Simulation of COVID-19 Symptoms in a Genetically Engineered Mouse Model

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Abstract

The ongoing infectious viral disease pandemic (also known as the *coronavirus* disease-19; COVID-19) by a constantly emerging viral agent commonly referred as the severe acute respiratory syndrome corona virus 2 or SARS-CoV-2 has revealed unique pathological findings from infected human beings, and the postmortem observations. The list of disease symptoms, and post-mortem observations is too long to mention; however, a few notable ones are worth mentioning to put into a perspective in understanding the malignity of this pandemic starting with respiratory distress or dyspnea, chest congestion, muscle or body aches, malaise, fever, chills, etc. We opine that further improvement for delivering highly effective treatment, and preventive strategies would be benefited from validated animal disease models. In this context, we designed a study and show that a genetically engineered mouse expressing the human angiotensin converting enzyme 2; hACE2 (the receptor used by SARS-CoV-2 agent to enter host cells) represents an excellent investigative resource in simulating important clinical features of the COVID-19 infection. The hACE2 mouse model (which is susceptible to SARS-CoV-2) when administered with a recombinant SARS-CoV-2 spike (S) protein intranasally exhibited a profound cytokine storm capable of altering the physiological parameters including significant changes in *in vivo* cardiac function along with multi-organ damage that was further confirmed via histological findings. More importantly, visceral organs from SARS-CoV-2 spike (S) treated mice revealed thrombotic blood clots as seen during postmortem examination of the mice. Thus, the hACE2 engineered mouse appears to be a suitable model for studying intimate viral pathogenesis paving the way for further identification, and characterization of appropriate prophylactics as well as therapeutics for COVID-19 management.

Introduction

All over the world humans have been affected by the constantly emerging new coronavirus agent. After the 1918 flu pandemic, it is the fifth pandemic that has proved so devastating. Officially, the very first report was traced in the Wuhan City of China during the month of December 2019, and the outbreaks are still being reported almost daily. Investigations are undergoing to the nature of its origin though [1, 2]. The causative infectious agent has been named as the severe acute respiratory syndrome-coronavirus 2019 (also known as SARS-CoV-2 or COVID-19, in short). People affected with this virus show various symptoms such as fever, malaise, dry cough, and dyspnea, and are also diagnosed with varying degree of pneumonia [3]. In fact, the SARS-CoV-2 is a retrovirus that can remain air-borne causing widespread infections. The SARS-CoV-2 generally spreads through coughing, talking, and sneezing but it can also spread from touching the items that might have been recently used by a COVID-19 infected individual. Currently there are no effective treatment(s) available for SARS-CoV-2; however, many vaccines are available that have been proven to be highly effective in preventing hospitalizations, and death. Researchers have been working hard to understand the disease mechanisms of SARS-CoV-2 infection so that they could design effective antiviral drugs, and to develop newer versions of the foolproof vaccines against COVID-19 to stop the pandemic.

While some mammalian viral agents such as the poxvirus exhibit a wide host-range for their transmissibility, and propagation including unrelated animal species but unfortunately the SARS-CoV-2 does not infect the laboratory mice strains unless they have been engineered genetically to express the human ACE2 gene; the receptor employed by SARS-CoV-2 agent to enter inside the host cells [4]. In fact, the laboratory mice have served as the 'workhorse' for advancing the biomedical research for devising newer therapies, and to test and validate underlying disease processes. The current study was designed using an engineered mouse model to simulate some, if not all, COVID-19 relevant disease symptoms, and to capture inflammatory signature markers. As we know that the SARS-CoV-2 virus interacts with angiotensin converting enzyme II (ACE2) receptor on the cell surface. The receptor is present on various cell types in many organs throughout the body e.g., lungs, heart, stomach, liver, kidney, etc. The SARS-CoV-2 virion surface is coated with a spike (S) protein that has two subunits: S1, and S2 (Fig. 1). The 'S' protein is known to induce both humoral, and cellular immune responses, and remains the target of new vaccines that are based on full-length S protein, and its receptor-binding domain, including DNA, viral vector, and subunit-based vaccines. In addition, the peptides, antibodies, organic compounds, and short interfering RNAs are additional therapeutics that target the S protein [5, 6]. Interestingly, the COVID-19 mRNA vaccines that are in use currently have been shown to induce the neutralizing antibody response against three SARS-CoV-2 variants [7].

The S1 subunit attaches to the host cell, and then subsequently recognized by the ACE2 receptor on the cell while the S2 subunit assists with viral fusion with the cell membrane [8]. The virus then triggers an intense inflammatory immune response in the infected host [9–15]. Once the immune system detects the presence of a virus, the cytokines, helper T-cells, and white blood cells become quite active [16–34]. If the immune system successfully creates antibodies in conjunction with white blood cells, and B-lymphocytes in time to stop spread of the virus then the host can make a successful recovery, however, if the immune system fails to contain the virus, then it will spread to the lower respiratory tract [35–45]. The immune system will then secret an army of potent cytokines that has been dubbed as the "cytokine storm". The cytokine storm contributes to multi-organ damage, and potentially can lead to death of the infected person [46, 47]. In many people the viral infection can be asymptomatic, or the virus just causes flu-like symptoms such as fever, headache, nausea, and the absence of taste and/or smell [48–56]. One of the symptoms that is important in terms of the pathophysiology is the shortness of breath which is essentially due to the extensive cell death since the virus destroys the lining of the lungs that eventually leads to further inflammatory changes in the lung causing improper alveoli function [57]. Excessive cells death can also lead to clogging, edema, and severe pneumonia. Individuals with prior health problems e.g., diabetes, hypertension, and cancer and the people who are over the age of 65 are more susceptible to developing the pneumonic symptoms due to their compromised immune system. Examples of individuals with a compromised immune system can be someone who smokes, has high blood pressure, cancer, diabetes, etc. [58].

