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D-Alanine as a biomarker and a therapeutic option for severe influenza virus infection and COVID-19

Shihoko Kimura-Ohba [a,b], Masamitsu N. Asaka [c], Daichi Utsumi [c], Yoshitsugu Takabatake [d], Atsushi Takahashi [d], Yasuhiro Yasutomi [c], Yoshitaka Isaka [d], Tomonori Kimura [a,b,d,*]

[a] Reverse Translational Research Project, Center for Rare Disease Research, National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN), 567-0085, Japan
[b] KAGAMI Project, National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN), 567-0085, Japan
[c] Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN), 305-0843, Japan
[d] Department of Nephrology, Osaka University Graduate School of Medicine, 565-0871, Japan

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ABSTRACT

Since the outbreak of coronavirus disease 2019 (COVID-19), biomarkers for evaluating severity, as well as supportive care to improve clinical course, remain insufficient. We explored the potential of D-amino acids, rare enantiomers of amino acids, as biomarkers for assessing disease severity and as protective nutrients against severe viral infections. In mice infected with influenza A virus (IAV) and in patients with severe COVID-19 requiring artificial ventilation or extracorporeal membrane oxygenation, blood levels of D-amino acids, including D-alanine, were reduced significantly compared with those of uninfected mice or healthy controls. In mice models of IAV infection or COVID-19, supplementation with D-alanine alleviated severity of clinical course, and mice with sustained blood levels of D-alanine showed favorable prognoses. In severe viral infections, blood levels of D-amino acids, including D-alanine, decrease, and supplementation with D-alanine improves prognosis. D-Alanine has great potentials as a biomarker and a therapeutic option for severe viral infections.

1. Introduction

The COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), posed a great threat to public health. Most patients were asymptomatic or had mild to moderate symptoms, whereas a subset of patients developed severe respiratory failure requiring artificial ventilation or extracorporeal membrane oxygenation (ECMO). Since the initial report of cases from Wuhan in 2019, over 590 million people have been infected with SARS-CoV-2 as of August 2022 [1]. The ongoing COVID-19 pandemic was foreshadowed by the Spanish flu in 1918 [2] and the 2009 influenza A virus (IAV) [3]. The pandemic, in association with severe viral infections, has been a great problem thus far.

The management of severe viral infections, including COVID-19 and IAV infection, involves three fundamental steps: early screening for early referral, prediction of prognosis for intensive care, and development of appropriate treatment. Within the last 2 years, diagnostic tests, such as polymerase chain reaction-based systems, have been introduced in clinics for diagnosis of COVID-19. Over 8000 clinical trials were initiated as of August 2022 to develop effective drugs for the treatment of COVID-19 [4]. However, it remains difficult to identify COVID-19 patients at high risk and provide the suitable care [5,6].

There is accumulating evidence supporting the use of D-amino acids in clinics. Amino acids have enantiomers, i.e., L- and D-amino acids, while L-amino acids have long been regarded to be exclusively present in nature. With the development of analytical methods, D-amino acids have been detected in the human body as sensitive biomarkers for several diseases [7–12]. New physiological functions of D-amino acids are also unravelling that may provide a new aspect of therapeutic strategy [13–16].

Abbreviations: COVID-19, coronavirus disease 2019; IAV, influenza A virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; ECMO, extracorporeal membrane oxygenation; 2D-HPLC, two-dimensional high-performance liquid chromatography; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole.

* Corresponding author at: Reverse Translational Research Project, Center for Rare Disease Research, National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN), 567-0085, Japan.
E-mail address: t-kimura@nibiohn.go.jp (T. Kimura).

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We aimed to apply D-amino acid research in the field of viral infections. Severe viral infections may greatly disrupt the homeostasis and potentially affect the dynamics of D-amino acids. D-Amino acids could modulate bacterial infections [17] or be used as a biosensor of bacteria in case of D-alanine-derivatives [18], whereas the significance of D-amino acids in the viral infections is still unresolved. We investigated whether D-amino acids would be useful in determining the disease severity of viral infection, and whether supplementation would improve the clinical condition.

2. Methods

2.1. Observational study

The observational study is a part of a clinical study to evaluate the efficacy and safety of a new virus adsorption therapy in patients with severe COVID-19 infection in Japan (CATCH-COVID, Japan Registry of Clinical Trials identifier: 052200134). This study is a single-arm, multicenter, open-blind trial and includes phase 1 and 2 clinical trials. Inclusion criteria included patient age over 18 years and severe cases of COVID-19 requiring either artificial ventilation or ECMO. As an exploratory study, the levels of D-amino acids were monitored in the patients. Blood samples were harvested in the morning. Patients with end-stage kidney disease were excluded from the analysis since D-amino acids accumulate in the blood owing to the lack of urinary excretion [9,11]. Approval for all facilities was obtained from the Central Ethics Review Committee of Osaka University (#CRB5180007). Reference data are composed of healthy volunteers [9] and living kidney donors before nephrectomy [11]. This study was conducted in compliance with the Declaration of Helsinki and the Ethical Guidelines for Medical Research Involving Human Subjects. Written informed consent for this study was obtained from all participants.

