



Published in final edited form as:

J Med Virol. 2022 December ; 94(12): 6097–6102. doi:10.1002/jmv.28094.

Inhibition of MEK signaling prevents SARS-CoV2-induced lung damage and improves survival of infected mice

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Abstract

Coronavirus disease 2019 (COVID-19) is the illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Over 500 million confirmed cases of COVID-19 have been recorded, with 6 million deaths. Thus, reducing the COVID-19-related medical burden is an unmet need. Despite a vaccine that is successful in preventing COVID-19-caused death, effective medication to relieve COVID-19-associated symptoms and alleviate disease progression is still in high demand. In particular, one in three COVID-19 patients have signs of long COVID syndrome and are termed long haulers. At present, there are no effective ways to treat long haulers. In this study, we determine the effectiveness of inhibiting mitogen-activated protein kinase (MEK) signaling in preventing SARS-CoV-2-induced lung damage in mice. We showed that phosphorylation of extracellular signal-regulated kinase (ERK), a marker for MEK activation, is high in SARS-CoV-2-infected lung tissues of mice and humans. We show that selumetinib, a specific inhibitor of the upstream MEK kinases, reduces cell proliferation, reduces lung damage following SARS-CoV-2 infection, and prolongs the survival of the infected mice. Selumetinib has been approved by the US Food and Drug Administration (FDA) to treat cancer. Further analysis indicates that amphiregulin (AREG), an essential upstream molecule, was upregulated following SARS-CoV-2 infection. Our data suggest that MEK signaling activation represents a target for

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AUTHOR CONTRIBUTION:

Jingwu Xie: Conceptualization, data collection, data analysis, and manuscript writing. **Michael J. Klemsz:** methodology, and manuscript review. **Melissa A. Kacena:** Data collection, and manuscript review. **George Sandusky:** Data collection and manuscript review. **Xiaoli Zhang:** Conceptualization, Methodology, data collection and analyses. **Mark Kaplan:** Methodology review, resources, and manuscript review.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS STATEMENT

We conducted the study after receiving institutional ethical committee clearance.

therapeutic intervention strategies against SARS-CoV-2-induced lung damage and that selumetinib may be repurposed to treat COVID-19.

Keywords

COVID-19; SARS-CoV-2; MEK; ERK phosphorylation; Ki67; lung; mouse model

INTRODUCTION

Common symptoms of COVID-19 include fever, fatigue, and dry cough, occurring in most patients. In contrast, severe symptoms such as pneumonia, acute respiratory distress syndrome (ARDS), and multiple organ failure are less common¹⁻⁴. Effective medication to relieve COVID-19-associated symptoms and slow disease progression is an unmet medical need, despite vaccination successfully reducing severe symptoms and mortality rate. Direct targeting antivirals (DTA) such as Remdesivir or monoclonal antibodies are more effective in the early stages of COVID-19, but ineffective in reducing late-stage symptoms. Increasing evidence indicate that both vaccination and DTA are not effective in combating illness caused by recent SARS-CoV-2 variants.

We hypothesize that targeting cellular factors required for symptom development after SARS-CoV-2 infection will allow us to identify novel strategies to relieve COVID-19-associated symptoms, including those in long haulers. Over the years, it has been shown that activation of the RAF/MEK/ERK signaling pathway is critical for the replication of DNA^{5,6} and RNA viruses, including SARS-CoV-2⁷⁻⁹. In this study, we demonstrated that selumetinib, a specific inhibitor of the upstream MEK kinases, approved by the US Food and Drug Administration (FDA) for cancer treatments, prevents phosphorylation of ERK, and reduces lung damage following SARS-CoV-2 infection, and prolongs the survival of infected mice. Thus, repurposing selumetinib may be a promising approach to reduce COVID symptoms and pathology.

