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Original Article

Angiotensin-(1–7) attenuates SARS-CoV2 spike protein-induced interleukin-6 and interleukin-8 production in alveolar epithelial cells through activation of Mas receptor

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KEYWORDS
ACE;
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Abstract *Background:* SARS-CoV-2 spike proteins (SP) can bind to the human angiotensin-converting enzyme 2 (ACE2) in human pulmonary alveolar epithelial cells (HPAEpiC) and trigger an inflammatory process. Angiotensin-(1–7) may have an anti-inflammatory effect through activation of Mas receptor. This study aims to investigate whether SARS-CoV-2 SP can induce

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COVID-19;
SARS-CoV-2;
Spike protein

inflammation through ACE2 in the alveolar epithelial cells which can be modulated through angiotensin-(1—7)/Mas receptor axis.
Methods: HPAEpiC were treated with SARS-CoV-2 SP in the presence or absence of ACE2 antagonist-dalbavancin and Mas receptor agonist-angiotensin-(1—7). Proinflammatory cytokine production (IL-6 and IL-8) were measured at mRNA and protein levels. MAP kinase phosphorylation and transcription factor activation was determined by Western Blot. Mas receptor was blocked by either antagonist (A779) or knockdown (specific SiRNA). Experiments were replicated using A549 cells.
Findings: SARS-CoV-2 SP (5 μg/mL) significantly induced MAP kinase (ERK1/2) phosphorylation, downstream transcription factor (activator protein-1, AP-1) activation and cytokine production (IL-6 and IL-8) at both mRNA and protein levels. Pretreatment with dalbavancin (10 μg/mL), or angiotensin-(1—7) (10 μM) significantly reduced ERK1/2 phosphorylation, AP-1 activation, and cytokine production. However, these angiotensin-(1—7)-related protective effects were significantly abolished by blocking Mas receptor with either antagonist (A799,10 μM) or SiRNA knockdown.
Interpretation: SARS-CoV-2 SP can induce proinflammatory cytokine production, which can be inhibited by either ACE2 antagonist or Mas receptor agonist-angiotensin-(1—7). Angiotensin-(1—7)-related protective effect on cytokine reduction can be abolished by blocking Mas receptor. Our findings suggest that ACE2/angiotensin-(1—7)/Mas axis may serve as a therapeutic target to control inflammatory response triggered by SARS-CoV-2 SP.

Introduction

The novel coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared a global pandemic by the World Health Organization (WHO) in March 2020. By October 2022, the WHO reported that the number of confirmed COVID-19 cases globally has exceeded 623 million, with over 6.5 million deaths.[1] Although the rapid development of COVID-19 vaccines and mass vaccination altered the course of pandemics and has reduced the risk of severe disease, hospitalization, and even death, we are still threatened by COVID-19, particularly in those who develop acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndromes (MODS).[2,3] COVID-19-related ARDS and MODS link to an increase in disease severity and mortality, which might result from cytokine storms.[4,5] Cytokine storm, triggered by SARS-CoV-2 infection, is characterized by systemic hyperinflammation with excessive circulating cytokines, including interleukin (IL)-2, IL-6, IL-1β, IL-8, IL-10, interferon-γ, and TNF-α.[5,6] Moreover, the proinflammatory cytokines, such as IL-6 and IL-8, are recognized as trigger signaling cascades of cytokines leading to tissue damage and organ failure as well as elevated serum levels of IL-6 and IL-8 have been the most frequently reported cytokines associated with worsening survival rate.[7,8] Discovering the pathogenesis of life-threatening cytokine storms in these important cytokines is urgently needed to develop therapeutics in COVID-19 pandemic era.

In the earlier SARS-CoV pandemic, angiotensin-converting enzyme 2 (ACE2) has been found to express in the airway epithelial cells, alveolar epithelial cells, vascular endothelial cells, and macrophages in the lung, and ACE2 has been identified as the entry receptor for SARS-CoV.[9—11] SARS-CoV-2 shares 79% genome sequence identity with SARS-CoV and uses ACE2 as the entry receptor.[12] The spike proteins (SP) are expressed on the surface of the virus particles with the characteristic of crown appearance. The receptor-binding domain in S protein binds to ACE2 in cells and allows viral entry with subsequent infection and inflammatory process. ACE2-associated lung injury has been studied in SARS-CoV infection.[13,14] SARS-CoV SP can downregulate ACE2 and induce the shedding of catalytically active ACE2 ectodomain.[15—17] Loss of ACE2 function tends to be associated with dysfunction of the renin-angiotensin system, enhancing inflammation and vascular permeability, subsequent pulmonary edema, and acute lung injury. In a murine model with loss of ACE2 expression, greater enhanced vascular permeability, increased lung edema, neutrophil accumulation, and diminished lung function are observed.[18]

ACE2 is a key regulatory enzyme in the renin-angiotensin system, responsible for the degradation of angiotensin II to angiotensin-(1—7). Angiotensin II increases blood pressure and inflammation through AT1 receptor (AT1R) expressed in pulmonary, renal, and cardiovascular systems. Activation of AT1R in human lung tissues has been reported to induce pulmonary fibrosis and tissue remodeling by forming reactive oxygen species.[19,20] Angiotensin-(1—7) has been identified as an endogenous ligand for the G-protein—coupled receptor- Mas receptor, a cell surface receptor highly expressed in the brain, heart, kidney, endothelium, and leucocytes.[21—23] Angiotensin-(1—7) have anti-thrombotic and antiproliferative properties. Indeed, angiotensin-(1—7) inhibits angiotensin II-stimulated ERK 1/2 and Rho kinase phosphorylation in the heart.[24,25] In previous studies, angiotensin-(1—7) inhibited allergic inflammation and attenuated airway remodeling in murine ovalbumin-

induced allergy models by suppressing ERK1/2- and NF-kB-dependent pathways.[26,27] Given that SARS-CoV-2 SP could trigger airway inflammation and acute lung injury via a similar inflammatory pathway and downregulation of ACE2/Angiotensin-(1—7) axis. The ACE2/Angiotensin-(1—7) axis might be the potential therapeutic strategy for SARS-CoV-2—induced lung injury.

This study aims to investigate whether SARS-CoV-2 SP can induce inflammation through angiotensin-converting enzyme 2 (ACE2) in the alveolar epithelial cells. The inhibitory effect of angiotensin-(1—7) through activation of Mas receptor is determined. The results of this study may provide further information to suppress lung inflammation in patients with SARS-CoV2-mediated acute lung injury.

