1 **TMPRSS2 contributes to virus spread and immunopathology in the**

2 **airways of murine models after coronavirus infection**

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14 Running head: Role of TMPRSS2 in coronavirus infection *in vivo*

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**Abstract**

20      Transmembrane serine protease TMPRSS2 activates the spike protein of highly

21    pathogenic human coronaviruses such as severe acute respiratory syndrome-related

22    coronavirus (SARS-CoV) and Middle East respiratory syndrome-related coronavirus

23    (MERS-CoV). *In vitro*, activation induces virus-cell membrane fusion at the cell surface.

24    However, the roles of TMPRSS2 during coronavirus infection *in vivo* are unclear. Here, we

25    used animal models of SARS-CoV and MERS-CoV infection to investigate the role of

26    TMPRSS2. Th-1-prone C57BL/6 mice and TMPRSS2-knockout (KO) mice were used for

27    SARS-CoV infection, and transgenic mice expressing the human MERS-CoV receptor,

28    hDPP4-Tg mice, and TMPRSS2-KO hDPP4-Tg mice were used for MERS-CoV infection.

29    After experimental infection, TMPRSS2-deficient mouse strains showed reduced body

30    weight loss and viral kinetics in the lungs. Lack of TMPRSS2 affected the primary sites of

31    infection and virus spread within the airway, accompanied by less severe immunopathology.

32    However, TMPRSS2-KO mice showed weakened inflammatory chemokine and/or cytokine

33    responses to intranasal stimulation with poly (I:C), a Toll-like receptor 3 agonist. In

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34    conclusion, TMPRSS2 plays a crucial role in viral spread within the airway of murine

35    models infected by SARS-CoV and MERS-CoV and in the resulting immunopathology.

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37 **Importance**

38 Broad-spectrum antiviral drugs against highly pathogenic coronaviruses and other

39 emerging viruses are desirable to enable a rapid response to pandemic threats.

40 Transmembrane protease serine type2 (TMPRSS2), a protease belonging to the type II

41 transmembrane serine protease family, cleaves the coronavirus spike protein, making it a

42 potential therapeutic target for coronavirus infections. Here, we examined the role of

43 TMPRSS2 using animal models of SARS-CoV and MERS-CoV infection. The results

44 suggest that lack of TMPRSS2 in the airways reduces the severity of lung pathology after

45 infection by SARS-CoV and MERS-CoV. Taken together, the results will facilitate

46 development of novel targets for coronavirus therapy.

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48    **Introduction**

49        Highly pathogenic human coronaviruses such as severe acute respiratory

50    syndrome-related coronavirus (SARS-CoV) (1-6) and Middle East respiratory

51    syndrome-related coronavirus (MERS-CoV) (7-9) cause severe infection of the lower

52    respiratory tract in humans. These zoonotic pathogens have mortality rates of >50% in aged

53    and immunosuppressed populations, making them potentially important emerging

54    pathogens (10, 11). Broad-spectrum antiviral drugs against these coronaviruses (and other

55    highly pathogenic viruses) will facilitate rapid responses to pandemic threats.

56    Transmembrane protease serine type2 (TMPRSS2), a protease belonging to the type II

57    transmembrane serine protease family, cleaves the influenza virus hemagglutinin (HA)

58    molecule in human airway epithelial cells (12); however, it can also cleave coronavirus

59    fusion glycoproteins, namely, the spike protein. The protease activates the spike protein to

60    induce virus-cell membrane fusion at the cell surface and facilitate entry of coronaviruses

61    into the host cell (13-16). Thus, active site inhibitors of TMPRSS2 are potential therapeutic

62    targets not only for influenza viruses but also coronaviruses (17). Some animal studies

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63    show that TMPRSS2-knockout (KO) mice are protected against severe pathology and death

64    after influenza virus infection (18-21). In addition, a genetic study revealed that those with

65    high expression of certain TMPRSS2 variants are at increased risk of severe outcomes after

66    infection with A (H1N1) pdm09 influenza (22). However, the roles of TMPRSS2 *in vivo*

67    during coronavirus infection are unclear. Here, we used animal models of coronavirus

68    infection to examine the role of TMPRSS2.

69        Previously, we established a murine model of SARS based on adult BALB/c mice. The

70    animals were moribund due to severe pulmonary edema caused by skewing the immune

71    response toward a Th2 profile after infection by mouse-adapted SARS-CoV (23, 24). We

72    used adult C57BL/6 mice because the TMPRSS2-KO mice were back crossed to this strain

73    (20). After infection with mouse-adapted SARS-CoV, Th-1-prone C57BL/6 mice developed

74    acute pneumonia, with around 15% body weight loss; however, this was not fatal. In

75    addition, we recently generated an animal model of MERS-CoV using transgenic mice

76    expressing hDPP4 (hDPP4-Tg mice) under the control of an endogenous promoter

77    (Iwata-Yoshikawa et al., Submitted). The hDPP4-Tg mice were susceptible to infection by

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78    MERS-CoV and developed acute pneumonia with transient loss of body weight. Next, we

79    generated TMPRSS2-KO hDPP4-Tg (TMPRSS2-KO Tg) mice by crossing male

80    hDPP4-Tg mice with female TMPRSS2-KO mice.

81        Here, we used these animal models to demonstrate a role for TMPRSS2 during infection

82    by SARS-CoV and MERS-CoV. TMPRSS2-deficient mice showed reduced body weight

83    loss and viral replication in the lungs. In addition, histopathological and

84    immunohistochemical analyses revealed that expression of TMPRSS2 influenced both the

85    primary site of infection and virus spread within the airways of both mouse models, which

86    was accompanied by different immunopathologies.

87

88    **Results**

89    **TMPRSS2-KO mice show no body weight loss and weak proinflammatory responses**

90    **after SARS-CoV infection**

91        To screen the generated TMPRSS2-KO mice, we confirmed the absence of the

92    TMPRSS2 gene by PCR analysis using a primer set specific for TMPRSS2 (Fig. 1). To

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93    examine the effect of TMPRSS2 expression during SARS-CoV infection, we infected

94    C57BL/6 wild-type (WT) and TMPRSS2-KO mice with $10^5$ TCID$_{50}$ F-musX

95    mouse-adapted SARS-CoV. WT mice showed clear loss of body weight from 2 to 4 days

96    post-injection (p.i.), but recovered later (the exception was a single moribund mouse at Day

97    5 p.i.); these symptoms were not observed in TMPRSS2-KO mice (Fig. 2a). Measurement

98    of the virus titer showed lower viral replication in the lungs of TMPRSS2-KO mice (Fig.

99    2b). There were no significant differences in the titers of neutralizing antibodies in serum

100    samples from either group (Fig. 2c).

101        Histopathological and immunohistochemical analyses revealed that lack of TMPRSS2

102    affected the primary infection sites in the airway. Immunohistochemical staining on Day 1

103    p.i. revealed strongly antigen-positive cells in the bronchiolar epithelium of WT mice

104    infected with SARS-CoV; however, only very weak antigen positivity was observed in

105    TMPRSS2-KO mice (Fig. 2d, left panels). Some antigen-positive cells were seen in alveoli

106    from both WT and TMPRSS2-KO mice on Day 3 p.i. (Fig. 2d, middle panels). On Day 3

107    p.i., cell debris and diffuse inflammatory infiltration by neutrophils and mononuclear cells

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108     were observed around bronchi and in the alveoli of WT mice; by contrast, focal

109     inflammatory infiltration was observed in the alveoli of TMPRSS2-KO mice (Fig. 2d, right

110     panels). On Day 10 p.i., formation of granulation tissue was observed in the healing

111     alveolar area of most WT mice (eight of nine mice) (Fig. 3, upper panel), whereas it was

112     observed in only a few TMPRSS2-KO mice (three of fourteen mice) (Fig. 3, lower panel).