2.2. Animal experiment

To induce severe influenza A virus infection, 1×10^3 $TCID_{50}$ of Influenza A/Puerto Rico/8/34 (PR8, H1N1; ATCC, Manassas, VA, USA) were inoculated to anesthetized four-week-old male C57BL/6 mice (SLC, Tokyo, Japan) through a nostril, mimicking childhood infection. Separately, the treatment effect of D-alanine was analyzed in mice with reduced viral load (1×10^2 $TCID_{50}$). Mice had free access to water containing low dose (0.1 %) of either D-alanine, D-serine (#2801, #2818; Peptide Institute, Ibaraki, Japan), or vehicle from 2 days before infection. Body weight at the time of death was imputed after the event.

SARS-CoV-2 infection experiments were performed as described previously [19]. In the analysis of D-alanine treatment, eight to twelve-week-old adult female and male CAG-hACE2 mice (ACE2 Tg #17; NIBIOHN, Ibaraki, Japan) were injected intraperitoneally with 1 % of D-alanine or vehicle control twice a day (0.2 g/kg per day) from −2 days post infection (dpi). Infection was performed intratracheally to anesthetized mice using 2×10^3 $TCID_{50}$ of SARS-CoV-2 Wuhan strain isolated in Tokyo (UT-NC-GM02; National Center for Global Health and Medicine).

Virus propagation was conducted using Madin-Darby Canine kidney cells (JCRB9029, NIBIOHN) for IAV, and VeroE6/TMPRSS2 cells (JCRB1819, NIBIOHN) for SARS-CoV-2. Viral titers were determined by plaque assay. Histological analyses were performed as previously described with modifications [20].

Blood samples were harvested between 9 and 12 am in mice experiments. All animal experiments were approved by the Animal Research Committee of NIBIOHN and were performed in accordance with the Guidelines for the Japanese Animal Protection and Management Law, and with the approved standard operating procedure of the biosafety level facility.

2.3. Determination of amino acid enantiomers using two-dimensional high-performance liquid chromatography

The preparation of samples and quantification of amino acid enantiomers by a two-dimensional high-performance liquid chromatography (2D-HPLC) system were performed as previously described [21,22]. Briefly, 20-fold volumes of methanol were added to the serum sample and an aliquot (10 μL of the supernatant obtained from the methanol homogenate) was placed in a brown tube. After drying the solution under reduced pressure, 20 μL of 200 mM sodium borate buffer (pH 8.0) and 5 μL of fluorescence labeling reagent (40 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) in anhydrous MeCN) were added, and then heated at 60 °C for 2 min. An aqueous 0.1 % (*v*/v) TFA solution (75 μL) was added, and 2 μL of the reaction mixture was subjected to the 2D-HPLC.

The enantiomers of amino acids were quantified using the 2D-HPLC platform. The NBD-derivatives of the amino acids were separated using a reversed-phase column (Singularity RP column, 1.0 mm i.d. × 50 mm; provided by KAGAMI Inc., Osaka, Japan) with the gradient elution using aqueous mobile phases containing MeCN and formic acid. The fractions of each amino acid were automatically collected using a multi-loop valve, and transferred to the enantioselective column (Singularity CSP-001S, 1.5 mm i.d. × 75 mm; KAGAMI Inc.). Then, D- and L-amino acids were separated in the second dimension by the enantioselective column. The mobile phases are the mixed solution of MeOH-MeCN containing formic acid, and the fluorescence detection of the NBD-amino acids was carried out at 530 nm with excitation at 470 nm using two photomultiplier tubes. Target peaks were quantified by scaling the standard peak shape [23]. The blood levels of relatively abundant four of D-amino acids were analyzed, and the blood levels of D-amino acids were presented as μM or ratios of D-amino acids to total amino acids (%).

2.4. Statistical analysis

Data were expressed as median ± interquartile range or mean ± standard error. Continuous variables between two groups were compared using two-tailed Student's *t*-test or two-way ANOVA followed by Bonferroni's multiple comparison test. Survival analysis was performed by Kaplan–Meier analysis and differences were determined by the log rank test. Statistical significance was defined as $p < 0.05$. STATA 15.0 and GraphPad Prism 9.0 were used for statistical analyses and data visualization.

3. Results

3.1. Blood levels of D-amino acids decrease with severe IAV infection

We first utilized a mouse model of severe influenza A virus (IAV) infection and examined the dynamics of D-amino acids. The mice were nasally infected with a lethal dose of PR8, which caused severe illness or death within a few days. The blood levels of relatively abundant D-amino acids, i.e., D-serine, D-proline, D-asparagine, and D-alanine, were analyzed 2 or 3 days after infection. Blood samples were harvested in the morning to minimize the intra-day variations. The ratios of D-amino acids to total amino acids of all measured D-amino acids were reduced after infection (Fig. 1, and Supplementary Fig. S1A). The reduction ratios were 85.3 % for D-alanine, 43.7 % for D-serine, 45.6 % for D-asparagine, and 58.2 % for D-proline compared to those in uninfected mice. The mice showed a significant reduction in body weight after infection, confirming the severity of the infection (Supplementary Fig. S1B). The blood levels of D-amino acids decreased with severe IAV infection.

