Early circulating strain of SARS-CoV-2 causes severe pneumonia distinct from that caused by variants of concern in mice transduced with an adenoviral vector expressing human ACE2

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Abstract

To analyze the molecular pathogenesis of SARS-CoV-2, a small animal model such as mice is needed: human ACE2, the receptor of SARS-CoV-2, needs to be expressed in the respiratory tract of mice. We conferred SARS-CoV-2 susceptibility in mice by using an adenoviral vector expressing hACE2 driven by an EF1 α promoter with a leftward orientation. In this model, severe pneumonia like human COVID-19 was observed in SARS-CoV-2-infected mice, which was confirmed by dramatic infiltration of inflammatory cells in the lung with efficient viral replication. An early circulating strain of SARS-CoV-2 caused the most severe weight loss when compared to SARS-CoV-2 variants of concern, although histopathological findings, viral replication, and cytokine expression characteristics were comparable. We found that a distinct proteome of an early circulating strain infected lung characterized by elevated complement activation and blood coagulation, which were mild in other variants, can contribute to disease severity. Unraveling the specificity of early circulating SARS-CoV-2 strains is important in elucidating the origin of the pandemic.

Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is currently causing a worldwide pandemic. Development of animal models that recapitulate coronavirus disease 2019 (COVID-19) are essential for evaluating vaccines and antivirals, and for understanding the pathogenesis of the disease. Although several SARS-CoV-2 animal models have been described to date, such as monkeys, ferrets, and hamsters^{1–6}, mice are considered to be better suited for use as animal models given their small size, rapid breeding cycles, and well-characterized immunological background. However, due to the lack of the viral entry receptor, human angiotensin converting enzyme (hACE2), laboratory mouse strains are non-permissive to the circulating SARS-CoV-2 infection^{7–9}. Several groups have reported mice that either permanently or transiently express hACE2. There are three transgenic mouse models using different promoters to drive permanent hACE2 expression, including a universal cytomegalovirus (CMV) enhancer/beta-actin promoter¹⁰, epithelial cell-specific promoter (K-18 or HFH4)^{11,12} and the endogenous mouse ACE2 promoter¹³. Nevertheless, the production of hACE2 transgenic mice is time-consuming and their versatility is limited as they are restricted to a single genetic background. hACE2 expression in mice can be achieved rather quickly by adeno-associated virus (AAV)- or adenovirus type 5 (Ad5)-mediated transduction. SARS-CoV-2 replication in mice sensitized by AAV-hACE2 transduction appears to be lower than that reported in other mouse models¹⁴. Transduction of mice with CMV promoter-driven hACE2-expressing Ad5 confers SARS-CoV-2 susceptibility, but only mild to moderate pulmonary pathogenesis has been induced by SARS-CoV-2 infection^{15,16}. The induction of an antiviral immune response to the adenovirus vector itself is supposed to have a negative effect on SARS-CoV-2 infection, which may inhibit severe disease in the mouse respiratory tract.

Results

Development of a severe pneumonia mouse model for SARS-CoV-2 infection using an hACE2 expressing adenoviral vector

The commonly used CMV-driven transgene-expressing Ad5 vectors lack the intrinsic viral E1 gene that is essential for adenovirus growth, where a transgene can be inserted. When the expression unit was inserted in the rightward orientation, a viral pIX gene located downstream of the inserted unit was co-expressed with the transgene, and a fusion protein consisting of the N-terminal part of transgene product was expressed. These pIX products may be one of the main causes of adenovirus-induced immune responses. Interestingly, the EF1 α promoter did not activate the pIX promoter in this adenoviral vector¹⁷. The EF1 α promoter with a leftward orientation resulted in a reduced antiviral response and maintained prolonged transgene expression¹⁷. Thus, we generated an adenoviral vector expressing hACE2 under the EF1 α promoter with a leftward orientation (rAd5 pEF1 α -hACE2-L) (Fig. 1a). We first examined whether intranasal administration of rAd5 pEF1 α -hACE2-L affects the body weight of BALB/c mice, and found that administration at 1×10^7 , 5×10^7 or 2.5×10^8 focus forming units (FFU) per mouse did not cause any decrease in body weight (Fig. 1b). The expression of hACE2 in the lungs of mice inoculated with rAd5 pEF1 α -hACE2-L was examined immunohistochemically using antibodies against hACE2 (Supplementary Fig. 1). In both 1×10^7 and 5×10^7 FFU administration groups, hACE2 expression was observed in alveolar macrophages at 1 to 3 days post infection (dpi), and in the type I and II alveolar epithelial cells thereafter. This expansion of hACE2 expression was seen rapidly in the 5×10^7 FFU group, with hACE2 expression observed throughout the tissue at 5 dpi and maintained until 6 dpi. In the 2.5×10^8 FFU group, hACE2 expression was observed in both alveolar macrophages and epithelial cells even at 1 dpi and was maintained up to 6 dpi. However, marked simultaneous thickening of the alveolar wall was observed in this administration group.

