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Simulation of COVID-19 symptoms in a genetically engineered mouse model: implications for the long haulers

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Abstract

The ongoing pandemic (also known as 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 postmortem observations is too long to mention; however, SARS-CoV-2 has brought with it a whole new clinical syndrome in "long haulers" including dyspnea, chest pain, tachycardia, brain fog, exercise intolerance, and extreme fatigue. We opine that further improvement in delivering 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; ACE-2 (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. The ACE-2 mouse model (which is susceptible to SARS-CoV-2) when administered with a recombinant SARS-CoV-2 spike protein (SP) intranasally exhibited a profound cytokine storm capable of altering the physiological parameters including significant changes in cardiac function along with multi-organ damage that was further confirmed via histological findings. More importantly, visceral organs from SP treated mice revealed thrombotic blood clots as seen during postmortem examination. Thus, the ACE-2 engineered mouse appears to be a suitable model for studying intimate viral pathogenesis thus paving the way for identification, and characterization of appropriate prophylactics as well as therapeutics for COVID-19 management.

Keywords Humanized mouse · SARS-CoV-2 spike protein · Clinical symptoms · Multi-organ damage · Disease management

Introduction

All over the world humans have been affected by the constantly emerging new coronavirus agent. Officially, the very first report was traced in Wuhan City of China during December 2019, and outbreaks are still being reported globally. 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). Infected people exhibit symptoms such as fever, malaise, dry cough, and dyspnea, and are also diagnosed with varying degree of pneumonia [3]. Currently there are not many effective treatment modalities or the cure available; however, vaccines are highly effective in preventing the hospitalization, severe disease, and death. Researchers are working to understand disease mechanism(s) of SARS-CoV-2 infection so that they could design more effective drugs, and develop newer versions of the foolproof vaccines against COVID-19 to stop the ongoing pandemic.

While some viral agents such as poxviruses exhibit a wide host-range for transmissibility, and propagation including propensity to infect unrelated animal species but

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unfortunately the SARS-CoV-2 does not infect laboratory mouse unless the mouse has been engineered genetically to express human ACE-2 gene; the receptor employed by SARS-CoV-2 agent to enter inside the human cells [4]. In fact, laboratory mouse has served as the 'workhorse' for advancing biomedical research, and for devising newer therapies, and also to test, and validate underlying disease processes. The current study was designed using an engineered mouse to simulate some of the COVID-19 relevant disease symptoms, and to capture inflammatory signature markers that appear to be relevant for COVID-19 long-haulers. SARS-CoV-2 virus interacts with angiotensin converting enzyme II (ACE-2) receptor on cell surface. The receptor is present on various cell types throughout body, e.g., lungs, heart, stomach, liver, and kidney. The virion surface is coated with a spike protein (SP) 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 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 (siRNAs) are additional therapeutics under development [5, 6]. Interestingly, the COVID-19 mRNA vaccines that are in use currently have been shown to induce neutralizing antibody response against the SARS-CoV-2 [7].

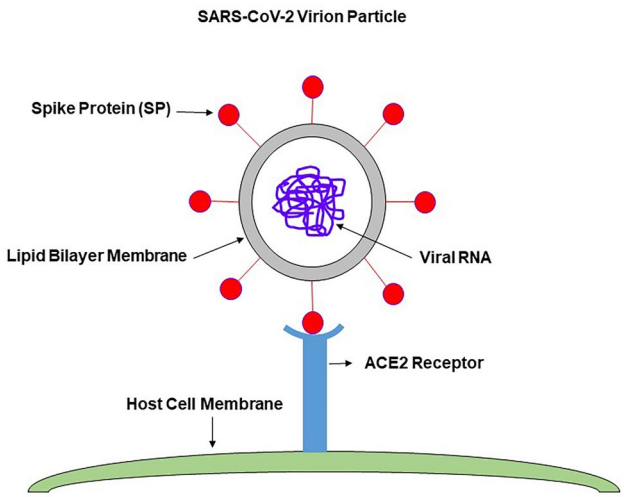

Fig. 1 A schematic depicting the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) virion and binding of its spike protein (SP) with the host cell receptor. The SARS-CoV-2 'SP' mediates binding of the virion with its receptor angiotensin converting enzyme 2 (ACE-2), and promotes fusion between the virion and host cell membrane thus allowing the virion entry into host cell. The viral ribonucleic acid (RNA) is a single stranded, and non-segmented that is ~ 30 kilobase in size is enclosed inside a protein coat known as the capsid. It is the capsid that is coated with 'SP' protein which has two subunits known as S1 and S2. The S2 subunit recognizes ACE-2 receptor on host cell membrane while S1 subunit helps mediate viral fusion with the cell