113        Next, we measured the concentrations of representative inflammatory growth factors,

114     chemokines, and cytokines in the lungs and observed transient elevation of FGF-basic,

115     MIP-1$\alpha$/CCL3, MIG/CXCL9, MCP-1/CCL2, IP-10/CXCK10, IL-1$\alpha$, IL-1$\beta$, IL-12, IL-6,

116     IL-4, and IL-10 in the lungs of WT mice at 3 days p.i. (Fig. 4a, black bars). Similar

117     responses were observed in the lungs of TMPRSS2-KO mice; however, the concentrations

118     of FGF-basic, KC/CXCL1, IL-12 (p40/p70), IL-4, and IL-10 were significantly lower than

119     those in WT mice at Day 2 or 3 p.i. (Fig. 4a, white bars).

120        Furthermore, we measured the expression of mRNA encoding the Toll-like receptor 3

121     (TLR3), which recognizes ds RNA and activates the NF-$\kappa$B pathway for the activation of

122     type 1 interferon (IFN), and type 1 IFN including IFN-$\alpha$4 and IFN-$\beta$ in the lungs of mice at

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123    6 h and at 1, 2 and 3 days p.i. by real-time reverse transcription RT-PCR (24, 25).

124    Interestingly, we found a transient increase in TLR3 expression in the lungs of

125    TMPRSS2-KO mice at 6 h p.i., but not in WT mice (Fig. 4b). However, IFN-α4 and IFN-β

126    mRNA expression was higher in WT mice than in TMPRSS2-KO mice (Fig. 4b).

127        Taken together, these results suggest that lack of TMPRSS2 affects both pathology and

128    immunopathology in the bronchi and/or alveoli after infection by SARS-CoV. Lower viral

129    replication in the lungs and less severe immunopathology observed in TMPRSS2-KO mice

130    resulted in no body weight loss and milder lung pathology.

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132    **TMPRSS2-KO hDPP4-Tg mice show weaker proinflammatory responses and less**

133    **severe lung pathology after infection with MERS-CoV**

134        TMPRSS2-KO hDPP4-Tg mice were generated as described in Materials and Methods.

135    To screen the generated Tg mice and TMPRSS2-KO hDPP4-Tg mice, we confirmed the

136    presence of the transgene of hDPP4 and absence of the TMPRSS2 gene by PCR analysis

137    using two primers sets specific for hDPP4 and TMPRSS2 (Fig. 1). The mice were then used

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138    to examine the role of TMPRSS2 after MERS-CoV infection. After intranasal inoculation

139    with HCoV-EMC 2012 (MERS-CoV), hDPP4-Tg mice showed a temporary and mild loss

140    of body weight (from 6 to 9 days p.i.); however, only very slight changes were observed in

141    TMPRSS2-KO Tg mice (Fig. 5a). The results of virus titer measurements suggested that the

142    virus replicated more slowly in the lungs of TMPRSS2-KO Tg mice (Fig. 5b). The lung

143    from one out of five Tg mice contained $10^{6.45}$TCID$_{50}$/g at Day 7 p.i. (two females and three

144    males), and that from three out of five TMPRSS2-KO mice contained $10^{4.2}$ or $10^{4.7}$

145    TCID$_{50}$/g at Day 7 p.i. (one female and four males). In addition, the titers of neutralizing

146    antibodies in serum from TMPRSS2-KO Tg mice were significantly lower than those in

147    serum from hDPP4-Tg mice (Fig. 5c).

148       More obvious histopathological differences were observed in MERS-CoV-infected

149    animals than in SARS-CoV-infected animals (Fig. 5d). On Day 1 p.i., many viral

150    antigen-positive cells were observed in the bronchi and alveolar areas of hDPP4-Tg mice;

151    there were none in the bronchi, and only a few in the alveoli, of TMPRSS2-KO Tg mice

152    (Fig. 5d, left panels). On Day 3 p.i., many viral antigen-positive cells were present in these

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153    areas in hDPP4-Tg mice, but fewer in TMPRSS2-KO Tg mice (Fig. 5d, middle panels). On

154    Day 7 p.i., thickening of the alveolar wall, with regenerated alveolar cells and prominent

155    cellular infiltration by macrophages and mononuclear cells, was observed in Tg mice, but

156    only mild infiltration (mainly by mononuclear cells) of the alveoli was observed in

157    TMPRSS2-KO Tg mice (Fig. 5d, right panels). On Day 14 p.i., lymphocyte aggregates and

158    cellular infiltrations were seen in the healing alveolar area of hDPP4-Tg mice (Fig. 6, upper

159    panel), whereas they were nearly absent from the healing alveolar area of TMPRSS2-KO

160    Tg mice (Fig. 6, lower panel). However, no granulation tissues were detected in either

161    mouse.

162      These data suggest that lack of TMPRSS2 has a marked effect on MERS-CoV infection

163    and replication in the bronchi and/or alveoli. Reduced viral replication in the lungs of

164    TMPRSS2-KO Tg mice resulted in only slight body weight loss and less severe lung

165    pathology.

166      Measurement of representative proinflammatory growth factors, chemokines, and

167    cytokines in hDPP4-Tg mice (i.e., GM-CSF, FGF-basic, MIP-1$\alpha$/CCL3, MIG/CXCL9,

168  MCP-1/CCL2, IL-1α, IL-1β, TNF-α, IL-12, IFN-γ, IL-17, and IL-13) revealed transient

169  elevation during the first 7 days p.i. (Fig. 7, black bars). TMPRSS2-KO Tg mice showed

170  similar responses, but they occurred later or were less pronounced than in hDPP4-Tg mice

171  (Fig. 7, white bars). The concentration of FGF-basic, GM-CSF, MIG/CXCL9, and TNF-α

172  in the lungs of TMPRSS2-KO Tg mice was lower than that in the lungs of hDPP4-Tg mice

173  on Days 5 and 7 p.i. Interestingly, expression of IL-6 and other inflammatory chemokines

174  (i.e., MIP-1α, IL-1α, and IL-1β) was higher in TMPRSS2-KO Tg mice than in hDPP4-Tg

175  mice on Day 7 p.i.

176     Furthermore, we measured the expression of mRNA encoding TLR3 and type 1 IFN,

177  including IFN-α4 and IFN-β, in the lungs of mice at 6 h and at 1, 2 and 3 days p.i. by

178  real-time reverse transcription RT-PCR (24, 25). No obvious increase in TLR3 expression

179  in the lungs of mice was observed (Fig. 7b); however, IFN-α4 and IFN-β mRNA

180  expression was higher in hDPP4-Tg mice than in TMPRSS2-KO Tg mice on days 2 and/or

181  3 p.i. (Fig. 7b).

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183 **Innate immune responses in TMPRSS2-KO mice induced by a Toll-like receptor 3**

184 **(TLR3) agonist**

185     Assay of chemokine and cytokine concentrations after coronavirus infection revealed

186 that proinflammatory immune responses in TMPRSS2-deficient mice were weaker than

187 those in WT mice. To assess the effect of knocking out TMPRSS2 on innate immune

188 responses in the absence of virus infection, we inoculated mice intranasally with poly (I:C),

189 a synthetic analog of double-strand RNA (26-28). Cytokine levels in the lungs were

190 measured at 24 h p.i. The concentrations of MCP-1/CCL2, KC/CXCL1, IL-1$\alpha$, IL-5, IFN-$\gamma$,

191 and IL-17 in TMPRSS2-KO mice were lower than those in WT mice (Fig. 8). In addition,

192 levels of FGF-basic and IL-6 in the TMPRSS2-KO Tg mice were lower than those in

193 hDPP4-Tg mice (Fig. 8). These results suggest that TMPRSS2-deficient mice intrinsically

194 exhibit weaker or delayed inflammatory chemokine and cytokine responses via TLR3.

