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Alcohol Increases Lung Angiotensin-Converting Enzyme 2 Expression and Exacerbates Severe Acute Respiratory Syndrome Coronavirus 2 Spike Protein Subunit 1—Induced Acute Lung Injury in K18-hACE2 Transgenic Mice

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During the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, alcohol consumption increased markedly. Nearly one in four adults reported drinking more alcohol to cope with stress. Chronic alcohol abuse is now recognized as a factor complicating the course of acute respiratory distress syndrome, and increasing mortality. To investigate the mechanisms behind this interaction, we developed a combined acute respiratory distress syndrome and chronic alcohol abuse mouse model by intratracheally instilling the S1 subunit of SARS-CoV-2 spike protein (S1SP) in K18—human angiotensin-converting enzyme 2 (ACE2) transgenic mice that express the human ACE2 receptor for SARS-CoV-2 and are kept on an ethanol diet. Seventy-two hours after S1SP instillation, mice on an ethanol diet showed a strong decrease in body weight, a dramatic increase in white blood cell content of bronchoalveolar lavage fluid, and an augmented cytokine storm, compared with S1SP-treated mice on a control diet.

Histologic examination of lung tissue showed abnormal recruitment of immune cells in the alveolar space, abnormal parenchymal architecture, and worsening Ashcroft score in S1SP- and alcohol-treated animals. Along with the activation of proinflammatory biomarkers (NF-κB, STAT3, NLRP3 inflammasome), lung tissue homogenates from mice on an alcohol diet showed overexpression of ACE2 compared with mice on a control diet. This model could be useful for the development of therapeutic approaches against alcohol-exacerbated coronavirus disease 2019. (Am J Pathol 2022, e11; https://doi.org/10.1016/j.ajpath.2022.03.012)

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak that began in December 2019 and spread rapidly across the globe causes acute lung injury, severe hypoxemia, and multiorgan failure. SARS-CoV-2 infects host cells by targeting the endothelial angiotensin-converting enzyme 2 (ACE2) in the lung, heart, kidney, and gastrointestinal tissues. The pathophysiology of acute respiratory distress syndrome (ARDS) in SARS-CoV-2 infection includes lung perfusion dysregulation and a cytokine storm that causes increased vascular permeability and disease severity. COVID-19 also can cause psychosocial problems, including increased alcohol consumption and consequent harms. Alcohol in the United States increased greatly immediately after the stay-at-home orders and relaxing of alcohol restrictions associated with the COVID-19 pandemic. An increase in the black marketing of alcohol also has been reported. Alcohol abuse increased so much that some countries even prohibited alcohol sales during the pandemic lockdown. Alcohol consumption is considered an independent factor that increases the incidence of ARDS, a severe form of acute lung disease.

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injury with a mortality rate of up to 50%. This translates to
tens of thousands of excess deaths in the United States each
year from alcohol-associated lung injury, which is compara-
tible with scarring of the liver (ie, cirrhosis) in terms of
alcohol-related mortality.8,15 Furthermore, people who drink
heavily are more likely to get pneumonia.8 Although acute
alcohol exposure (<24 hours) favors anti-inflammatory re-
sponses, chronic alcohol consumption favors proin-
flammatory cytokine release.9 Notwithstanding that alcohol
consumption alone does not cause ARDS, it makes the
lungs susceptible to dysfunction induced by pathologies,
such as the inflammatory stresses of sepsis, trauma, and so
forth.10 Studies on human monocytes have shown that
several pathogens, when combined with chronic ethanol
consumption, promote the production of inflammatory cyto-
kines. In the lung, cytokine production is augmented by
ethanol, exacerbating respiratory distress syndrome and
greatly increasing the expression of transforming growth
factor β (TGF-β).11

We recently developed an animal model to study acute
lung injury caused by subunit 1 (S1) of the SARS-CoV-2
spike protein (S1SP) using K18–human ACE2 (hACE2)
transgenic mice.12 Intratracheal instillation of S1SP induced
coronavirus disease 2019 (COVID-19)–like lung and sys-
temic inflammatory responses, including a cytokine storm in
bronchoalveolar lavage fluid (BALF) and serum. In this
study, we used this model to interrogate how chronic
alcohol consumption may worsen the development of
COVID-19–like ARDS.