Once the S1 subunit attaches to cell, it is recognized by the ACE-2 receptor while the S2 subunit assists with fusion with cell membrane [8]. Virus then triggers an intense immune response [9–15]. The immune system detects the virus, and then cytokines, helper T-cells, and white blood cells become active [16–34]. This leads to the "cytokine storm" that contributes to multi-organ damage and can lead to death [35, 36]. In many cases the infection can be asymptomatic, or the virus just causes flu-like symptoms [37–45]. One of the symptoms is the shortness of breath due to extensive lung-cell death causing alveoli dysfunction [46]. Cells' death can also lead to edema, vessels clogging, and pneumonia. Individuals with co-morbidities, e.g., diabetes, hypertension, and cancer and people over the age of 65 are highly susceptible to developing pneumonia due to their compromised immune system [47].

Surprisingly, COVID-19 also causes clots inside the blood vessels of lungs, heart, kidney, and other vital organs, and these blood clots can induce additional medical emergencies like stroke or a heart attack, potentially resulting in death [48]. It is believed that clots are the results of SARS-CoV-2 induced damage in the lining of blood vessels. This damage can induce platelets recruitment to prevent the blood leaking out into the surrounding tissues. In fact, clots are formed to fix the damaged blood vessels; however, excessive clotting could block vessels though, thus disrupting the blood flow [48]. Research has shown that the number of available ACE-2 receptors can influence clots formation. In that context, more ACE-2 receptors can increase viral fusion events, thus potentially increasing blood vessels' injury, hence paving the way for more clot formation. Unfortunately, excessive coagulation/coagulopathy could result in a "clotting cascade" leading to thrombosis (blood clot within a blood vessel). SARS-CoV-2 also causes acute cardiovascular injury. The proposed cause of cardiovascular injury is myocarditis because of the SARS-CoV-2 led systemic inflammation. Protein–protein interactions during infection lead to not only formation new virus particles but also cause tissue (blood vessel, myocardium, etc.) injury [49]. When spike protein binds to ACE-2 receptor in the heart, it alters cell-signaling process thus causing myocardial injury [48]. 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 heart failure, therefore, expediting myocardial injury further. In short, COVID-19 can severely affect heart's potential long-term effects from myocarditis that essentially include "arrhythmia, heart failure, and increased risk of stroke or subsequent heart attacks".

Another side effect of COVID-19 is excess fluid accumulation within body. When blood vessel encounters a foreign

pathogen then endothelial cells react by changing from a squamous shape to a columnar shape that helps "adhesion molecules" attract cells such as leukocytes, and chemokines thus allowing the immune system to fight off the pathogen. When helper cells are recruited then shape of the endothelial lining is altered that 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 basement membrane of the arteries, and capillaries in the lungs causing fluid leakage [50]. 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 more newer variants, and sub-variants of "Omicron" and its progeny have been identified. The variants/sub-variants such as BA.1, BA.2, and their respective lineages are the modified forms of the original virion wherein mutations arise that raise public health concerns since they tend to spread easier and faster, causing worse symptoms, making testing less accurate, and that basically "escape" the immune surveillance provided by the COVID-19 vaccines or by natural infection [51–54]. The alpha, beta, gamma, and delta variants were first detected in the United Kingdom, South Africa, Brazil, and India, respectively. The delta along with other such variants/sub-variants are the current mutant virions that are present in the USA. These have been shown to be "more transmissible" than the alpha variant that had swept through the world [51–55]. New immune-evading Omicron variants such as BA.4, BA.5 are most likely present in many U.S. states [56].

There are currently not many known effective treatments or cure available for SARS-CoV-2, and if one gets infected there are only a few palliative measures that can be taken to decrease the symptoms. Convalescent sera, and the monoclonal antibodies have been shown to impart some protection during the early phase of the infection. Since there is no universal known treatment or cure, thus it is highly recommended that one gets vaccinated to decrease the chances of contracting COVID-19. In the present study, we treated the engineered ACE-2 mouse as well as human cells with SARS-CoV-2 spike protein (SP) and collected multiple data sets. The study paradigm turned out to be highly encouraging in understanding the COVID-19 in a much more elaborate way, and we believe that the results might help in devising better tools in diagnosing, treating, and preventing breakthrough infections, and managing COVID-19 symptoms in the long-haulers.