MATERIALS AND METHODS

Mouse model of SARS-CoV-2 infection

The Indiana University School approved all animal studies of the Institutional Animal Care and Use Committee (IACUC) and the Indiana University Institutional Biosafety Committee (IBC). Cg-Tg(K18-ACE2)2PrImn/J mice (Jackson Laboratory, Bar Harbor, ME, Stock No: 034860) were used in this study. At approximately 8 weeks of age, K18-ACE2 mice were either mock infected (media only) or were infected (media containing virus) intranasally (20µl) with SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, Manassas, VA), n=4-5/group using methods described previously^{10,11}. Mice were administered with a viral concentration of 0 (mock) or 1×10^4 (plaque-forming unit). As detailed below, several additional assessments were also completed each day, including body weight and body condition score (BCS). Criteria for humane endpoints were established in consultation with IACUC and collaborating veterinarians as described previously¹¹.

Discarded human specimens from COVID-19 patients were used to detect expression of ERK^{1/2} phosphorylation. Use of human materials has been approved by Institutional Review Board (IRB).

Body Condition Scoring

Body condition scores (BCS) were performed as previously described¹¹ (see Table below). Mice with a score of 0-7 will continue with daily scoring. Mice with a score of 8 or greater or any category of a score of 3 will be euthanized.

Score	Activity	Posture	Respiration
0	Normal	Normal	Normal
1	Slightly reduced/mild gait abnormality	Slightly hunched	Slight increase in rate or effort
2	Moving very slowly/severely altered gait	Moderately hunched	Slight to moderate increase in rate and/or effort
3	Reluctant to move or did not move	Severely hunched	Noticeable increase in rate and/or effort

Virus Preparation

SARS-CoV-2 isolate USA-WA1/2020 was propagated in Vero-furin cells, and progeny virions were collected in the supernatant 2-3 days postinfection. Vero-furin cells were used for virus production [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO₂]. SARS-CoV-2 was quantified by a plaque assay as previously described¹¹.

Histology and immunofluorescent staining

All mice were either euthanized at day 14 +/- 5 days or found dead, and lung tissues were placed in 10% NBF for at least 72 hours as per CDC recommended guidelines. They were paraffin-embedded, sectioned, stained with Hematoxylin and eosin stain (H&E), and examined by pathologist Dr. George Sandusky. Immunofluorescent staining was performed as described previously¹². Antigen retrieval was performed with tissue sections from mouse and human COVID-19 lung tissues. Non-specific staining was blocked with 10% FBS in PBS for 30 min and was then incubated with anti-phosphor-ERK^{1/2} (1:100, cat# 4376S, lot#10, Cell Signaling Inc.) or AREG antibodies (1:100, Cat# bs-3847R, lot# AH10221226, Bioss Inc) in a blocking buffer at 4°C overnight. After washing with PBS, cells were incubated with Alexa Fluor 488 goat anti-rabbit (1:300, Thermo Scientific) for 30 min at room temperature. Nuclei were mounted with DAPI (Invitrogen). Cross sections of lung from all mice were used to count the number of thrombi and fibrin thrombi after H&E staining, and the average per section was used to compare the control group with selumetinib treatment group. The number of lymphocytes, endothelial and bronchiolar epithelial cells were counted from the areas with bronchioles and blood vessels. The ratios were calculated for comparison between the control group and the treatment group.

Statistical analysis

Data were presented as mean \pm SD. from at least three independent experiments. Statistical comparisons between two groups were performed using a two-tail unpaired *t-test*¹³. $P < 0.05$ was indicated as the statistically significant difference.

RESULTS

Activation of mitogen-activated protein kinases (MAPKs), including MEK^{1/2}, represents critical signal transducers that respond to viral infection, resulting in alteration of cell functions, such as cell differentiation, proliferation, survival, and apoptosis¹⁴. Activation of physiological intracellular signaling cascades caused by SARS-CoV-2 infection may lead to the phosphorylation and activation of downstream molecules, and specific inhibitors may help reduce SARS-CoV-2-induced symptoms. We examined MEK^{1/2} activity by detecting ERK^{1/2} phosphorylation in patients with SARS-CoV-2 infection. As indicated in Fig.1A, we detected a high level of ERK^{1/2} phosphorylation in COVID-19 patients, suggesting that MEK^{1/2} activation occurs in patients with COVID-19.