Next, BALB/c mice were intranasally administered with rAd5 pEF1 α -hACE2-L at 1×10^7 , 5×10^7 or 2.5×10^8 FFU/animal. Five days after administration, mice were further intranasally inoculated with an early circulating strain of SARS-CoV-2 isolated in Japan (originated from Wuhan; Wu-2020 strain) at 1×10^5 plaque forming units (PFU) per mouse (Fig. 1c). As a result, the 5×10^7 FFU rAd5/mouse group showed clinical symptoms which manifested as ruffled fur, reduced activity, and marked reduction in body weight, which peaked at 5-6 dpi with SARS-CoV-2 Wu-2020. Although the 2.5×10^8 FFU rAd5/mouse group showed weight loss until 4 dpi of SARS-CoV-2 infection, this group showed recovery of body weight after 5 dpi. The 1×10^7 FFU rAd5/mouse group showed a slight reduction of weight followed by rapid recovery. Therefore, we determined that 5×10^7 FFU rAd5/animal was the most suitable dose for assessing disease severity. Comparison of low (1×10^4 PFU/mouse) and high (1×10^5 PFU/mouse) titers of SARS-CoV-2 infection demonstrated that weight loss was more apparent in the high-titer group, approximately 25% maximum (Fig. 1d). Dark red and brown lesions were observed on the surfaces of lungs infected with 1×10^5 PFU of SARS-CoV-2 at 7 dpi (Fig. 1e). Histopathologically, SARS-CoV-2 infected lungs (1×10^5 PFU/mouse) showed severe pneumonia characterized initially by thickened epithelium (2 dpi) and further with profound infiltration of leukocytes including macrophages, neutrophils and lymphocytes,

hemorrhaging and increased blood coagulation (4 to 7 dpi) (Fig. 1f and Supplementary Fig. 3). The expression of SARS-CoV-2 nucleoprotein (N) was evident on alveolar epithelial cells at 2 dpi, but attenuated from 4 to 7 dpi (Fig. 1f). In the mock group, hACE2 expression in the alveolar epithelial cells persisted up to 7 dpi, but in the SARS-CoV-2 infected group, the hACE2-expressing cells disappeared at 4 dpi and were almost completely depleted at 7 dpi (Fig. 1f).

Comparison of pathogenesis of SARS-CoV-2 variants in the mouse model

Variants of concern (VOCs) have emerged showing evidence of altered virus characteristics¹⁸. VOCs have been associated with increased transmissibility, evasion of immunity from infection and vaccination, and reduced susceptibility to antibody therapies^{19–21}. Large population studies have observed a significant trend toward increased mortality associated with B.1.1.7 (Alpha)^{22,23}. We studied the pathogenesis of VOCs [QHN001 (B.1.1.7, Alpha), TY7-501 (P.1, Gamma) and TY8-612 (B.1.351, Beta)], which were clinically isolated in Tokyo. We first examined the growth characteristics of these VOCs and the Wu-2020 strain in Vero E6/TMPRSS2 cells²⁴ at a multiplicity of infection (MOI) of 0.001, and found that the growth kinetics were almost comparable among strains (Fig. 2a). To analyze their replication in mouse lungs, we inoculated the rAd5 pEF1 α -hACE2-L administered BALB/c mice with SARS-CoV-2 via the intranasal route. Viral replication in the lungs was examined by quantitative real-time RT-PCR (qRT-PCR) for the detection of SARS-CoV-2 genome, and by plaque assay using Vero E6/TMPRSS2 cells. A clear increase in viral replication was observed with a peak at 2 dpi in all strains (Fig. 2b and 2c), followed by a gradual decrease towards 7 dpi. The Beta strain showed reduced genome copy numbers (significantly at 7 dpi), but other strains showed comparable genome copy numbers throughout the course of infection. Macroscopically, in Wu-2020- and Alpha strain-infected lungs, multiple dark red and brown lesions appeared on the surfaces of all lung lobes from 4 to 7 dpi (Fig. 2d, arrows). In contrast, the discolored lesions were restricted to the upper left lobe in Gamma and Beta strain-infected lungs at 7 dpi. Mice inoculated with all but the Beta strain of SARS-CoV-2 exhibited clinical signs of ruffled fur, reduced activity and loss of body weight. The loss of body weight in mice did not correspond to the extent of viral replication and lung lesions (Fig. 2e). The Beta strain, an exception, showed less viral replication and lesions in the lungs, so the degree of weight loss was lower throughout the course of infection. Although viral replication was similar in the Alpha and Gamma strains, the appearance of lung lesions was more pronounced in Alpha, however, weight loss was relatively more pronounced in Gamma. Mice infected with all four strains continued to lose weight until 5 dpi, but mice infected with three VOCs began to recover thereafter. Though viral replication and the extent of lung lesions were comparable in Alpha- and Wu-2020-infected mice, mice infected with Wu-2020 did not regain weight, resulting in significant weight loss at 7 dpi compared with mice infected with VOCs (Fig. 2e). To examine whether viral replication and clinical manifestations by infection with SARS-CoV-2 Wu-2020 and VOCs were dependent on the exogenously expressed hACE2, we prepared an adenovirus vector that expresses β -galactosidase (LacZ) instead of hACE2 (rAd5 pEF1 α -LacZ-L)²⁵. BALB/c mice were administered with rAd5 pEF1 α -LacZ-L at 5×10^7 FFU/animal, and five days later, they were inoculated with SARS-CoV-2 Wu-2020, Alpha, Gamma and Beta at 1×10^5 PFU/animal. The Wu-2020- and Alpha-infected mice showed no weight loss, while the