COVID-19 can also cause clots inside the blood vessels of lungs, heart, and kidney. These blood clots can induce additional medical emergencies like stroke or a heart attack, potentially resulting in death of the infected host [59]. The clots are the results of SARS-CoV-2 induced damage caused in the lining of blood

vessels. This damage in the blood vessels can induce platelets recruitment to prevent the blood leaking out from vessels into the surrounding tissues. Clots are formed to fix the damaged blood vessels, excessive clotting could block the blood vessels though, thus disrupting the blood flow [59]. Research has also shown that the number of available ACE2 receptors can influence blood clots formation. In that context, more ACE2 receptors can increase viral fusion, thus potentially increasing blood vessels' injury, hence paving the way for more clots' formation. Unfortunately, excessive coagulation could result in a "clotting cascade" then thrombosis (blood clot within a blood vessel). SARS-CoV-2 also affects the heart as it has been shown to cause acute cardiovascular injury. The proposed major cause of cardiovascular injury is myocarditis because of the SARS-CoV-2 led systemic inflammation. Myocardial injury can also occur when spike protein binds to ACE2 receptor in the heart leading to altered signaling thus causing myocardial injury [59]. COVID-19 not only causes *de novo* myocardial injury but also puts individuals on a serious health risk trajectory who happen to have diabetes, are obese, have coronary artery disease, or the heart failure, therefore, expediting myocardial injury further. It is important to note that obesity can result in constant inflammation of tissues which results in cell death. In short, the COVID-19 can severely affect the heart's potential long-term effects from myocarditis that essentially include "arrhythmia, heart failure, and increased risk of stroke or heart attack".

Another harmful impact COVID-19 is accumulation of excess fluid within the body. When blood vessel encounters a foreign pathogen such as the SARS-COV-2 virus then endothelial cells lining the vessels react by changing from a squamous shape to a columnar shape that helps "adhesion molecules" attract helper cells such as leukocytes and chemokines to cause an inflammatory response and allow the immune system to attempt to fight off the virus. Not only are helper cells recruited, but when the shape of the endothelial lining is altered it can result in "thrombogenic basement membrane" leading the neutrophils to expand under the effects of cytokines, specifically IL-1a, and when this inflammatory process is further activated then endothelial lining gets disrupted. Furthermore, the endothelial cells containing metalloproteinases (MMPs) can destroy the basement membrane of the arteries, and capillaries in the lungs causing leakage of the fluid [60]. It is important to remember that there are many variants of the SARS-CoV-2 such as alpha (B.1.1.7), beta (B.1.351), gamma (P.1), the commonest one delta (B.1.617.2), but very recently one more newer variant "Omicron" has been identified in South Africa. A variant is a modified form of the original virion particle wherein mutations arise that raises public health concerns because the new variant(s) seems to spread easier and faster, cause worse symptoms, making testing less accurate, and that basically "escape" the immune surveillance system provided by the currently available COVID-19 vaccines or by natural infection with the original virus. The alpha, beta, gamma, and delta variants were first detected in the United Kingdom, South Africa, Brazil, and India, respectively. The delta variant is the current variant that is most present in the United States. The variant has been shown to be "60% more transmissible" than the alpha variant that has been sweeping through the world [61].

There are currently no known treatments available for SARS-CoV-2, and if one gets infected there are a few palliative measures that can be taken to decrease some symptoms such as fever or pain with hydrocortisone, ibuprofen, or aspirin. Convalescent sera, and the monoclonal antibodies have been

shown to impart some protection during the early phase of the infection. Out of the listed medications hydrocortisone has been noted to cause the least amount of side effects. Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen have been shown to alter the pentose phosphate pathway, and therefore should not be used for more than 2-3 days in a row. Also, acetaminophen has been shown to create reactive oxygen species (ROS) which can be harmful to the cells, and therefore should not be used on a long-term basis. Also, proposed immunomodulators include azithromycin, and clarithromycin [62]. In addition to taking medication, one should ensure that he/she quarantines for 14 days after beginning to experience symptoms of COVID-19. Since there is no known effective form of treatment, thus it is highly recommended that one gets vaccinated to decrease the chances of contracting COVID-19. And if one is to contract COVID-19 after being vaccinated it has been noted that one's symptoms should not be as severe and is less likely to be hospitalized. It is also important to keep in mind that being vaccinated means one has received at least two doses of vaccine and waited 2 weeks since their last vaccination to be considered fully vaccinated. Although one's chance of getting sick after being vaccinated has decreased tremendously, one should still carry out safety protocols as recommended by health agencies. This means maintaining a 6 feet distance between others when in public, wearing a mask, washing hands frequently, and practicing sanitary guidelines. On a general note, physical exercise has been shown to help in decreasing one's chance of contracting COVID-19 or decreasing the severity of the symptoms because exercise seems to assist in building the immune system, and helping one remain healthy. In the present study, we treated the engineered hACE2 mouse as well as human cells with SARS-CoV-2 spike protein (S) and collected multiple data sets. The study paradigm turned out to be highly encouraging in understanding the COVID-19 infection in a much more elaborate way, and we believe that the results might help in devising better tools in diagnosing, treating, and preventing corona virus disease, and other similar infections.