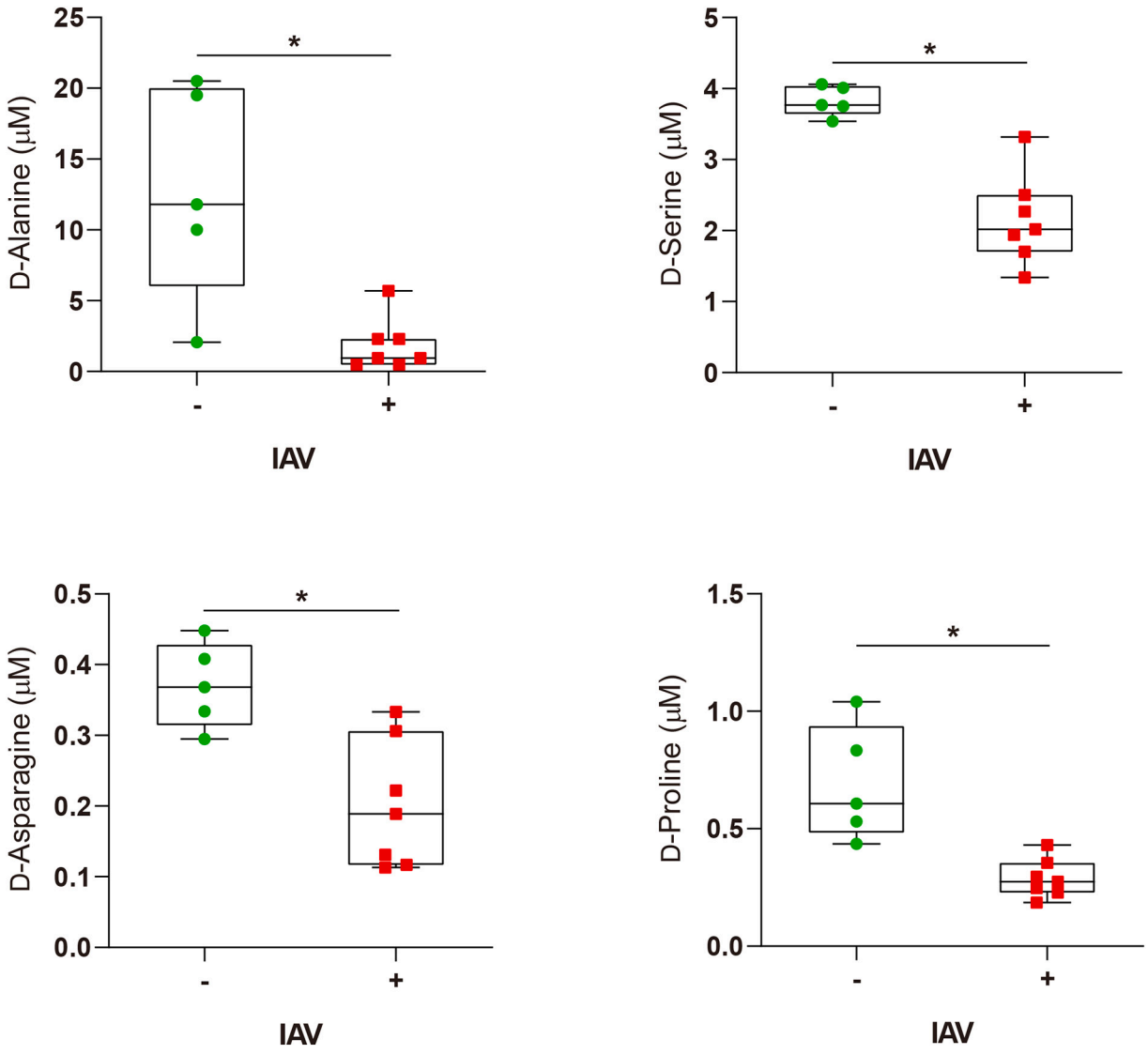

Fig. 1. Effects of influenza A virus (IAV) infection on D-amino acid dynamics in mice. The levels of D-amino acids in serum from mice 2 or 3 days after infection. $n = 5–7$. Data, median ± interquartile range. $^*P < 0.05$.

3.2. Blood levels of D-amino acids decrease in patients with severe COVID-19

To expand these findings to other viral infections, we explored whether D-amino acids also reflect the disease severity of the COVID-19 in human patients. The blood levels of four major D-amino acids were monitored in severe COVID-19 patients with respiratory failure requiring artificial ventilation or ECMO. In the analyzed 5 patients, 2 patients had diabetes mellitus, a risk factor for worse prognosis [24]. The D-amino acids ratios and the blood levels of D-amino acids were lower in patients with COVID-19 than in normal controls, as observed in the mouse IAV infection model (Fig. 2 and Supplementary Fig. S2). Of note, blood levels of L-amino acids behaved differently between IAV mouse model (i.e., low in infected mice) and patients with COVID-19 (i.e., high in patients), suggesting that D-amino acids, but not L-amino acids, are suitable to monitor viral infections.