195

196 **Discussion**

197     Mouse models of SARS-CoV and MERS-CoV infection allow us to investigate disease

198    pathogenesis and vaccine applications, and to evaluate antiviral drugs and other therapies.

199    hDPP4-Tg mice are susceptible to infection by a MERS-CoV isolate, resulting in acute

200    pneumonia but no brain disease (Iwata-Yoshikawa N, submitted). Here, we generated a

201    TMPRSS2-KO mouse bearing hDPP4. After infection with SARS-CoV or MERS-CoV,

202    TMPRSS2-deficient mice were protected from body weight loss. The results suggest that

203    TMPRSS2 plays an important role in the early phase of disease (lung infection); in

204    particular, SARS-CoV and MERS-CoV replicated in the bronchioles.

205        In humans, TMPRSS2 is expressed widely in epithelial tissues, including that lining the

206    upper airways, bronchi, and lung (29). The protein sequence of human and mouse

207    TMPRSS2 is conserved, with 78% sequence identity between the two species. *In situ*

208    hybridization analyses of mouse embryos and adult tissues reveal that TMPRSS2 is

209    expressed in the epithelium lining the gastrointestinal, urogenital, and respiratory tracts,

210    including the bronchi and bronchioles, but not in alveolar epithelium (30). Kim et al.

211    showed that depletion of TMPRSS2 (the molecule was inactivated by disrupting the serine

212    protease domain through homologous recombination) from mice did not affect

213    development or survival to adulthood; neither were there abnormalities in organ histology

214    or function (31). While the physiological function of TMPRSS2 is unclear, data suggest

215    that it does regulate sodium currents in lung epithelial cells through proteolytic cleavage of

216    the epithelial sodium channel (32). We also made the interesting observation that

217    TMPRSS2-deficient mice show weaker, or delayed, inflammatory chemokine and cytokine

218    responses mediated by Toll-like receptor 3.

219    Host cellular proteases such as trypsin, tryptase Clara, miniplasmin, human airway

220    trypsin-like protease, and TMPRSS2 cleave the HA glycoprotein of influenza A viruses.

221    Cleavage of HA is critical for viral entry into cells during fusion between the viral and host

222    cell membranes (33). Serine protease inhibitors such as camostat and aprotinin inhibit both

223    influenza virus replication in human airway epithelial cells and the release of cytokines

224    (IL-6 and TNF-α) into cell supernatants (34). In addition, animal studies using

225    TMPRSS2-KO mice reveal that TMPRSS2 is essential for the spread and pathogenesis of

226    influenza viruses such as emerging H7N9 and seasonal H1N1 and H3N2 (18-21).

227    TMPRSS2 also cleaves the coronavirus spike protein to generate unlocked,

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228     fusion-catalyzing forms at the cell surface and facilitate rapid "early" entry (14, 15, 35-39).

229     In addition, SARS-CoV and MERS-CoV enter cells via two distinct pathways: TMPRSS2

230     via the cell surface and cathepsin L via the endosome (13, 14, 16, 36, 40, 41). A previous

231     antiviral study revealed that a serine protease (TMPRSS2) rather than a cysteine protease

232     (Cathepsin L) facilitated the spread of SARS-CoV in the infected mouse (42). Our findings

233     are in agreement with this study; coronavirus replication in the lungs, especially in the

234     bronchioles, was less pronounced in TMPRSS2-deficient mice. However, viral spread and

235     inflammatory infiltration were still detected in the alveoli. Several proteases, including

236     other serine proteases and the cysteine protease, cathepsin L, may activate both SARS-CoV

237     and MERS-CoV, allowing the virus to spread to alveolar areas in TMPRSS2-deficient mice.

238     In addition, TLR3 mRNA expression in the lungs at 6 h p.i. of SARS-CoV-inoculated

239     TMPRSS2-deficient mice suggested that TLR3, which recognizes specifically dsRNA and

240     localizes to endosomes (43), recognized viral RNA within the endosomal component. Thus,

241     we speculate that the pathway employing cathepsin L and the endosome mainly contributed

242     to SARS-CoV infection in the TMPRSS2-deficient mice.

243     MERS-CoV infected animals had more obvious histopathological differences than

244     SARS-CoV infected animals in this animal model. In addition, weak-positive SARS-CoV

245     antigens at 1 day p.i. and a few virus antigen positive cells at 3 days p.i. were detected in

246     the bronchi of TMPRSS2-KO mice, but not in MERS-CoV-inoculated TMPRSS2-KO Tg

247     mice. Thus, MERS-CoV may rely more on TMPRSS2 during early infection than

248     mouse-adapted SARS-CoV, although differences in viral passage history and in the genetic

249     backgrounds of the animals should also be considered. More work will be required to test

250     the possibility that viral mutations are acquired during virus spreading in TMPRSS2

251     deficient mice.

252     TMPRSS2-deficient mice, including TMPRSS2-KO and TMPRSS2-KO Tg mice,

253     showed less severe loss of body weight after infection. Peak expression of FGF-basic, also

254     known as FGF2, after infection synchronized with peak body weight loss in WT and

255     hDPP4-Tg mice. FGFs play a role in tissue repair after pneumonia, including bronchiolitis

256     obliterans organizing pneumonia (BOOP) and interstitial pneumonia (both fibrous

257     pulmonary disorders), by promoting proliferation of fibroblasts (44). In fact, formation of

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258    granulation tissue was observed in WT mice after SARS-CoV infection. BOOP and

259    pulmonary fibrosis are common in those with SARS, including patients who survive the

260    infection (45). While there is limited evidence for development of fibrosis during end-stage

261    acute respiratory distress syndrome induced by MERS-CoV, clinical data from MERS

262    patients suggest that the situation is similar to that observed for SARS (45, 46). In addition,

263    some metabolic FGFs cause body weight loss (47).

264        As expected, lower expression of cytokines and chemokines was observed in

265    TMPRSS2-deficient mice than in TMPRSS2-competent mice after coronavirus infection.

266    This result is similar to those reported for TMPRSS2 and TMPRSS4 double-KO mice on

267    Day 3 post-H3N2 influenza A virus infection (48). High levels of virus replication very

268    likely induce severe tissue damage and increased cellular infiltration by immune cells. Viral

269    replication is likely a major cause of the elevated inflammatory chemokine levels observed

270    in WT mice; nevertheless, we assessed the possibility that TMPRSS2, a serine protease,

271    may also contribute to inflammatory reactions after TLR3 stimulation. Intranasal

272    administration of poly (I:C) induced expression of MCP-1, KC, IL-1$\alpha$, IL-1$\beta$, and IL-12 in

273    the lungs of WT mice, but not in those of TMPRSS2-KO mice. The genetic backgrounds of

274    WT and TMPRSS2-KO mice were the same because the TMPRSS2-KO mice were

275    produced from TMPRSS2 gene knockout C57BL/6 ES cells. The physiological function of

276    TMPRSS2 remains unclear; however, TMPRSS2 may contribute to more severe or rapid

277    immunopathology in WT mice by increasing the levels of inflammatory cytokines and

278    chemokines after TLR3 stimulation.

279        The immune responses to poly I:C treatment in 14-16 weeks-old hDPP4-Tg mice were

280    quite different from those in 14-16 weeks-old WT C57BL/6 mice. The hDPP4-Tg mice

281    were produced from BDF1×C57BL/6 mice; however, the Tg mice were backcrossed with

282    C57BL/6 mice for eight generations. Thus, the genetic backgrounds were almost the same

283    between these strains. On the other hand, hDPP4 expression did not have a marked effect

284    on basal innate immune responses in 10-week-old C57BL/6 and hDPP4-Tg mice; however,

285    hDPP4-Tg show slightly stronger or earlier innate immune responses than C57BL/6 mice

286    (Iwata-Yoshikawa et al., Submitted). In addition, Simeoni et al reported that hDPP4/CD26

287    transgene expression induced major phenotypic changes in T-cell populations within the

288    thymus and peripheral blood of their Tg mice, and that peripheral blood T-cell reduction

289    was age-dependent (49). Thus, the compromised immune responses in our hDPP4-Tg mice

290    were possibly due to hDPP4.