Materials and Methods

Animals and Treatment Groups

All animal studies were approved by the Old Dominion
University Institutional Animal Care and Use Committee
and adhered to the principles of animal experimentation as
published by the American Physiological Society. Healthy
male K18-hACE2 transgenic mice (Jackson Laboratories), 8
to 10 weeks old, 20 to 25 g body weight, were placed on the
Lieber-DeCarli ‘82 control liquid diet for 5 days after arrival
at the animal facility and then divided randomly into four
groups (Figure 1), as follows: i) vehicle (VEH) group: mice
continued on the Lieber-DeCarli ‘82 control liquid diet for 14
days and then on day 19 were instilled intratracheally (i.t.)
with vehicle (sterile saline) at 2 mL/kg body weight; ii)
S1SP group: mice on a control diet for 14 days and then on
day 19 instilled i.t. with SARS-CoV-2 S1SP at 400 µg/kg at
2 mL/kg body weight; iii) ethanol VEH group: mice trans-
ferred to the Lieber-DeCarli ‘82 ethanol liquid diet, consist-
ing of 5% to 6% ethanol, for 2 weeks and then on day 19
instilled i.t. with vehicle (sterile saline) at 2 mL/kg body
weight; and iv) ethanol S1SP group: mice transferred to the
Lieber-DeCarli ‘82 ethanol liquid diet for 2 weeks, then on
day 19 instilled i.t. with SARS-CoV-2 S1SP at 400 µg/kg at
2 mL/kg body weight. The Lieber-DeCarli liquid diet
contains 36% of calories from either ethanol (ethanol diet)
or isocaloric maltose dextrin (control diet), 35% of calories
from fat, 11% of calories from carbohydrate, and 18% of
calories from protein.13 All animals consumed liquid food
ad libitum (approximately 20 to 30 mL/day). Treatment with
the ethanol diet produces blood alcohol concentrations of
approximately 180 mg/dL by day 10.14 Mice did not receive
water during days 5 to 19. Groups 3 and 4 were transferred
to the ethanol diet gradually to minimize stress, as follows:
days 5 to 7: mixture of one-third ethanol diet and two-thirds
control diet; days 8 to 10: mixture of two-thirds ethanol diet
and one-third control diet; and days 11 to 22: ethanol diet
only. After i.t. instillation on day 19, mice also were given
free access to water. All mice were euthanized on day 22
(72 hours after i.t. instillation).

Histology, Lung Injury Scoring, Fibrosis Scoring, and
Steatosis Scoring

Immediately after euthanasia, the chest was opened,
and the lungs were inflated through the trachea with 10% formal-
dehyde to a pressure of 15 cm H2O and then immersed in the
same solution. Seventy-two hours later, samples were
embedded in paraffin. Sections (5-µm thick) were stained
with hematoxylin and eosin (H&E) and Masson’s trichrome
stains. Twenty randomly selected fields from each slide were
examined under immersion (magnification, ×100). Fields
from H&E-stained sections were scored according to the
Lung Injury Score15 method to estimate the severity of lung
inflammation; this method takes into account histologic ev-
idence of injury, including accumulation of neutrophils in
the alveolar or the interstitial space, formation of hyaline
membranes, presence of proteinaceous debris in the alveolar
space, thickening of the alveolar walls, hemorrhage, and
atelectasis. In addition, fields from Masson’s trichrome–
stained sections were scored according to the Ashcroft score to
quantify lung architectural changes and estimate overall collagen deposition.16

Livers also were collected and fixed with 10% formal-
dehyde in the same way and paraffin sections were stained
with H&E and Masson’s trichrome. Twenty randomly
selected fields from each slide were examined. The Hepatic
Steatosis Scoring was performed according to the General
Nonalcoholic Fatty Liver Disease Scoring System for Ro-
dent Models,17 which takes into account hepatocellular
steatosis, hypertrophy, and inflammation.

