The Pathogenicity of 2019 Novel Coronavirus in hACE2 Transgenic Mice

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24 **Abstract**

25 2019-nCoV caused pneumonia cases in China has become a public health emergency of

26 international concern (PHEIC). The first priority for prevention and treatment of the

27 disease is to find the pathogenicity of 2019-nCoV *in vivo*. Weight loss and virus

28 replication were detected in infected-hACE2 mice. The typical histopathology was

29 interstitial pneumonia with significant inflammatory cells infiltration around the

30 bronchioles and blood vessels, and viral antigens were observed in bronchial epithelial

31 cells and alveolar epithelial cells. The phenomenon was not found in wild type mice

32 infected with 2019-nCoV and the mock-infected hACE2 mice. The pathogenicity of

33 2019-nCoV in hACE2 mice was clarified and the Koch's postulates was fulfilled as well,

34 and the model may facilitate the development of therapeutics and vaccines against 2019-

35 nCoV.

36

37 In late December of 2019, a cluster of severe pneumonia cases caused by 2019 novel

38 coronavirus (2019-nCoV), linked to a seafood market in which exotic animals were also

39 sold and consumed, were identified and reported from Wuhan City, Hubei Province,

40 China[1,2]. The number of infections has since soared, with almost 10,000 cases reported

41 and over 200 deaths as of January 31, 2020 in China[3], and imported cases from travelers

42 of mainland China in several other countries. It is critical to find the pathogenicity and

43 biology of the virus for prevention and treatment of the disease.

44   Because 2019-nCoV was highly homologous with Severe acute respiratory

45 syndrome coronavirus (SARS-CoV), human Angiotensin-converting enzyme 2 (hACE2),

46 which was the entry receptor of SARS-CoV, was also considered to have a high binding

47 ability with the 2019-nCoV by molecular biological analysis[4,5]. Therefore, we used the

48 hACE2 transgenic and wild type mice infected with or without 2019-nCoV infection to

49 study the pathogenicity of the virus. Specific pathogen-free, 6-11-month-old, male and

50 female hACE2 mice and wild type mice (n=7) were inoculated intranasally with 2019-

51 nCoV stock virus at a dosage of 10^5 $TCID_{50}$ per mouse, and the mock-infected hACE2

52 mice (n=3) were used as control. Weight loss of up to 5% was observed for 10 dpi only in

53 the 2019-nCoV-infected hACE2 mice (Figure 1a), and other clinical symptoms were not

54 observed. Major organs were harvested at 3 dpi and 5 dpi to assess for biodistribution of

55 2019-nCoV in infected hACE2 mice. Viral RNA was positive by RT-PCR (Figure 1b)

56 and identified by sequencing only in the lung of infected-hACE2 mice at 3 dpi and 5 dpi.

57 Meanwhile, the virus was successfully isolated by Vero cells culture (Figure 1d) and

58 observed by an electron microscope (Figure 1e). However, the virus was not found in the

59 PBS-infected hACE2 mice or infected-wild type mice (data not shown).

60   The typical pneumonia was demonstrated as bilateral ground-glass opacity and

61   subsegmental areas of consolidation by image in patient[6], but no histopathological results

62   were reported until now. The major organs of mice were examined by histopathology and

63   immunofluorescence. Compared to PBS-infected hACE2 mice or 2019-nCoV-infected-

64   wild type mice (Figure 2a and b), focal lesions were observed in the dorsal of the right

65   middle lobe in 2019-nCoV-infected hACE2 mice (Figure 2c). Consistently, lung tissues

66   from 2019-nCoV-infected hACE2 mice had multifocally mild or moderate pneumonia

67   with interstitial hyperplasia. And significant inflammatory cells infiltration around the

68   bronchioles and blood vessels (Figure 2f) were found. The alveolar interstitium is also

69   expanded with inflammatory cells, and the alveolar lumen contains cell debris mixed

70   with leukocytes. Bronchial epithelial cells showed swelling, degeneration, and some of

71   them dissolved and necrotic foci (Figure 2g, h and i). Meanwhile, 2019-nCoV antigens

72   were observed in the bronchial epithelial cells (Figures 3h and i) and alveolar epithelial

73   cells (Figures 3n, o, p and q) of lungs in 2019-nCoV-infected hACE2 mice. In addition,

74   the co-localization of 2019-nCoV S protein and hACE2 receptor was demonstrated in

75   alveolar epithelial cells of infected-hACE2 mice by immunofluorescence (Figures 3n, o,

76   p and q). The phenomenon was not found in the PBS-infected hACE2 mice (Figures 3k, i

77   and m) or infected-wild type mice (data not shown).

