda A, Suzuki T, Tsunetsugu-Yokota Y, Sato Y,**
790   **Morikawa S, Tashiro M, Sata T, Hasegawa H, Nagata N.** 2014. Effects of
791   Toll-like receptor stimulation on eosinophilic infiltration in lungs of
792   BALB/c mice immunized with UV-inactivated severe acute respiratory
793   syndrome-related coronavirus vaccine. Journal of virology **88:**8597-8614.

794 32. **Memish ZA, Zumla AI, Assiri A.** 2013. Middle East respiratory syndrome
795   coronavirus infections in health care workers. The New England journal
796   of medicine **369:**884-886.

797 33. 2013. State of Knowledge and Data Gaps of Middle East Respiratory
798   Syndrome Coronavirus (MERS-CoV) in Humans. PLoS currents **5**.

799 34. **O'Hagan JJ, Carias C, Rudd JM, Pham HT, Haber Y, Pesik N, Cetron MS,**
800   **Gambhir M, Gerber SI, Swerdlow DL.** 2016. Estimation of Severe Middle
801   East Respiratory Syndrome Cases in the Middle East, 2012-2016.
802   Emerging infectious diseases **22:**1797-1799.

803 35. **Omrani AS, Matin MA, Haddad Q, Al-Nakhli D, Memish ZA, Albarrak**
804   **AM.** 2013. A family cluster of Middle East Respiratory Syndrome
805   Coronavirus infections related to a likely unrecognized asymptomatic or
806   mild case. International journal of infectious diseases : IJID : official
807   publication of the International Society for Infectious Diseases
808   **17:**e668-672.

809 36. 2013. Update: Severe respiratory illness associated with Middle East
810   Respiratory Syndrome Coronavirus (MERS-CoV)--worldwide, 2012-2013.
811   MMWR. Morbidity and mortality weekly report **62:**480-483.

812 37. **Assiri A, Abedi GR, Bin Saeed AA, Abdalla MA, al-Masry M, Choudhry**

813   AJ, Lu X, Erdman DD, Tatti K, Binder AM, Rudd J, Tokars J, Miao C,
814   Alarbash H, Nooh R, Pallansch M, Gerber SI, Watson JT. 2016.
815   Multifacility Outbreak of Middle East Respiratory Syndrome in Taif,
816   Saudi Arabia. Emerging infectious diseases **22:**32-40.
817   38. Cockrell AS, Peck KM, Yount BL, Agnihothram SS, Scobey T, Curnes NR,
818   Baric RS, Heise MT. 2014. Mouse dipeptidyl peptidase 4 is not a
819   functional receptor for Middle East respiratory syndrome coronavirus
820   infection. Journal of virology **88:**5195-5199.
821   39. Pascal KE, Coleman CM, Mujica AO, Kamat V, Badithe A, Fairhurst J,
822   Hunt C, Strein J, Berrebi A, Sisk JM, Matthews KL, Babb R, Chen G, Lai
823   KM, Huang TT, Olson W, Yancopoulos GD, Stahl N, Frieman MB,
824   Kyratsous CA. 2015. Pre- and postexposure efficacy of fully human
825   antibodies against Spike protein in a novel humanized mouse model of
826   MERS-CoV infection. Proceedings of the National Academy of Sciences of
827   the United States of America **112:**8738-8743.
828   40. Chien CH, Huang LH, Chou CY, Chen YS, Han YS, Chang GG, Liang PH,
829   Chen X. 2004. One site mutation disrupts dimer formation in human
830   DPP-IV proteins. The Journal of biological chemistry **279:**52338-52345.
831   41. Tynell J, Westenius V, Ronkko E, Munster VJ, Melen K, Osterlund P,
832   Julkunen I. 2016. Middle East respiratory syndrome coronavirus shows
833   poor replication but significant induction of antiviral responses in human
834   monocyte-derived macrophages and dendritic cells. The Journal of
835   general virology **97:**344-355.
836   42. Zhou J, Chu H, Li C, Wong BH, Cheng ZS, Poon VK, Sun T, Lau CC,
837   Wong KK, Chan JY, Chan JF, To KK, Chan KH, Zheng BJ, Yuen KY. 2014.
838   Active replication of Middle East respiratory syndrome coronavirus and
839   aberrant induction of inflammatory cytokines and chemokines in human
840   macrophages: implications for pathogenesis. The Journal of infectious
841   diseases **209:**1331-1342.
842   43. Ng DL, Al Hosani F, Keating MK, Gerber SI, Jones TL, Metcalfe MG,