3.3. Supplementation of D-alanine alleviates the severity of IAV infection in mice

Decreased blood levels of D-amino acids are common phenomena in the studied severe viral infections. We investigated whether supplementation of D-amino acids have protective effects on the course of IAV infection in mouse models. For this purpose, we utilized IAV infection model with reduce viral load. We selected D-alanine as a supplement based on the prominent reduction upon infection with IAV. We also tested D-serine for its relative abundance in blood [8]. To increase the supplementation effects of D-amino acids, the mice were treated with D-amino acids by providing free access to water containing a low dose (0.1 %) of either D-alanine or D-serine from 2 days before infection until the end of the experiment (Fig. 3A). After the preliminary experiment, we selected D-alanine for the analysis, based on its better protective effects against body weight reduction (Supplementary Fig. S3, A–C). The severe body weight reduction observed in IAV infection model mice was mitigated upon supplementation with D-alanine (Fig. 3B). To analyze the correlates of body weight protection, blood and lung samples were harvested at 5 days post-infection. Unlike vehicle-treated mice, histological lesions such as obstructive alveolar spaces, hemorrhage, and immune cell infiltration, were less prominent in the lungs of D-alanine-treated mice (Fig. 3C). D-Alanine treatment reduced the viral titers in the lungs (Fig. 3D).

The effects of D-alanine on survival rate in the studied mice were limited (Fig. 3E). These results were attributed to the variable efficacy of D-alanine supplementation. The blood level of D-alanine increased upon

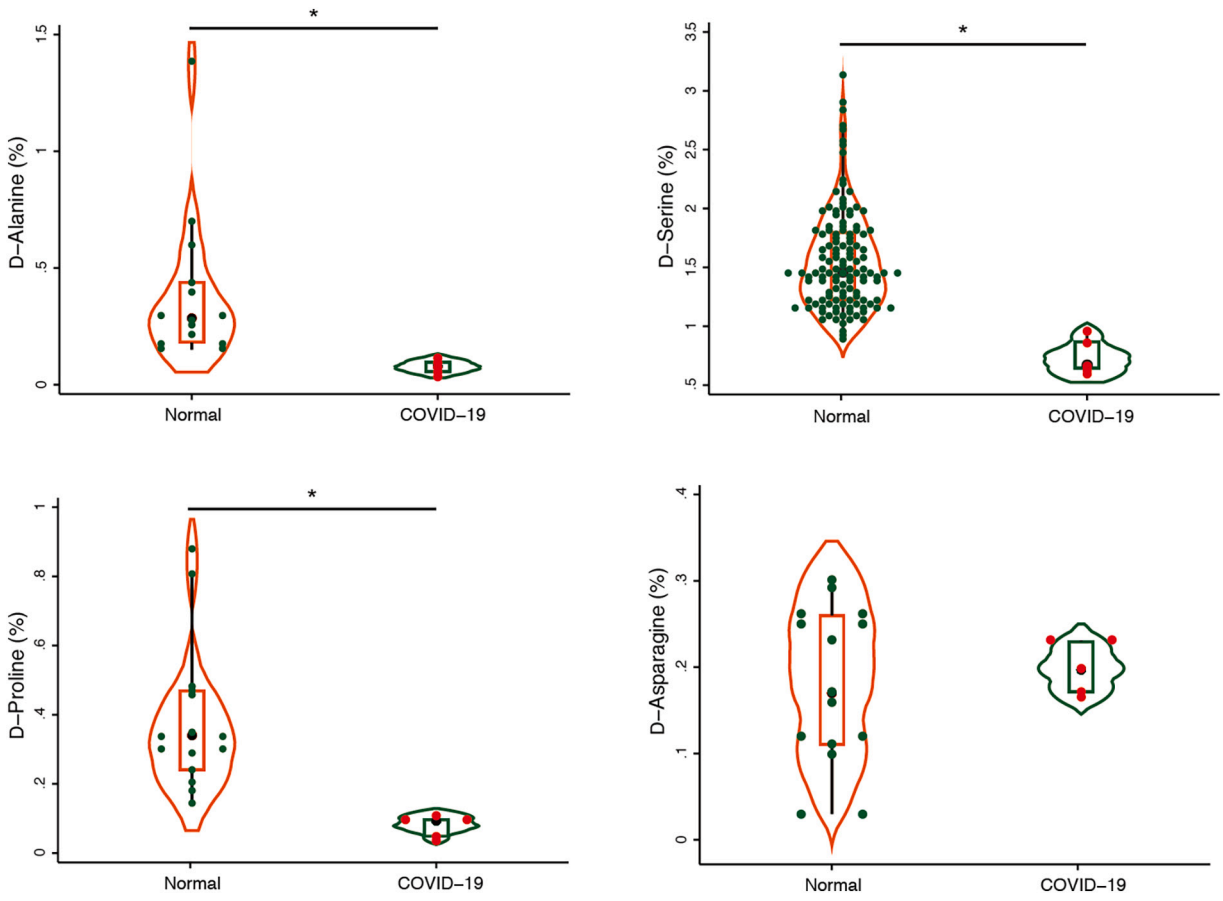

Fig. 2. The levels of D-amino acids in the serum of patients with severe COVID-19. $n = 5$ (COVID-19), 15 (reference in D-Pro, D-Ala and D-Asn) and 60 (reference in D-Ser). Central box, inter-quartile range; whisker, 95th percentile; black dot, median. *$P < 0.05$.

supplementation, with some exceptions, and the level of D-alanine after infection were lower in mice that exhibited a reduction of body weight (Fig. 3F and Supplementary Fig. S4, A and B). Results of the survival analysis confirmed that the mice without elevation of blood D-alanine level in D-alanine treated group exclusively showed body weight reduction (Fig. 3G). Besides D-alanine, lower blood levels of other D-amino acids were associated with body weight reduction after infection (Supplementary Fig. S4B). Overall, D-alanine showed a protective effect against IAV infection, and the blood levels of D-alanine and other D-amino acids also reflected the therapeutic effects.