78   The speed of geographical spread of severe viral pneumonia disease caused by

79   2019-nCoV has been declared as public health emergency of international concern

80   (PHEIC), with cases reported on multiple continents only weeks after the disease was

81   first reported[7]. Although it has been determined by bioinformatics that the pathogen of

82   this epidemic is a novel coronavirus, it is necessary to be confirmed by animal

83   experiments following Koch's principles. After experimental infection of transgenic

84   hACE2 mice with one of the earliest known isolates of 2019-nCoV, the mice lost weight

85   and showed interstitial pneumonia, which are comparable with initial clinical reports of

86   pneumonia caused by 2019-nCoV[6]. In addition, the 2019-nCoV S protein and hACE2

87   receptor were found to co-localize in alveolar epithelial cells, supporting that the 2019-

88   nCoV, similar to SARS-CoV, also utilizes the hACE2 as a receptor for entry[4]. Therefore,

89   the present study clarified the pathogenicity of 2019-nCoV in hACE2 mice and fulfills

90   the Koch's postulates as well, and the model may facilitate the development of drugs and

91   vaccines against 2019-nCoV.

92

93   **Materials and methods**

94   *Ethics statement*

95   Murine studies were performed in an animal biosafety level 3 (ABSL3) facility using

96   HEPA-filtered isolators. All procedures in this study involving animals were reviewed

97   and approved by the Institutional Animal Care and Use Committee of the Institute of

98   Laboratory Animal Science, Peking Union Medical College (BLL20001).

99

100 *Viruses and cells*

101 The 2019-nCoV (strain HB-01) was kindly provided by Professor Wenjie Tan[1], from the

102 China Centers for Disease Control and Prevention (China CDC). The complete genome

103 for this 2019-nCoV was submitted to GISAID (BetaCoV/Wuhan/IVDC-HB-

104 01/2020|EPI_ISL_402119), and deposited in the China National Microbiological Data

105 Center (accession number NMDC10013001 and genome accession numbers

106 MDC60013002-01). Seed 2019-nCoV stocks and virus isolation studies were performed

107 in Vero cells, which are maintained in Dulbecco's modified Eagle's medium (DMEM,

108 Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml

109 penicillin, and 100 µg/ml streptomycin, and incubated at 37°C, 5% CO_2. Titers for 2019-

110 nCoV were determined using a standard 50% tissue culture infection dose ($TCID_{50}$) assay.

111

112 *Animal experiments*

113 For the animal experiments, specific pathogen-free, 6-11-month-old, male and female

114 transgenic hACE2 mice were obtained from the Institute of Laboratory Animal Science,

115 Peking Union Medical College, China. Transgenic mice were generated by

116 microinjection of the mice ACE2 promoter driving the human ACE2 coding sequence

117 into Institute of Cancer Research (ICR) or C57BL/6J mice[8]; the presence of human

118 ACE2 in the mice used for these experiments was confirmed by PCR (data not shown).

119 The hACE2 mice (n=7) or ICR mice (n=5) were respectively inoculated intranasally with

120 2019-nCoV stock virus at a dosage of 10^5 $TCID_{50}$ per mouse. As a control, hACE2 mice

121 (n=3) were mock-infected with an equivalent challenge volume of PBS. PBS- and 2019-

122 nCoV-infected animals were continuously observed daily to record body weights, clinical

123 symptoms, decreased responsiveness to external stimuli and death. Two mice from the

124 2019-nCoV-infected group were dissected at 3 days post-infection (dpi) and at 5 dpi to

125 collect trachea, lung, kidney, brain, spleen, and liver tissues for the determination of viral

126 load after 2019-nCoV infection. One mouse from the PBS-infected group was dissected

127 at 3 dpi as a control. One mouse from the ICR mice group inoculated with 2019-nCoV

128 was dissected at 3 dpi as the receptor control.

129

Preparation of Homogenate Supernatant

Tissues homogenates were prepared by homogenizing perfused whole tissue using an electric homogenizer for 2 min 30 s in 1 ml of DMEM. The homogenates were centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was collected and stored at −80°C for viral isolation and viral load detection.