Gamma- and Beta-infected mice showed slight weight loss at 3 dpi, but recovered quickly (Supplementary Fig. 2a). Infectious SARS-CoV-2 was detected in the lungs of Alpha-, Gamma-, and Beta-infected mice at 2 dpi, but it eventually disappeared more rapidly than in animals inoculated with rAd5 pEF1 α -hACE2-L (Supplementary Fig. 2b). Proliferation of these viruses in the lung did not appear to lead to the development of clinical symptoms.

Next, we performed histopathological analysis of Wu-2020, Alpha, Gamma, and Beta-infected lungs in rAd5 pEF1 α -hACE2-L inoculated mice, and found little difference among these four strain-induced diseases (Fig. 3a). In all SARS-CoV-2-infected lungs, thickening of the alveolar wall was observed at 2 dpi, where the SARS-CoV-2 N expression was specifically detected. The differences in the distribution of viral antigens and host cell specificity among virus strains were not clear. Viral antigens then began to diminish by 4 dpi and almost disappeared by 7 dpi in all virus strains. Increases in inflammatory cell infiltration, as well as hemorrhaging and blood coagulation were observed in all strain-infected lungs at 4 and 7 dpi (Fig. 3a and Supplementary Fig. 3).

Cytokine expression in the SARS-CoV-2-infected lung

Next, we examined cytokine expression in the lung using a multiplex bead array. Inflammatory cytokine, such as IL-6, was significantly elevated in all strains compared to mock-infected animals (Fig. 3b). Furthermore, IL-1b, IFN-g, IL-12, MIP-1b, MIP-2, LIF, KC, IL-10, MCP-1, M-CSF, G-CSF, and GM-CSF were significantly elevated and VEGF was decreased, at least in a strain compared to mock-infected mice. In Beta-infected lungs, the expression of some cytokines, such as MIP2, LIF, and MCP-1, was relatively low compared to other strains. This is correlated with the manifestation of low viral replication and mild weight loss. For infection by other strains, there was no clear evidence of a relationship between cytokine levels and disease severity.

Proteome analysis of SARS-CoV-2-infected lungs

The clinical severity of COVID-19 is not always associated with increased levels of pro-inflammatory cytokines and other inflammation markers²⁶. To survey the molecules associated with disease severity, tandem mass tag (TMT) peptide labeling, combined with mass spectrometry (MS) quantitative proteomics in mouse lung at 7 dpi with SARS-CoV-2 was performed. The TMT-based quantitative proteomic method was approved previously for comparison of protein levels across multiple organs in human COVID-19 autopsy cases²⁷. TMTpro 12-plex MS revealed distinct lung proteomes associated with infection by SARS-CoV-2 strains (Fig. 4a and Supplementary Fig. 4a). Gene ontology (GO) enrichment analysis of significantly ($p < 0.05$) up- and down-regulated (2-fold) proteins showed that the proteome of Wu-2020-infected lung was distinct from those of other variants (Fig. 4b). Immune response-related factors, such as regulation of complement activation, immune effector process, as well as platelet degranulation and regulation of blood coagulation, were enriched in the proteins that changed significantly in the proteome of Wu-2020-infected lungs (Fig. 4b). In contrast, the proteomes of VOC-infected lungs were associated with structural organization, such as the development of extracellular