3.4. *Supplementation of D-Alanine alleviates the severity of COVID-19 mouse models*

To exemplify this protective effect, D-alanine was treated to a mouse model of infection with SARS-CoV-2 Wuhan strain isolated in Tokyo. We utilized CAG-hACE2 mice, which are vulnerable to SARS-CoV-2 infection and show a reduction in body weight between 5 and 7 dpi upon infection with moderate (2×10^3 $TCID_{50}$) or severe (2×10^4 $TCID_{50}$) viral doses (Fig. 4A) [19]. In this model, the blood D-amino acids levels initially increased until 4 dpi, and then decreased on 7 dpi with the change in D-alanine level statistically significant (Fig. 4B). Since the blood levels of D-amino acids decreased as the body weight decreased, the blood D-amino acids levels were also considered to reflect the severity of infection in this model.

Then, we treated moderate SARS-CoV-2 infection model mice with a D-alanine dose of 0.2 g/kg per day (Fig. 5A). To increase the D-alanine level in blood, the mice were injected intraperitoneally with D-alanine. Treatment with D-alanine improved the survival rate and suppressed the reduction in the body weight (Fig. 5B and Supplementary Fig. S5A and B). Albeit statistically significant, the effects of D-alanine on survival rate were limited, potentially because of the variable efficacy of supplementation as seen in IAV model mice. The blood D-alanine level decreased at 6 dpi despite treatment (Fig. 5C and Supplementary Fig. S5C), and lower blood levels of D-alanine and other D-amino acids were associated with a worse prognosis (Fig. 5D and E; Supplementary Fig. S5D). The mice whose blood levels of D-alanine were maintained by supplementation were better in prognoses. In summary, D-alanine was protective against SARS-CoV-2 infection, and the blood levels of D-amino acids reflected the therapeutic effects.

4. Discussion

This study revealed the two-way functions of D-alanine as a biomarker and as a therapeutic agent for severe viral infection. The blood levels of D-amino acids decreased in mice after developing severe viral infection, and these levels were lower than the normal range of D-amino acids in severe human cases of COVID-19. Supplementation with D-alanine improved the prognosis of IAV infection and COVID-19 mouse models, while blood levels of D-amino acids were also associated with the treatment effects. D-Alanine and other D-amino acids are biomarkers that reflect the severity of IAV infection and COVID-19, and supplementation with D-alanine reduces the consequences of severe viral infections.

Low D-amino acid levels in blood have rarely been reported. The amounts of D-amino acids are extremely low in human blood by a few percent than that of L-amino acids [8,25], and the blood levels of D-amino acids further decreased in patients and mice with severe viral infections. The blood D-alanine level fluctuates with circadian rhythm [26], whereas the blood D-alanine level seen in severe viral infections is