291       Broad-spectrum antiviral drugs against coronaviruses and other highly pathogenic

292    viruses will enable a rapid response to pandemic threats. Here, we demonstrate a role of

293    TMPRSS2 during infection by SARS-CoV and MERS-CoV. TMPRSS2 played an active

294    role at primary infection sites and influenced the spread of coronaviruses within the airways

295    of both mouse models, modulating the eventual immunopathology. Interestingly,

296    inflammatory chemokine and cytokine levels in TMPRSS2-KO mice were lower even after

297    intranasal stimulation by poly (I:C), suggesting an as-yet-unidentified physiological role for

298    TMPRSS2. In conclusion, we show that TMPRSS2 plays a role in the spread and

299    immunopathology of coronaviruses in the airways.

300

301    **Materials and methods**

302    **Ethics statements**

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303    Experiments using recombinant DNA and pathogens were approved by the Committee

304    for Experiments using Recombinant DNA and Pathogens at the National Institute of

305    Infectious Diseases, Tokyo, Japan. All animal experiments were approved by the Animal

306    Care and Use Committee of the National Institute of Infectious Diseases and were

307    conducted in accordance with institutional Guidelines for the Care and Use of Animals. All

308    animals were housed in a Japan Health Sciences Foundation-certified facility.

309

310    **Cells and viruses**

311    Vero E6 cells (American Type Cell Collection, Manassas, VA) were cultured in Eagle's

312    MEM containing 5% fetal bovine serum (FBS), 50 IU/ml penicillin G, and 50 μg/ml

313    streptomycin (5% FBS-MEM). Stocks of a mouse-passaged Frankfurt 1 isolate of

314    SARS-CoV and F-musX-VeroE6 were propagated twice and titrated on Vero E6 cells prior

315    to cryopreservation at 80°C, as previously described  (23). MERS-CoV (HCoV-EMC 2012

316    strain) was kindly provided by Dr. Bart Haagmans and Dr. Ron Fochier (Erasmus Medical

317    Center, Rotterdam, The Netherlands). Stocks of MERS-CoV were propagated twice,

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318     titrated on Vero E6 cells, and cryopreserved at -80°C. Viral infectivity titers are expressed

319     as the $TCID_{50}$/ml on Vero E6 cells and were calculated according to the Behrens-Kärber

320     method. All work with infectious SARS-CoV and MERS-CoV was performed under

321     biosafety level 3 conditions.

322

323     **Generation of mice.**

324        TMPRSS2-/- mice were established from TMPRSS2 gene knockout C57BL/6

325     embryonic stem (ES) cells (product number VG13341), which were obtained from the

326     Knockout Mouse Project (KOMP) Repository (UC Davis). The ES cells were injected into

327     C57BL/6 mouse blastocysts, and chimeric mice with a complete C57BL/6 genetic

328     background were generated. TMPRSS2-/- mice with a homologous genotype were obtained

329     by crossing male and female TMPRSS2+/- C57BL/6 mice (20).

330        The transgenic mice expressing human DPP4 gene (hDPP4-Tg mice) were generated by

331     microinjection of the purified BAC clones carrying hDPP4 gene into the pronuclei of

332     fertilized eggs from BDF1×C57BL/6NCr mice (Iwata-Yoshikawa et al., submitted). The

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333    transgenic mice were then backcrossed with C57BL/6NCr mice for eight generations, and

334    subsequently crossed with homozygous TMPRSS2 knockout (TMPRSS2-/-) mice.

335    Genomic DNA isolated from ear punch tissues was subjected to genotyping by PCR

336    analysis using hDPP4-specific primers (Forward; 5'-ACACACACACTCTCACACACT-3',

337    Reverse; 5'-TCTCAGTGCCATAAAAGCCCA-3') (Iwata-Yoshikawa N et al., submitted)

338    or TMPRSS2-specific primers P11 (5'-ACCTGGAGTATACGGGAACGTGA-3') and P12

339    (5'-GTGAGTGGGTGAAGGTTGGGTAG-3') (31).

340

341    **Animal experiments**

342    Mice were anesthetized by intraperitoneal injection of a mixture of 1.0 mg of ketamine

343    and 0.02 mg of xylazine (0.08 ml/10 g of body weight). TMPRSS2-KO mice, C57BL/6

344    mice lacking a homologous genotype of the TMPRSS2 gene (TMPRSS2$^{-/-}$) (20), and

345    C57BL/6 mice (WT mice; TMPRSS2$^{+/+}$) were inoculated intranasally with SARS-CoV ($10^5$

346    TCID$_{50}$ in 30 µl of F-musX). Human DPP4-expressing transgenic mice (hDPP4-Tg mice:

347    C57BL/6 mice heterozygous for the human DPP4 gene [hDPP4$^{+/-}$ TMPRSS2$^{+/+}$])

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348    (Iwata-Yoshikawa et al., manuscript in preparation) and hDPP4$^{+/-}$ TMPRSS2$^{-/-}$ C57BL/6

349    mice (TMPRSS2-KO Tg mice) were obtained by crossing hDPP4-Tg mice with

350    TMPRSS2-KO mice. These mice were inoculated intranasally with MERS-CoV ($10^6$

351    TCID$_{50}$ in 30 µl of HCoV-EMC 2012). Infected mice were observed for clinical signs of

352    infection, and body weight was measured daily, for 10 days or 14 days (n = 6–14 mice, all

353    aged 12 to 28 weeks). For analysis of virus replication, cytokine expression, and pathology,

354    animals were sacrificed at various time points after inoculation (n = 3–4 mice per group, all

355    aged 13 to 30 weeks).

356        WT, TMPRSS2-KO, hDPP4-Tg, and TMPRSS2-KO Tg mice (n = 4 mice per group, all

357    aged 14 to 16 weeks) were anesthetized by intraperitoneal injection of a mixture of 1.0 mg

358    of ketamine and 0.02 mg of xylazine (0.08 ml/10 g of body weight). Mice then received 20

359    µg of poly (I:C) (Invitrogen, San Diego, CA) in 20 µl of PBS (intranasally) (25). All mice

360    were sacrificed 24 h after administration for analysis of cytokine expression.

361

362    **Virus titration**

363  Lung tissue homogenates (10% [wt/vol]) were prepared in MEM containing 2% FBS, 50

364  IU/ml penicillin G, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. Samples were

365  clarified by centrifugation at $740 \times g$ for 20 min, and the supernatant was inoculated onto

366  Vero E6 cell cultures for virus titration.

367

368  **Neutralizing antibody test**

369  Serum was collected from mice sacrificed on Day 10 or 14 p.i. After inactivation at 56°C

370  for 30 min, Vero E6 cells were infected with virus (100 $TCID_{50}$ per well) in the presence of

371  plasma (serially diluted 2-fold), incubated for 3 or 5 days, and then examined for cytopathic

372  effects. Plasma titers of neutralizing antibodies were calculated as the reciprocal of the

373  highest dilution at which no cytopathic effect was observed. The lowest and highest

374  dilutions tested were 4 and 256 or 64, respectively.