structures and changes in matrix organization, as well as nuclear DNA replication. Up-regulation of proteins associated with complement activation, e.g., C3a anaphylatoxin chemotactic receptor, complement components and complement factors was prominent in Wu-2020-infected lungs (Fig. 4c). The complement system has been shown to be involved in the severity of human COVID-19^{28,29}. Up-regulation of proteins involved in platelet degranulation and blood coagulation, e.g., kininogen, fibronectin, plasminogen activator inhibitor-1, coagulation factor XII and plasma kallikrein, was also remarkable in Wu-2020-infected lung tissue (Fig. 4d and f). These factors are considered to work in concert and contribute to COVID-19 pneumonia via dysregulation of thrombus formation. Up-regulation of minichromosome maintenance complex component (MCM)2, 3, 4, 5, 6, and 7, which are related to nuclear DNA replication, was observed in SARS-CoV-2 infection, regardless of strain (Fig. 4g). MCM2-7 act as replicative DNA helicases that unwind the DNA duplex template as a hetero-hexameric complex³⁰. The involvement of the MCM family in immune responses against viral infection is still poorly characterized. However, MCM up-regulation is correlated with proliferation and maintenance of leukocytes^{31,32}, suggesting that the MCM family is involved in the activation of infiltrating cells in the COVID-19 pneumonia observed in lungs infected with all of the virus strains. Structural organization-related proteins, such as collagens, were down-regulated in VOC-infected lungs (Fig. 4e). Collagen deposition is a hallmark of lung fibrosis³³ and has been confirmed in the lungs of COVID-19 patients³⁴. It is considered that collagen deposition may be correlated with mild disease onset based on the recovery of body weight in VOC-infected mice. Pathway analysis based on Wikipathways (<https://www.wikipathways.org/index.php/WikiPathways>) supports the enrichment of complement and coagulation cascades, as well as the blood-clotting cascade, in Wu-2020-infected lungs (Supplementary Fig. 6).

Discussion

We established a system to recapitulate COVID-19-like pneumonia in mice infected with SARS-CoV-2 after inducing hACE2 with rAd5 pEF1 α -hACE2-L. When rAd5 pEF1 α -hACE2-L was used, there were few abnormalities in protein expression (Supplementary Fig. 4a), suggesting that this adenoviral vector has low cytotoxicity. Mice infected with the Wu-2020 strain developed diffuse pneumonia. Histopathologically, thickened alveolar walls, hemorrhaging, and infiltration of inflammatory cells were prominent. The SARS-CoV-2 N antigen was found in alveolar epithelial cells early in the course of the disease, but decreased as the pneumonia progressed. These immunohistopathological findings appear to be similar to human COVID-19 autopsy cases in early 2020^{35–37}.

Although the viral vector-mediated hACE2 delivery system results in mild clinical signs of SARS-CoV-2 infection compared to those of K18-hACE2 Tg mice³⁸, it has the advantage of rapidly sensitizing various laboratory mouse strains, leading to a reduction in costly and time-consuming genetic manipulation procedures for transgenic animals. The present mouse model using rAd5 pEF1 α -hACE2-L exhibited more severe weight loss and pulmonary pathogenesis after SARS-CoV-2 early circulating strain inoculation than the previously reported COVID-19 mouse model using CMV promoter-driven hACE2 expressing

adenoviral or AAV vectors^{15,16,39}. This model has the potential to accelerate the pace of research into viral replication and pathogenicity *in vivo* and facilitate the development of vaccines and therapeutics.