Materials And Methods

Animals, and the human primary cells

The human umbilical vein endothelial cells; HUVEC, and human coronary artery endothelial cells; HCAEC were treated with SARS-CoV-2 spike (S) protein. Male and female transgenic mice expressing the human ACE2 receptor were purchased from the Jackson Laboratory ((Bar Harbor, ME, USA), (B6. Cg-Tg(K18-ACE2)2Prlmn/J, Genotype: hemizygous for Tg(K18-ACE2)2Prlmn). The mice (in short, as the ACE2 mice) were housed in a pathogen-free environment under conditions of 20°C ± 2°C, 50% ± 10% relative humidity, 12 h light/dark cycles, and they were provided with food and water *ad libitum*. The animal procedures were reviewed and subsequently approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Louisville School of Medicine, Louisville, Kentucky, USA. Further, the animal care and guidelines of the National Institutes of Health (NIH, USA) were also adhered to. The male, and female mice approximately of the same age (8–12 weeks) were recruited. The mice were fed standard chow diet and water *ad libitum*. Mice were anesthetized with Ketamine/Xylazine (50/10 mg/Kg). Mice were administered intranasally while the cells were treated in petri dish either with SARS-CoV-2 recombinant

spike (S) protein (ECD-His-tag, Genescript, cat# Z03481) or with freshly mixed poly(I:C) poly[I:C]-HMW, Invivogen, tlrl-pic) @ 2.5 mg/ml and SARS-CoV-2 recombinant spike protein (SP) (ECD-His-tag, Genescript, Z03481) 5-15 µg (in 10 µL sterile phosphate buffered saline; PBS followed by 100 µL air) [63]. The respective control mice, and cells were treated with either @ 2.5 mg/kg poly (I:C) or PBS at the same volumes. Post treatment mice were followed up to 5 days while the cells were harvested at 6 hours or 24 hours.

Measurement of physiological parameters

Body temperature, body weight, respiration rate, heart rate, systolic and diastolic pressure, and the intraocular pressure (IOP) were recorded in the SARS-CoV-2 spike (S) protein treated and untreated human ACE2 receptor engineered mice groups as reported earlier in our published work [64] [65, 66]. Only measurements that were judged by data analytical system to be within its acceptable parameters were recorded as valid.

Creatine kinase isoform measurements

The blood levels of creatine kinase (CK) activity were also measured. In brief, the tissue-specific injury was determined by measuring the CK isoforms in the serum samples from each group of mice. The CK-MM represents the cardiac- and skeletal-muscle-specific isoform, while the CK-BB is primarily a nerve-specific and kidney-specific isoform, respectively. From each mouse, 10 µl of plasma was mixed with 1 µl of activator and loaded onto the CK gel as instructed by the manufacturer (QuickGel® CK Vis Isoenzyme Procedure; Helena Laboratories, TX, United States). The gels were run at 400 V for 4:15 min. The standard (ST) amounts of CK isoforms were also loaded in parallel to the samples [67, 68].

Cytokine profiling, and Western blotting

The relative expression profile of cytokines was performed using a proteome profiler antibody array (R&D Systems, ARY015; Minneapolis, MN). The array was hybridized with an equal amount of total protein human primary umbilical vein endothelial cells (HUVEC) or human primary coronary artery endothelial cells (HCAEC) from treated and untreated SARS-CoV-2 spike (S) protein. The assay was performed according to the manufacturer's protocol. For the Western blotting antibodies IL6 (Cat. #12153), IL8 (Cat. #94407), MIG (Cat. #30327, and uPAR (Cat. #12863) were purchased from the Cell Signaling Technology (Danvers, MA) while CD147 (Cat. #ab64616) and GAPDH (Cat. #SC-365062) were purchased from Abcam (Waltham, MA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-rabbit IgG-HRP conjugate and anti-mouse IgG-HRP conjugate, both were from Cell Signaling Technology (Cat. #7074, and Cat. # 7076 respectively). For GAPDH, the primary antibody dilution used was 1:3000, and the secondary antibodies with HRP conjugation, the dilutions used were 1:5000, respectively. The protein was isolated using protein extraction buffer (RIPA lysis buffer, protease inhibitor cocktail and PMSF). Lysates were spun in extraction buffer for 12 h and then centrifuged at 12,000 × g for 15 min. Supernatants at

different time points either from human primary umbilical vein endothelial cells (HUVEC) or human primary coronary artery endothelial cells (HCAEC) were transferred to new tubes and protein concentrations were analyzed via Bradford protein estimation assay. Samples (total protein (50 µg) were run on a 10/12% sodium dodecyl sulfate (SDS)-polyacrylamide gel with Tris–glycine SDS buffer. The gel was transferred electrophoretically overnight onto a PVDF membrane at 4°C. The membranes were blocked with a 5% milk solution for 1 h. Primary antibodies were diluted at a concentration 1:1000 in TBST and incubated on the membrane overnight. All membranes were washed in TBST solution four times and then incubated with secondary HRP conjugated antibody solution for 1 h at room temperature. Four TBST washing steps followed before membranes were developed using a chemiluminescent substrate in a BioRad Chemidoc (Hercules, Calif.). Band intensities were determined using densitometry analysis. The relative optical densities of protein bands were analyzed using gel software Image Lab 3.0. The membranes were stripped and re-probed with GAPDH as the loading control. The expression levels of each protein were also quantified as shown in the respective bar charts, n=3-5 petri dish/group.

Visceral organ observation, and histopathological investigation

Mice visceral organs were collected and observed for their appearance. The heart and lung samples were collected in 4% buffered paraformaldehyde for fixation and were processed after embedding in paraffin. A 5-µm-thick section from each sample was cut and stained with hematoxylin and eosin (H&E). The detailed methods have been previously described [69].

Statistical analysis

Data from mice and cells were collected and statistically analyzed using the GraphPad Prism 9.0 (GraphPad Software, United States). Multiple comparisons were performed using one-way ANOVA with Bonferroni, as appropriate.to analyze the difference between the groups, including a Tukey's post hoc analysis for groups' comparison. The comparison between two groups were performed by unpaired Student’s t-test. The *p<0.05 was regarded as statistically significant, data was reported as mean ± SEM, and error bars indicate SEM, n = 3–5 petri dish or animals/group,

Results

As per our “*a priori*” belief that biding of the SARS-CoV-2 virion’s spike (S) protein to the host cell is associated with downstream cellular and molecular signaling events, we were able to show many of the salient features that are generally seen in the COVID-19 infected patients. To our knowledge, this study is the first one of its kind wherein we have attempted to capture some of the clinical features, and postmortem observation as seen in patients. In addition, we were able to collect data points both at the whole organism level as well as under *in vitro* conditions employing a range of tools such as cellular, biochemical, physiological, and histopathological approaches. While many studies have sought to simulate infection related observations; however, we are unaware of the similar attempts by others of using an engineered animal species and other similar resources to specifically study a rage of parameters

that are highly relevant to the actual clinical scenario. The findings from this study could help learn and gain newer insights towards improving the efficacy of the currently available diagnostic, therapeutic, and prophylactic strategies.