BALF White Blood Cell Count

BALF was collected by instilling and withdrawing 1 mL
sterile 1× phosphate-buffered saline via the tracheal can-
nula. The BALF was centrifuged at 2400 × g for 10 minutes
at 4°C (5417R centrifuge; Thermo Fisher) and the sup-
natant was collected and stored immediately at −80°C.
The cell pellet was resuspended in 1 mL sterile
phosphate-buffered saline and the total number of white blood cells was determined using a hemocytometer, differential analysis was performed with the Wright-Giemsa stain kit.

All histopathologic and morphologic analyses were performed by an investigator blinded to the study groups.

Total Protein and Cytokine Analysis in BALF

BALF supernatant was collected and prepared as described above. Protein concentration was determined using the micro-bicinchoninic acid assay according to the manufacturer’s protocol. BALF supernatant IL-6, KC, MCP-1, TGF-β1, and tumor necrosis factor α (TNFα) were analyzed in triplicate via mouse/human enzyme-linked immunosorbent assay kits.

Lung Tissue Collection

Immediately after euthanasia, the thorax was opened, blood was drawn from the heart through the right ventricle, and the pulmonary circulation was flushed with sterile phosphate-buffered saline containing EDTA. The lungs were dissected from the thorax, snap-frozen in liquid nitrogen, and kept at -80°C for subsequent analysis.

Western Blot Analysis

Proteins in lung tissue homogenates were extracted from frozen lungs by ultrasonic homogenization (50% amplitude, 3 times for 10 seconds) in ice-cold lysing RIPA buffer with added protease inhibitor cocktail (100:1). The protein lysates were gently mixed under rotation for 3 hours at 4°C, and then centrifuged twice at 14,000 × g for 10 minutes at 4°C. The supernatants were collected, and the total protein concentration was analyzed using the micro-bicinchoninic acid assay. Equal amounts of proteins from all samples (1000 μg/mL) were used for Western blot analysis. The lysates were first mixed with tricine sample buffer 1:1, boiled for 5 minutes, and then separated on a 10% polyacrylamide SDS gel by electrophoresis. Separated proteins then were transferred to a nitrocellulose membrane, incubated overnight at 4°C with the appropriate primary antibody, diluted in the blocking buffer, followed by a 1-hour incubation with the secondary antibody at room temperature, and scanned by digital fluorescence imaging (Odyssey CLx; LI-COR, Dallas, TX). β-actin was used as loading control. ImageJ software version 1.8.0 (NIH, Bethesda, MD; http://imagej.nih.gov/ij), last accessed July 18, 2021, was used to perform densitometry of the bands from the Western blot membranes. Some membranes were stripped for 5 minutes and incubated with other primary and secondary antibodies.

RNA Isolation and Quantitative Real-Time PCR

Lung tissue, stored in RNAlater solution for at least 24 hours, was dried and homogenized in TRIzol, followed by a cleaning step using the RNeasy Mini Kit. The purified RNA was transcribed into cDNA using the SuperScript IV VILO Reverse Transcription Kit and analyzed by real-time quantitative PCR with SYBR Green Master Mix on a StepOne Real-Time PCR System (version 2.3; Applied Biosystems). Results were evaluated using the standard curve method and expressed as fold of control values. β-actin mRNA expression was used for the normalization of all samples.

Statistical Analysis

Statistical significance of differences among groups was determined by one-way or two-way analysis of variance followed by the Tukey post hoc test using GraphPad Prism Software (GraphPad Software, San Diego, CA). Differences among groups were considered significant at P < 0.05.

Results

To make sure that 14 days of ethanol diet is enough for the development of chronic alcohol abuse symptoms, we first investigated morphologic changes in liver samples stained
with H&E and Masson’s trichrome. K18-hACE2 transgenic mice on 14 days of an alcohol diet showed prominent signs of severe fatty liver disease (steatosis) (Figure 2A), that were reflected in the profoundly increased hepatic steatosis score (Figure 2B). Mice on a control diet showed healthy liver architecture.