SARS-CoV-2 Wu-2020, an early circulating strain, was shown to be highly pathogenic in mouse lung. The Wu-2020, Alpha and Gamma strains have comparable replication potentials in both Vero E6/TMPRSS2 cells and mouse lung (Fig. 2a-c). In addition, these strains induced a marked cytokine response (Fig. 3b), and infection led to similar histopathological findings (Fig. 3a and Supplementary Fig. 3). However, there was a clear difference in the lung proteome (Fig. 4b) between the Wu-2020 strain, which induced prolonged weight loss, and the other strains, which induced weight loss followed by recovery (Fig. 2e). The findings showed that proteins involved in the complement system were elevated most markedly in cases of Wu-2020 infection (Fig. 4b and c). The release of proinflammatory complement peptides helps to recruit leukocytes to the lung and aids in the assembly of the terminal complex that damages vascular endothelium^{28,29,40}. Increased levels of complement fragments is related to disease severity in COVID-19 patients^{28,41}, which suggests that they are well suited for use as a marker for serious injury in Wu-2020-infected lung. The altered blood coagulation system, which is manifested by the up-regulation of thrombosis-associated proteins, such as tissue factor (TF), coagulation factor XII and plasma kallikrein (Fig. 4f), can also be involved in disease severity in Wu-2020-infected lungs. TF initiates the extrinsic coagulation pathway to form thrombin in response to tissue injury and inflammation^{42–45}. Coagulation factor XII is activated by polyphosphates released from platelets, and initiates an intrinsic coagulation cascade.^{46–48}, which occurs with disease onset in acute respiratory distress syndrome⁴⁹. Factor XII also activates plasma kallikrein, thereby increasing the formation of the proinflammatory peptide bradykinin⁵⁰. Simultaneously, several inhibitory factors for complement activation, such as complement factor H and vitronectin (Fig. 4c), and for coagulation, such as plasminogen which activates fibrinolysis (Fig. 4d), were up-regulated in Wu-2020-infected lungs; these factors may play a role for prevention of excessive tissue injury. These results indicate that molecular events in pneumonia lesions are altered in ways that cannot be observed by morphological observation. Even in these cases, the lung damage associated with elevated proteins that are related with complement activation, platelet degranulation and blood coagulation may result in the manifestation of severe symptoms, such as unrecovered weight loss after Wu-2020 infection.

In this study, levels of pro-inflammatory cytokines could not be used as markers of disease severity. Rather, the findings showed that complement-related and blood coagulation factors may be key factors associated with COVID-19 severity. In addition, we observed 35-fold and 9-fold up-regulation of metallothionein-2 (Mt2) and Mt1, respectively, in Wu-2020-infected lung (Supplementary Fig. 5). Mt1/2, which are potently induced by heavy metals, other sources of oxidative stress and cytokines, facilitate metal binding and detoxification⁵¹. In response to GM-CSF, macrophages express Mts (Mt2 rather than Mt1), which are involved in antimicrobial responses and contribute to the production of reactive oxidative species⁵². We observed a correlation between disease severity and Mt1/2 amount, suggesting that Mt1/2 may act as a marker for COVID-19 severity. Additionally, we identified other potential biomarkers that may be correlated with disease severity, including tenascin, membrane-spanning 4-domains subfamily A

member 6C and stefin-1/3 (Supplementary Fig. 5), whose associations with COVID-19 have not been studied to date. Furthermore, abundance of the SARS-CoV-2 N protein in lungs infected with Wu-2020 was markedly higher than that in other strains (Supplementary Fig. 5). SARS-CoV-2 N protein has been shown to promote NLRP3 inflammasome activation⁵³, and it is possible that SARS-CoV-2 N protein remaining in the lung may stimulate excessive inflammation. The amount of residual SARS-CoV-2 N protein in lesions may also be indicative of lung injury. It is possible that a comparison of SARS-CoV-2 strains that exhibit different pathogenicity may reveal the existence of novel biomarkers for disease severity.

Intrinsic etiological differences in VOCs have not been demonstrated. Although the Alpha, Gamma, and Beta strains do not show increased pathogenicity in hamsters^{54–57} or rhesus macaques⁵⁸, Alpha and Beta strains showed high pathogenicity in the K-18 hACE2 Tg mouse model^{59,60}. Our findings revealed that there is a difference in the manifestation of symptoms associated with SARS-CoV-2 strains, and that an early isolated strain was highly pathogenic in the lung. The major difference between our findings and previous studies is that the respiratory-specific pathogenesis of SARS-CoV-2 was recapitulated in the mouse model using rAd5 pEF1α-hACE2-L in this study. SARS-CoV-2 infection involves extra-respiratory manifestations, including cardiac, gastrointestinal, hepatic, renal, and neurological symptoms. Disease severity in K-18 mice infected with Alpha and Beta strains may be due to these extra-respiratory symptoms, as shown by the presence of neurological pathogenesis^{61,62}. Comparative studies of human autopsy cases of each variant have not yet been performed. Some autopsy cases of patients infected with the Alpha and Gamma strains revealed no significant morphological or histopathological differences compared to early circulating strains^{63,64}. However, our findings showed that the elevation of several potent biomarkers may affect lung pathogenesis. Understanding the overall differences in an organ's proteome can help to unravel the pathology of emerging variants. With the accumulation of autopsy cases infected with VOCs, these changes in pathologies will be revealed.