To test the role of MEK^{1/2} activity in SARS-CoV-2-induced lung damage, we need to use a model system and a specific inhibitor. K18-hACE2 transgenic mice are susceptible to SARS-CoV-2 infection and are a commonly used mouse model for COVID-19^{15–17}. We first tested whether SARS-CoV2 infection in K18-hACE2 transgenic mice causes activation of MEK^{1/2}, as indicated by ERK^{1/2} phosphorylation. We monitored ERK^{1/2} phosphorylation in the lung tissue following SARS-CoV2 infection and detected a high phosphorylated ERK^{1/2} (Fig.1B–C).

To demonstrate that selumetinib is effective in suppressing MEK^{1/2} activity, we examined the level of ERK^{1/2} phosphorylation by immunofluorescent staining. Phospho-ERK was highly detectable in lung tissues following SARS-CoV-2 infection (Fig.1C). In contrast, selumetinib injection nearly diminished phospho-ERK^{1/2} in the lung tissues (Fig.1D), indicating that selumetinib effectively suppresses MEK^{1/2} activation.

We performed histological analyses and found significant differences between selumetinib treatment group and the control group in the infected mice (Fig. 2). Mice infected with SARS-CoV-2 exhibit chronic interstitial pneumonia, fibrin thrombi (indicated as # in Fig. 2A), thrombi (indicated by ** in Fig. 2B), perivascular and peribronchiolar lymphocytic infiltration (indicated by an orange * in Fig. 2C), alveolar macrophages (see green arrows in Fig. 2D), active bronchiolar epithelial cell degeneration (a green arrow in Fig. 2B indicated degenerated cells). Collapsed alveoli were common in SARS-CoV-2 infected lung tissues (indicated by * in Fig.2A). With selumetinib treatment, the SARS-CoV-2 infected lung tissues looked essentially normal (Fig. 2E–H), but contained multifocal lymphoid nodules. Thrombi and congestion were less severe in selumetinib-treated mice (shown in Fig. 2E–H). We observed significant reduction in the number of thrombi and fibrin thrombi in the selumetinib treatment group (Fig. 2I–J, with p-values of 0.04 and 0.03 respectively). Similarly, the ratio of perivascular lymphocytes to endothelial cells in the areas with blood vessels was significantly lower in selumetinib treatment group Fig. 2K, $p=0.0002$). Samples from selumetinib treatment group also have a significant lower ratio of peribronchiolar

lymphocytes to bronchiolar epithelial cells (Fig. 2L, $p=0.0005$). These data strongly indicate that selumetinib reduces SARS-CoV-2-induced lung tissue damage.

It is known that MEK^{1/2} plays an essential role in cell proliferation, and nuclear ki67 expression is a marker for cell proliferation¹⁴. After immunofluorescent staining with ki67 antibodies, we observed >25% of ki67 positivity in SARS-CoV-2 infected lung tissues. After selumetinib treatment, ki67 positivity was reduced to <5% ($p=0.007$) (Fig. 3). This result suggests that selumetinib reduces cell proliferation in the infected lung tissue. Further examination suggested that ki67 positive cells are mostly lymphocytes.

Next, we determined whether selumetinib effectively reduces mouse mortality after SARS-CoV-2 infection. In the K18-hACE2 mouse model, most mice die in days 5-7 after virus infection. We observed a tendency of better survival for mice treated with selumetinib. At day 11, we found that the survival rate of the mice treated with selumetinib was twice that of the control mice (67% vs. 33%). This result suggests that selumetinib may be effective in reducing COVID19-related mortality.

There are numerous mechanisms by which MEK^{1/2} is activated, including activation of upstream growth factors and signaling crosstalk. We detected elevated expression of the growth factor amphiregulin (AREG) in SARS-CoV2-infected mouse lung tissues compared with mock-infected tissues (see Fig.4). This result suggests that elevated AREG expression may be partly responsible for MEK^{1/2} activation in the lung tissues after SARS-CoV2 infection.