375

376  **Histopathology and immunohistochemistry**

377  Mice were anesthetized and perfused with 2 ml of 10% phosphate-buffered formalin.

378    The lungs were harvested, fixed, embedded in paraffin, sectioned, and stained with

379    hematoxylin and eosin. Masson's trichrome staining was also conducted to detect fibrosis

380    in the lungs. Immunohistochemical analysis was performed using a polymer-based

381    detection system (Nichirei-Histofine Simple Stain Mouse MAX PO(R); Nichirei

382    Biosciences, Inc., Tokyo, Japan). Antigen retrieval from formalin-fixed mouse tissue

383    sections was performed by autoclaving in retrieval solution (pH 6.0; Nichirei Biosciences)

384    at 121°C for 10 min. Hyper-immune rabbit serum raised against SARS-CoV (23) or an

385    anti-MERS-CoV nucleocapsid antibody (Sino Biological Inc., Beijing, China) was used as

386    the primary antibody to detect viral antigens. Peroxidase activity was detected with

387    3,3'-diaminobenzidine (Sigma-Aldrich). Hematoxylin was used for counterstaining.

388

389    **Detection of inflammatory cytokines and chemokines**

390    Cytokines and chemokines in mouse lung homogenates (10% wt/vol) were measured

391    using a commercial Mouse Cytokine 20-Plex antibody bead kit (Thermo Fisher Scientific)

392    and a Luminex 100™ apparatus (Luminex Co, Austin, TX), as described previously (23). A

28

393  panel of inflammatory cytokines and chemokines (bFGF, GM-CSF, IFN-γ, IL-1α, IL-1β,

394  IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 [p40/p70], IL-13, IL-17, IP-10, KC, MCP-1, MIG,

395  MIP-1α, TNF-α, and VEGF) was detected according to the manufacturer's protocols.

396

397    **Quantitative real-time RT-PCR.** To measure the levels of type I IFN and TLR3

398  mRNA expression, RNA was extracted from 20% (w/v) lung of mice infected with viruses

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

400  instructions. mRNAs encoding IFN-α, IFN-β, and TLR3 were examined by real-time

401  RT-PCR using an ABI Prism 7900HT Fast real-time PCR system (Applied Biosystems,

402  Foster City, CA). The TaqMan probes and primers, and the reaction conditions, have been

403  described previously (24, 25). Expression of each gene was normalized to that of β-actin.

404

405  **Statistical analysis**

406    Data are expressed as the mean and standard error of the mean. Statistical analyses were

407  performed using Graph Pad Prism 7 software (GraphPad Software Inc., La Jolla, CA).

408    Body weight curves, virus titers, and multiplex assay results were analyzed using one-way

409    or two-way analysis of variance (ANOVA). Significant effects of viral titers in different

410    animal strains at different time points were assessed by two-way ANOVA, and p-values

411    were calculated using Bonferroni's multiple comparisons test. The results of the

412    neutralizing antibody titer assays were analyzed using the Mann-Whitney test. A p-value <

413    0.05 was considered statistically significant.

414

421

429

430

431    **References**

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613

614 **Figure legends**

615 **FIG 1.** Genotyping of C57BL/6 (WT), TMPRSS2-KO (KO), hDPP4-Tg (Tg) and

616 TMPRSS2-KO hDPP4 (KO-Tg) mice by PCR analysis. PCR analysis was performed on

617 the genomic DNA from ear punches taken from WT, KO, Tg, and KO-Tg mice (WT,

618 4-5-week-old, n=3, [male=3]; KO, 4-5-week-old, n=3, [male=3]; Tg, 4-5-week-old, n=3,

619 [male=1, female=2]; KO-Tg, 4-5-week-old, n=3, [male=1, female=2]), and the resulting

620 products (391 bp for hDPP4 and 388 bp for TMPRSS2) are shown. Numbers indicate

621 positions of the 500 bp standard molecular weight marker ladder. M, 100 bp ladder; P,

622 positive controls for hDPP4 or TMPRSS2; N, negative control without the ear punch

623 template.

624

625 **FIG 2.** Experimental infection of TMPRSS2-knockout (KO) mice with SARS-CoV.

626 C57BL/6 (WT) and TMPRSS2-KO mice were inoculated with F-musX (SARS-CoV). (a)

627 Body weight curve during the first 10 days post-infection (p.i.). Numbers of animals per

628 group were as follows: KO, n=14 (male=11, female=3); WT, n=10 (male=5, female=5).

629     Mice of 22-28 weeks-old were used. Error bars represent standard errors. *, p<0.05; ****,

630     p<0.0001 (one-way ANOVA.). (b) Virus titer in lungs from SARS-CoV-inoculated animals

631     at 6 h and at 1, 2, and 3 days p.i. Numbers of animals per group were as follows: KO, n=4-5

632     per time point (male=0-1, female=3-4); WT, n=4-5 per time point (male=0-1, female=3-4).

633     Mice of 14-30 weeks-old were used. Error bars represent standard errors. The dotted line

634     indicates the limit of detection. P-values in the graph were calculated by two-way ANOVA

635     to determine significant effects of viral titers in different animal strains at different time

636     points. (c) Neutralizing antibody titer in serum on Day 10 p.i. The data are from the same

637     animals used in (a), except for one mouse that died. Each symbol represents an individual

638     mouse. Numbers of animals per group were as follows: KO, n=14 (male=11, female=3);

639     WT, n=9 (male=4, female=5). Mice of 22-28 weeks-old were used. Error bars represent

640     standard errors. P-values for the graph were calculated by the Mann-Whitney test. The

641     dotted line indicates the limit of detection. (d) Histopathological examination of the lungs

642     from WT and TMPRSS2-KO mice after infection by SARS-CoV. Numbers of animals per

643     group were as follows: KO, n=3 per time point (male=1-2, female=1-2); WT, n=3 per time

644    point (male=1-2, female=1-2). Mice of 15-20 weeks-old were used. Representative images

645    of lungs from mice on Days 1 and 3 days p.i. Immunohistochemical analysis using an

646    anti-SARS-CoV polyclonal antibody (at 1 and 3 days p.i.). Hematoxylin and eosin staining

647    at 3 days p.i. On Day 1 p.i., viral antigen-positive cells are seen mainly in the bronchi of a

648    WT mouse, whereas very weakly positive cells are seen in a TMPRSS2-KO mouse (left

649    panels, brown color). On Day 3 p.i., several alveolar cells around the bronchi in both WT

650    and TMPRSS2-KO mice are positive for viral antigen (middle panels, brown color). Cell

651    debris and diffuse inflammatory infiltration by neutrophils and mononuclear cells are seen

652    around bronchi and in the alveolar area of WT mice, whereas focal inflammatory

653    infiltration is observed in the alveoli of TMPRSS2-KO (right panels, inset). Br, bronchi; Al,

654    alveolar area; V, vein. Bars, 20 or 200 μm.

655

656    **FIG 3.** Formation of granulation tissue in TMPRSS2-knockout (KO) mice after infection

657    with SARS-CoV. Histopathological examination of the lungs from WT and TMPRSS2-KO

658    mice at 10 days after infection with SARS-CoV. Representative images of lungs are from

659    the same animals in Fig. 2a. HE, Hematoxylin and eosin staining; MT, Masson's trichrome

660    staining. Granulation tissue, known as Masson bodies (blue allows in HE; Red arrows in

661    MT) was located in the alveolar duct walls of WT mice, but to a lesser extent in

662    TMPRSS2-KO mice. Br, bronchi; Ad, Alveolar duct; Al, alveolar area. Bars, 200 or 100

663    μm.

664

665    **FIG 4.** Immune responses in TMPRSS2-knockout (KO) mice after infection with

666    SARS-CoV. C57BL/6 wild-type (WT) and TMPRSS2-KO mice were inoculated with

667    F-musX (SARS-CoV). Cytokine and chemokine responses (a) and the levels of type I IFN

668    and TLR3 mRNA expression (b) in the lungs at 6 h and at 1, 2, and 3 days p.i. The lung

669    homogenates were from the same animals in Fig 1(b) and assays were done using unicate

670    samples per animal. Expression of each gene was normalized to that of β-actin in (b).