843   Tong S, Tao Y, Alami NN, Haynes LM, Mutei MA, Abdel-Wareth L, Uyeki
844   TM, Swerdlow DL, Barakat M, Zaki SR. 2016. Clinicopathologic,
845   Immunohistochemical, and Ultrastructural Findings of a Fatal Case of
846   Middle East Respiratory Syndrome Coronavirus Infection in the United
847   Arab Emirates, April 2014. The American journal of pathology
848   **186:**652-658.

849   44.   Poissy J, Goffard A, Parmentier-Decrucq E, Favory R, Kauv M, Kipnis E,
850   Mathieu D, van der Werf S, Guery B. 2014. Kinetics and pattern of viral
851   excretion in biological specimens of two MERS-CoV cases. Journal of
852   clinical virology : the official publication of the Pan American Society for
853   Clinical Virology **61:**275-278.

854   45.   Drosten C, Seilmaier M, Corman VM, Hartmann W, Scheible G, Sack S,
855   Guggemos W, Kallies R, Muth D, Junglen S, Muller MA, Haas W,
856   Guberina H, Rohnisch T, Schmid-Wendtner M, Aldabbagh S, Dittmer U,
857   Gold H, Graf P, Bonin F, Rambaut A, Wendtner CM. 2013. Clinical
858   features and virological analysis of a case of Middle East respiratory
859   syndrome coronavirus infection. The Lancet. Infectious diseases
860   **13:**745-751.

861   46.   Seeley EJ. 2013. Updates in the management of acute lung injury: a focus
862   on the overlap between AKI and ARDS. Advances in chronic kidney
863   disease **20:**14-20.

864   47.   Park JE, Jung S, Kim A, Park JE. 2018. MERS transmission and risk
865   factors: a systematic review. BMC public health **18:**574.

866   48.   Faure E, Poissy J, Goffard A, Fournier C, Kipnis E, Titecat M, Bortolotti
867   P, Martinez L, Dubucquoi S, Dessein R, Gosset P, Mathieu D, Guery B.
868   2014. Distinct immune response in two MERS-CoV-infected patients: can
869   we go from bench to bedside? PloS one **9:**e88716.

870   49.   Guan WD, Mok CK, Chen ZL, Feng LQ, Li ZT, Huang JC, Ke CW, Deng X,
871   Ling Y, Wu SG, Niu XF, Perera RA, Da Xu Y, Zhao J, Zhang LQ, Li YM,
872   Chen RC, Peiris M, Chen L, Zhong NS. 2015. Characteristics of Traveler

873   with Middle East Respiratory Syndrome, China, 2015. Emerging

874   infectious diseases **21:**2278-2280.

875   50.   **Min CK, Cheon S, Ha NY, Sohn KM, Kim Y, Aigerim A, Shin HM, Choi JY,**

876   **Inn KS, Kim JH, Moon JY, Choi MS, Cho NH, Kim YS.** 2016.

877   Comparative and kinetic analysis of viral shedding and immunological

878   responses in MERS patients representing a broad spectrum of disease

879   severity. Scientific reports **6:**25359.

880   51.   **Shin HS, Kim Y, Kim G, Lee JY, Jeong I, Joh JS, Kim H, Chang E, Sim**

881   **SY, Park JS, Lim DG.** 2018. Immune responses to MERS coronavirus

882   during the acute and convalescent phases of human infection. Clinical

883   infectious diseases : an official publication of the Infectious Diseases

884   Society of America.

885   52.   **Mahallawi WH, Khabour OF, Zhang Q, Makhdoum HM, Suliman BA.**

886   2018. MERS-CoV infection in humans is associated with a

887   pro-inflammatory Th1 and Th17 cytokine profile. Cytokine **104:**8-13.

888   53.   **Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S,**

889   **Takahashi H, Sawa H, Chiba J, Kurata T, Sata T, Hasegawa H.** 2005.