From these data, we conclude that MEK^{1/2} inhibitor selumetinib effectively reduces SARS-CoV-2 infection-induced lung damage and could be repurposed to treat COVID-19 patients.

DISCUSSION

This study identified MEK^{1/2} as a targetable vulnerability for SARS-CoV-2-induced symptoms in a mouse model of COVID-19. The MEK^{1/2} inhibitor selumetinib is well-tolerated in cancer patients, with very low toxicity. In fact, selumetinib has been approved by FDA to treat uveal melanoma, NF1-related inoperable plexiform neurofibromas, and subsets of liver cancer and acute myeloid leukemia. Our data indicate that selumetinib could be repurposed to treat COVID-19 patients to reduce SARS-CoV-2-induced symptoms, including for long haulers. While our studies provide insightful information on how SARS-CoV-2 induces lung damage, we anticipate more in-depth studies will follow. In addition to mechanistic studies, there are several strategies available to block SARS-CoV-2-induced activation of the AREG-MEK signaling axis, such as neutralizing antibodies against AREG, RAF and MEK inhibitors. We also anticipate that alternatives to the K18-hACE2 transgenic mice may be useful for better understanding of the pathological process of SARS-CoV-2 infection.

The relevance of this study to human COVID-19 may be further studied using the real-world data from the Optum dataset.

This study has limitations. All mouse studies used only one mouse model. While K18-hACE2 mice are commonly used model for studying COVID-19 pathogenesis, they do not exactly represent all the symptoms in human COVID-19.

ACKNOWLEDGMENTS

This project was supported, in part, by IU TRANSLATIONAL RESEARCH GRANT (TReG) PROGRAM FY22 (JX), R01 AI057459 (MHK), the Cooperative Center of Excellence in Hematology (CCEH) Award, funded in part by NIH 1U54DK106846 (MAK) and the Indiana Clinical and Translational Sciences Institute, funded in part by NIH UL1TR002529 (MAK). The views expressed in this article are solely those of the authors and do not necessarily represent the official position or policy of NIH.

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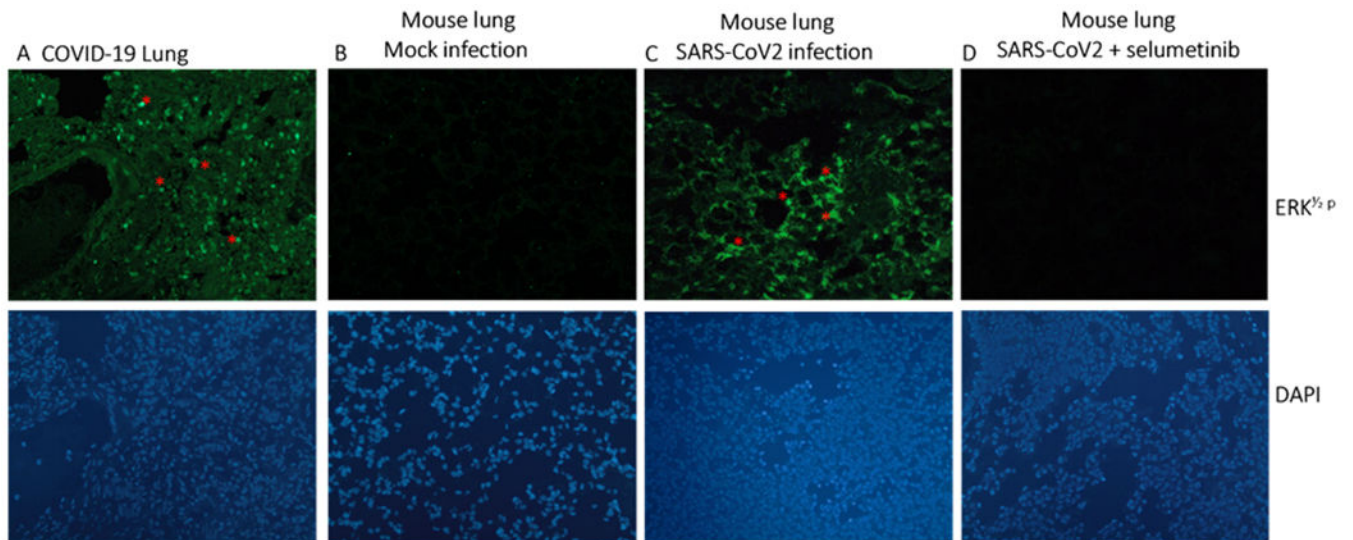