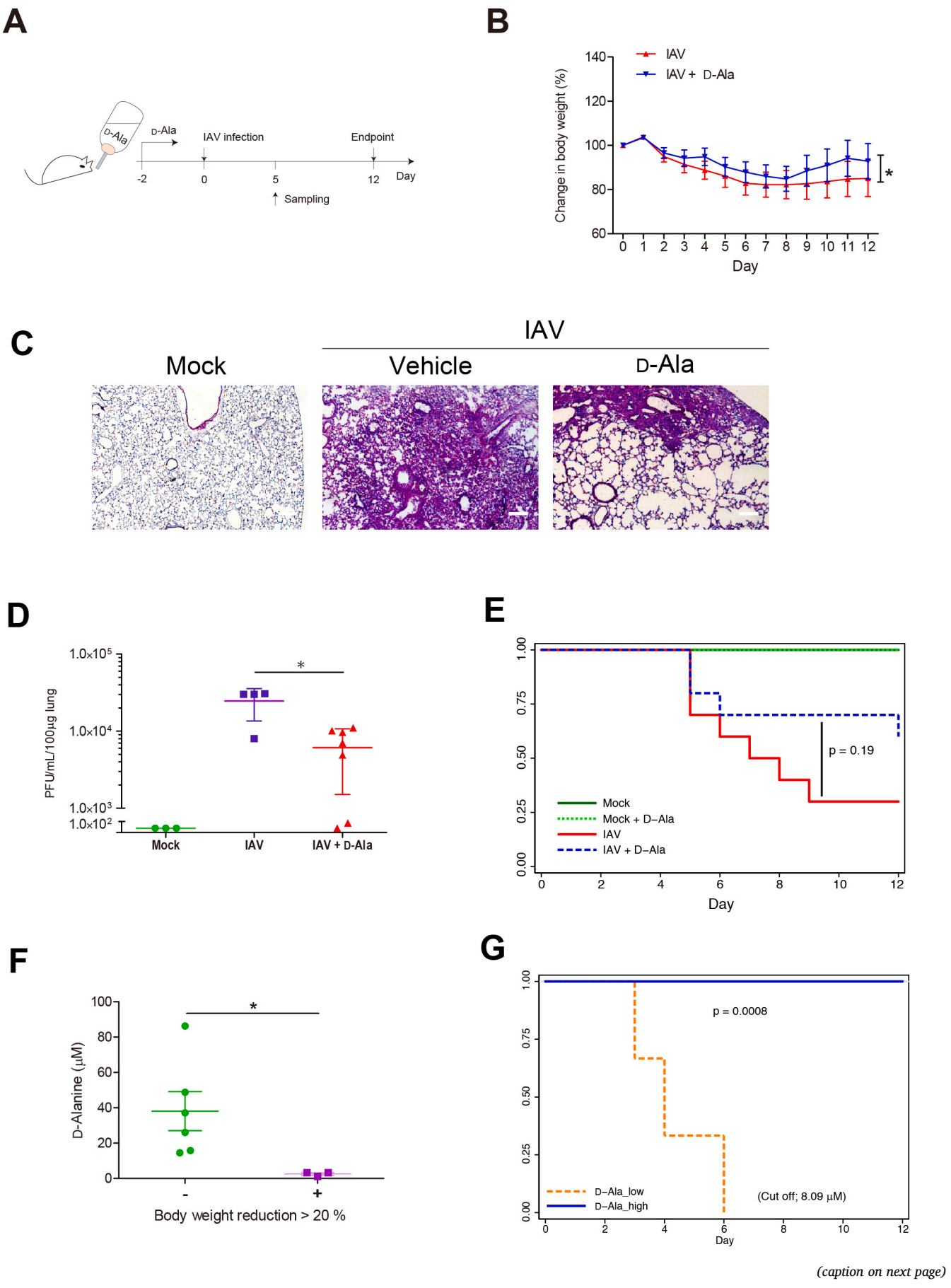

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Fig. 3. Supplementation of D-alanine alleviates influenza A virus (IAV) infection in mice. (**A**) Schematic diagram of experimental schedule. (**B**) Change in body weight of mice models of IAV infection with or without D-alanine. $n = 10$. (**C**) Representative hematoxylin and eosin-stained lung tissues at day 5 after infection. Bars, 200 μm. $n = 3$–5. (**D**) IAV load assessed by plaque quantification assay of homogenized lung tissue at day 5 after infection. $n = 3$–7. (**E**) Kaplan-Meier analysis of the survival rate. $n = 10$ (IAV) and 3 (Mock). (**F**) Blood level of D-alanine at 5 days after infection in D-alanine-treated IAV-infected mice with or without body weight reduction. (**G**) Kaplan-Meier analysis of body weight reduction of >20 % in IAV infected mice with lower (<8.09 μM) or higher (above 8.09 μM) level of blood D-alanine. Data, (**B**) mean ± SEM or (**D**, **F**) median ± interquartile range. $^*P < 0.05$.