Materials and methods

Measurement of physiological parameters in animals

Male, and female transgenic mice expressing the human ACE-2 receptor (B6.Cg-Tg(K18-ACE2)2Prlmn/J, Genotype: Hemizygous genotype, Hemizygous for Tg(K18-ACE2)2Prlmn were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice (in short, we will refer them as the ACE-2 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 standard chow diet, 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 (10–12 weeks) were recruited. The mice were anesthetized with Ketamine/Xylazine (50/10 mg/Kg), and then administered intranasally with the SARS-CoV-2 spike protein (ECD-His-tag, Genescript, Cat# Z03481), SP in short, and the followed by 100 µL air [57]. Post treatment mice were followed up to 5 days. Their body temperature, body weight, respiration rate, heart rate, systolic and diastolic pressure, and the intraocular pressure (IOP) were recorded in the SP treated and untreated mice groups as reported earlier in our published work [58–60]. Only measurements that were judged by data analytical system to be within the acceptable parameters were recorded, as valid.

Echocardiography of the SARS-CoV-2 spike protein (SP) treated ACE-2 mice versus untreated ACE-2 mice groups

Ultrasound was performed using Vevo 2100 imaging system; cardiac and aortic data were collected as described [61]. Mice were placed supine on a warm platform (37 °C) under isoflurane anesthesia. Using a MS550D (22–25 MHz) transducer, thoracic cavity was imaged. Aortic arch velocity, and cardiography function were assessed in pulse wave, and color Doppler modes. The transducer probe was placed on left hemithorax of the mice in the partial left decubitus position. Two-dimensionally targeted M-mode echocardiograms were obtained from a short-axis view of the left ventricle at or just below the tip of mitral-valve leaflet and were recorded. LV size, and the thickness

of LV wall were also measured. Only the M-mode ECHO with well-defined continuous interfaces of the septum, and posterior wall were collected indicating the diastolic (longer), and systolic (shorter) chamber lengths of the ACE-2 mice treated with spike protein (SP) in comparison to the untreated control ACE-2 mice.

Creatine kinase isoform measurement

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 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 serum 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 [62, 63].

Experiments on human cells for the cytokine profiling, and Western blotting

Human umbilical vein endothelial cells (HUVEC), and human coronary artery endothelial cells (HCAEC) were treated either with SP or with freshly mixed poly(I:C) poly[I:C]-HMW, Invivogen, tlrl-pic) @ 2.5 mg/ml and SP (5–15 µg) in 10 µl sterile phosphate buffered saline (PBS). The respective control cells were treated with either @ 2.5 mg/kg poly (I:C) or PBS using the same volume, and cells were harvested at 6 h or 24 h post treatment. The relative expression profile of cytokines was performed using a proteome profiler antibody array (R&D Systems, ARY015; Minneapolis, MN) post 24 h of treatment. The arrays were hybridized with an equal amount of total protein from HUVEC treated and untreated SP, and control reagents. Assay was performed according to the manufacturer's protocol. For Western blotting, antibodies such as IL6 (Cat. #12153), IL8 (Cat. #94407), MIG (Cat. #30327, and uPAR (Cat. #12863) were purchased from Cell Signaling Technology (Danvers, MA) while CD147 antibody (Cat. #ab64616) and GAPDH (Cat. #SC-365062) were purchased from Abcam (Waltham, MA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Anti-rabbit IgG-HRP conjugate and anti-mouse IgG-HRP conjugate, both were bought from Cell Signaling Technology (Cat. #7074, and Cat. # 7076, respectively). For GAPDH, primary antibody dilution used was 1:3000, and secondary antibodies with HRP conjugation, dilutions used were 1:5000, respectively. 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 from HUVEC and HCAEC were transferred to new tubes and protein concentrations were analyzed via Bradford protein estimation assay. Protein samples (a total protein of 50 µg) were run on a 10/12% sodium dodecyl sulfate (SDS)-polyacrylamide gel with Tris–glycine SDS buffer. Proteins from the gel were transferred electrophoretically overnight onto a PVDF membrane at 4 °C. Membranes were blocked with a 5% milk solution for 1 h. Primary antibodies were diluted at a concentration 1:1000 in TBST buffer and incubated on membrane overnight. All membranes were washed in TBST buffer 4× and then incubated with secondary HRP conjugated antibody solution for 1 h at room temperature. Four TBST buffer washing steps followed before membranes were developed using a chemiluminescent substrate in a BioRad Chemidoc (Hercules, Calif., USA). Band intensities were determined using densitometry analysis. Relative optical densities of protein bands were analyzed using gel software Image Lab 3.0. Membranes were stripped and re-probed with GAPDH as the loading control. Expression levels of each protein were also quantified as shown in the respective bar charts, n = 3–5 petri dish/group. For molecules that were difficult to demonstrate via Western blotting, were subjected for an extra step of immunoprecipitation assay to before visualizing them on the blots.