Fig.1. ERK^{1/2} phosphorylation in SARS-CoV-2-infected lung tissues of mice and the humans. ERK^{1/2} phosphorylation was detected by immunofluorescent staining using specific antibodies (shown in green and highlighted by red*). Positive staining was observed in lung tissues from COVID-19 patients (A), SARS-CoV-2-infected K18-hACE2 transgenic mice (shown in C), not in the lung tissues of mock-infected lung tissues (Shown in B). When K18-hACE2 transgenic mice was treated with MEK inhibitor selumetinib, ERK phosphorylation was not detectable (shown in D), indicating effective suppression of MEK activity by selumetinib. ERK^{1/2} phosphorylation was shown in red (top panels), and nuclear staining was done with DAPI (shown in blue, bottom panels).

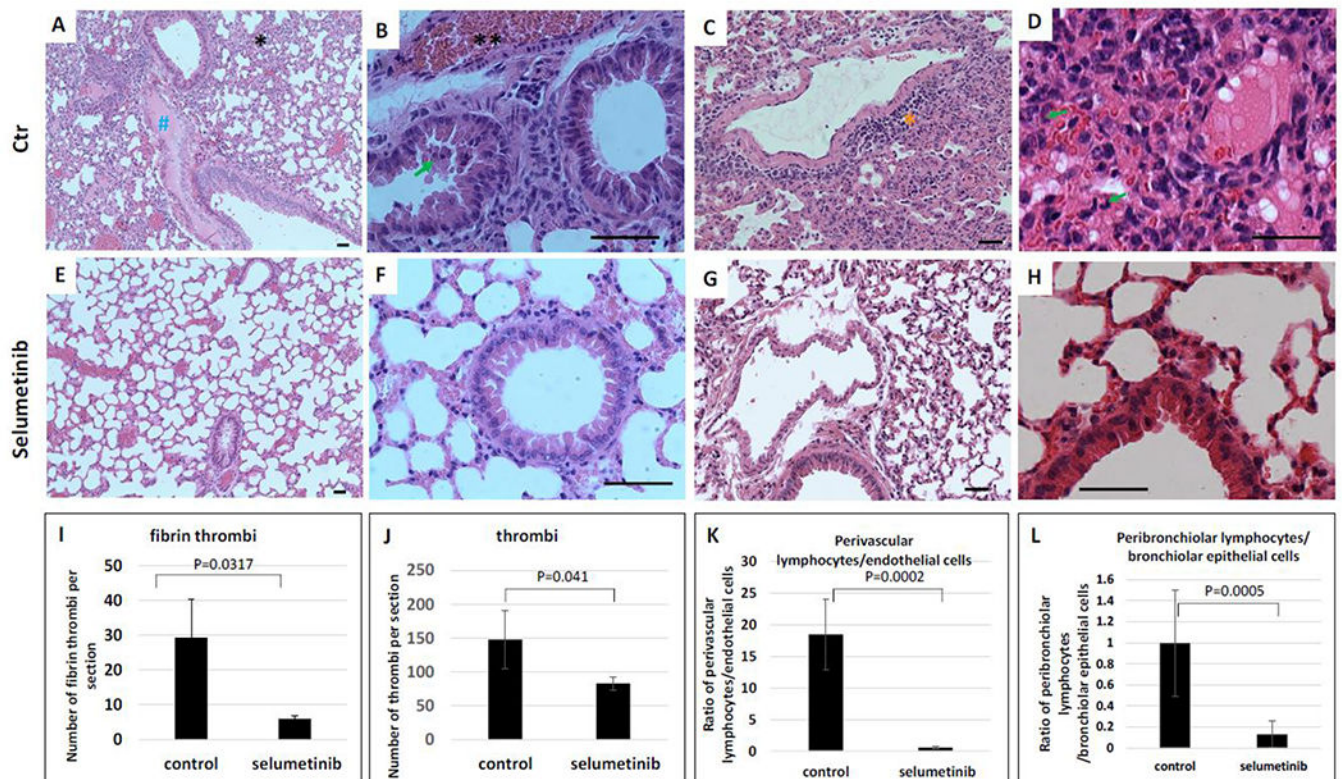


Fig.2. Histological changes of lung tissues by selumetinib.