RNA extraction and RT-PCR

Total RNA was extracted from organs using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcription was performed using the PrimerScript RT Reagent Kit (TaKaRa, Japan) following manufacturer instructions. RT-PCR reactions were performed using the PowerUp SYBG Green Master Mix Kit (Applied Biosystems, USA), in which samples were processed in duplicate using the following cycling protocol: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s, and then 95°C for 15 s, 60°C for 1 min, 95°C for 45 s. The primer sequences used for RT-PCR are targeted against the envelope (E) gene of 2019-nCoV and are as follows: Forward: 5'-TCGTTTCGGAAGAGACAGGT-3', Reverse: 5'-GCGCAGTAAGGATGGCTAGT-3'.

Laboratory preparation of the antibody of 2019-nCoV Spike-1 (S1) protein

Mice were immunized with purified 2019-nCoV S1 protein (Sino biological) and splenocytes of hyper immunized mice were fused with myeloma cells. Positive clones were selected by ELISA using 2019-nCoV S1 protein (Supplementary Figure 1). The cell

152 supernatant of 7D2 clone, binding to 2019-nCoV S1 protein, was collected for

153 immunofluorescence analysis.

154

155 *Hematoxylin and Eosin Staining*

156 For each mouse, the whole left lung was embedded in Cryo-Gel for histological

157 examination by frozen section method. The lung tissue sections (10 um) were fixed in

158 100% acetone for 15 minutes and stained with Hematoxylin and Eosin (H&E). The

159 histopathology of the lung tissue was observed by light microscopy.

160

161 *Immunofluorescence and confocal microscopy*

162 For viruses and ACE2 receptor localization analysis, the lung tissue sections (10 um)

163 were washed twice with PBS, fixed by Immunol Staining Fix Solution (P0098), blocked

164 1 hour at room temperature by Immunol Staining Blocking Buffer (P0102) and then

165 incubated overnight at 4°C with the appropriate primary and secondary antibodies. The

166 nuclei were stained with DAPI. First, anti-2019-nCoV S protein (laboratory preparation)

167 and sera of patient in convalescent phase were used to test the 2019-nCoV, respectively.

168 Secondly, for analysis the relationship between 2019-nCoV and ACE2 receptor, anti-

169 2019-nCoV S protein (mouse monoclonal 7D2, laboratory preparation, 1:200) and anti-

170 hACE2 antibody (rabbit polyclonal, ab15348, Abcam1:200) were used. The sections was

171 washed with PBS and incubated with secondary antibodies conjugated with FITC (goat

172 anti-human, ab6854, Abcam, 1:200), TRITC (goat anti rabbit, ZF-0317, Beijing ZSGB

173 Biotechnology, 1:200), or FITC (goat anti-mouse, ZF-0312, Beijing ZSGB

174   Biotechnology, 1:200), respectively, dried at room temperature and observed via

175   fluorescence microscopy.

176

177   *Statistical analysis*

178   All data were analyzed with GraphPad Prism 6.0 software. Statistically significant

179   differences between the virus HB-01-infected hACE2 mice and other mice with or

180   without HB-01 infection were determined using Welch's t-test. The level of statistical

181   significance is designated as $*p<0.05$, $**p<0.01$ or $^{\#}p<0.05$, $^{\#\#}p<0.01$.