Visceral organ observation, and histopathological investigation

Mice vital visceral organs were collected, and observed for their appearance after the experiments. The heart, lung, and kidney samples were also collected in 4% buffered paraformaldehyde for fixation, and were processed after embedding in paraffin. After that 5 µm-thick sections from each sample were cut, and stained with hematoxylin and eosin (H&E). The detailed methods for tissue processing, and staining have been described [64].

Statistical analysis

Data from mice, and human 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 the groups' comparison. The comparisons between two groups were performed by unpaired Student's *t*-test. The $*p < 0.05$ was regarded as statistically significant. The data are reported

as mean ± SEM, and error bars indicate SEM, $n = 3–5$ petri dish or 3-5 animals/group.

Results

Animals, especially transgenic strains such as mice have served excellent disease models in dissecting out the complex disease processes, and in testing new therapeutic compounds [65]. As per our "a priori" belief that binding of the SARS-CoV-2 virion's spike protein (SP) to the host cell receptor, i.e., angiotensin converting enzyme 2 (ACE-2) in humans is associated with downstream cellular, and molecular signaling events. We could show many of the salient features that are generally seen in the COVID-19 patients in the clinic. To our knowledge, this study is one of the first disease modeling investigations in an experimental setting wherein we attempted to capture some of the clinical features, and postmortem observations that are seen in COVID-19 patients. In addition, we were able to collect data points both at whole organism level, as well as, under in vitro human cell culture 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, and a humanized animal species, and other similar resources to specifically study a rage of parameters that are highly relevant to the actual COVID-19 clinical scenario. We believe that the findings from this study could help us learn further and gain newer insight(s) toward improving the efficacy of the currently available diagnostic, therapeutic, and prophylactic strategies to control the ongoing pandemic.

Measurement of physiological parameters, and echocardiography in mice

In some mice the body temperature post administration of the SARS-CoV-2 spike protein (SP) appeared to be little high but was not significant; however, during the next few days the temperature dropped down significantly in comparison to the control/untreated mice. Similarly, the body weight in the treated mice group was found to be less (Fig. 2A). Future work should focus whether temperature variation could potentially determine the disease outcome in models but in COVID-19 infected humans hypothermia displayed abnormal markers of coagulopathy thus clearly suggesting a hypercoagulable phenotype; however, hyperthermic slow resolvers did exhibit elevated inflammatory markers and the highest odds of mortality [66]. It is worth mentioning that COVID-19 is associated with clinically significant weight loss and risk of hospitalization in human subjects since the disease negatively impacts body weight and the nutritional status [67]. The respiration and heart rates were found to be not significantly affected in the SP treated ACE-2 mice in comparison to the untreated ACE-2 mice which contrasts with the observations in human patients (Fig. 2A) [68]. Likewise, the systolic and diastolic pressures were not affected much (Fig. 2B). This finding was in direct contrast to the clinical observation in human patients wherein COVID-19 increased both systolic, and diastolic blood pressures, and thus became a new onset of hypertension [69, 70]. Interestingly, the intraocular pressure (IOP) in SP treated ACE-2 mice was significantly affected than the untreated ACE-2 mice (Fig. 2B). COVID-19-related ocular hypertension has also been reported in human subjects [71].