890   Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine

891   protects against influenza virus infection. Journal of virology

892   **79:**2910-2919.

893

894 **FIGURE LEGENDS**

895 **Fig. 1.** Generation of transgenic mice expressing human dipeptidyl peptidase 4 (hDPP4).

896 (A) Schematic diagram showing a bacterial artificial chromosome clone (BAC clone

897 RP11-345G-9) containing the hDPP4 gene used to produce the transgenic mice. The open

898 and closed circles denote the centromere (Cen) and telomere (Tel) of human chromosome 2,

899 respectively. The gray arrows indicate the genes located downstream of the hDPP4 gene

900 (white arrow). The cloned region in the BAC construct is denoted by a black line. GCG,

901 glucagon gene; FAP, fibroblast activation protein; IFIH1, interferon induced with helicase

902 C domain 1. (B) Expression of hDPP4 on peripheral blood CD3-positive T-lymphocytes

903 from the transgenic (Tg) mice. Tg1 and Tg2, hDPP4$^{+/-}$ transgenic mouse lines 1 and 2;

904 non-Tg, hDPP4$^{-/-}$ mouse. (C) Genomic DNA was extracted from Tg2 mice, and human

905 DPP4 exons 1–26 were subjected to PCR using specific primers. M: marker; Lane 1: exon

906 1; Lane 2: exon 2; Lane 3: exon 3; Lane 4: exon 4; Lane 5: exon 5; Lane 6: exon 6 to 7;

907 Lane 7: exon 8; Lane 8: exon 9; Lane 9: exon 10; Lane 10: exon 11; Lane 11: exon 12;

908 Lane 12: exon 13 to 14; Lane 13: exon 15 to 16; Lane 14: exon 17 to 18; Lane 15: exon 19;

909 Lane 16: exon 20; Lane 17: exon 21; Lane 18: exon 22; Lane 19: exon 23; Lane 20: exon

910 24; Lane 21: exon 25; Lane 22-23: exon 26.

911

912 **Fig. 2.** Expression of human dipeptidyl peptidase 4 (hDPP4) in tissues from humans and

913 mice. Tg2, hDPP4$^{+/-}$ transgenic mouse line 2; non-Tg, hDPP4$^{-/-}$ mouse. (A) Western blot

44

914   analysis of homogenized human and mouse tissues with an anti-hDPP4 polyclonal antibody

915   or an anti-β-actin polyclonal antibody (internal control). Arrows indicate the positions of

916   hDPP4 (110 kDa). (B) Immunohistochemical analysis of hDPP4 expression in human, Tg2,

917   and non-Tg mice tissues stained with an anti-hDPP4 polyclonal antibody. Sections were

918   counterstained with hematoxylin. Scale bars, 50 µm for large images of liver, kidney, small

919   intestine, pancreas, spleen, and lymph node; and 20 µm for large images of lung and brain;

920   insets, 25 µm.

921

922   **Fig. 3.** Innate immune responses in Tg2, non-Tg, and C57BL/6 mice. C57BL/6, non-Tg,

923   and Tg2 mice received an intranasal inoculation of poly(I:C) or saline and were sacrificed

924   24 h later (n = 4/group). Expression of pro-inflammatory cytokines and chemokines in

925   saline- and poly(I:C)-inoculated animals. P-values were calculated using one-way ANOVA,

926   followed by Tukey's post-test; ns: not significant; *$P < 0.05$. Error bars indicate the

927   standard deviation.

928

929   **Fig. 4.** Permissiveness of transgenic mice to infection by MERS-CoV. (A) The body

930   weight of 10-week-old mice was monitored daily after intranasal inoculation of

931   MERS-CoV at a dose of 10^5 TCID$_{50}$ (n = 8 for hDPP4-transgenic mouse line 2 (Tg2), n =

932   10 for C57BL/6 mice). Error bars represent the standard deviation. **$P < 0.01$, ***$P < 0.001$

933   (two-way ANOVA). (B) Seroconversion of Tg2 mice inoculated with MERS-CoV. Titer of