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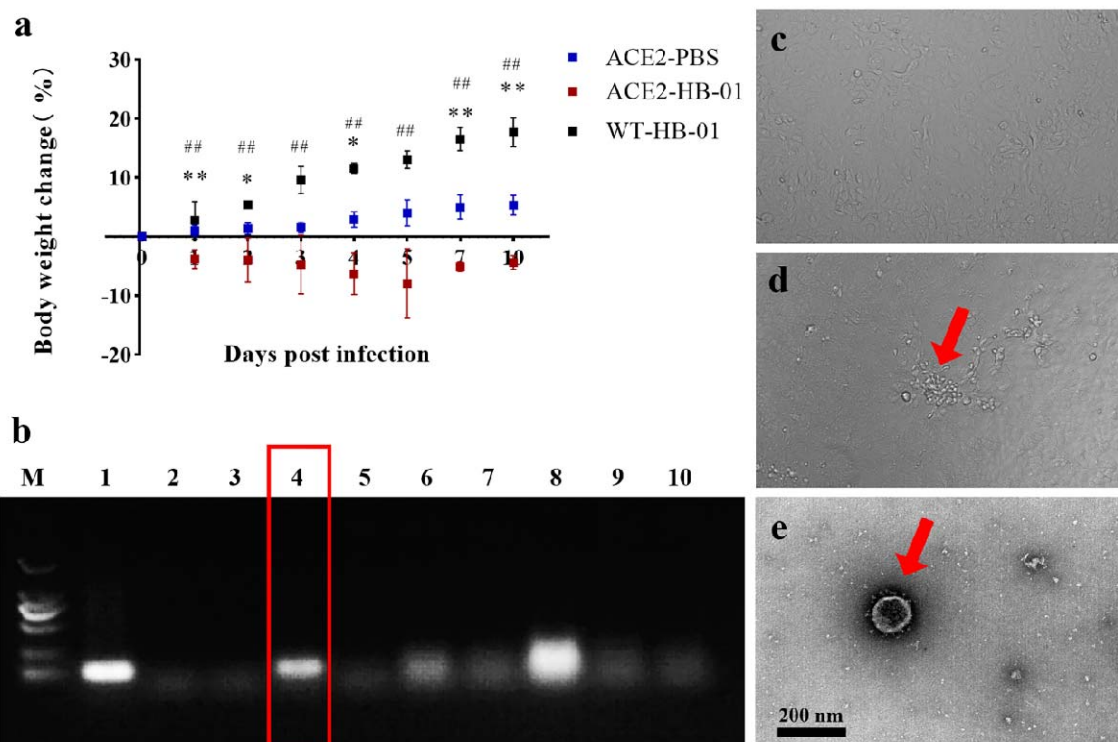210 SARS coronavirus infection. Comp Med. 2007 Oct;57(5):450-9.