Alcohol consumption had no effect on the body weight of transgenic mice instilled with saline. A decrease in appetite after anesthesia was reflected in a slight weight loss during the first 24 hours. Both groups of transgenic mice instilled with S1SP showed a rapid decrease in body weight, unlike control groups. However, mice on a control diet started to recover 48 hours after instillation, while alcohol-consuming animals continued to lose weight (Figure 3).

Mice on a regular diet instilled with S1SP showed a significant increase in leukocyte content of BALF compared with the VEH group. Mice on an alcohol diet and treated with S1SP showed a dramatic increase in the white blood cell content of BALF compared with S1SP-instilled mice on a normal diet (Figure 4A). There was no difference between control and ethanol diets in the two VEH groups. A similar profile also was observed in the total protein levels in BALF, suggesting exacerbated capillary permeability and further confirming the presence of strong acute inflammation (Figure 4B). A BALF white blood cell differential analysis showed an upward shift of mononuclear cell content in ethanol-fed, S1SP-instilled mice, while neutrophils primarily increased in S1SP-instilled mice on a control diet (Figure 4C).

H&E-stained lung sections from mice on a control diet instilled with S1SP showed recruitment of neutrophils and a higher lung injury score than vehicle-instilled mice on a control diet. Mice on an ethanol diet and instilled with saline showed a higher number of interstitial mononuclear cells compared with mice on a control diet, altered parenchymal architecture, and a higher lung injury score (Figure 5). VEH, vehicle.

IL-6 and TNFα concentrations in BALF increased in the VEH-instilled group on an alcohol diet compared with the normal diet VEH group (Figure 6). Both S1SP-instilled groups showed increased levels of IL-6 and TNFα compared with their respective controls, however, S1SP-instilled mice on an ethanol diet showed even higher

![Figure 2](image-url) A: Histologic analysis [hematoxylin and eosin (H&E) and Masson’s trichrome] of the liver on day 19, after 14 days on a control or ethanol (EtOH) Lieber-DeCarli ’82 liquid diet. Mice on an alcohol diet show extensive fields of fatty liver; macrovesicular steatosis (dashed arrow); large lipid droplets are present in hepatocytes: microvesicular steatosis (bold arrow); small lipid droplets are present in hepatocytes: inflammatory cells (open arrow). B: Hepatic steatosis score. n = 4 to 5 mice per group. ****P < 0.0001 with analysis of variance and the Tukey test. Scale bars = 50 mm. Original magnification: ×20.

![Figure 3](image-url) Body weight changes in mice on alcohol (EtOH) or control diets after intratracheal instillation of the severe acute respiratory syndrome coronavirus 2 S1 subunit spike protein (S1SP). n = 5 mice per group. *P < 0.05, ***P < 0.001, and ****P < 0.0001, with analysis of variance and the Tukey test, VEH, vehicle.
values of both cytokines. Similar results were observed with TGF-β1 (Figure 6E). Similar to other cytokines, MCP-1 exacerbated increase in the ethanol S1SP group, however, significant up-regulation of KC was observed in S1SP-treated mice on a control diet only, in agreement with histologic and BALF neutrophil concentration data that depicted much lower lung and BALF neutrophil presence in ethanol-S1SP–treated mice (Figure 6C).

Figure 4  A–D: White blood cells (WBCs) (A), total protein concentration (B), and leukocyte differentials (C and D) in bronchoalveolar lavage fluid (BALF) 72 hours after intratracheal instillation of severe acute respiratory syndrome coronavirus 2 S1 subunit spike protein (S1SP). Means ± SEM. n = 4 to 5 per group. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 with analysis of variance and the Tukey test. Scale bars = 10 mm. Original magnification, ×100. EtOH, alcohol; VEH, vehicle.