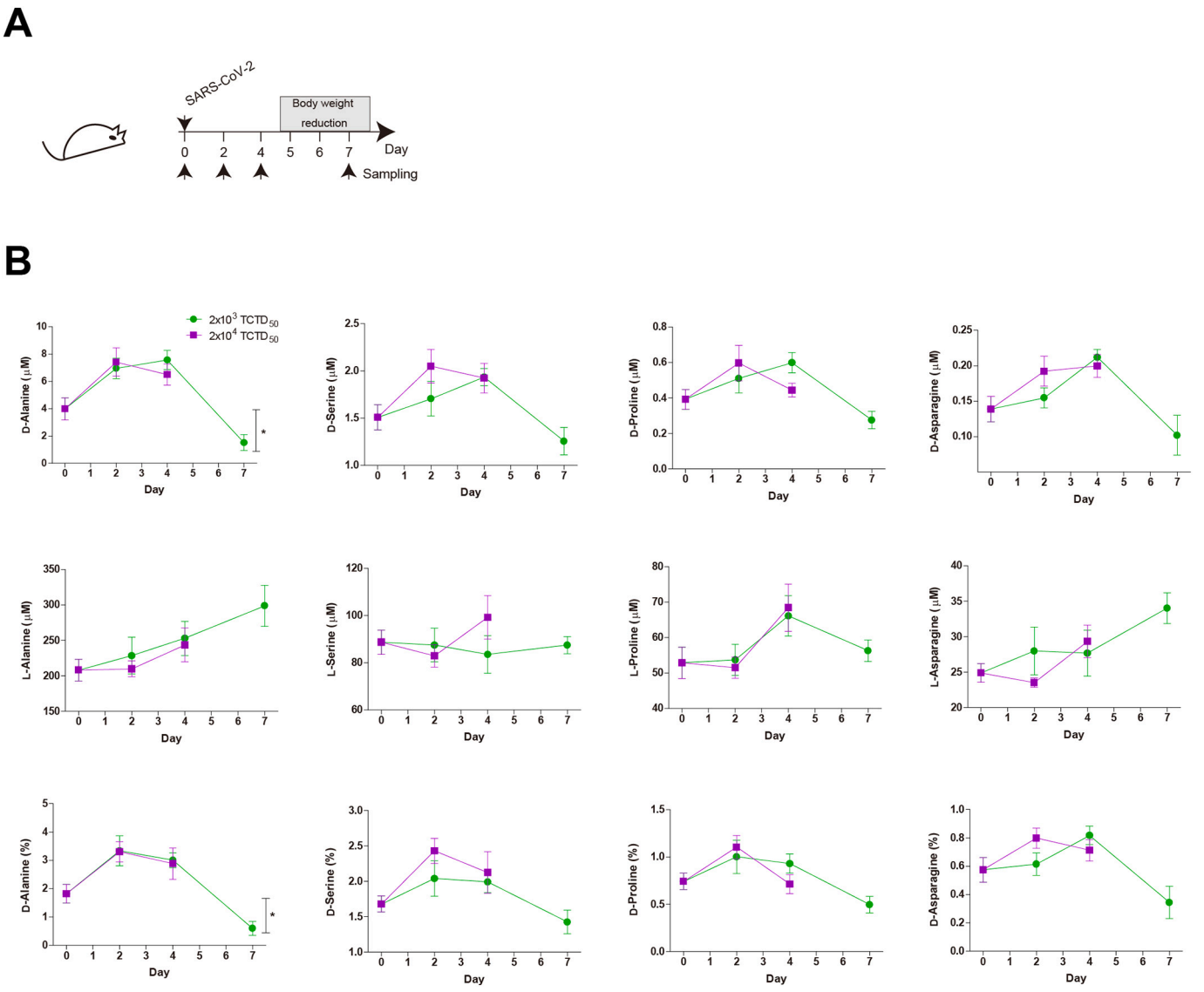

Fig. 4. Effects of SARS-CoV-2 infection on D-amino acid dynamics in mice. (**A**) Schematic diagram of experimental schedule. (**B**) Changes in blood levels of D-amino acids after infection. Of note, blood samples from severe mouse models (2×10^4 $TCID_{50}$) were unobtainable at 7 dpi. $n = 12$. Data, mean ± SEM. $^*P < 0.05$ versus day 0.

much lower than the normal range. In this study, the blood samples were consistently harvested in the morning, and thus, the variations in the levels of D-alanine are derived from the pathological conditions rather than the circadian clock. The reported mean blood levels of D-alanine in daytime and nighttime were approximately 8 and 1.5 μM in mice, and 0.7 and 1.5 μM in human, respectively [27,28]. The median blood levels of D-alanine were 15.7 and 0.95 μM for IAV uninfected and infected mice, and 0.93 and 0.40 μM for normal control and COVID-19 patients, respectively. The low blood level of D-alanine in viral infections likely corresponds to, or is even lower than, its lowest level in the circadian rhythm.

Despite supplementation, it was difficult to maintain the blood D-alanine level during fulminant infection, suggesting the increased consumption. D-alanine may be used for host defense [13] or for the improvement of the systemic condition as indicated by the maintenance of body weight or blood levels of other D-amino acids. The mechanism of D-alanine in the reduction of viral titers and alleviation of the lung lesions in IAV-infected mice remains elusive. Other D-amino acids, including D-serine, could also have the protective effects in viral infections, and the combinational usage of D-amino acids may be beneficial. On the other hand, complication of diabetes may worsen the prognosis of COVID-19 [24], potentially through the aberrant metabolism of D-alanine [10,29]. Further studies will elucidate the mechanisms and effective treatment.

D-Amino acids are natural nutrients present in daily foods or produced from the gut microbiota [13]. In the presence of severe viral