Methods

Drugs, reagents, and antibodies

Dalbavancin, an antibiotic known as an inhibitor of SARS-CoV-2 SP-ACE2 interactions by directly binding to ACE2 receptor, was purchased from Sigma—Aldrich (catalog number SML2378, St. Louis, MO, USA). The other drugs and reagents used in this study are listed in the following: SP from SARS-CoV-2 (recombinant S1 protein; catalog number PKSR030480, Elabscience Biotechnology, Houston, Texas, USA), Angiotensin-(1—7) and Mas receptor blocker A779 (catalog number 1562 and 5937, respectively, both from Tocris Bioscience, Bristol, UK), enzyme-linked immunosorbent assay (ELISA) for interleukin (IL)-6 and IL-8 (R&D systems, Minneapolis, MN, USA). Antibodies against extracellular signal-regulated kinase (ERK), phospho-ERK, P38, phospho-P38, Jun N-terminal kinase (JNK), phospho-JNK, activator protein-1 (AP-1), nuclear factor kappa B (NF-κB), phospho—NF—κB and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA).

Human pulmonary alveolar epithelial cells

This study was approved by the Institutional Review Board of Taipei Veterans General Hospital (VGHIRB No.2020-07-006AE). Both primary cells (human pulmonary alveolar epithelial cells, HPAEpiC, catalog No. 3200, purchased from

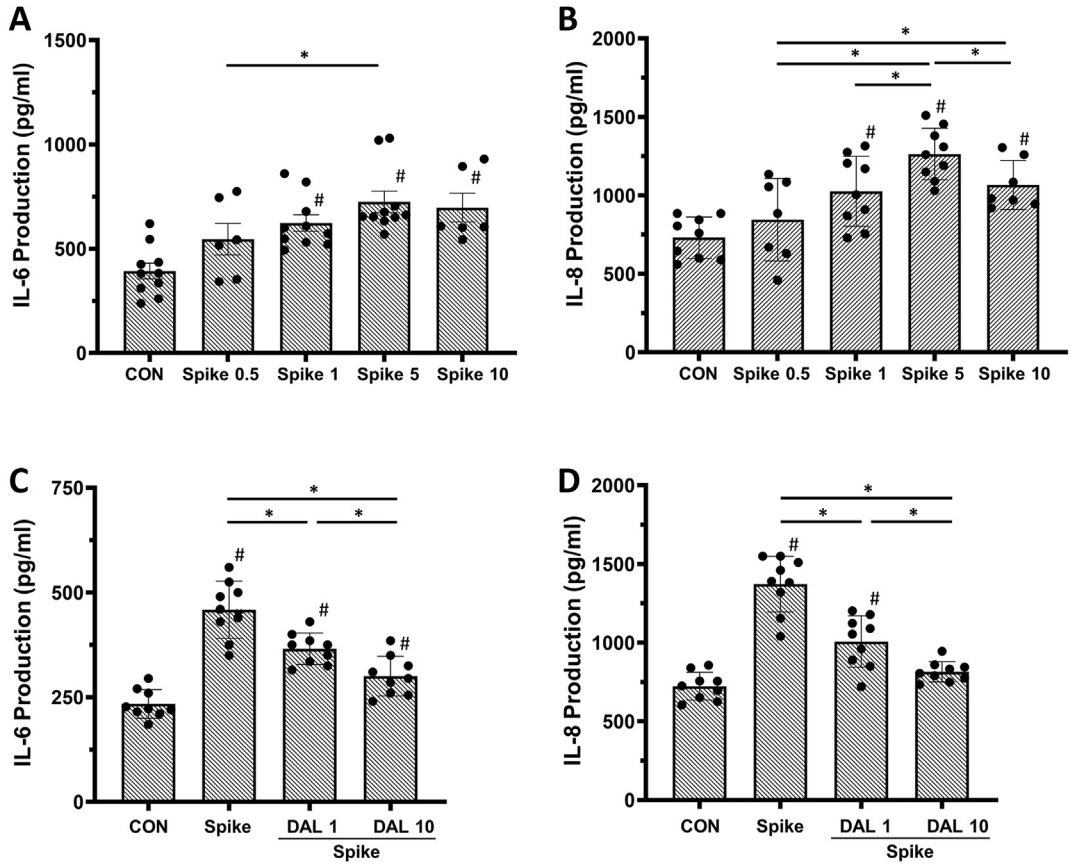

Figure 1. Spike protein induced IL-6 and IL-8 production in human pulmonary alveolar epithelial cells, and these reactions were suppressed by pretreatment of ACE2 antagonist-dalbavancin. Human pulmonary alveolar epithelial cells (HPAEpiC) were stimulated with SARS-COV-2 spike protein (0.5—10 μg/mL) for 24 h, followed by collection of supernatants in culture plates for measurement of IL-6 (A) and IL-8 (B) concentration (pg/mL). HPAEpiC were stimulated with spike protein (5 μg/mL) in the presence or absence of 2-h pretreatment with ACE2 antagonist-dalbavancin (1 or 10 μg/mL) for 24 h, followed by measurement of IL-6 (C) and IL-8 (D). The bar charts with dot plots represent the mean and the standard deviation in error bar from 3 to 10 repetitive experiments. $^{*}P < 0.05$, one-way ANOVA followed by pairwise comparisons with LSD test. $^{\#}P < 0.05$, compared to control. CON, Control; Dal, dalbavancin; Spike: SARS-COV-2 spike protein.

ScienCell Research Laboratories, San Diego, California) and A549 cells (No. 60074, purchased from Bioresource Collection and Research Center, Hsin-Chu, Taiwan) were applied in this study. Epithelial cells were thawed at 37 °C and cultured in an alveolar epithelial cell medium (No. 3201, ScienCell Research Laboratories for HPAEpiC and No. 12491015, Gibco for A549 cells, respectively) containing essential and non-essential amino acids, vitamins, hormones, growth factors, and trace minerals. Cells were grown in the poly-L-lysine-coated culture plates (2 μg/cm^2) and seeded onto 6-well culture plates (1 cc, cell density 5×10^5 cells/mL) for Western blots or onto 24-well culture plates (0.5 cc, cell density 1×10^5 cells/mL) to collect supernatants for measurement of IL-6 and IL-8. Usually, cells were incubated for 3—5 days until a confluent monolayer was formed and used for experiments.