Figure 5  A and B: H&E staining of lung sections (A) and the Lung Injury Score (B) from K18–human angiotensin-converting enzyme 2 transgenic mice on normal and alcohol (EtOH) diets 72 hours after intratracheal instillation of either saline or severe acute respiratory syndrome coronavirus 2 S1 subunit spike protein (S1SP). Green arrows indicate the recruitment of neutrophils in the alveolar spaces. Means ± SEM. n = 4 to 5 per group. ***P < 0.001 with analysis of variance and the Tukey test. Scale bars = 10 mm. Original magnification, ×100. VEH, vehicle.
To explore the potential effect of increased TGF-β levels on fibroblast activation, fixed lung sections were additionally stained with Masson’s trichrome to visualize collagen deposition. Significant changes in parenchymal architecture, including thickening of the alveolar walls as well as multiple segments with significant collagen deposition, were observed in S1SP-instilled mice that received alcohol (Figure 7).

![Figure 6](https://example.com/figure6.png)

**Figure 6** A–E: Expression levels of inflammatory cytokines IL-6 (A), MCP-1 (B), KC (C), TNFα (D), and TGFβ1 (E) in bronchoalveolar lavage fluid (BALF) 72 hours after intratracheal instillation of severe acute respiratory syndrome coronavirus 2 S1 subunit spike protein (S1SP). Means ± SEM. n = 4 to 5 per group. *P < 0.05, **P < 0.01, and ***P < 0.001 with analysis of variance and the Tukey test. EtOH, alcohol; VEH, vehicle.

![Figure 7](https://example.com/figure7.png)

**Figure 7** A and B: Masson’s trichrome staining of lung sections (A) and the Ashcroft score (B) from K18–human angiotensin-converting enzyme 2 transgenic mice on normal and alcohol (EtOH) diets 72 hours after intratracheal instillation of either saline or severe acute respiratory syndrome coronavirus 2 S1 subunit spike protein (S1SP). Red arrows indicate the deposition of collagen in the alveolar spaces. Means ± SEM. n = 4 to 5 per group. ***P < 0.001 with analysis of variance and the Tukey test. Scale bars = 50 mm. Original magnification, ×20. VEH, vehicle.
Lung tissue homogenates from mice on an alcohol diet showed overexpression of ACE2 compared with mice on a control diet. S1SP did not affect ACE2 expression further (Figure 8).

As we recently published, intratracheal instillation of a single element of SARS-CoV-2, S1SP, into K18-hACE2 transgenic mice increased the expression of proinflammatory biomarkers in the lung. This was confirmed here, in which Western blot analysis of lung homogenates showed significant increases in the phosphorylation of both STAT3 and IkBα in transgenic mice on a control diet instilled with S1SP. Alcohol significantly amplified the inflammatory effect of S1SP. Moreover, S1SP significantly increased the expression of inflamasome NLRP3, and even more so in mice on an ethanol diet (Figure 9). Profound activation of both extracellular signal-regulated kinase and AKT signaling was observed in mice on an alcohol diet. This occurred in both VEH- and S1SP-instilled groups (Figure 10).

**Figure 8** K18-human angiotensin-converting enzyme 2 (ACE2) transgenic mice on an alcohol (EtOH) diet instilled with the S1 subunit spike protein (S1SP) show overexpression of ACE2 in lung tissue homogenates. Means ± SEM. n = 3 to 4. **P < 0.01, ***P < 0.001 with analysis of variance and the Tukey test. VEH, vehicle.