671    Numbers of animals per group were as follows: KO, n=4 per time point (male=0-1,

672    female=3-4); WT, n=4 per time point (male=0-1, female=3-4). Mice of 14-30 weeks-old

673    were used. Error bars represent standard errors. P-values for the graph were calculated by

674    ANOVA. *, p<0.05; **, p<0.01; ****, p<0.0001.

675

676    **FIG 5.** Experimental infection of TMPRSS2-knockout hDPP4-transgenic (KO-Tg) mice

677    with MERS-CoV. hDPP4-Tg (Tg) and KO-Tg mice were inoculated with EMC-HCoV

678    (MERS-CoV). (a) Body weight curve during the 14 days post-infection (p.i.). Numbers of

679    animals per group were as follows: KO-Tg, n=8 (male=2, female=6); Tg, n=6 (male=3,

680    female=3). Mice of 12-14 weeks-old were used. Error bars represent standard errors. ***,

681    p<0.001 (one-way ANOVA) (b) Virus titer in the lungs of MERS-CoV-inoculated animals

682    at 6 h and at 1, 2, and 3 days p.i. Numbers of animals per group were as follows: KO-Tg,

683    n=4 per time point (male=0-1, female=3-4); Tg, n=4 per time point (male=1-2, female=2-3).

684    Mice of 13-22 weeks-old were used. Error bars represent standard errors. The dotted line

685    indicates the limit of detection. P-values indicated in the graph were calculated by two-way

686    ANOVA for significant effects of viral titers in different animal strains at different time

687    points. (c) Neutralizing antibodies in serum from mice on Day 14 p.i. The sera were from

688    the same animals in (a). Numbers of animals per group were as follows: KO-Tg, n=8

43

689    (male=2, female=6); Tg, n=6 (male=3, female=3). Mice of 12-14 weeks-old were used.

690    Error bars represent standard errors. *, p<0.05. P-values in the graph were calculated by the

691    Mann-Whitney test. The dotted line indicates the limit of detection. (d) Histopathological

692    examination of the lungs of Tg mice and TMPRSS2-KO Tg mice after infection with

693    MERS-CoV. Numbers of animals per group were as follows: KO-Tg, n=3 per time point

694    (male=1-2, female=1-2); WT, n=3 per time point (male=2-3, female=0-1). 19-25 weeks-old

695    mice were used. Representative images from mice taken on Days 1, 3, and 7 p.i.

696    Immunohistochemical analysis at 1 and 3 days p.i. using an anti-MERS-CoV nucleocapsid

697    polyclonal antibody. Hematoxylin and eosin staining at Day 7 p.i. Viral antigen-positive

698    cells are seen both in the bronchi and alveoli of a Tg mouse (left panels, brown color).

699    Some pneumocytes in the Tg mouse are positive for viral antigen, but negative in a

700    TMPRSS2-KO Tg mouse (Day 1 p.i.; left panels, brown color). Several viral

701    antigen-positive cells are seen in the alveoli and bronchi of a Tg mouse on Day 3 p.i., but

702    fewer are present in a TMPRSS2-KO Tg mouse (middle panels, brown color). On Day 7,

703    massive cellar proliferation is observed in the alveoli of a Tg mouse, with numerous

44

704    macrophages and mononuclear cells (right panels, inset). By contrast, multinuclear cells

705    (including neutrophils and eosinophils) are seen in the alveoli of a TMPRSS2-KO Tg

706    mouse (right panels, inset, arrows). Br, bronchi; Al, alveolar area; V, vein. Bars, 20 or 200

707    μm.

708

709    **FIG 6.** Recovery from acute pneumonia without granulation tissue in TMPRSS2-knockout

710    hDPP4-transgenic (KO-Tg) mice with MERS-CoV. Histopathological examination of the

711    lungs from hDPP4-Tg (Tg) and KO-Tg mice 14 days after infection with MERS-CoV.

712    Representative images of lungs are from the same animals in Fig. 5a. HE, Hematoxylin and

713    eosin staining; MT, Masson's trichrome staining. A lymphocyte aggregate (*) and cellular

714    infiltrations persisted in the alveolar area of the Tg mouse, whereas infiltrations in KO-Tg

715    mice were mild. No granulation tissues were detected in these mice. Br, bronchi; Al,

716    alveolar area; Bars, 200 or 100 μm.

717

718    **FIG 7.** Immune responses in TMPRSS2-knockout (KO) hDPP4-transgenic mice after

45

719    infection with MERS-CoV. hDPP4-Tg (Tg) and TMPRSS2-KO hDPP4 (TMPRSS2-KO

720    Tg) mice were inoculated with EMC-HCoV (MERS-CoV). (a) The lung homogenates were

721    from the same animals in Fig 5(b) and assays were done using unicate samples per animal.

722    The dotted line indicates the limit of detection. (b) The levels of type I IFN and TLR3

723    mRNA expression in the lungs at 6 h and at 1, 2, and 3 days p.i. Numbers of animals per

724    group were as follows: KO-Tg, n=4 per time point (male=0-1, female=3-4); Tg, n=4 per

725    time point (male=1-2, female=2-3). Mice of 13-22 weeks-old were used. Expression of

726    each gene was normalized to that of β-actin. Error bars represent standard errors. P-values

727    for the graph were calculated by ANOVA. *, p<0.05; **, p<0.01.

728

729    **FIG 8.** Immune responses after intranasal inoculation of mice with poly (I:C).

730    Concentrations of inflammatory cytokines and chemokines in the lungs at 24 h

731    post-infection. The assays were done using unicate samples per animal. Numbers of