SP-related production of inflammatory cytokine

Confluent monolayer of HPAEpiC in 24-well culture wells were stimulated with the SP at different concentrations (0.5, 1, 5, and 10 μg/mL). After incubation for the specific

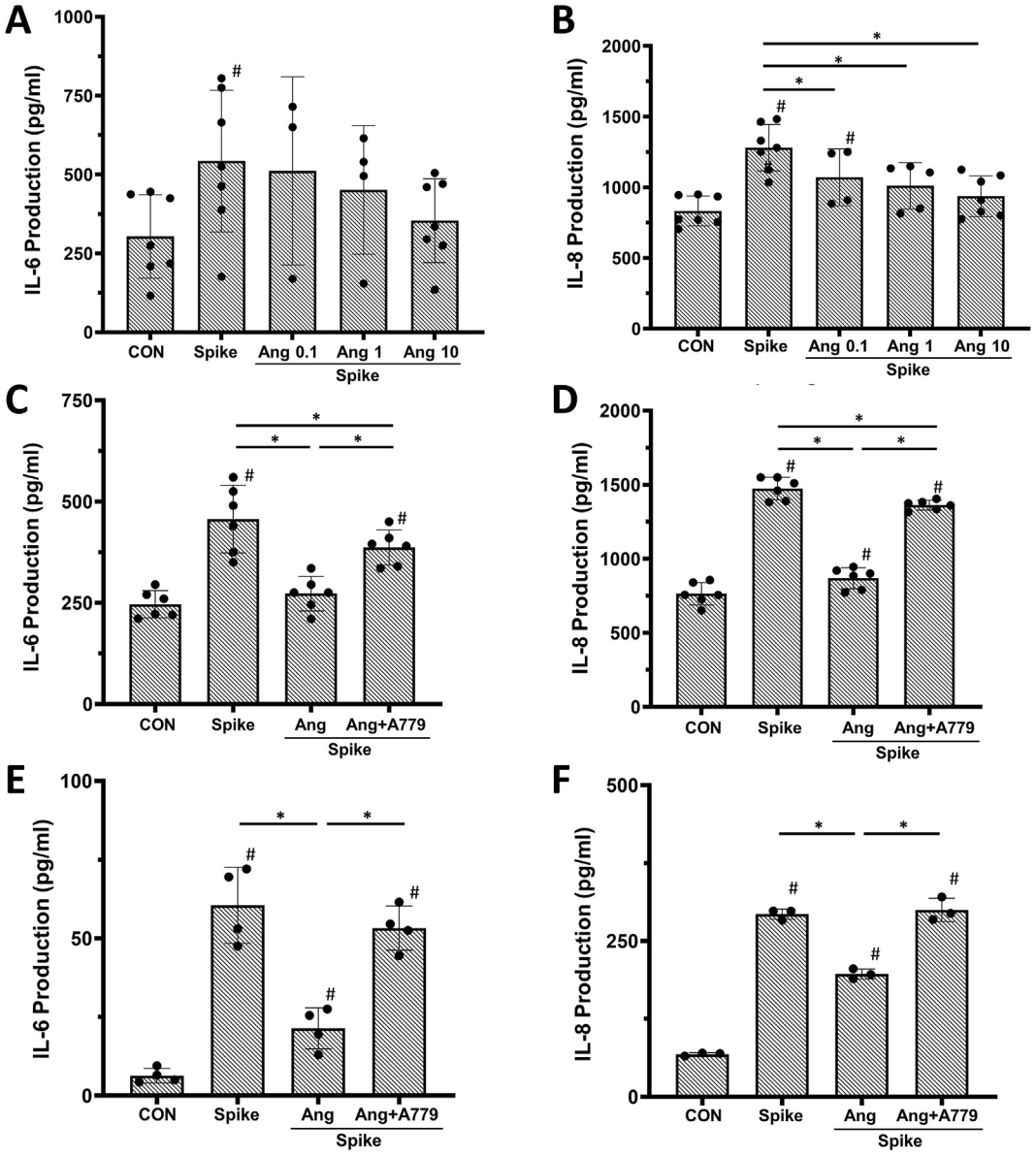

Figure 2. Angiotensin-(1—7) suppressed SP-induced production of IL-6 and IL-8, and these suppressive effects were reversed by pretreatment with Mas receptor antagonist- A779. HPAEpiC were pretreated with or without angiotensin-(1—7) (0.1—10 μM) for 2 h, followed by adding spike protein 5 μg/mL for 24 h and measurement of IL-6 (A) and IL-8 (B) concentration (pg/mL) in supernatants. HPAEpiC were pretreated with angiotensin-(1—7) (10 μM) alone or combined angiotensin-(1—7) (10 μM) and A779 (10 μM) for 2 h, followed by adding spike protein 5 μg/mL for 24 h and measurement of IL-6 (C) and IL-8 (D), which experiments were replicated using A549 cells (E and F). The bar charts with dot plot represent the mean and the standard deviation in error bar from 3 to 7 repetitive experiments. *$P < 0.05$, one-way ANOVA followed by pairwise comparisons with LSD test. $^{\#}P < 0.05$, compared to control. Ang, angiotensin-(1—7); CON, Control; Spike: SARS-COV-2 spike protein.

period, the supernatants were collected at the end of experiments and frozen at −80 °C until the measurement of IL-6 and IL-8 by the specific ELISA Kits according to the manufacturer's instructions. Therefore, the optimal stimulation concentration of SP was determined as 5 μg/mL, which was applied for further experiments. To test the effect of comparative antagonist (dalbavancin) to ACE2 receptor, HPAEpiC were treated with SP 5 μg/mL in the absence or presence of 2-h preincubation of various concentrations of dalbavancin (1 or 10 μM) for 24 h, followed by measurement of IL-6 and IL-8. Cell viability was determined by light microscopy and dye exclusion with trypan blue.