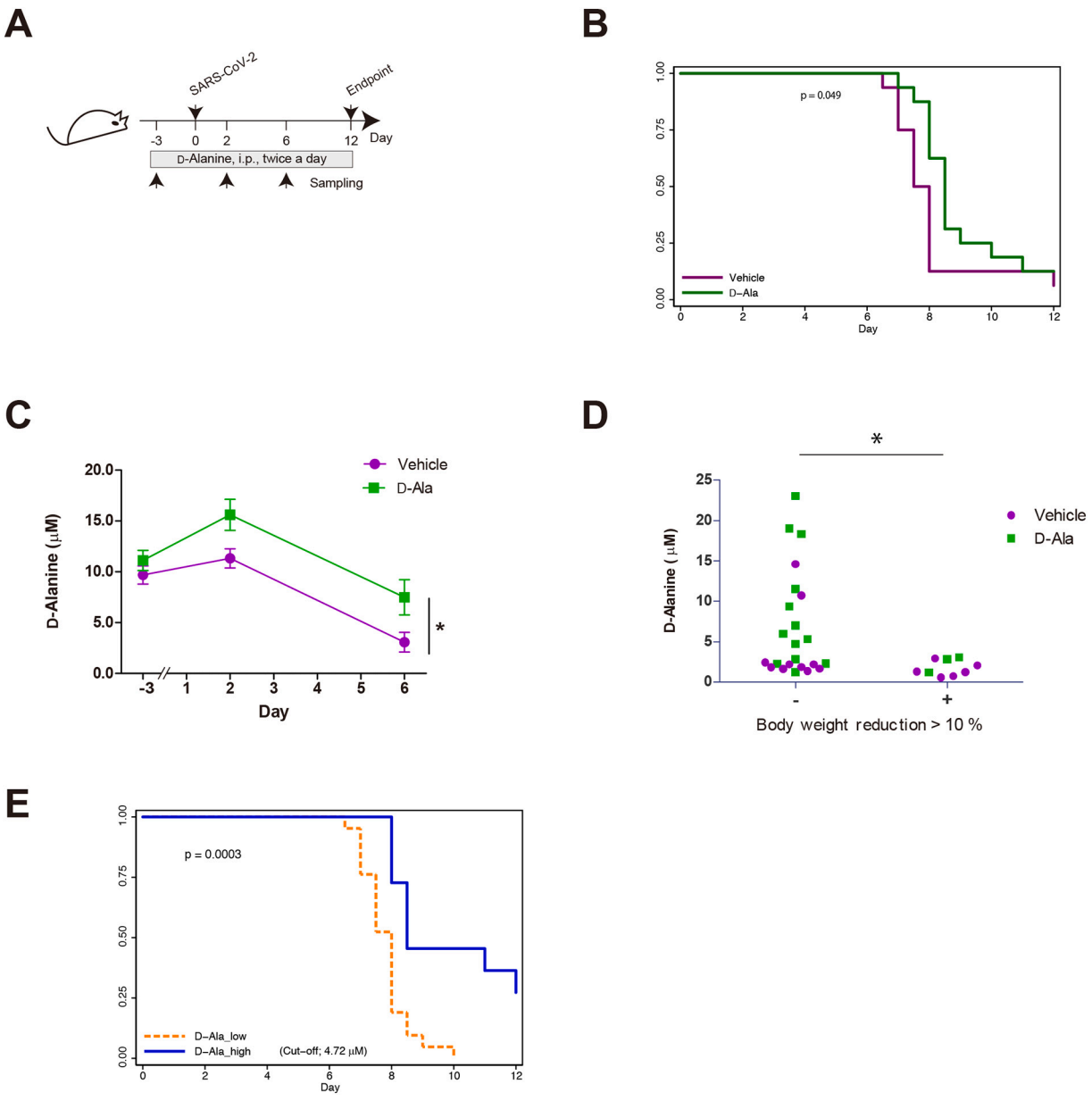

Fig. 5. Supplementation of D-alanine alleviates SARS-CoV-2 infection in mice. (**A**) Schematic diagram of experimental schedule. (**B**) Kaplan-Meier analysis of the survival rate. $n = 16$ for each group. (**C**) Blood levels of D-alanine at indicated days after infection. $n = 16$. (**D**) Blood level of D-alanine at 6 days after infection in vehicle- or D-alanine-treated COVID-19 mouse model with or without body weight reduction. (**E**) Kaplan-Meier analysis of the survival rate in COVID-19 mouse model with lower (<4.72 μM) or higher (above 4.72 μM) level of blood D-alanine at dpi 6. Data, (**C**) mean ± SEM. $^*P < 0.05$.

infection, the intake of D-amino acids from foods is likely insufficient or deregulated microbiota may not produce sufficient D-amino acids. D-Alanine is suitable for the supportive care of viral infections, although its protective effect was limited. This may partially be attributable to the difficulty in maintaining its blood level. The dose of D-alanine should be adjusted to avoid its decline in blood level, whereas excessive amounts of D-amino acids could be biphasic [30]. Monitoring the blood D-alanine level helps adjust the dosing of D-alanine and thus may maximize the supplemental effect of D-alanine. Besides D-alanine, the blood levels of other D-amino acids also reflected severity of viral infections even after supplementation with D-alanine. To monitor the safety, treatment efficacy and activity of infections, the blood D-amino acid levels should be measured upon supplementation of D-alanine.

There are limitations to this study. This study mainly bases preclinical investigations and the effects of D-alanine were tested only in mouse models. The clinical cohort was small and the presence of comorbidity may form a limitation in the interpretation of the results. An ancestral strain of SARS-CoV-2 was used for the COVID-19 model, and the evaluation on new variants of SARS-CoV-2 could be interesting.

The results of this study will provide key information for clinics and public health. Monitoring D-alanine enables the stratification of COVID-19 patients at high risk, rendering the triage of patients and optimization of medical resources. Provision of D-alanine, a basically safe nutrient, as a therapeutic option will facilitate the patient care. For the next pandemic of viral infections, D-alanine could be a therapeutic candidate for the prompt clinical application. Preclinical and clinical studies will be promoted by using D-alanine as a sensitive biomarker. We believe that the dual function of D-alanine, as a biomarker and a therapeutic option for severe viral infections, will be utilized efficiently in the clinical settings and for the public health.

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CRediT authorship contribution statement

Conceptualization, TK; Methodology, SKO, MNA, DU, YY, YI, TK; Investigation: SKO, MNA, DU, YT, AT, YY, YI, TK; Visualization: TK; Funding acquisition: SKO, MNA, DU, YI, TK; Project administration: YY, YI and TK; Supervision: YY, YI and TK; Writing–original draft: TK; Writing–revised draft: SKO and TK.

Declaration of competing interest

TK has an equity in KAGAMI Inc., a startup company for the medical implications of chiral amino acid research. SKO, MNA, DU, YY, YI, and TK are preparing for the patent application, which is related with this work. All other authors declare no competing interests.

Data availability

All of the data analyzed in this study are included in this published article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2022.166584.

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