**Figure 9** A and B: K18—human angiotensin-converting enzyme 2 transgenic mice on an alcohol (EtOH) diet instilled with S1 subunit spike protein (S1SP) show activation of STAT3 (A) and IkBα (B) in lung tissue homogenates. C: S1SP also increased inflamasome NLRP3 expression, especially in mice on an EtOH ethanol diet. D: Western blot analysis; protein band density was normalized to that of β-actin. For IkBα and STAT3, the ratio of phosphorylated to total then was calculated and all three are presented as fold of control (VEH). Means ± SEM. n = 3 to 4. *P < 0.05, **P < 0.01, and ***P < 0.001 with analysis of variance and the Tukey test. pIkBα, phospho-IkBα; pSTAT3, phospho-STAT3; VEH, vehicle.
Discussion

We used a novel mouse model of SARS-CoV-2 in combination with an established model of chronic and binge ethanol feeding (the NIAAA model) to study the exacerbations of ARDS induced by SARS-CoV-2 S1SP in vivo, thereby simulating the pathogenesis of COVID-19 disease in alcoholics. The NIAAA model is widely recognized and useful for the study of alcoholic liver disease and systemic damage by alcohol consumption. This model is similar to the drinking pattern in patients with alcoholic hepatitis, who have a background of chronic alcoholism and a record of recent excessive alcohol consumption in anamnesis. The model suggests using 8- to 10-week-old male C57BL/6 mice because they are an alcohol-prefering strain and have shown the best survival rate. Other strains either refuse the alcohol diet or are affected too adversely by the 5% ethanol density was normalized to that of ß-actin. C: The ratio of phosphorylated to total then was calculated and presented as fold of control (VEH). Means ± SEM. n = 3 to 4. *P < 0.05, **P < 0.01 with analysis of variance and the Tukey test. pAKT, phospho-AKT; pERK, phospho-ERK; S1SP, S1 subunit spike protein; VEH, vehicle.

Figure 10 A and B: K18–human angiotensin-converting enzyme 2 transgenic mice on an alcohol (EtOH) diet instilled with either vehicle or S1SP show increased phosphorylation of extracellular signal-regulated kinase (ERK) (A) and AKT (B) in lung tissue homogenates. Western blot analysis; protein band density was normalized to that of ß-actin. C: The ratio of phosphorylated to total then was calculated and presented as fold of control (VEH). Means ± SEM. n = 3 to 4. *P < 0.05, **P < 0.01 with analysis of variance and the Tukey test. pAKT, phospho-AKT; pERK, phospho-ERK; S1SP, S1 subunit spike protein; VEH, vehicle.
reported to independently cause hyperactivation of STAT3, IKBz, and NLRP3 inflamasome both in vitro and in vivo.\textsuperscript{23–25}

We observed monocyte, macrophage, and especially neutrophil recruitment in the BALF, and alveolar space of mice instilled with S1SP. In intensive care units, COVID-19 patients present excessive alveolar infiltration of neutrophils.\textsuperscript{26} Neutrophil recruitment to the focus of infection is fundamental for the fight against the invading pathogens.\textsuperscript{27} Chronic alcohol ingestion disturbs both immunologic and nonimmunologic host defense mechanisms within the airway.\textsuperscript{28} Neutrophil recruitment into the airways is reduced in alcohol-exposed mice infected with \textit{A. fumigatus}.\textsuperscript{20} Importantly, no pathohistologic differences between alcoholic and nonalcoholic groups were observed in the first 2 days after infection. In agreement, we observed predominantly mononuclear cell recruitment in alveoli of alcohol-treated mice receiving S1SP, in contrast to S1SP-instilled mice on a normal diet who showed primarily neutrophil infiltration. At the same time, spike protein—altered lung parenchymal structure was not significantly different between mice on control and ethanol diets. Monocytes and macrophages play an important role in the pathogenesis of both alcoholic liver disease\textsuperscript{29} and acute lung injury.\textsuperscript{30} These cells, infected via ACE2-independent and ACE2-dependent pathways, lose their ability to fight the virus and induce adaptive immune responses.\textsuperscript{31,32} Their impaired functions can lead to multiple organ damage, mainly owing to exacerbation of ALI, provocation of a cytokine storm, and development of fibrosis.\textsuperscript{33} Patient BALF analysis has shown previously that alcohol causes alveolar macrophage dysfunction and an alcohol-induced increase in oxidative stress.\textsuperscript{34,35} Here, we observed hyperexpression of ACE2 in lung homogenates of K18-hACE2 transgenic mice on an alcohol diet, suggesting an additional mechanism of exacerbation of COVID-19 by ethanol. Additional evidence of worsening of COVID-19—related ARDS by alcohol consumption is provided by the dramatic increase of cytokine concentration in BALF. Compared with controls, mice instilled with S1SP show overexpression of