The impact of angiotensin-(1—7)/Mas receptor axis on SP-related cytokine production

To investigate the impact of angiotensin-(1—7)/Mas receptor axis on the SP-related cytokine production, HPAEpiC were treated with SP 5 μg/mL in the absence or presence of 2-h preincubation of various concentrations of Mas agonist-angiotensin-(1—7) (0.1, 1, and 10 μM) for 24 h. Therefore, angiotensin-(1—7) 10 μM was determined for further experiments. Subsequently, the inhibitory effect of angiotensin-(1—7)/Mas axis were testified by blocking the Mas receptor. HPAEpiC were concurrently pretreated with angiotensin-(1—7) 10 μM and the Mas receptor antagonist-A779 10 μM for 2 h, followed by incubation with SP 5 μg/mL for 24 h. Additionally, identical experiments were replicated using A549 cells. Furthermore, the effect of Mas receptor knockdown was performed using specific small interfering RNAs (SiRNA, No. D-001830-10-05, purchased from Dharmacon, Lafayette, Colorado, USA) in A 549 cells. The Target sequence of siRNA were 5′-CGACUAUGCUUUAGAUUAU-3′, GCCAGAAGACAAUUGUAA-3′, 5′-ACACAAUUGUCACAUUAUC-3′, and 5′-CAUCAUUUGUUGUUGAGGA-3'. A549 cells were transfected with the Mas specific siRNA (25 μM) for 24 h according to the manufacturer's protocol. GAPD siRNA group was also performed as transfection control. Transfected A549 cells were treated with SP 5 μg/mL in the presence of 2-h preincubation of angiotensin-(1—7) 10 μM for 24 h, followed by measurement of IL-8.

Real-time quantitative PCR (qPCR) for IL-6 and IL-8 mRNA

The response of SP-related cytokine production at mRNA levels was determined by qPCR. HPAEpiC were treated with SP 5 μg/mL in the presence or absence of 2 h-preincubation of angiotensin-(1—7) 10 μM for 6 h. At the end of treatment, total RNA of the HPAEpiC was isolated by the Trizol reagent (Invitrogen) and was used for cDNA synthesis performing reverse transcriptase (Promega Corporation, Madison, WI, USA) with oligo-dT as the primer. The obtained cDNAs were then used as templates for semi-quantitative PCR. PCR was performed in a real-time PCR system (LightCycler® 480 System, Roche Diagnostics, Mannheim, Germany). The PCR program was 95 °C for 2 min; 45 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 15s; and one cycle of 72 °C for 10 min. The levels of cytokine mRNA in each sample were calculated on the basis of the relative standard curve generated with the RNA from HPAEpiC. The data were normalized to the expression of β-actin. The primers for IL-6, IL-8 and β-actin were 5′-AAAGCAGCAAAGAGGCAC-3'/5′-CCTCAAACTCCAAAGACCAG-3′,5′-AGCTGGCCGTGGCTCTCT-3'/5′-CTGACATCTAAGTTCTTTAGCACTCCTT-3′, and 5′-TGGCATTGCCGACAGGAT/3′- GCTCAGGAGGAGCAATGATCT, respectively.

Western Blot analysis

SP or angiotensin-(1—7)-related activation of mitogen-activated protein kinase (MAPK, including ERK, JNK, and p38) in HPAEpiC were tested. The procedure to detect MAPK activity has been described in detail previously.[28] Briefly, SP 5 μg/mL was added to HPAEpiC, followed by incubation for different periods (5—60 min) to test the optimal time point of MAPK phosphorylation. Subsequently, HPAEpiC were treated with SP 5 μg/mL in the absence or presence of 2 h-preincubation of dalbavancin 10 μg/mL, or angiotensin-(1—7) 10 μM, or combined angiotensin-(1—7) 10 μM and A779 10 μM for the indicated period. In A 549 cells, ERK experiments were replicated in identical steps. Additionally, the downstream transcriptional factors AP-1 and NF-κB were also examined. At the end of each treatment, cells were lysed on ice in a lysis buffer. Aliquots of cell lysates were separated on 8—12% SDS-PAGE and then transblotted onto ImmobilonTM-P membrane (Millipore). After being blocked with 5% skim milk, the blots were incubated with specific primary antibodies (β-actin

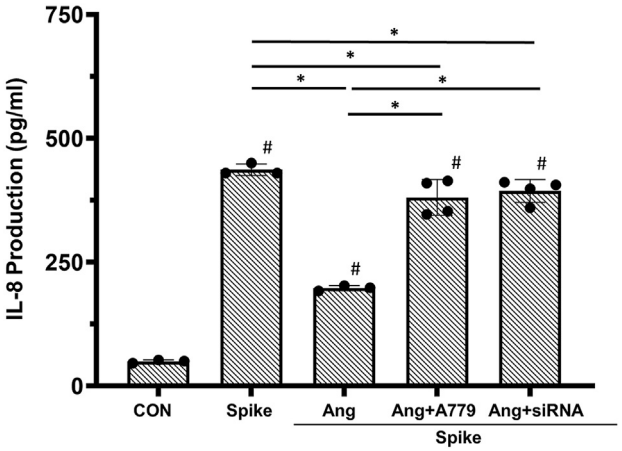

Figure 3. Effect of Mas receptor-specific small interfering RNAs on angiotensin-(1—7)-related protective effect. A549 cells were transfected with Mas receptor-specific SiRNAs (25 μM) for 24 h, followed by adding spike protein 5 μg/mL with 2-h pretreatment with angiotensin-(1—7) (10 μM) for 24 h. Concurrently, A549 cells (without SiRNA transfection) were treated with spike protein 5 μg/mL in the absence or presence of 2-h pretreatment with angiotensin-(1—7) (10 μM) alone or with combined angiotensin-(1—7) (10 μM) and A779 (10 μM) for 24 h and then measurement of IL-8. The bar charts with dot plot represent the mean and the standard deviation in error bar from 3 to 4 repetitive experiments. $^{*}P < 0.05$, one-way ANOVA followed by pairwise comparisons with LSD test. $^{\#}P < 0.05$, compared to control. Ang, angiotensin-(1—7); CON, Control; siRNA: small interfering RNAs specific to MAS receptor; Spike: SARS-COV-2 spike protein.

1:10,000 dilution; all others 1:1000), and the appropriate secondary antibodies. The specific protein bands were detected using an enhanced chemiluminescence kit (PerkinElmer). The densitometric analysis of immunoblots was performed by using the Quantity One Software (version 4.6.2, *Bio-Rad*, Hercules, CA, USA).