More importantly, the echocardiography findings; however, did reveal alterations in cardiac functions as seen in the representative M-mode echocardiography images from each group, i.e., SP administered, and control (saline) administered (CTL) indicating diastolic (longer) and systolic (shorter) chamber lengths in the ACE-2 mice treated with SP in comparison to the untreated control ACE-2 mice. The contraction and relaxation of the myocardium are found to be attenuated in the SP treated mice in comparison to the untreated control ACE-2 mice (Fig. 3). Clinical studies in human subjects have reported an association between COVID-19 and cardiovascular disease. Notably, the pre-existing cardiovascular disease appears to be strongly linked with worse outcomes such as death in patients with COVID-19. Nonetheless, COVID-19 itself can also induce cardiac injury, acute coronary syndrome, arrhythmia, and venous thromboembolism [72].

Creatine kinase assay

When the serum samples were subjected to assess the relative activities of various isoforms of the phospho-creatine kinase (CK) employing a gel-based assay from the SP treated ACE-2 mice, and untreated ACE-2 control mice groups, the tissue-specific injury was evident in the treated group as determined by the measurement of respective CK isoforms. For example, the muscle (CK-MM) injury was maximum, and significant followed by heart (CK-MB), and brain (CK-BB) (Fig. 4A and B). In fact, COVID-19 is accompanied by multiorgan failure in many patients, and that is strongly associated with increasing mortality rate [73, 74].