Measurement of physiological parameters, and echocardiography of mice

The body temperature, body weight, and respiration rate were found to be less in the SARS-CoV-2 spike (S) protein treated hACE2 (Cg-Tg) mice in comparison to the untreated ACE2 mice while the heart rate (systolic and diastolic) was not affected much (Fig. 2A). Interestingly, the intraocular pressure (IOP) in the SARS-CoV-2 spike (S) protein treated hACE2 mice was significantly affected than the untreated ACE2 mice (Fig. 2B).

More importantly, the echocardiography findings; however, did reveal alterations in diastolic and systolic chambers of the hACE2 mice treated with the spike (S) protein in comparison to the untreated control hACE2 mice. Furthermore, contraction and relaxation of the myocardium was attenuated in spike (S) protein treated mice in comparison to the untreated control hACE2 mice (Fig. 3) as seen via the M-mode echocardiography images from each group of mice.

Creatine kinase assay

When blood level of the creatine kinase (CK) activity was assayed in the SARS-CoV-2 spike (S) protein treated hACE2 (Cg-Tg) mice, and untreated mice groups the tissue-specific injury was evident in the treated group as determined by the measurement of CK isoforms in the serum sample. The muscle injury was maximum, and significant followed by heart and brain (Fig. 4).

3.3 Cytokine proteome profiling, and analysis of key target proteins

The expression profile of cytokines captured by the array analysis, and the important protein targets investigated via the Western blotting either from the cell culture supernatants or the cell lysates that were treated with SARS-CoV-2 spike (S) protein alone or with Poly: IC for different timer points, the cytokines and the key protein molecules revealed robust modulation of their respective expression levels in the SARS-CoV-2 spike (S) protein treated cells than the non-treated cells (Fig. 5A-F).

Visceral organ observation, and histopathological investigation

When the mice visceral organs when collected, and observed for their appearance, it became abundantly clear that there was significant change in their appurtenance and there were blood clots, and thrombi in them. Most importantly, the organs looked dark in color (Fig. 6). The H&E-stained lung, heart, and kidney samples revealed extensive infiltrations of the immune cells in the SARS-CoV-2 spike (S) protein treated

mice in comparison to the untreated mice. Interestingly, kidney exhibited extensive tissue damage in the treated mice than the non-treated mice (Fig. 7A, B).

Discussion

In this study we show that upon SARS-CoV-2 virion's spike (S) protein treatment of the mice, and human cells led to the hyperinflammatory state relative to untreated mice or human cells. The spike (S) protein elicited secretome from inflamed targets, that is, from the *in vivo* (mice) or *in vitro* (human cells) systems causing an increased expression of the important proteins/targets. The significant increase in cytokines or alterations in mouse organs relative to untreated cells or mice confirm our hypothesis that biding of the spike (S) protein to the host cell is associated with downstream cellular processes/events. Such pathological events/processes might be happening in the COVID-19 patients during viral pathogen replication in the target host cells. We recognize that our work has some limitations such as: 1) we did not use the infectious virus particles (virions) simply because of the fact that we are not allowed to handle, and culture or grow the SARS-CoV-2 agent in our laboratory since we are not equipped for experimenting on infectious agents, 2) although we did use human cells in conducting *in vitro* experiments, and an engineered mouse model expressing the human angiotensin converting enzyme 2 (hACE2) receptor to obtain the data, we were again limited in not using the human serum/plasma samples because of the associated risk of inadvertent SARS-CoV-2 viral transmission/spread to others while using the human origin samples from the clinic/hospital patients who are being tested or treated for the COVID-19 in our medical school's affiliated clinics/hospitals.

In conclusion, we present interesting evidence that interaction between the SARS-CoV-2 virion's spike (S) protein with that of human angiotensin converting enzyme 2 (hACE2) receptor leads to robust cellular events. If further research can validate or extend our findings then certainly such small, engineered animal models could serve as important tools in fighting, and winning this non-stop COVID-19 pandemic, and other related infectious diseases. As shown by others that a heightened pathological response in the form of increased cytokine storm and multi-organ damage can lead to vital organ failure, and ultimately death in some COVID-19 patients as already revealed during the last ~2 years since the start of the pandemic [70–82]. To dissect out further the physiological and pathological implications of the SARS-CoV-2 induced changes, we set out this important study to captures some of the initial/beginning phase of the intimate interaction(s) between the host cell receptor with the SARS-CoV-2 spike (S) protein employing a humanized/genetically engineered mouse model expressing the human angiotensin converting enzyme 2 (hACE2) and the recombinant SARS-CoV-2 spike (S) that was delivered via the intranasal route [83]. The SARS-CoV-2 spike (S) protein binding to ACE2 receptor amplify susceptibility to COVID-19 virion-induced inflammation in various organs along with occurrence of the cytokine storm in the patients. Accordingly, the antibodies specific to the spike (S) protein have been shown to be highly protective, and thus reducing not only the clinical symptoms but also the cytokine storm, and the subsequent organ inflammation or damage, therefore; reducing the mortality after SARS-CoV-2 exposure.