Statistical analysis

Data in the bar charts are expressed as mean and SD in error bar. The comparisons between different experimental conditions were evaluated by one-way analysis of variance (ANOVA) followed by a post-hoc test with LSD method. A P value < 0.05 were considered significant for all tests.

Results

Effect of SARS-CoV-2 SP on IL-6 and IL-8 production

Adding SP to HPAEpiC resulted in significant production of IL-6 and IL-8 in a dose—response relationship in the supernatants at 24 h (Fig. 1A and B), and SP 5 μg/mL reached the maximal increase both in IL-6 and IL-8. Thus SP 5 μg/mL was used for further experiments. With 2-h pretreatment of SP-ACE2 binding antagonist, dalbavancin also showed a significant and dose-dependent reduction in SP-induced IL-6 and IL-8 production at 24 h (Fig. 1C and D). Dalbavancin 10 μM exerted the maximal extent of cytokine reduction and was opted for following experiments. Cell viability was greater than 98% in each experiment. The production of IL-8 was more prominent than IL-6, thus IL-8 was opted in A549 cell experiments.

Effect of angiotensin-(1—7) on IL-6 and IL-8 production via Mas receptor

In HPAEpiC, pretreatment with angiotensin-(1—7) attenuated SP-induced production of IL-6 and IL-8 in a dose—response fashion at 24 h (Fig. 2A and B). Angiotensin-(1—7) 10 μM had the maximal reduction, and thus was used for further experiments. Compared to pretreatment with angiotensin-(1—7) 10 μM alone, combined pretreatment with angiotensin-(1—7) 10 μM and A779 10 μM, angiotensin-(1—7)-related suppression on SP-induced cytokine production was attenuated and IL-6 and IL-8 significantly re-

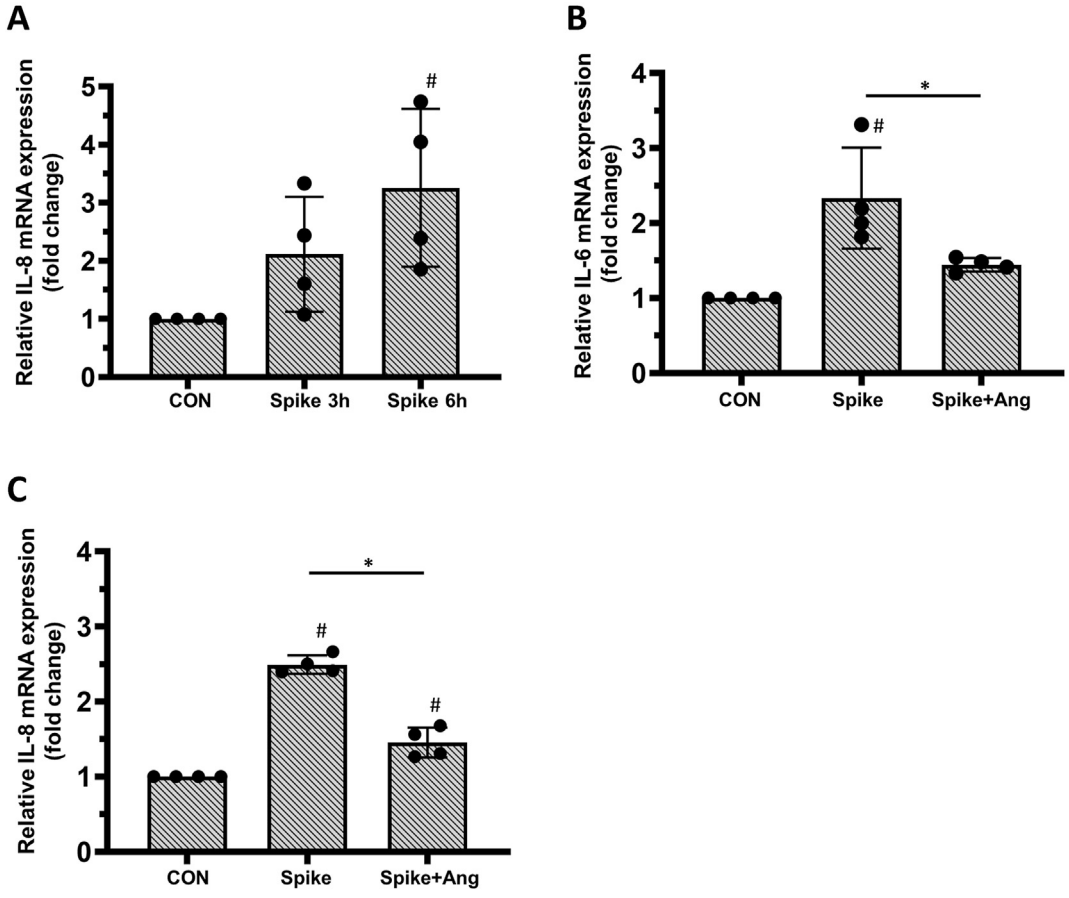

Figure 4. Spike protein significantly enhanced IL-6 and IL-8 mRNA expression at 6 h. HPAEpiC were stimulated with SARS-CoV-2 spike protein (5 μg/mL) for 3 or 6 h, followed by cell lysis for measurement of IL-8 mRNA expression by real-time quantitative PCR (A). HPAEpiC were stimulated with spike protein (5 μg/mL) in the presence or absence of 2-h pretreatment with angiotensin-(1—7) (10 μM) for 6 h, followed by measurement of IL-6 (B) and IL-8 (C) mRNA expressions. The bar charts with dot plot represent the mean and the standard deviation in error bar from 4 repetitive experiments. *$P < 0.05$, one-way ANOVA followed by pairwise comparisons with LSD test. #$P < 0.05$, compared to control. Ang, angiotensin-(1—7); CON, Control; Spike: SARS-COV-2 spike protein.

increased in HPAEpiC (Fig. 2C and D). In A549 cells, similar results were replicated (Fig. 2E and F). As to knockdown of Mas receptor in A 549 cells, the angiotensin-(1—7)-related suppression on SP-induced IL-8 production was significantly neutralized by SiRNA, which results were similar to the effect of Mas receptor antagonist- A779 (Fig. 3). Regarding the mRNA expression of these proinflammatory cytokines, adding SP to HPAEpiC significantly enhanced IL-8 mRNA expression at 6 h (Fig. 4A), which time point was used in the following experiments. The mRNA expression trend was consistent with cytokine production in that angiotensin-(1—7) 10 μM also significantly reversed the SP-induced mRNA expressions for IL-6 and IL-8 at 6 h, respectively (Fig. 4B and C). Cell viability was greater than 98% in each experiment.