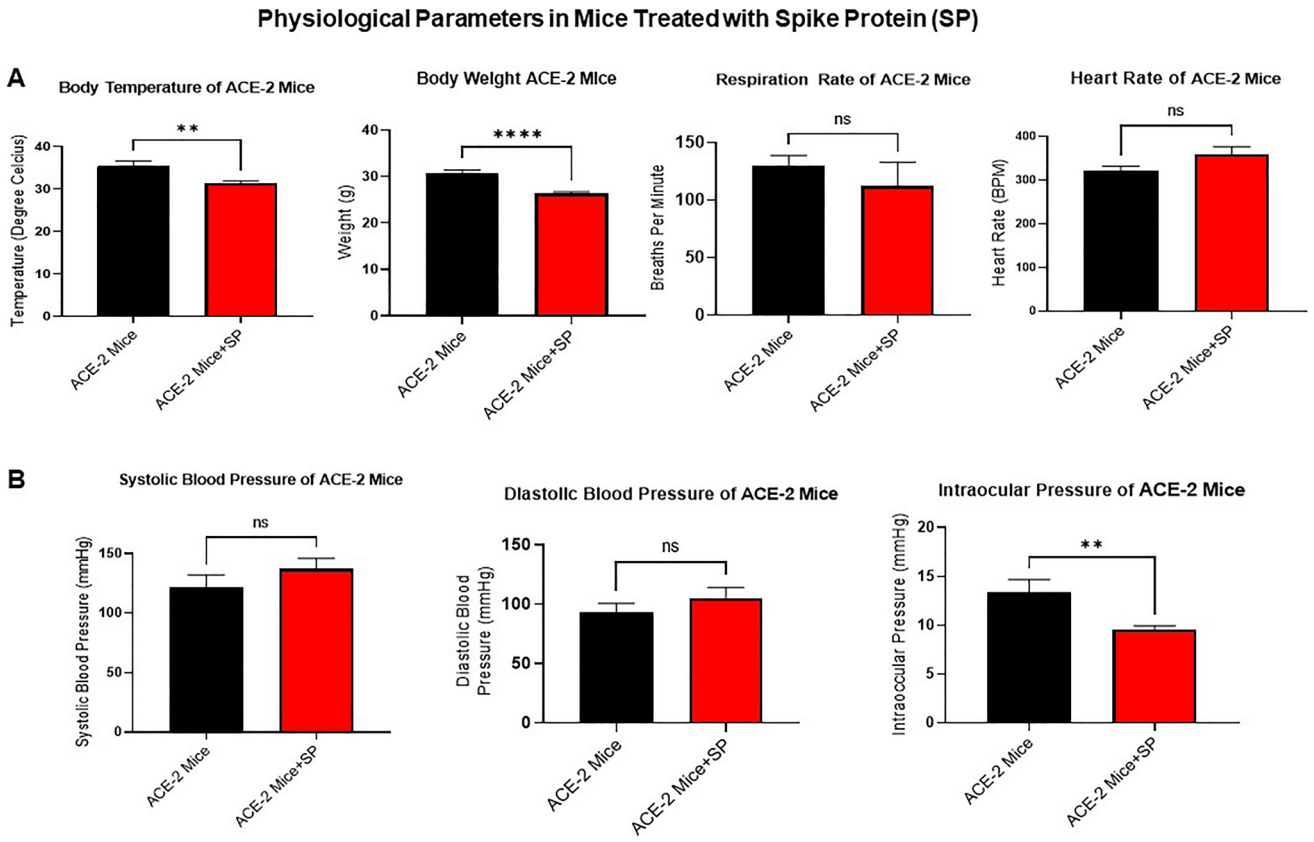

Fig. 2 **A** Measurement of the body temperature, weight, respiration, heart rate, blood pressure (systolic, and diastolic), and intraocular pressure (IOP). The human angiotensin converting enzyme 2 transgenic mice (B6.Cg-Tg(K18-ACE2)2Prlmn/J, Genotype: Hemizygous genotype, Hemizygous for Tg(K18- ACE2)2Prlmn; in short, ACE-2 mice) were treated or untreated with the SARS-CoV-2 spike protein (SP). **A** Daily body temperature, and the body weight of the ACE-2 mice administered with SP via the nasal route were compared to the control mice (without SP). Unpaired *t*-tests were performed, $*p<0.01$, $**p<0.001$ $***p<0.001$, $****p<0.0001$, respectively, $n=3$–5 mice/group. The respiration rate, and heart rate of ACE-2 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 was measured by Coda non-invasive instrument. Unpaired *t*-tests were performed, $p<0.3890$, $n=3$–5 mice/group. Systolic, and diastolic pressure were measured, and the unpaired *t*-test were performed, $p<0.4696$, $n=3$–5 mice/group. Similarly, IOP was also measured by iCareLab tonometer, and unpaired *t*-test was performed, $p<0.0023$, $n=3$–5 mice/group. The body temperature is significantly decreased in mice treated with SP (35.50 ± 1.01 vs 31.39 ± 0.52), the body weight of those treated with SP were found to be significantly less (30.68 ± 0.73 vs 26.27 ± 0.42); however, the respiration rate (130.00 ± 7.4 vs 112.40 ± 18.7 breaths per minute), heart rate (321.70 ± 8.9 vs 358.00 ± 16.7 beats per minute), systolic (122.00 ± 8.3 vs 136.90 ± 8.56 mmHg), and diastolic blood pressure (93.33 ± 6.0 vs 105.00 ± 8.26 mmHg) were not significantly different. Interestingly, IOP was significantly decreased in mice treated with SP (13.43 ± 1.2 vs 9.500 ± 0.43)

Protein array profiling, and Western blotting for cytokines/inflammatory molecules, on human cells

The expression profile of cytokines was captured by array analysis, and important protein targets were investigated via Western blotting either from cell culture supernatants or cell lysates that were treated with SP alone or with Poly: IC for different time points. Poly I:C is a synthetic polyinosinic-polycytidylic acid double-stranded RNA and has been used to stimulate release of cytokines and interferon-gamma production [57, 75]. The results revealed significant changes in the levels of cytokines and key protein molecules in the SP treated cells than the non-treated cells (Figs. 5, 6, and 7). Cytokines such as IL-6 have been considered as a potential COVID-19 early disease biomarker, and relevant prognostic tool for the development of fatal pneumonia in patients [76–79]. Interestingly, high CD47 levels seems to contribute to vascular disease, vasoconstriction, and hypertension thus predisposing individuals to serious complications like pulmonary hypertension, lung fibrosis, myocardial injury, stroke, and acute kidney injury [80, 81]. The role of urokinase plasminogen activator receptor (uPAR) has been suggested as one of the main orchestrators of fatal progression to pulmonary, kidney, and heart failure in COVID-19 patients. Newer drugs that could regulate uPAR system may help treat severe complications COVID-19 [82]. Because lack of therapeutic options for tackling acute respiratory