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Figure 1. Weight loss and virus detection in hACE2 mice post infection with 2019-nCoV. Two groups of mice were experimentally challenged intranasally with a dose of 10^5 TCID$_{50}$ 2019-nCoV, and the PBS-infected hACE2 mice were used as control. WT mice were inoculated with 2019-nCoV as receptor control. And then the weight loss was recorded over 10 days (a). Mice were sacrificed and their major organs harvested for viral load and virus isolation at 3 and 5 dpi, the distribution of 2019-nCoV in various organs of infected hACE2 mice was detected by RT-PCR and the results of 3 dpi was representatively shown (b) (line 1: positive control, line2: negative control, line 3-10: trachea, lung, heart, spleen, intestine, liver, kidney and brain in turn). (c) Live virus could not be isolated from the lungs of infected-WT mice on Vero cells, but (d) cytopathic effects were observed from Vero cells infected with homogenate from the lungs of infected-hACE2 mice. Electron microscope pictures of 2019-nCoV from infected-hACE2 mice lung tissue culture homogenate at 3 dpi (e). Significant differences are indicated with different asterisks (Welch's t-test, ACE2-HB-01 vs ACE2-PBS, *p<0.05, **p<0.01*; ACE2-HB-01 vs WT-HB-01, [#]p<0.05, [##]p<0.01*).
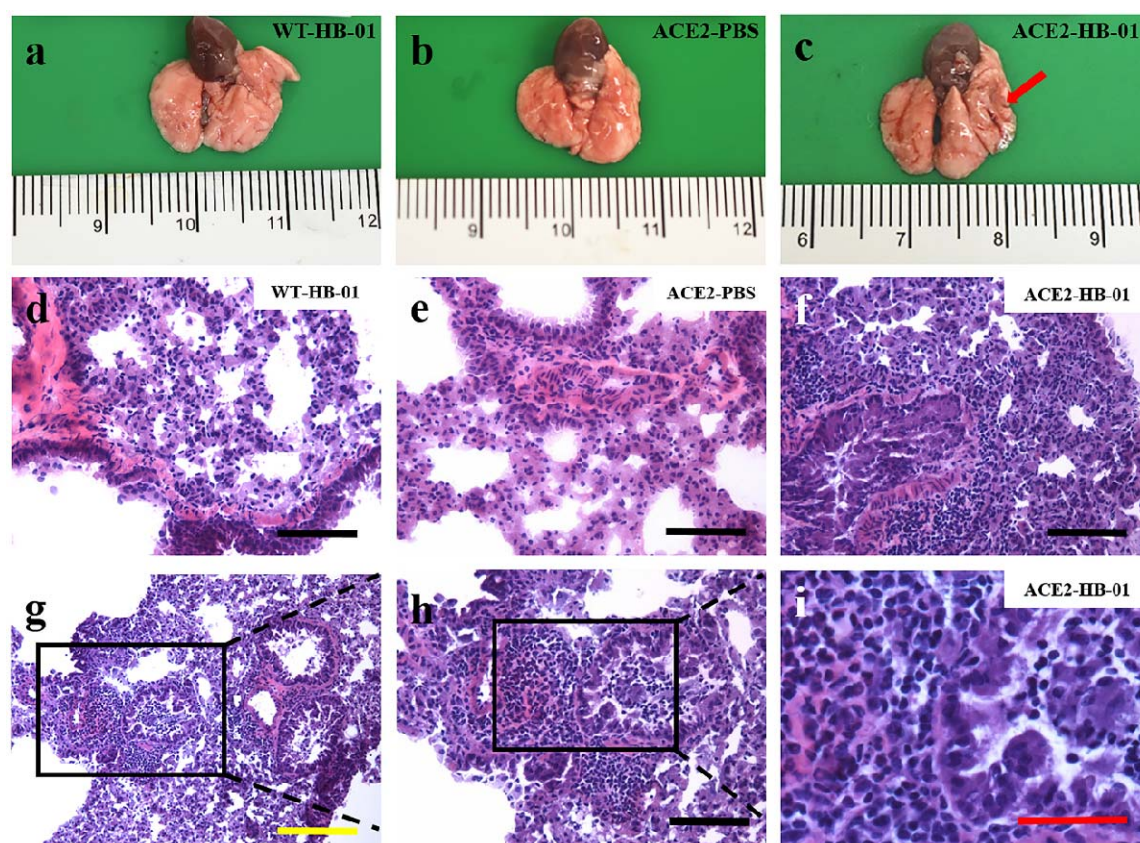
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Figure 2. Gross pathology and histopathology of lungs of 2019-nCoV-infected hACE2 mice. Gross pathology of lungs from infected-WT mice (a), PBS-infected hACE2 mice (b) and 2019-nCoV-infected hACE2 mice (c) at 3 dpi. (d-i) Histological examination of lungs of infected-WT mice (d), PBS-infected hACE2 mice (e), and 2019-nCoV-infected hACE2 mice (f-i). Histopathological examination of bronchioles and blood vessels (f) and alveolar interstitum (g-i) from infected-hACE2 mice. Yellow bar = 200 µm, Black bar = 100 µm, Red bar = 50 µm.
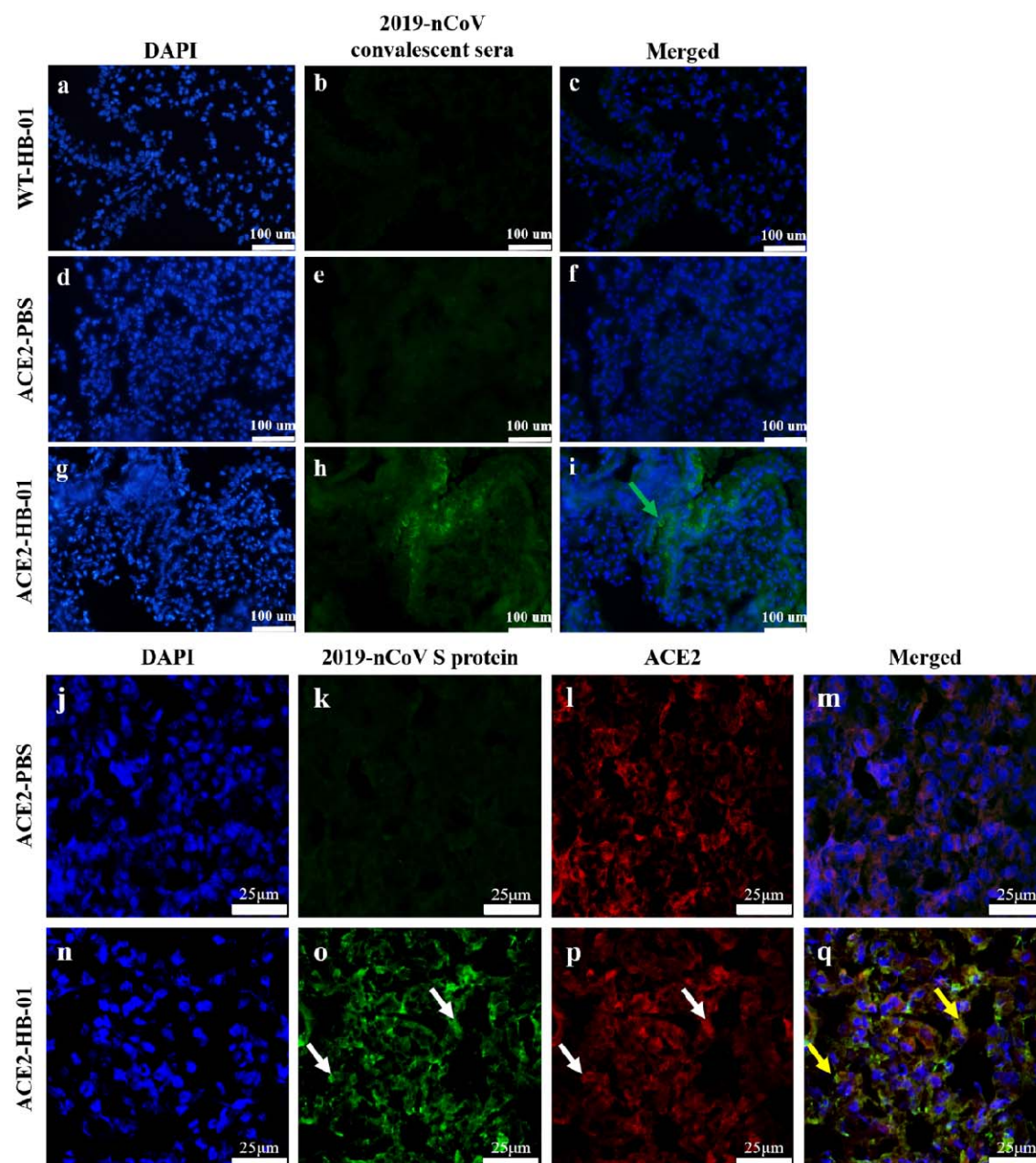
Figure 3. Immunofluorescence analysis of viral antigens in lungs of 2019-nCoV-infected hACE2 mice. (a-i): Fluorescence of sections of mice lungs after incubation with DAPI or antisera of 2019-nCoV-convalescent patients respectively. The lung sections of infected-WT mice (a-c), PBS-infected hACE2 mice (d-f) and infected-hACE2 mice (g-i). Green arrows indicate presence of 2019-nCoV in the alveolar epithelial cells. (j-q): Co-localization of 2019-nCoV S protein and hACE2 receptor in hACE2 mouse lungs, the sections were incubated with DAPI, a polyclonal antibody against 2019-nCoV S protein