SARS-CoV-2 spike protein-induced ERK1/2 phosphorylation and AP-1 activation

We further investigated whether SARS-CoV-2 SP-induced cytokine production through MAP kinase phosphorylation, a step necessary for MAP kinase activation. In HPAEpiC, ERK1/2 MAP kinase was rapidly phosphorylated after stimulation with SP (5 μg/mL), and the peak effect was observed at 5 min and declined over time (Fig. 5A and B). However, neither p38 nor JNK phosphorylation was observed in response to SP stimulation until 60 min of incubation (data not shown). Similar to the trend of cytokine production, ERK1/2 phosphorylation was significantly enhanced after treatment with SP 5 μg/mL at 5 min, which was significantly suppressed with 2-h pretreatment with angiotensin-(1—7) 10 μM, and dalbavancin 10 μg/mL (Fig. 5C and D). In addition, the angiotensin-(1—7)-related suppression of ERK1/2 phosphorylation was abolished by concurrent treatment with A779 10 μM. In A 549 cells, the similar trend of ERK phosphorylation was replicated (please see Appendix Fig. S1). As to the downstream transcriptional factor in response to SP (5 μg/mL) stimulation in A549 cells, the peak activation was observed at 60 min for AP-1 (Fig. 6A and B), but was absent for NF-κB (data not shown). Furthermore, SP-related AP1 activation was suppressed by angiotensin-(1—7) 10 μM, and dalbavancin 10 μg/mL. However, the

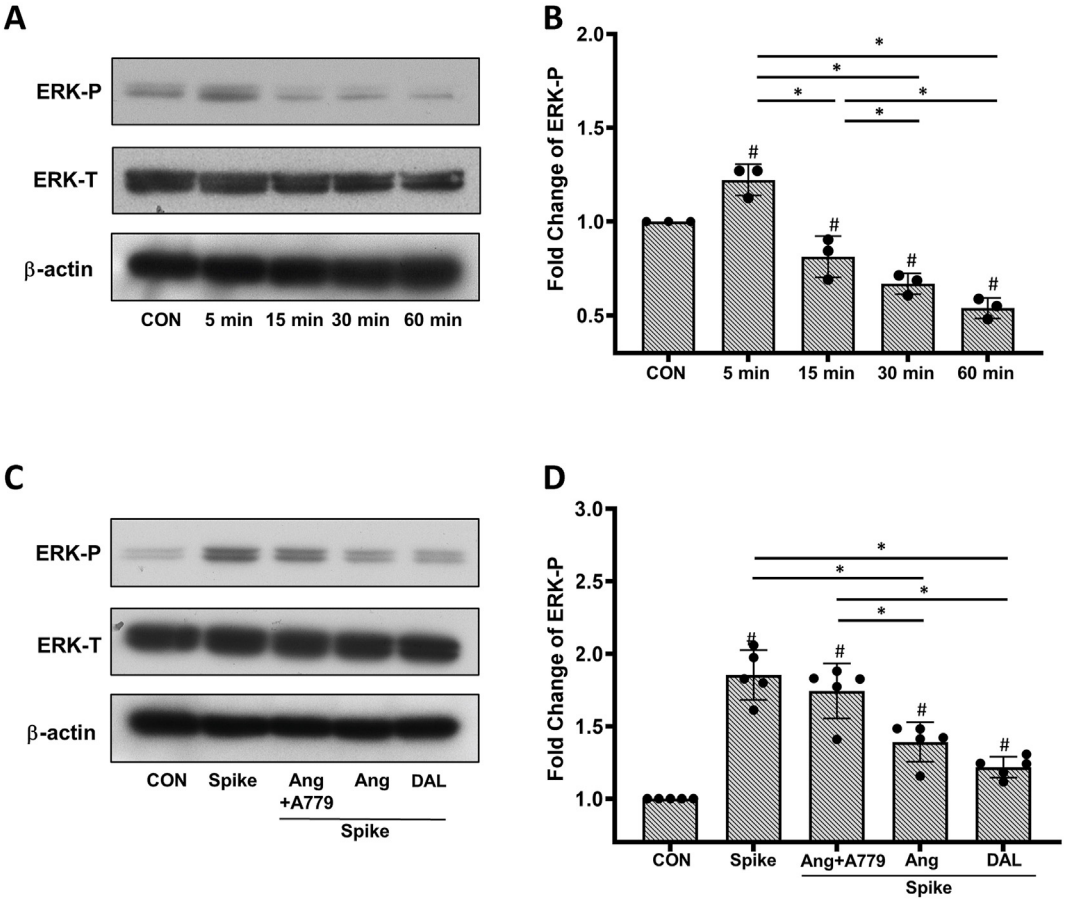

Figure 5. Effect of Spike protein on ERK1/2 phosphorylation. HPAEpiC were stimulated with SARS-CoV-2 spike protein (5 μg/mL) for various time periods (5—60 min), followed by cell lysis, Western blotting for ERK1/2 phosphorylation (A) and densitometric analysis of immunoblots (B). HPAEpiC were pretreated with or without dalbavancin (10 μg/mL), or angiotensin-(1—7) (10 μM), or combined angiotensin-(1—7) (10 μM) and A779 (10 μM) for 2 h, followed by stimulated with spike protein (5 μg/mL) for 5 min, and Western blotting for ERK1/2 phosphorylation (C) and densitometric analysis (D). The bar charts with dot plot represent the mean and the standard deviation in error bar from 3 to 4 repetitive experiments. $^{*}P < 0.05$, one-way ANOVA followed by pairwise comparisons with LSD test. $^{\#}P < 0.05$, compared to control. Ang, angiotensin-(1—7); CON, control; Dal, dalbavancin; ERK-P, phosphorylation of ERK; ERK-T, total ERK; Spike: SARS-COV-2 spike protein.

angiotensin-(1—7)-related AP-1 suppression was abolished by Mas receptor antagonist- A779 (Fig. 6C and D). This trend is identical to that observed in ERK phosphorylation (Fig. 5C and D).