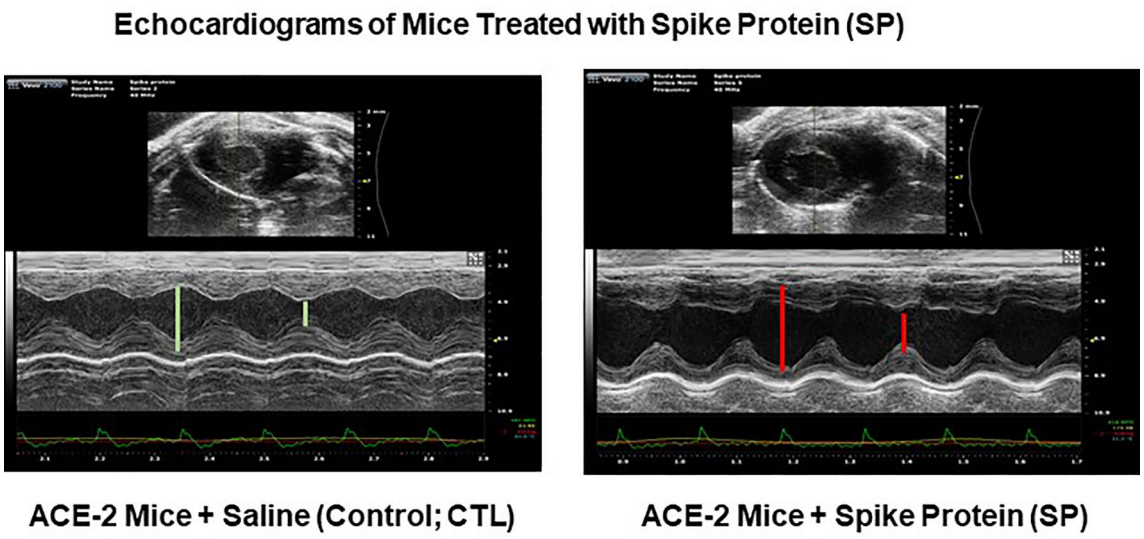

Fig. 3 Echocardiography of the SP treated ACE-2 mice versus untreated ACE-2 mice groups. Representative M-Mode image of parasternal long-axis view images from each group are presented indicating diastolic (longer) and systolic (shorter) chamber lengths in the ACE-2 mice treated with SP in comparison to the untreated control ACE-2 mice. The contraction and relaxation of the myocardium are attenuated in SP treated mice in comparison to the untreated control ACE-2 mice, $n = 3$–5 mice/group

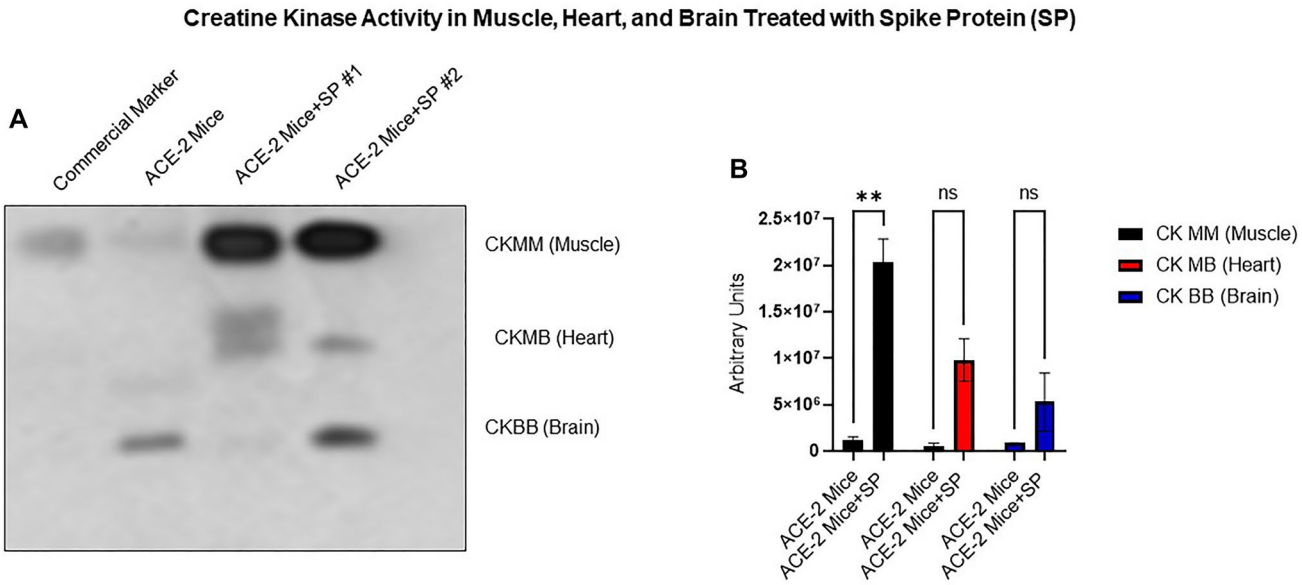

Fig. 4 Creatine phosphokinase (CK) levels in SP treated ACE-2) mice versus untreated ACE-2 mice. SP treated (SP #1 and SP #2) were higher compared to the control mice. Differences in CK levels (**A**) is due to multi-organ damage such as skeletal muscle (CK-MM), heart (CK-MB), and the brain (CK-BB) (**B**). The binding of SP to ACE-2 receptor causes multi-organ damage. Two-Way ANOVA with multiple comparisons, $^{**}p < 0.009$, $n = 3$–5 mice/group