![Figure 11](image-url)

\textbf{Figure 11}  Signaling pathways in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) S1 subunit spike protein (S1SP)— (right) and alcohol-exacerbated SARS-CoV-2 S1SP—induced acute lung injury (left). ACE2, angiotensin-converting enzyme 2; PMN, ______.; VEGF, vascular endothelial growth factor.
cytokines, in agreement with our previous data. Here, even saline-instilled mice on an ethanol diet showed significant increases in IL-6 and TNF-α compared with control mice. A few clinical studies have indicated anti-inflammatory properties of alcohol, including a reduction in IL-6, while animal studies have suggested a linear relationship between alcohol drinking and IL-6. Ethanol consumption alters both IL-6 and TNF-α expression in lipopolysaccharide-challenged Kupffer cells. Similarly, modest alcohol consumption suppresses TNF-α levels in monocytes, probably by suppressing post-transcriptional TNF-α production. However, mice who received daily 2.5 g/kg ethanol by gavage for 4 days (acute model) showed increased TNF-α and decreased NF-κB activity in plasma, thus unleashing the apoptotic effects of TNF-α. Here, we showed that the cytokine storm, associated with COVID-19—like ARDS, is more pronounced in alcohol-consuming animals. A recent meta-analysis of gene expression profiles in COVID-19 patients predicted that ethanol may augment systemic inflammation by enhancing the activity of IL-1β, IL-6, and TNF. The lack of KC activation in BALF taken from S1SP mice on an alcohol diet compared with S1SP mice on a control diet is consistent with the observed monocyte/neutrophil shift in BALF. This finding suggests that chronic alcohol consumption may change the immune response in ARDS. The activation of TGF-β is critical to the development of pulmonary edema in ALI and also plays an important role in the development of pulmonary fibrosis. The expression of TGF-β1, CD44v6, MMP-9, caveolin-1, and other tissue biomarkers of the TGF-β signaling pathway, along with the deposition of extracellular matrix components, collagen I, collagen III, and α-smooth muscle actin, have been detected in lung sections from COVID-19 patients. In the present model, alcohol did not increase the expression of TGF-β1 in mice instilled with saline, but amplified it in S1SP-instilled animals.

It was reported previously that the SARS-CoV-2 spike protein leads to the induction of transcriptional regulatory molecules, such as NF-κB and mitogen-activated protein kinase/extracellular signal-regulated kinase 42/44. Activation of mitogen-activated protein kinase by COVID-19 plays an important role in the survival of the virus. Modulation of the mitogen-activated protein kinase pathway by alcohol is variable and depends on the organ, cell type, and acute or chronic exposure, but its mechanism has been poorly studied in lungs. SARS-CoV-2 endocytosis occurs through a clathrin-mediated pathway, regulated by phosphatidylinositol 3-kinase/AKT signaling. The AKT signaling pathway was activated by the N protein of SARS-CoV in Vero E6 cells. Activation of the AKT also has been linked to the induction of lung fibrosis in patients with COVID-19. Here, we observed a dramatic activation of extracellular signal-regulated kinase 42/44 and AKT, which may in part explain the associated pathologies.

In summary, our data show that K18-hACE2 transgenic mice on an alcohol diet exhibit a more severe S1SP-induced ARDS than corresponding mice on a control diet, and that overexpression of ACE2 may play a critical role in this process (Figure 1). It is not clear how alcohol consumption will affect the lungs in the late stages of COVID-19, especially considering that the proinflammatory pathways studied here also are involved in the development of pulmonary fibrosis. Thus, this model could be useful for the development of therapeutic interventions against alcohol-exacerbated COVID-19.

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