45

934   MERS-CoV-specific neutralizing antibodies in mouse serum on Days 7, 14, and 35

935   post-inoculation with MERS-CoV (Tg2, n = 3–5; C57BL/6, n = 4–6). The dotted line

936   denotes the detection limit of the assay. Error bars represent the standard deviation. (C)

937   Viral load in the respiratory tract of mice inoculated with MERS-CoV. NW, nasal wash

938   fluid; Maxilla, maxilla including nostril; LW, lung wash fluid. Mice were euthanized at the

939   indicated times post-viral inoculation (n = 3–4 per time point). Viral titer was expressed as

940   the mean ± standard deviation. The dotted line denotes the detection limit of the assay. **P

941   < 0.01, ***P < 0.001 (two-way ANOVA). (D) Quantitative real-time RT-PCR analysis of

942   MERS-CoV viral RNA in splenocytes isolated from Tg2 and C57BL/6 mice. RNA was

943   extracted from splenocytes infected with MERS-CoV at a multiplicity of infection of 1.

944   RNA levels were normalized against β-actin (endogenous control).

945

946   **Fig. 5.** Histopathological changes in the lungs of human dipeptidyl peptidase 4

947   (hDPP4)-transgenic mice inoculated with MERS-CoV. Representative images of lungs

948   from hDPP4$^{+/-}$ transgenic mouse line 2 on Days 1, 3, 5, 7, 14, and 35 post-inoculation (p.i.).

949   Mild but progressive interstitial infiltration was seen within 7 days p.i. (left column). IHC

950   staining of sequential sections revealed abundant MERS-CoV antigen-positive cells in

951   affected areas (middle column). Severe inflammation, with many mononuclear cells in the

952   alveolar spaces and regenerated type II pneumocytes in the alveolar wall, was observed

953   within 7 days p.i. (right column). Scale bars: left and middle columns, 100 μm; right

954   column, 50 μm; insets of middle column, 20 μm. HE, hematoxylin and eosin staining; IHC,

955   immunohistochemistry using an anti-MERS-CoV nucleocapsid protein polyclonal antibody.

956

957   **Fig. 6.** Double immunofluorescence images taken at 1 day p.i. showing human dipeptidyl

958   peptidase 4 (hDPP4) (green) and MERS-CoV antigen (red) in the lungs of Tg2 mice

959   infected with MERS-CoV. Viral antigen-positive cells in the lungs were hDPP4-positive

960   bronchiolar epithelial cells (upper panels) and alveolar epithelial cells (lower panels).

961   Original magnification, × 600.

962

963   **Fig. 7.** Histopathological changes in the brain of human dipeptidyl peptidase 4

964   (hDPP4)-transgenic mice inoculated with MERS-CoV. A, D, G show sagittal sections of the

965   head, including the nasal cavity, olfactory bulb, and brain, of a Tg2 mouse infected with

966   MERS-CoV (images taken at 3, 7, and 35 days p.i.). Right panels show the brain cortex

967   from A, D, and G, respectively (B, E, F: hematoxylin and eosin staining; C, F, I:

968   immunohistochemical analysis of MERS-CoV antigen). Neither lesions nor MERS-CoV

969   antigen-positive cells were detected in the brain. Scale bars in A, D, G = 1 mm; scale bars

970   in B, C, E, F, H, and I = 20 μm.

971

972   **Fig. 8.** Susceptibility of adult human dipeptidyl peptidase 4 (hDPP4)-transgenic mice to

973   MERS-CoV infection. Tg2, hDPP4$^{+/-}$ transgenic mouse line 2; non-Tg, hDPP4$^{-/-}$ mouse.

47

974   (A) The body weight of 25-week-old mice was monitored daily after intranasal inoculation

975   with MERS-CoV (n = 6 Tg2 mice; and n = 7 non-Tg mice). *P < 0.05 and ***P < 0.001

976   (two-way ANOVA). (B) Seroconversion of Tg2 mice inoculated with MERS-CoV. Titer of

977   MERS-CoV-specific neutralizing antibodies in mouse serum on Days 7, 14, and 35

978   post-inoculation with MERS-CoV (Tg2, n = 4–6; non-Tg, n = 4). The dotted line denotes

979   the detection limit of the assay. Error bars represent the standard deviation. (C) Viral titer in

980   nasal wash fluid (NW), maxilla (including nostril), lung wash fluid (LW), and lungs of

981   25-week-old Tg2 and non-Tg mice at 3 days post-inoculation (p.i.) (n = 4 mice per group).