In conclusion, we demonstrated that an early circulating SARS-CoV-2 specifically induces the manifestation of severe symptoms and is associated with dramatically altered host responses. How pathogenicity was transformed from the initial strains to that observed in the VOCs needs to be elucidated. Detailed analyses of the pathogenicity of the early circulating strains will lead to a better understanding of the origin of the pandemic.

Methods

Ethics statement

All experiments using mice were approved by the Tokyo Metropolitan Institute of Medical Science Animal Experiment Committee and were performed in accordance with the animal experimentation guidelines of the Tokyo Metropolitan Institute of Medical Science.

Cells and viruses

Vero E6/TMPRSS2 cells, which constitutively express human TMPRSS2²⁴, and human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, and G-418 (1 mg/mL, only in VeroE6/TMPRSS2 cells). All cells were cultured at 37°C in 5% CO₂.

SARS-CoV-2 early circulating strain (TY/WK-521) and VOCs [QHN001 (B.1.1.7 Alpha strain, GISAID ID: EPI_ISL_804007), TY7-501 (P.1, Gamma strain, GISAID ID: EPI_ISL_833366) and TY8-612 (B.1.351, Beta strain, GISAID ID: EPI_ISL_1123289)] were obtained from the National Institute of Infectious Diseases, Japan (passage 1). A passage 2 of SARS-CoV-2 was used in all study that was generated in Vero E6/TMPRSS2 cells by infecting viruses with the passage 1 at an MOI of 0.001. Cell culture media was harvested at 2 to 3 dpi. The sequence of the S gene of all stock viruses was confirmed to be similar to those annotated in the GISAID database. All replication-competent SARS-CoV-2 experiments were performed in a biosafety level-3 (BSL-3) laboratory in the Tokyo Metropolitan Institute of Medical Science.

Generation of rAd5 pEF1α-hACE2-L

E1- and E3-deleted adenovirus derived from human adenovirus type 5 encoding expression units with a leftward orientation were used in this study, as described previously^{17,25}. The hACE2 gene was cloned in the antisense orientation into pAxCawtit2, the adenoviral cosmid vector that contains the left end of adenovirus type 5 with the E1 region substituted with an expression cassette containing the EF1α promoter and a multicloning site using an Adenovirus Dual Expression Kit (Takara Bio, Tokyo, Japan). The rAd5 pEF1α-hACE2-L was generated by transfecting pAxCawtit2 encoding hACE2 into HEK293 cells by using CalPhos Mammalian Transfection Kit (Takara Bio). The rAd5 pEF1α-hACE2-L were purified using two rounds of cesium chloride gradient centrifugation, and the titers of the concentrated and purified virus stocks were determined using HEK293 cells and an Adeno-X Rapid Titer Kit (Takara Bio) according to the manufacturer's instructions.

Plaque formation assay

Vero E6/TMPRSS2 cells in six-well plates were washed with DMED-GlutaMAX, and inoculated with serially diluted SARS-CoV-2, and incubated at 37°C for 60 min with rocking every 15 min. After removing the viruses, cells were washed with DMEM-GlutaMAX and overlaid with agarose medium. After incubation of cells at 37°C for 2 days, the plaques were visualized with crystal violet staining and counted.

Mouse study

Specific pathogen-free 7-8-week old female BALB/c CrSLC mice were purchased from the Japan SLC (Hamamatsu, Japan), housed in a temperature-controlled BSL-3 animal facility in the Tokyo Metropolitan Institute of Medical Science. All of the animals were given free access to food and water and were maintained on a 12 h light/12 h dark cycle.

Mice were intranasally inoculated with 1, 2.5 or 5×10^7 FFU rAd5 pEF1 α -hACE2-L/mouse in a 50 μ L volume. Five days after inoculation, SARS-CoV-2 was intranasally inoculated in a 50 μ L volume. Prior to inoculation, mice were anesthetized by intraperitoneal administration of ketamine-xylazine mixture. Mice were euthanized by exsanguination under isoflurane anesthesia before autopsy for tissue collection.

Viral RNA quantification

The left lung lobe from each mouse was homogenized in nine volumes of Leibovitz's L-15 medium (Thermo Fisher Scientific, Waltham, MA, USA) using a Multi-Bead Shocker (Yasui Kikai, Osaka, Japan). Total RNA samples were extracted from 50 μ L of the supernatant of lung homogenates using Isogen LS (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Fifty nanograms of total RNA was used for quantitating the SARS-CoV-2 genome. Viral RNA was quantified using a 1-step reverse transcription qRT-PCR, as described previously⁶⁵. Viral loads were calculated as copies per 1 μ g of total RNA.