H&E slides were examined for changes in lung tissues. Figures 2A–D were from the control group, and 2E–H were from selumetinib treatment group. Figures 2I–L are summaries of data analyses as described below. We detected fibrin thrombi (indicated by # in A), thrombi (indicated by ** in B) in SARS-CoV-2-infected mouse lung tissues. We also observed active bronchiolar epithelial cell degeneration (indicated by a green arrow in B), perivascular (indicated by red * in C) and peribronchiolar lymphocytic infiltration (shown in D) and collapsed alveoli (indicated by a * in A). All these symptoms were less severe in selumetinib-treated mice (as shown in E–H). We found that the number of fibrin thrombi (shown in I, $p=0.0317$) and thrombi (shown in J, $p=0.041$) per lung tissue section was significantly lower in selumetinib-treated mice. The biggest difference was the ratio of perivascular lymphocytes to endothelial cells between the control group and the selumetinib-treated group (shown in K, $p=0.0002$). A lower ratio between peribronchiolar lymphocytes to bronchiolar epithelial cells was also observed between the control group and the selumetinib treatment group (shown in L, $p=0.0005$).

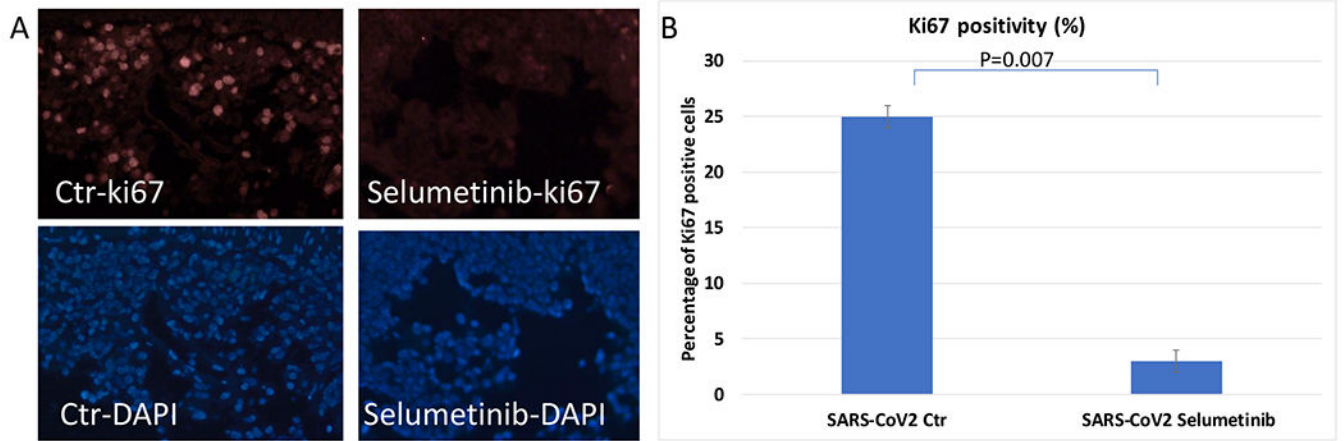


Fig.3. The efficacy of selumetinib on ki67 positivity of lung tissues from SARS-CoV-2 infected mice.

A shows typical images of Ki67 staining and DAPI staining. Presence of Ki67 positive cells (in red) was observed in SARS-CoV-2 infected K18-hACE2 mice, but not in selumetinib (shown as drug)-treated mice. **B** shows the summary of ki67 staining from 3 mice in each group. Three fields were counted from each mouse lung tissue, and the number is the average from mice. $P=0.007$.