The proposed signal pathway

Based on our findings, the signal pathway of SP-induced production of proinflammatory cytokine is illustrated in Fig. 7. SP stimulates HPAEpiC to produce IL-6 and IL-8 through ACE2, downstream ERK1/2 phosphorylation and activation of AP-1. This pathway could be blocked by adding ACE2 antagonist-dalbavancin. In addition, the Mas antagonist-angiotensin-(1—7) might suppress SP-induced ERK1/2 phosphorylation, AP-1 activation and downstream pro-inflammatory cytokine production. However, these effects might be abolished by antagonizing or knocking down Mas receptor. Taken together, angiotensin-(1—7)/Mas axis can mediate SP-related inflammatory cascades.

Discussion

In this study, we demonstrated that SARS-CoV-2 SP can induce proinflammatory cytokine IL-6 and IL-8 in human alveolar epithelial cells via ACE2 receptor, ERK1/2 phosphorylation, and downstream AP-1 activation. The action can be attenuated by ACE2 antagonist dalbavancin and angiotensin-(1—7) via activation of Mas receptor. Angiotensin-(1—7) may become an alternative measure to control inflammatory response mediated by SARS-CoV-2. These findings suggest that the axis of ACE2/angiotensin-(1—7)/Mas/ERK/AP-1 serves as a potential therapeutic target in the COVID-19 pandemic era.

The functional receptor ACE2 for SARS-CoV has been identified on lung alveolar epithelial cells, enterocytes of the small intestine, and endothelial cells of arteries and veins decades ago.[9] In 2020, Wrapp et al. reported that SARS-CoV-2 trimeric SP binds ACE2 with higher affinity than SARS-CoV.[29] ACE2 is a counter-regulatory enzyme to convert angiotensin II into angiotensin-(1—7). Dalbavancin,

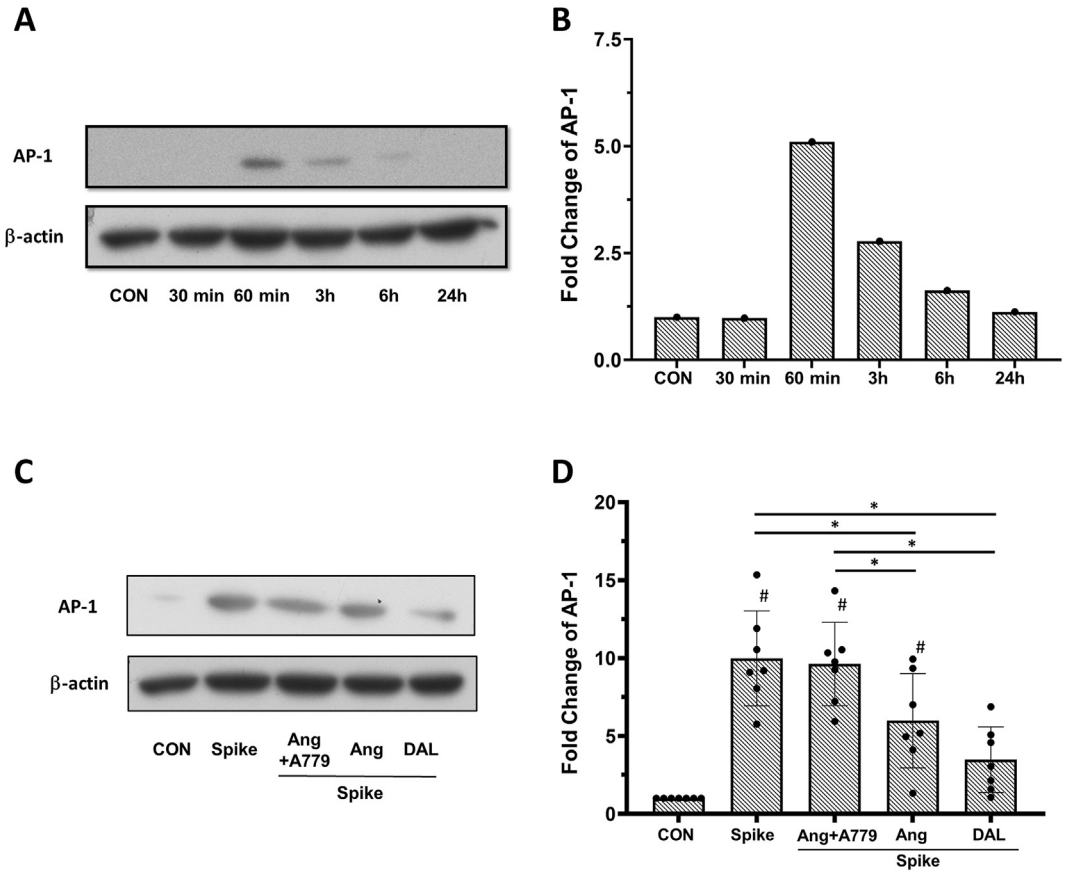

Figure 6. Effect of spike protein on transcriptional factor activation. A549 cells were stimulated with SARS-CoV-2 spike protein (5 μg/mL) for various time periods (5 min—24 h), followed by cell lysis, Western blotting for AP-1. AP-1 activation peaked at 60 min after SP stimulation on Western blotting (A) and densitometric analysis of immunoblots (B). Then A549 cells were pretreated with or without dalbavancin (10 μg/mL), or angiotensin-(1—7) (10 μM), or combined angiotensin-(1—7) (10 μM) and A779 (10 μM) for 2 h, followed by stimulated with spike protein (5 μg/mL) for 60 min, and Western blotting for AP-1 (C) and densitometric analysis (D). The bar charts with dot plot represent the mean and the standard deviation in error bar from 7 repetitive experiments. $^{*}P < 0.05$, one-way ANOVA followed by pairwise comparisons with LSD test. $^{\#}P < 0.05$, compared to control. Ang, angiotensin-(1—7); CON, control; Dal, dalbavancin; Spike: SARS-COV-2 spike protein.

binding ACE2 to block its interaction with SARS-CoV-2 SP in mouse model,[30] also significantly inhibited IL-6 and IL-8 production from alveolar cells observed in the study. In contrast to angiotensin II, angiotensin-(1—7) exerts vasodilating, anti-inflammatory, anti-oxidative, and anti-fibrotic effects by activating Mas receptor.[31] Angiotensin-(1—7) can attenuate lung inflammation, airway remodeling, and hyperresponsiveness in an ovalbumin-sensitized murine model, suggesting a protective role of angiotensin-(1—7).[26] In addition, it has been shown that ACE2/Angiotensin-(1—7)/Mas receptor can protect against bleomycin-induced pulmonary fibrosis.[32] Mas receptor antagonist A779 significantly inhibited the protective effect of angiotensin-(1—7) on reducing cytokine release, suggesting the critical role of Mas receptor in SARS-CoV-2-mediated lung inflammation.