245   or human ACE2 protein respectively. The lung sections of PBS-infected hACE2 mice (j-

246   m). The lung sections of infected-hACE2 mice (n-q). The white arrows showed the viral

247   S protein (o) and hACE2 (p) respectively, the yellow arrows showed the merge of viral S

248   protein and hACE2 (q).

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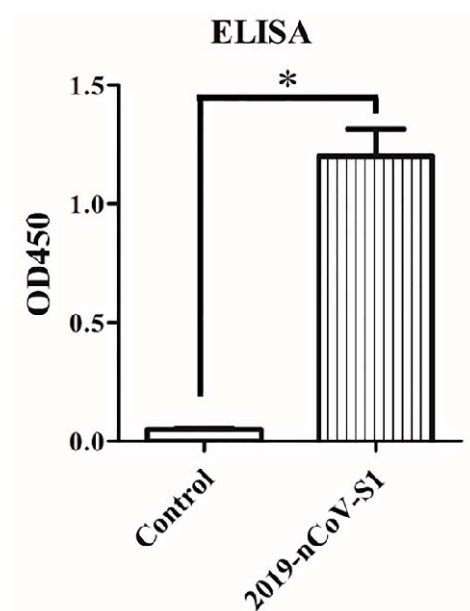
Supplementary Figure 1. Identification of 7D2 antibody against 2019-nCoV S1 protein. The plate coated by 0.2 ug 2019-nCoV S1 protein was incubated with 7D2 antibody as primary antibody (1:200) and detected using HRP-conjugated goat anti-mouse secondary antibody. The titer of antibody was determined using enzyme-linked immunosorbent assay (ELISA) assay. Significant differences are indicated with an asterisk (unpaired t-test, *$p<0.05$*).

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