Multiple cytokine expression analyses

The left lung lobes were lysed in lysis buffer (1% Triton-X100, 20 mM EDTA, 50 mM Tris-Cl pH 7.5, 150 mM NaCl) containing cOmplete protease inhibitor (Sigma Aldrich, MO, USA), and were assayed using the Bio-plex Suspension Array System, which utilizes Luminex-based technology. Mouse Cytokine/Chemokine Magnetic Bead Panel (32-plex) was used according to the manufacturer's instructions (Merck KGaA, Darmstadt, Germany).

Immunohistochemistry

The mice lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 4- μ m, stained with HE, and subjected to routine histological examination. Paraffin block sections were also used for staining of the hACE2 and SARS-CoV-2 N protein. Antigen retrieval was performed by autoclaving sections in 10 mM citrate buffer (pH 6.0) for 10 min, and then the sections were immersed in 0.3% hydrogen peroxide in methanol at room temperature for 30 min to inactivate

endogenous peroxidase. The sections were blocked with BlockAce (DS Pharma Biomedical, Osaka, Japan) for 15 min, and incubated overnight at 4°C with 0.2 mg/mL of rabbit anti-ACE2 polyclonal antibody [N1N2] (GenTex, Inc., CA, USA) or 2 mg/mL of rabbit anti-SARS-CoV-2 N protein monoclonal antibody [HL344] (GenTex). Secondary labeling was performed by incubation at RT for 30 min with EnVision+ System-HRP labeled Polymer Anti-Rabbit (Dako Denmark A/S, Glostrup, Denmark), followed by color development with ImmPACT DAB Peroxidase Substrate (Vector Laboratories, Burlingame, CA, USA) at RT for 10 min. Nuclear staining was performed with hematoxylin solution.

TMTpro 12-plex MS analysis

Lysates extracted from left lung lobes were processed and digested by using an EasyPep Mini MS Sample Prep kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Three mouse lungs were pooled, and 25 µg of peptides from each sample were labeled with 0.25 mg of TMTpro mass tag labeling reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. After TMT labeling, the 8 sample channels were combined in equal proportions, dried using a speed-vac, and resuspended in 0.1% TFA. Samples were fractionated into 8 fractions using High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. One microgram of peptide from each fraction was analyzed by LC-MS/MS on an EASY-nLC 1200-connected Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) equipped with an FAIMS-Pro ion mobility interface (Thermo Fisher Scientific). Peptides were separated on an analytical column (C18, 1.6 µm particle size ´ 75 µm diameter ´ 250 mm, Ion Opticks) using 4-hr gradients (0% to 28% acetonitrile over 240 min) with a constant flow of 300 nL/min. Peptide ionization was performed using a Nanospray Flex Ion Source (Thermo Fisher Scientific). FAIMS-Pro was set to three phases (-40, -60, and -80 CV) and a '1 sec cycle for a phase' data-dependent acquisition method was used where the most intense ions in every 1 sec were selected for MS/MS fragmentation by HCD. MS raw files were analyzed using a Sequest HT search program in Proteome Discoverer 2.4 (Thermo Fisher Scientific). MS/MS spectra were searched against the SwissProt reviewed mouse reference proteome (UniProt). TMTpro-based protein quantification was performed using the Reporter Ions Quantifier node in Proteome Discoverer 2.4.

Statistical analysis

Statistical analyses were performed with Prism software (version 9.1.2; GraphPad, San Diego, CA, USA). Statistical significance was assigned when p values were <0.05. Inferential statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Tukey's test or Mann–Whitney *U* test, as appropriate.

Declarations

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Competing interests

The authors declare no competing financial or non-financial interests.

Author Contributions

Y.M., F.Y. and M.K. conceived, designed, coordinated and performed the study, contributed to data interpretation, data presentation and manuscript writing. T.S., T.M., K.Y. and N.Y. assisted with the animal experiments. N.Y. assisted with the qRT-PCR experiments for quantitation of the viral genome. A.T. assisted with the generation of the adenoviral vector. Y.M., A.E., K.Y. and Y.S. performed proteome analyses and contributed to data presentation.