732    animals per group were as follows: WT, n=4 per group (male=2, female=2); KO, n=4 per
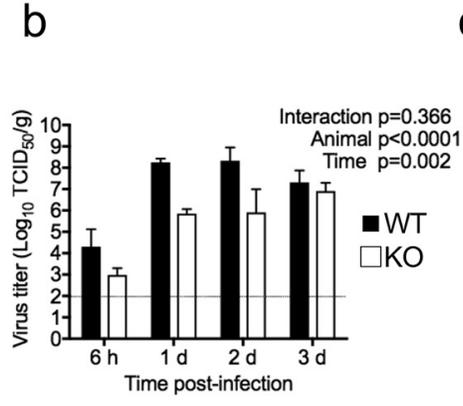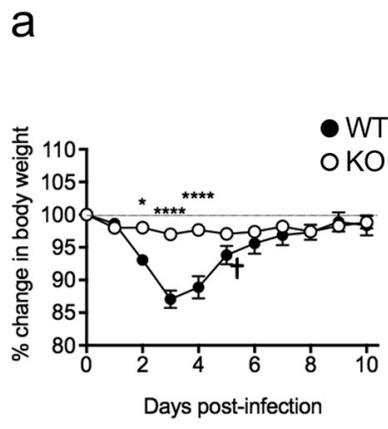
733    group (male=2, female=2); Tg, n=4 per group (male=2, female=2); KO-Tg, n=4 per group
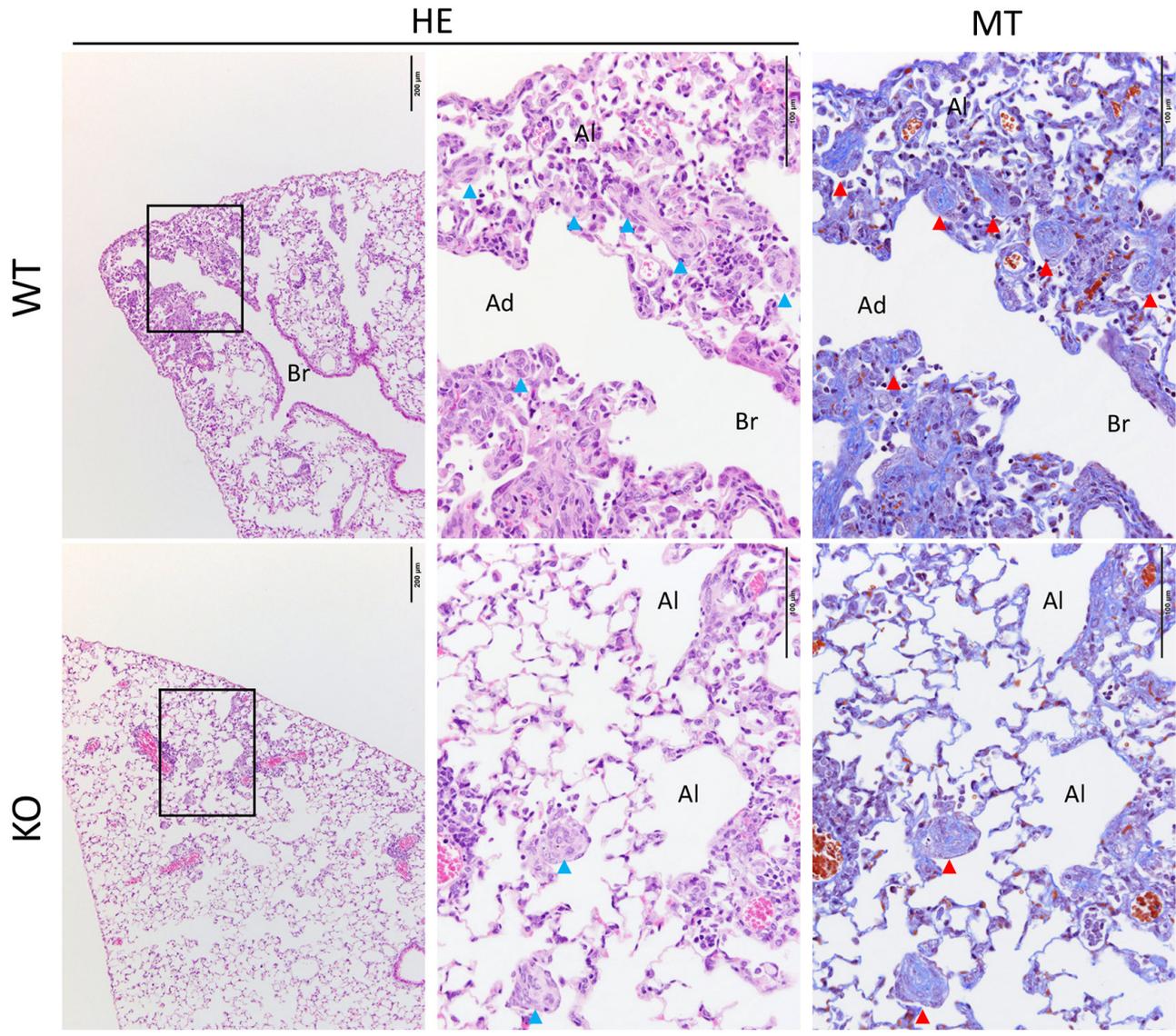
46

734     (poly I:C, male=2, female=2; PBS, male=1, female=3). Mice of 14-16 weeks-old were used.