Our findings support the hypothesis and corroborates the clinical observation of targeting the spike protein as a COVID-19 preventing strategy for safeguarding human health against this deadly disease in susceptible population. The same is true for the fact that therapeutically targeting of the spike (S) protein via specific monoclonal antibodies in the initial phase of the COVID-19 can prevent serious organ damage, and related health issues in the COVID-19 affected patients with alleviation of both the morbidity and mortality. Our result from the preclinical model also suggests that creatinine kinase-based assays, and other blood biomarkers may be developed, and employed to not only protect other individuals who are vulnerable to adverse COVID-19 outcomes in whom there are increased chances of occurring serious COVID-19 symptoms such as in obese or numerous chronic diseases affected individuals. In short, animal models such as engineered ones may play an important role(s) in studying in-depth disease mechanisms towards developing lifesaving therapeutics, and preventative measures. Finally, we hypothesize about the most plausible mechanisms(s) by which SARS-CoV-2 most likely induce the visceral organ damage in the infected host. Post internalization of the SARS-CoV-2 virions via the human angiotensin converting enzyme 2 (hACE2) receptor, it causes a robust surge of the inflammatory markers, epithelial barrier dysfunction, and the multi-organ damage and congestive (cardio-pulmonary) heart failure (CHF) [47, 84]. Interestingly, the hACE2 is highly expressed in the renal tubular epithelial cells and podocytes. Studies have shown robust increase of neopterin (NPT) in COVID-19 patients. Interestingly, NPT is generated by IFN-γ-induced inflammatory macrophage (M1Q) in response to viral infection. COVID-19 infection causes recruitment of inflammatory cells and a further robust surge of the inflammatory cytokines, epithelial barrier dysfunction, podocyte and endothelial damage leading to AKI. As reported in this study, the SARS-CoV-2 spike (S) protein induced cytokine storm. We strongly believe that M1Q activation leads to oxidative stress, and peroxynitrite/nitrosylation. The resultant NLRP3 inflammasome formation activates the apoptosis pathways leading to T cell lymphopenia (that is decrease in CD4+, and CD8+ cells) thus inciting the proximal tubular epithelial cell/podocyte injury and leakage (Fig. 8). It is known that the COVID-19 activates innate immune system causing the acute kidney injury (AKI) as reported in 27-40% of the ICU admissions [84–98]. More importantly, the hACE2 engineered mouse model can also be used to identify the potential safety issues that may be associated with COVID-19 inhibitors that are being developed by the pharmaceutical industry.

Declarations

Author contributions MS conceived the idea of the research plan, designed experiments, helped analyze data, and wrote the manuscript's draft. MS and SCT edited and finalized the manuscript. NB, YZ, RPH, and SP performed the experiments, and helped write the materials, and methods section and figure legends in the initial draft of the manuscript.

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Data availability statement The datasets generated and analyzed during this study are available upon request as per the data sharing policies of NIH.

Competing Interests The authors have no conflict of interest, financial or otherwise.

Consent to participate Not Applicable.

Ethics approval The study protocol was approved by the University of Louisville School of Medicine, Louisville, Kentucky.