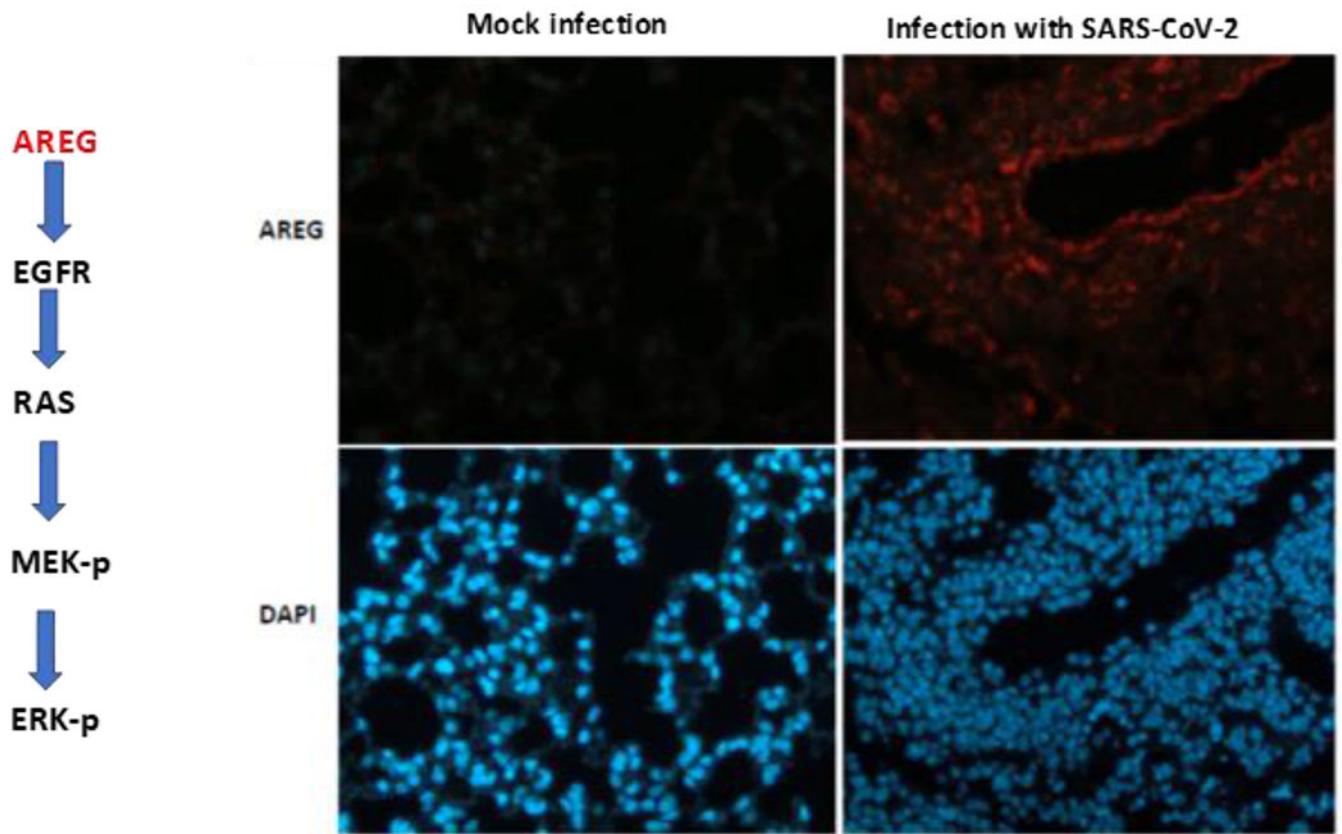


Fig.4. Detection of amphiregulin (AREG) expression in K18hACE2 transgenic mice with or without SARS-CoV-2 infection. AREG protein was detected by immunofluorescent staining (shown in red) with specific antibodies. No AREG expression was observed in mock infected mouse lung tissues whereas high expression of AREG was seen in SARS-CoV-2- infected mouse lung tissues.