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Figures

Figure 1

Development of a severe pneumonia mouse model following SARS-CoV-2 infection transduced with rAd5 pEF1α-hACE2-L

(a) Schematic diagram of rAd5 pEF1α-hACE2-L. ITR indicates an inverted terminal repeat. Δ E1 and Δ E3 indicate the deletion of E1 and E3 genes from the adenoviral genome. (b) Changes in body weight of rAd5 pEF1α-hACE2-L-infected BALB/c mice. Data represent means and standard deviations (SD), $n=3-18$. (c) Changes in body weight of SARS-CoV-2 (Wu-2020, 1×10^5 PFU/animal)-infected BALB/c mice. Day 0 equals 5 dpi of rAd5 pEF1α-hACE2-L at the indicated FFU/mouse. Data represent means and SD, $n=6$. (d) Changes in body weight of SARS-CoV-2 Wu-2020-inoculated BALB/c mice at the indicated PFU/mouse. Day 0 equals 5 dpi of rAd5 pEF1α-hACE2-L (5×10^7 FFU/animal). Data represent means and SD, $n=6$. (e) Lung macroscopic appearance. (f) Histopathological analyses of Mock- or SARS-CoV-2-infected lungs with Hematoxylin-Eosin (HE) staining and immunohistochemistry using antibody against hACE2 and SARS-CoV-2 N. Scale bars represent 100 μm.

Fig. 2

Figure 2

Fig. 2. Comparison of pathogenesis of SARS-CoV-2 variants in the mouse model

(a) Viral growth in Vero E6/TMPRSS2 cells at an MOI of 0.001. Viral titers are shown in PFU/mL, which were calculated by a standard plaque assay using Vero E6/TMPRSS2 cells. Data represent means and SD, $n=3$. (b) Viral loads in lung homogenate determined by qRT-PCR to detect the SARS-CoV-2 genome.

(c) Viral titers in lung homogenate determined by a plaque assay using Vero E6/TMPRSS2 cells. Data represent means and SD, $n=5$ (2d and 4d) and $n=6$ (7d). * $p<0.05$, ** $p<0.001$ (one-way ANOVA followed by Tukey's test). The colors of the asterisks indicate the following: black (vs Wu-2020) and pink (vs Alpha). (d) Macroscopic appearance of lung. Yellow arrows indicate lesions with a dark red and brown color. (e) Changes in body weight of SARS-CoV-2 variant-infected BALB/c mice. Day 0 equals 5 dpi of rAd5 pEF1 α -hACE2-L (5×10^7 FFU/animal). The p value is shown (Wu-2020 vs mock and all VOCs) (one-way ANOVA followed by Tukey's test). Data represent means and SD, $n=6$.

Figure 3

Histopathological analyses and cytokine levels of mouse lung infected with SARS-CoV-2 variants

(a) Histopathologic findings with HE staining and detection of SARS-CoV-2 N protein in mouse lungs infected with SARS-CoV-2. Scale bars represent 100 μm . **(b)** Left lung homogenates were used for measurement of multiplex cytokines and chemokines using the Bio-plex suspension array system. Data represent means and SD, $n=5$. * $p<0.05$, ** $p<0.01$ (one-way ANOVA followed by Tukey's test). The colors of the asterisks indicate the following: black (vs mock) and gray (vs Wu-2020).

Fig. 4

Figure 4

Proteomic landscape of SARS-CoV-2 infected mouse lungs

(a) Volcano plots for mouse lung proteome of the indicated group compared adenovirus-infected/SARS-CoV-2 non-infected (mock) mice. Up-regulated ($\text{Log}_2 \geq 1$) and down-regulated ($\text{Log}_2 \leq -1$) proteins and p value < 0.05 indicate threshold lines. The numbers of up- and down-regulated proteins are 403 and 411

(Wu-2020 vs Mock), 206 and 289 (Alpha vs Mock), 283 and 236 (Gamma vs Mock), 249 and 189 (Beta vs Mock). The lung lysates used for proteomics were generated by combining three mouse lung homogenates, and the two combined lysates per each group were used for analysis. **(b)** GO enrichment analysis in proteomes of SARS-CoV-2-infected mouse lungs. Top 20 terms of each group are shown. Terms were further categorized in immune response (orange), blood system (pink), DNA replication (green) and structure organization (blue). **(c-g)** Intrinsic relative protein expressions categorized in regulation of immune effector process **(c)**, platelet degranulation **(d)**, extracellular structure organization **(e)**, regulation of blood coagulation **(f)** and nuclear DNA replication **(g)**. X axes indicate relative values shown as fold change when that of mock equals 1. The dashed lines represent the thresholds of up-regulation (>2) and down-regulation (<0.5).

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