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Figures

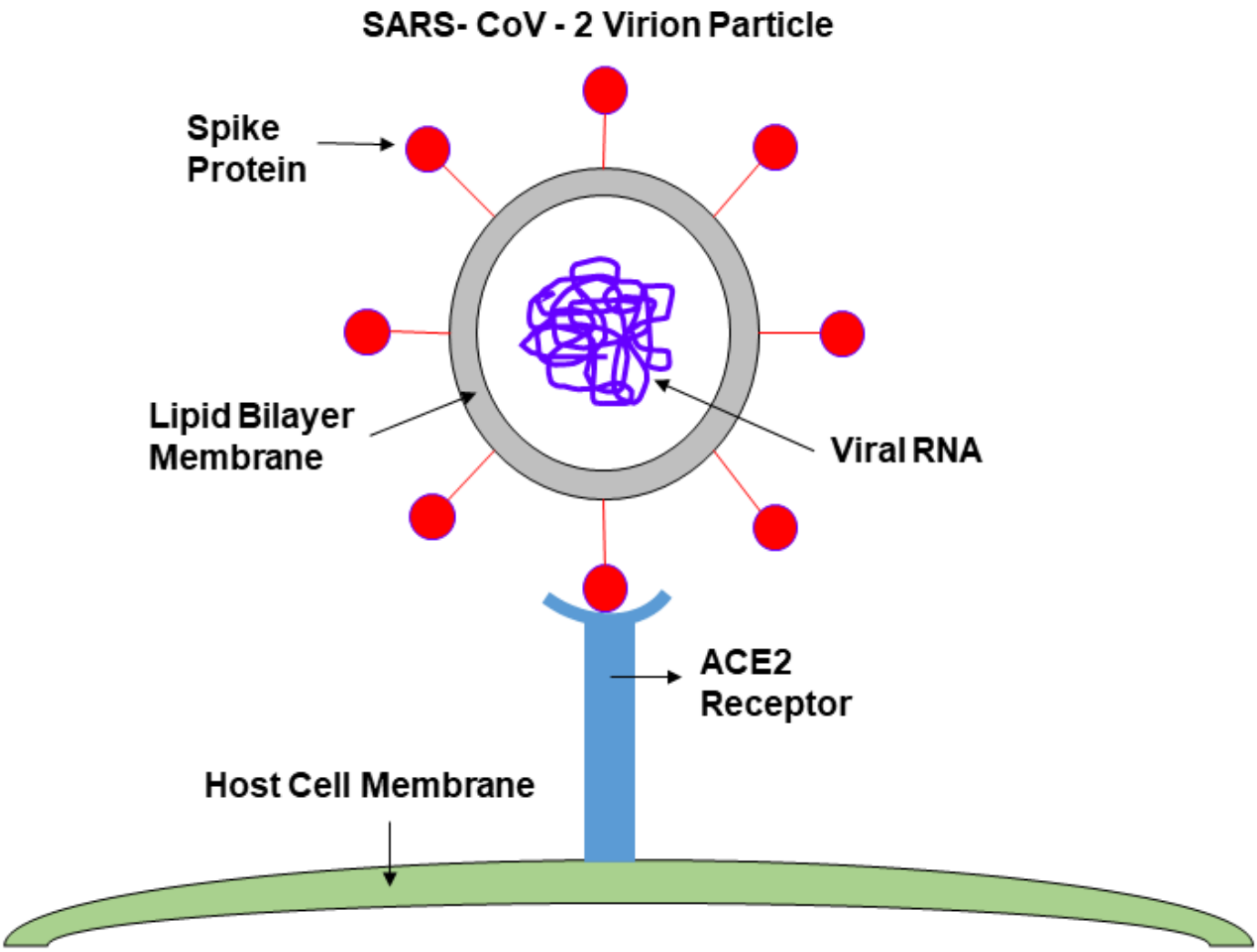

Figure 1

A schematic depicting the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) virion and binding of its spike (S) protein with the host cell receptor. The SARS-CoV-2 'S' protein mediates binding of the virion with its receptor angiotensin-converting enzyme 2 (ACE2) and promotes the fusion between the virion and host cell membrane thus allowing the virion entry into the host cell. The viral ribonucleic acid (RNA) is a single stranded, non-segmented that is ~ 30 kilobase in its size is enclosed inside a protein coat known as capsid. It is the capsid that is coated with 'S' protein which has two subunits known as S1 and S2. The S2 subunit recognizes the ACE2 receptor on the host cell membrane while the S1 subunit helps mediate viral fusion with the cell.

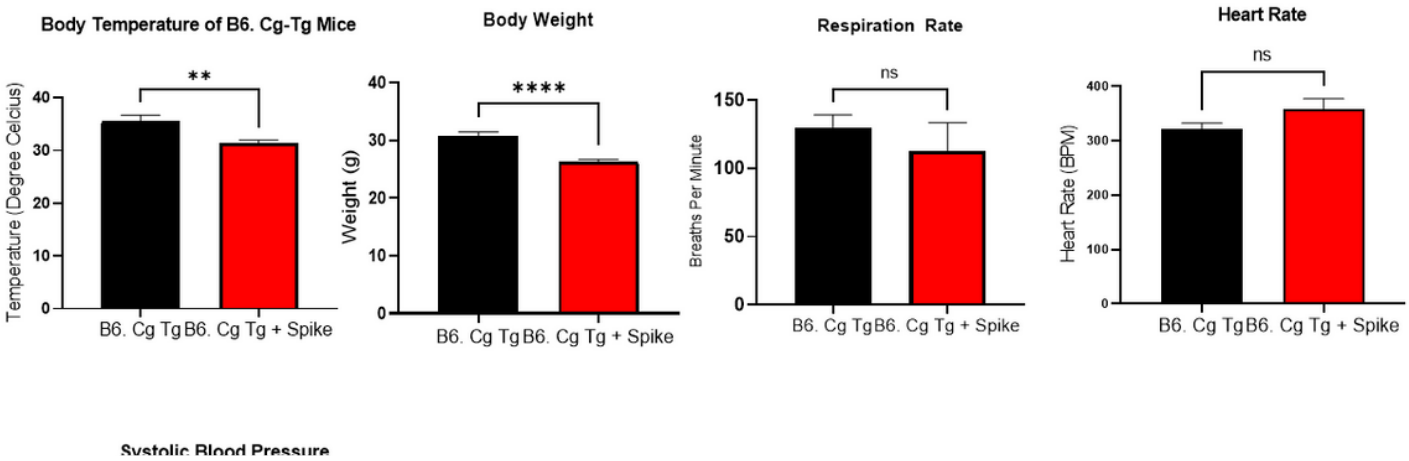

Figure 2

(A) Body temperature, weight, respiration, and heart rate, blood pressure (systolic, diastolic), and intraocular pressure of the SARS-CoV-2 spike (S) protein treated hACE2 (Cg-Tg) mice versus untreated ACE2 mice (B6. Cg TgB6). (A) Daily body temperature, and the body weight of the ACE2 mice administered with the spike (S) protein via the nasal route were compared to the control mice (without spike; S protein administration). Unpaired t-tests were performed, *<p0.01, **p<0.001 ***p<0.001, ****p<0.0001 respectively, n=3-5 mice/group. The respiration rate, and heart rate of ACE2 mice (measured while doing the echocardiography) were also recorded. Unpaired t-test were performed, p<0.2056, p<0.2002 respectively, n=4. (B) Blood pressure were measured by Coda Non-Invasive Instrument. Unpaired t-test were performed, p<0.3890, n=3-5 mice/group. Systolic, and diastolic pressure were measures and the unpaired t-test were performed, p<0.4696, n=3-5 mice/group. Similarly, intraocular pressure was measured by iCareLab Tonometer, and unpaired t-test were performed, p<0.0023, n=3-5 mice/group.

Figure 3

Echocardiography of the SARS-CoV-2 spike (S) protein treated hACE2 (Cg-Tg) mice versus untreated ACE2 mice (B6. Cg TgB6) groups. Representative M-mode echocardiography images from each group are

presented indicating diastolic (longer) and systolic (shorter) chamber lengths in the hACE2 mice treated with the spike (S) protein in comparison to the untreated control hACE2 mice. The contraction and relaxation of the myocardium are attenuated in spike (S) protein in comparison to the untreated control hACE2 mice, n=3-5 mice/group.