982   Viral titer is expressed as the mean ± standard deviation. The dotted line denotes the

983   detection limit of the assay. **P < 0.01 and ***P < 0.001 (two-way ANOVA).

984

985   **Fig. 9.** Histopathological changes in the lungs of human dipeptidyl peptidase 4

986   (hDPP4)-transgenic mice inoculated with MERS-CoV. Representative histopathological

987   images of the lungs from 25-week-old Tg2 mice at 1, 3, 5, 7, 14, and 35 days

988   post-MERS-CoV infection. Time-dependent recruitment of inflammatory cells to the lung

989   (left and right columns). Marked inflammatory cell infiltration was noted at 7 days

990   post-inoculation (p.i.) (J and L). Middle column, immunohistochemical staining for

991   MERS-CoV antigen. Scale bars: left and middle columns = 100 μm; right column = 50 μm;

992   insets of middle column = 20 μm. HE, hematoxylin and eosin staining; IHC,

993   immunohistochemistry using an anti-MERS-CoV nucleocapsid protein polyclonal antibody.

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994

995 **Fig. 10.** Identification of cells infiltrating the lung of Tg2 mice infected with MERS-CoV.

996 Representative images of lungs from 10-week-old (Young) and 25-week-old (Adult)

997 hDPP4$^{+/-}$ transgenic mice (line 2) on Day 7 post-inoculation (p.i.). Infiltrating cells were

998 positive for Iba-1 (green) or CD3 (brown). Bars, 20 μm. Upper panels: hematoxylin and

999 eosin staining (HE); Middle and lower panels: immunohistochemistry (IHC) using an

1000 anti-Iba-1 polyclonal antibody and an anti-CD3 monoclonal antibody.

1001

1002 **Fig. 11.** Cytokine and chemokine levels and expression of type I interferon (IFN) genes in

1003 the lungs of Tg2 mice infected with MERS-CoV. Cytokine and chemokine levels in lung

1004 samples from 10-week-old (A) and 25-week-old (B) mice. Tg2 mice were inoculated with

1005 MERS-CoV or cell culture medium containing 2% FBS. Lungs were collected at the

1006 indicated times post-viral inoculation (n = 3–4 mice per time point). Data represent the

1007 mean ± standard deviation. The dotted line denotes the detection limit of the assay. *P <

1008 0.05, **P < 0.01, and ***P < 0.001 (two-way ANOVA). (C) Quantitative real-time RT-PCR

1009 analysis of type I IFN gene expression in lung homogenates from Tg2 mice inoculated with

1010 MERS-CoV or cell culture medium containing 2% FBS. RNA levels were normalized

1011 against β-actin (endogenous control). Data represent the mean ± standard deviation. The

1012 dotted line denotes the detection limit of the assay. *P < 0.05 and ***P < 0.001 (two-way

1013 ANOVA).

49

1014 **Table 1.** Primers used for PCR of human DPP4 exons

Target	Size (bp)	Sequence (5'→3')	
		Forward	Reverse
Exon 1	130	AATGTTTAACTCGGGGCCGA	CGGAAGTGAGCGTTCAGAGA
Exon 2	162	GGACTTGATCTGCTCGGCTT	CCTGACCTGAGCTCCAACTG
Exon 3	391	ACACACACACTCTCACACACT	TCTCAGTGCCATAAAAGCCCA
Exon 4	468	GTGCAAAGGGAGAAAGACTGA	CCACTTTGCCATATGCTGCA
Exon 5	562	GTGCACAGTGATGGCAATGA	CCACCATGCCCGACTTTAAC
Exon 6–7	330	GTCTCCTATAGTGAGTGGCCA	TCTGACAACTGGAGAGACTCAC
Exon 8	565	GGTCAGCCTTCTCGGTCTTC	GGAGACATCTGGTGCTGTGA
Exon 9	440	AGCCCAGCAAATGCAAAGTG	GCCAGATGCTGTTGACTTCAG
Exon 10	296	TGCAGACGTTTTTGTGCAGT	GGCTGTGATCCACTTTGCCA
Exon 11	274	CCAAGGTCTGGCAATAGTCA	TTCACTCTCCCCAACTGCAC
Exon 12	299	GAGCTTCCAGAAGGACCCAG	GCTGACTCATCCATAAAACCCC
Exon 13–14	848	TGCTTGCAGCCAGAAGTCAT	CTTCTGGGCAAAGAGGGCAT
Exon 15–16	708	CTCCGTGCACACTTAGGCTT	GGAGCTGCTTCGAAGTGAGT