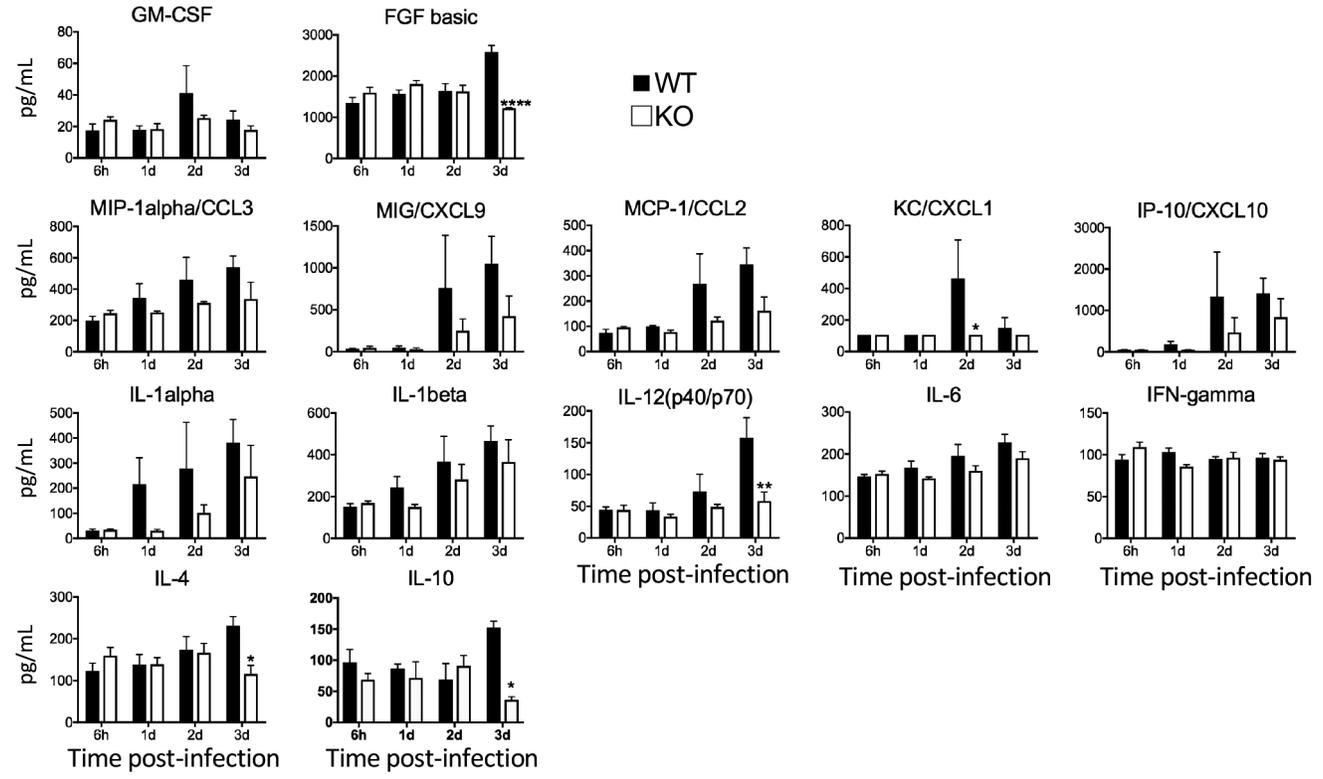735     Error bars represent standard errors. P-values for the graph were calculated by ANOVA. *,
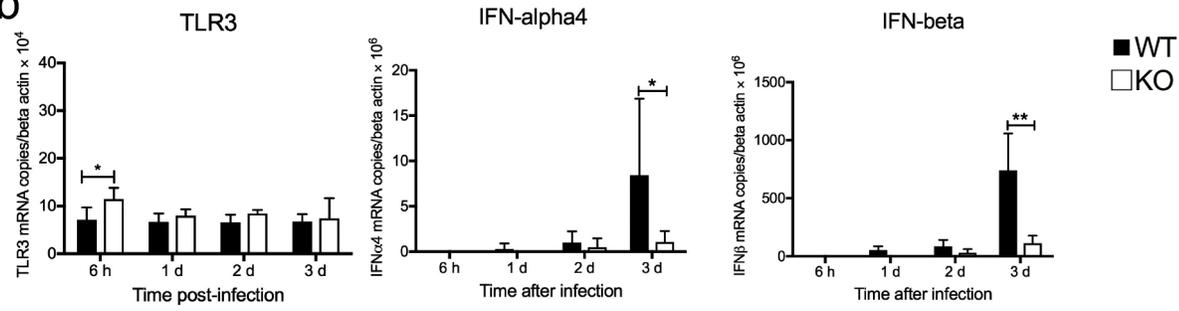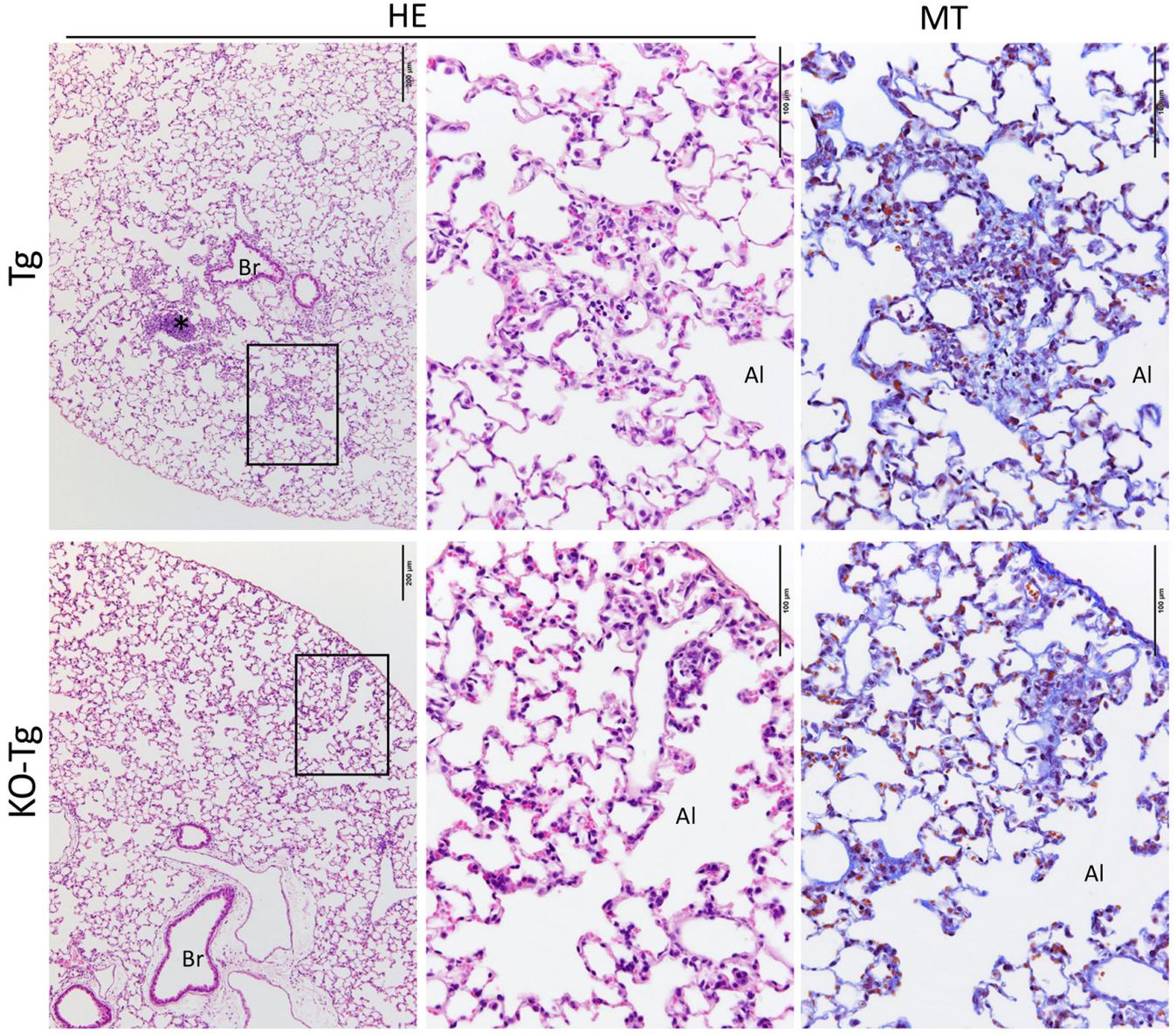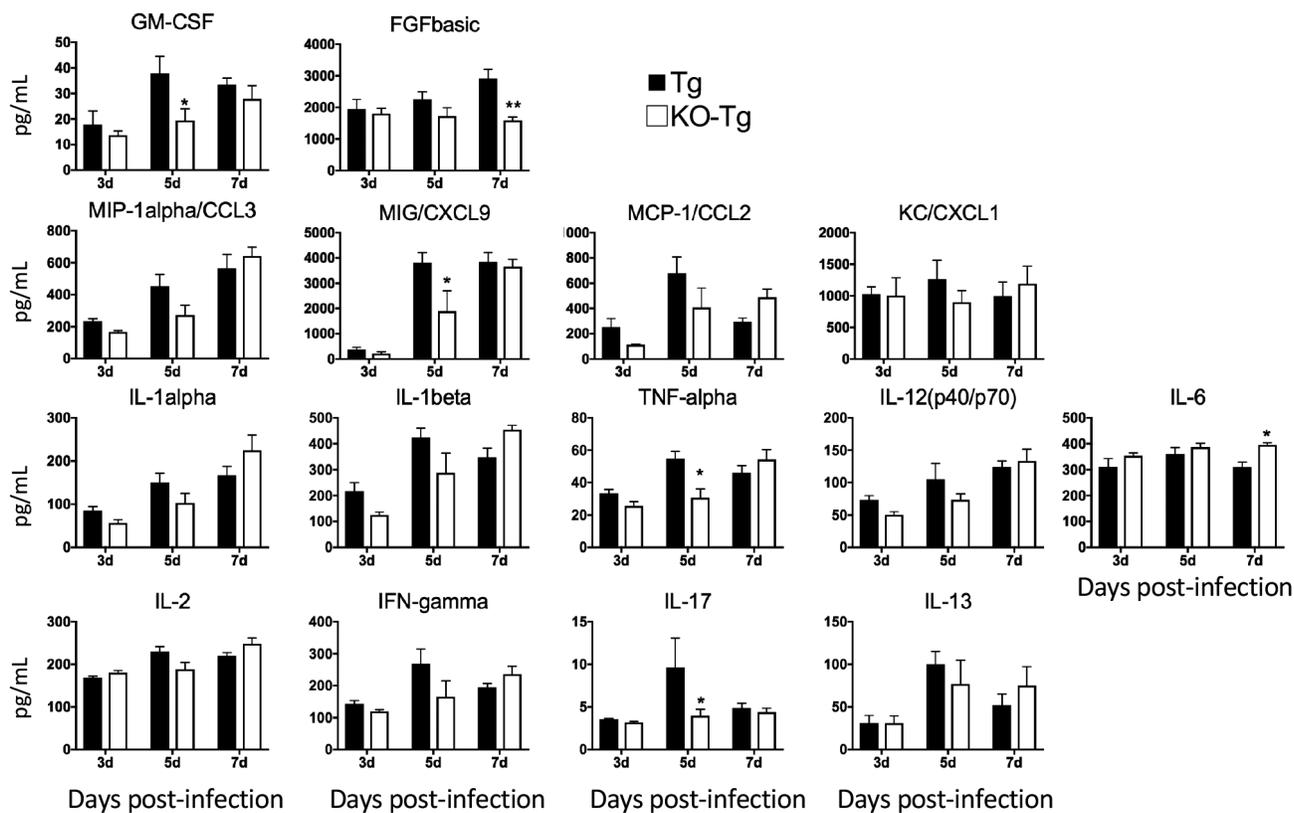
736     $p < 0.05$.

a



b

Interaction p=0.366
Animal p<0.0001
Time  p=0.002

c



d

HE                                          MT

WT

KO

HE

MT

Tg

KO-Tg

a



b