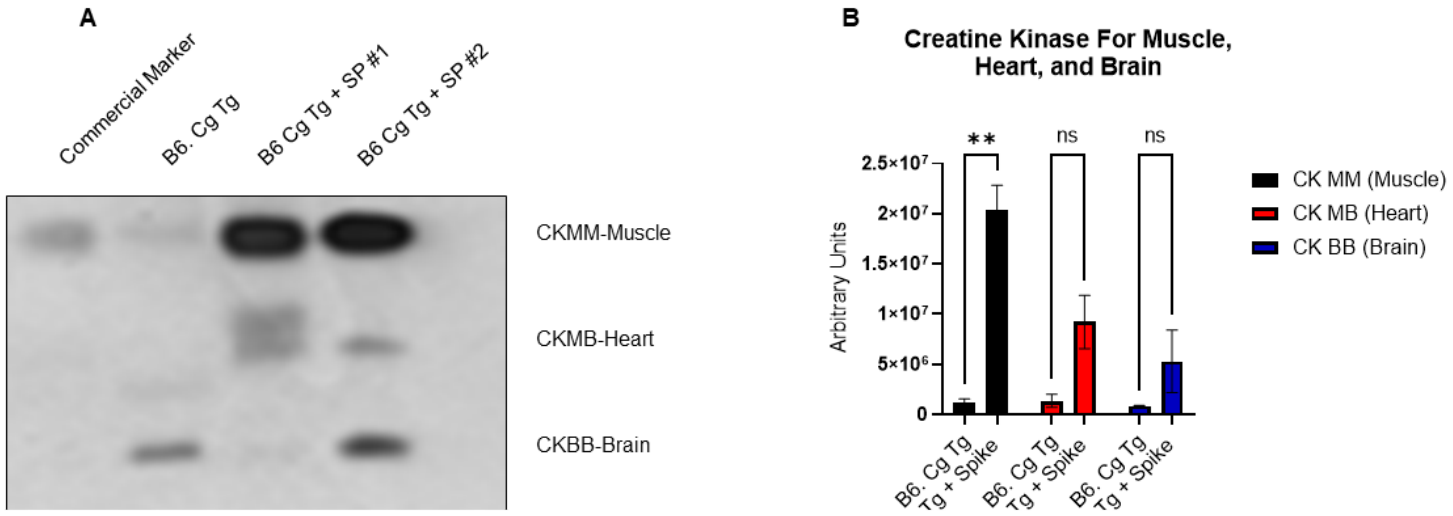

Figure 4

Creatine phosphokinase (CK) levels in the SARS-CoV-2 spike (S) protein treated hACE2 (Cg-Tg) mice versus untreated ACE2 mice (B6. Cg TgB6). The spike (S) protein treated (SP #1 and SP #2) were higher compared to the control mice. Differences in CK levels (A) in due to multi-organ damage such as skeletal muscle (CK-MM), heart (CK-MB), and the brain (CK-BB) were quantified (B). The binding of spike (S) protein to hACE2 receptor causes multi-organ damage. Two-Way ANOVA with multiple comparisons, **p<0.009, n=3-5 mice/group.

Figure 5

(AB) The proteome profiler with antibodies arrays reveals induction of cytokines on primary human umbilical vein endothelial cells (HUVEC). The cells were treated either with sterile phosphate buffered saline (PBS) alone or SARS-CoV-2 spike (S) protein; or with 10 mg of Poly I:C, or both SARS-CoV-2 spike (S) protein and Poly I:C (A). The quantitation and comparison of spike protein induced CD147, IL-6 & IL-8, MIG, and uPAR are shown in comparison with respective control (B). Depicted the different fluorescence intensity of the different proteins measured, n=3-5 petri dish/group.

(C) Western blot analysis of the key targets. Supernatants from human primary umbilical vein endothelial cells (HUVEC) at 6 and 24 hours post treatment using the control (ctl), sp (spike protein), sp-poly (spike protein and poly I:C), and poly (poly I:C). The primary antibodies used were Interleukin-6 (IL-6), CD147 (EMPERIN), and uPAR and protein bands were normalized with GAPDH. The expression levels of each protein were also quantified as shown by the bar charts, n=3-5 petri dish/group, ns= not significant, *p<0.01, ****=p<0.0001

(D) Western blot analysis of the key targets. Supernatants from human primary coronary artery endothelial cells (HCAEC) at 6 and 24 hours post treatment were performed using the control (ctl), sp (spike protein), sp-poly (spike protein and poly I:C), and poly (poly I:C). The primary antibodies used were Interleukin-6 (IL-6), CD147 (EMPERIN), and uPAR, and the protein bands were normalized with GAPDH. The expression levels of each protein were also quantified as shown by the bar charts. n=3-5 petri dish/group, ns= not significant, *p<0.01, ****=p<0.0001

(E) Western blot analysis of the key molecules. Immunoprecipitants from the human primary coronary artery endothelial cells (HCAEC) and human primary umbilical vein endothelial cells (HUVEC) post 24 hours treatment were used from the samples: control (ctl), sp (spike protein), sp-poly (spike protein and poly I:C), and poly (poly I:C). The primary antibodies used were for Interleukin-8 (IL-8), and MIG (cxcl9). Expression levels of the proteins are shown in the bar charts after the bands were normalized with GAPDH, n=3-5 petri dish/group, ns= not significant, *p<0.01, ****=p<0.0001

(F) Western blot analysis of the key molecules and their quantitation. Lysates from the human primary coronary artery endothelial cells (HCAEC) and human primary umbilical vein endothelial cells (HUVEC) were probed 24 hours post treatment using the samples: medium as control (ctl), SP (spike protein), SP-Poly (spike protein and poly I:C), and Poly (poly I:C). The primary antibody used were Interleukin-6 (IL-6), CD147 (EMPERIN), and uPAR. The protein bands were normalized with GAPDH, n=3-5 petri dish/group.