Emerging evidence suggested IL-6 might be a useful prognostic marker in critical-ill patients with SARS-CoV-2 infection.[33] Not only IL-6, elevated proinflammatory cytokines (IL-2, 8, 10, 1β, tumor necrosis factors (TNF), interferons (IFN), colony-stimulating factors (GM-CSF), and chemokines (CCL2, CCL3, CCL4) play a significant role in disease prognosis.[34—36] The alveoli are the primary target for SARS-CoV-2, which may contribute to the development of acute respiratory distress syndrome with alveolar inflammation, damage, hyaline membrane formation, and epithelial and microvascular injury.[37] Alveolar epithelial cells exposed to SARS-CoV-2 infection increased expression of proinflammatory cytokines including IL-6, IL-8, CXCL-2, and TNF-α 24 h after inoculation.[38] Herein, we demonstrated that SARS-CoV-2 SP stimulated alveolar epithelial cells to produce IL-6 and IL-8 at both mRNA and protein levels. MAP kinase ERK1/2 and AP-1 was activated after the binding of SP. ERK1/2 involves a variety of cellular responses, including cell growth, differentiation, and response to stress stimuli[39] and AP-1 mediated proinflammatory cytokine production. The activation of ERK1/2 and AP-1 in alveolar epithelial cells was significantly attenuated by treatment with angiotensin-(1—7) and these protective effects were abolished by Mas receptor antagonist A779 (Figs. 5D and 6D). These trends were replicated in proinflammatory cytokine production (Fig. 2E and F), indicating that the Mas-ERK-AP1 pathway modulated SP-induced proinflammatory cytokine production. Additionally, both antagonism and knockdown of Mas receptor resulted in the loss of angiotensin1-(1—7)-related protection of cytokine production, which further potentiate the role of Mas receptor mediating SP-induced inflammation in alveolar epithelial cells.

In this in vitro study, blocking ACE2 and activating Mas receptor via angiotensin-(1—7) can attenuate SARS-CoV-2 SPs-mediated inflammation in alveolar epithelial cells. This could be a new therapeutic strategy for the treatment of COVID-19 patients. As for in-vivo evidence, spike protein-induced lung inflammation was detected in C57BL/6 J mice via intratracheal instillation in our preliminary experiments. Lung inflammation scores and increased macrophages in the bronchoalveolar lavage fluid were suppressed by treatment with angiotensin-(1—7) via intraperitoneal injection (Appendix Fig. S2 and S3). The therapeutic effect compared to the other treatment regimens requires further investigation. At present, transfusion of convalescent plasma to neutralize viral particles and block subsequent entry into cells via ACE2 appears to benefit COVID-19 patients with more severe conditions. The treatment is limited because of its availability, transmission of harmful pathogens, and transfusion-related lung injury. Angiotensin-(1—7) peptide or analogs may benefit patients with vascular dysfunction and is currently in several clinical trials.[40] However, no clinical studies have validated the safety and efficacy of such a combination strategy in COVID-19 patients currently. It deserves further clinical studies to prove the values of the combination of ACE2 antagonist and Mas receptor activation in treating COVID-19.

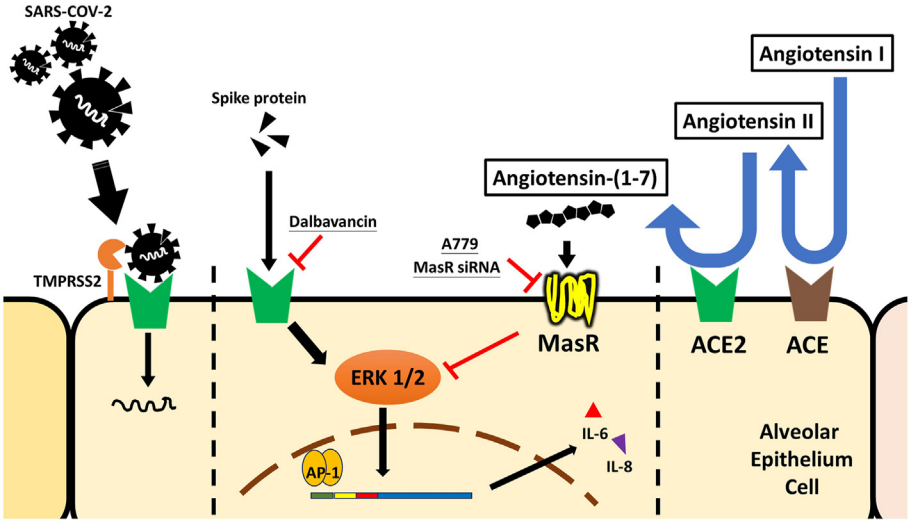

Figure 7. The proposed signal pathway of spike protein-related proinflammatory cytokine production. Spike protein stimulates human alveolar epithelial cells to produce IL-6 and IL-8 through ACE2, ERK1/2 phosphorylation and downstream AP-1 activation. This pathway can be blocked by adding ACE2 antagonist, dalbavancin. In addition, angiotensin-(1—7) can counterbalance spike protein-induced ERK1/2 phosphorylation, AP-1 activation and production of IL-6 and IL-8. This effect is mediated by Mas receptor since the Mas receptor antagonist (A779) and Mas receptor-specific siRNA restores SP-related inflammatory cascades. The experiments in this study is illustrated in the area between the 2 dotted lines.

In conclusion, SARS-CoV-2 SP can induce proinflammatory cytokine (IL-6 and IL-8) production via ACE2 receptor in vitro. The action can be attenuated directly by ACE2 receptor antagonism or indirectly by angiotensin-(1—7) through activation of Mas receptor. Angiotensin-(1—7) may become an alternative measure to control inflammatory response mediated by SARS-CoV-2.

Data sharing statement

All reagents, experimental protocols, and the data that support the findings of this study are available from the corresponding authors, Su and Perng, upon reasonable request.

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Declaration of competing interest

The authors have no conflicts of interest.

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Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.09.003.

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