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Exon 17–18	847	GCCCTGTGCCTTTCCAGTAA	GCATGTTCTCCAAATCCCTTCC
Exon 19	247	TGCTACTGACGGACATGAGG	GAAAGGACGCATTTGGCTCC
Exon 20	549	GGATGCATACTTCTCCACGG	AGGACATATGCCAACTCCCT
Exon 21	281	GCAGAGAACAAATGGCAGGG	ACTGCCCAGAGACCTAAGACT
Exon 22	206	AATGTGGAAACTGCGACTCG	TCTCTTTGTACCTGGCAGCA
Exon 23	567	AGGTGCTTTAGCCACCCTTT	GAGAGTCTTCTGGGCTCTAAAGG
Exon 24	364	TCCCTTCCAGTCCTGTCTCC	CAGTCTTGCCCCTCATGCTT
Exon 25	239	CTTTCCCACCCCTTGGTACC	CCTGTCTGTGGCACTGCTAA
Exon 26 (1)	784	TAGACCCCCTCTTTGACCCC	GAACAGCTCTTCTCCGAGGG
Exon 26 (2)	723	AAGGGATGGCAAGATGTGGG	TCCATATGCCAGTGCGGTTT

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1021 **Table 2.** Virus isolation, detection of viral antigens by immunohistochemistry, and detection of the MERS-CoV genome by real-time

1022 PCR in tissues from Tg2 and non-Tg mice inoculated with MERS-CoV

	Liver			Spleen			Kidney			Heart			Lung			Intestine			Brain			Blood		
Animal	I	A	G	I	A	G	I	A	G	I	A	G	I	A	G	I	A	G	I	A	G	I[1]	A	G
Tg2	-	-	NE	-	-	-	-	-	-	-	-	NE	+	+	NE	-	-	NE	-	-	-	-	-	+[2]
Non-Tg	-	-	NE	-	-	-	-	-	-	-	-	NE	-	-	NE	-	-	NE	-	-	-	-	-	-

1023 I: virus isolated; A: viral antigens; G: viral genome.

1024 +: virus was isolated, or viral antigens or the viral genome was detected, in tissues.

1025 -: no virus, antigen, or genome was detected in tissues.

1026 Tg2 = hDPP4$^{+/-}$ transgenic mouse line 2; non-Tg = hDPP4$^{-/-}$ mouse.

1027 NE: not examined.

1028 [1]: serum samples used for analysis.

1029 [2]: viral genome was detected in samples at 3 and 5 days p.i.

52

A

Human
chromosome 2
BAC clone
RP11-345J-9

B

Gated on
CD3+ cells

non-Tg   Tg1   Tg2

hDPP4

% of Max

C

(bp) M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

500-

100-

A

Human

(Size kDa)

DPP4

β-actin

Tg2

(Size kDa)

DPP4

β-actin

non-Tg

DPP4

β-actin

	Lower magnification (HE)	MERS-CoV Ag (IHC)	Higher magnification (HE)
1 dpi	A	B	C
3 dpi	D	E	F
5 dpi	G	H	I
7 dpi	J	K	L
14 dpi	M	N	O
35 dpi	P	Q	R

	hDPP4	MERS-CoV NP	Merge
Bronchioles			
Alveoles			

3 dpi

7 dpi

35 dpi

A

B

C

NW   Maxilla   LW   Lung

Lower magnification (HE)   MERS-CoV Ag (IHC)   Higher magnification (HE)

1 dpi

3 dpi

5 dpi

7 dpi

14 dpi

35 dpi

Young

Adult

HE

Iba-1 x CD3