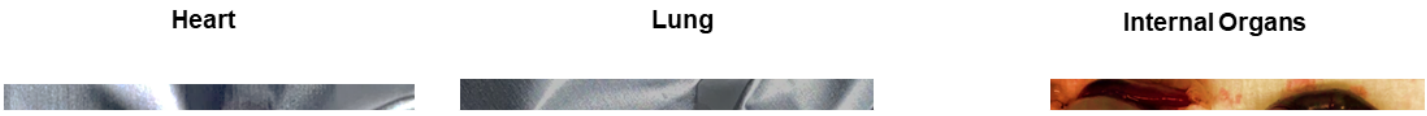

Figure 6

Representative pictures of the mice heart, lungs, and internal visceral organs. The heart, and intestine clearly show evidence of blood clot, and thrombosis formation. Some of the vital organs look very dark in

color indeed, n=3-5 mice/group.

Figure 7

(A) Hematoxylin and eosin (H&E) staining of the lung, heart, and kidney samples. from the humanized ACE2 (B6. Cp-Tg) and ACE2 (B6.Cp-Tg) + Spike protein. Black circle clearly depicts a cluster of infiltrated immune cell populations while heart section shows diffused inflammatory cells throughout the parenchyma, magnification x 20, scale bar – 50 μm n=3-5 mice/group. **(B)** Kidney sections (5 μm thickness) were stained with H&E (A) Representative image of control kidney and (B) Spike (S) protein treated kidney showing loss of glomerular tuft and hyaline deposit (green arrows), desquamation of tubular epithelium and necrosis (red arrow heads), inflammatory cell infiltration (yellow arrows) and tubular necrosis (purple arrow). Magnification x 60, scale bar – 50 μm. The control mice received saline, n=3-5 mice/group.

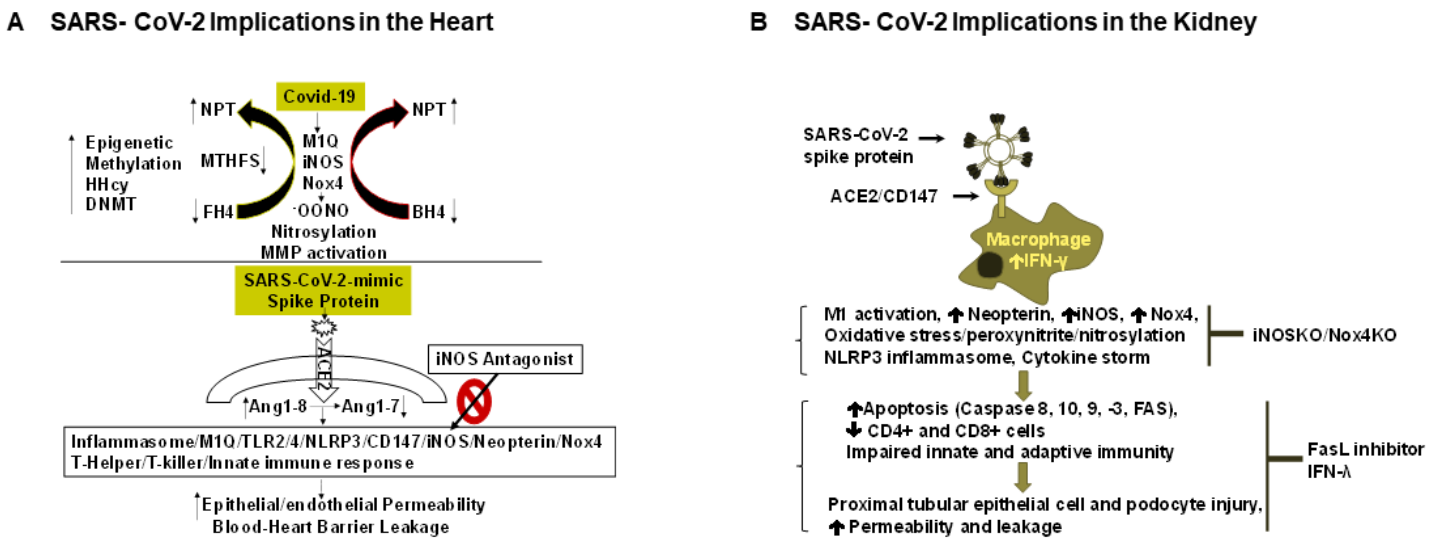

Figure 8

(A) plausible hypothesis regarding SARS-CoV-2 induced visceral organ damage. The binding of SARS-CoV-2 spike (S) protein with ACE2 receptor mimics SARS-CoV-2 infection and causes the accumulation of Ang1-8, activation of inflammasome and M1Q macrophages via the "TLR4/NLRP3/CD147/Nox4/iNOS/neopterin" axis in the heart. This cascade of events leads to endothelial blood-heart barrier (BHB) leakage however; the iNOSKO/Nox4KO and iNOS antagonists may mitigate the inflammasome/NLRP3/M1Q mediated endothelial BHB leakage (**A**), as reported earlier by **Tyagi and Singh**, Multi-organ damage by COVID-19: Congestive (cardio-pulmonary) heart failure, and blood-heart barrier leakage, Mol Cell Biochem. 2021;476 (4):1891-1895). Similarly, biding of the SARS-

CoV-2 spike (S) protein to ACE2/CD147 on macrophages can cause M1Q activation by IFN-γ towards generating the neopterin and thus stimulating the iNOS, Nox4 and NLRP3 inflammasome pathway in the kidney that in turn can trigger apoptosis which may lead to CD4+ and CD8+ cell lymphopenia. These alterations might inflict the proximal tubular epithelial cell/podocyte damage and the resultant parenchymal leakage. In that case, the iNOSKO/Nox4KO and Fas/FasL antagonists (Kp7-6)/IFN-λ treatment could help mitigate the cytokine storm and T cell lymphopenia thus protecting the proximal tubular epithelial/podocyte function **(B)**. M1; inflammatory macrophage (M1Q), iNOS; inducible of nitric oxide synthase, BH4; FH4; tetrahydrobiopterin, tetrahydrofolate.