distress syndrome (ARDS) in COVID-19 patients, attention has now focused on differentiating hyper- and hypo-inflammatory phenotypes of ARDS to help develop effective therapeutic interventions. In this regard, IL-8 which is a pro-inflammatory cytokine performs an important role in neutrophil activation and has been identified for the progression of COVID-19 disease [83, 84]. Furthermore, it has been reported that COVID-19 patients with severe outcome also display higher plasma levels of chemokines such as CXCL9/MIG, CXCL8/IL-8, and CXCL10/IP10 along with cytokines IL-6 and IL-10 than the patients with the milder form of the COVID-19 [85]. From our animal disease modeling study, it is apparent that molecules such as IL-6 and IL-8 can be used

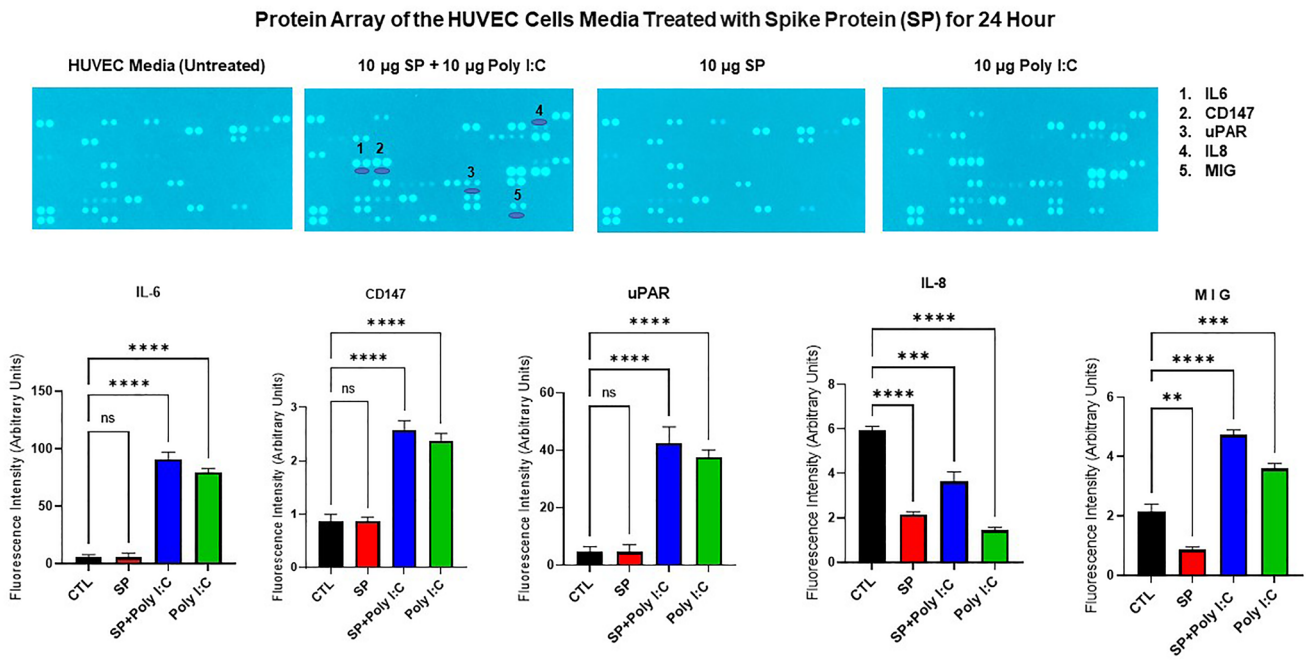

Fig. 5 The proteome profiler with antibodies arrays reveals induction of cytokines in primary human umbilical vein endothelial cells (HUVEC). The cells were treated either with sterile phosphate buffered saline (PBS/saline) alone or SP; or with 10 mg of Poly I:C, or both SP and Poly I:C. The quantitation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

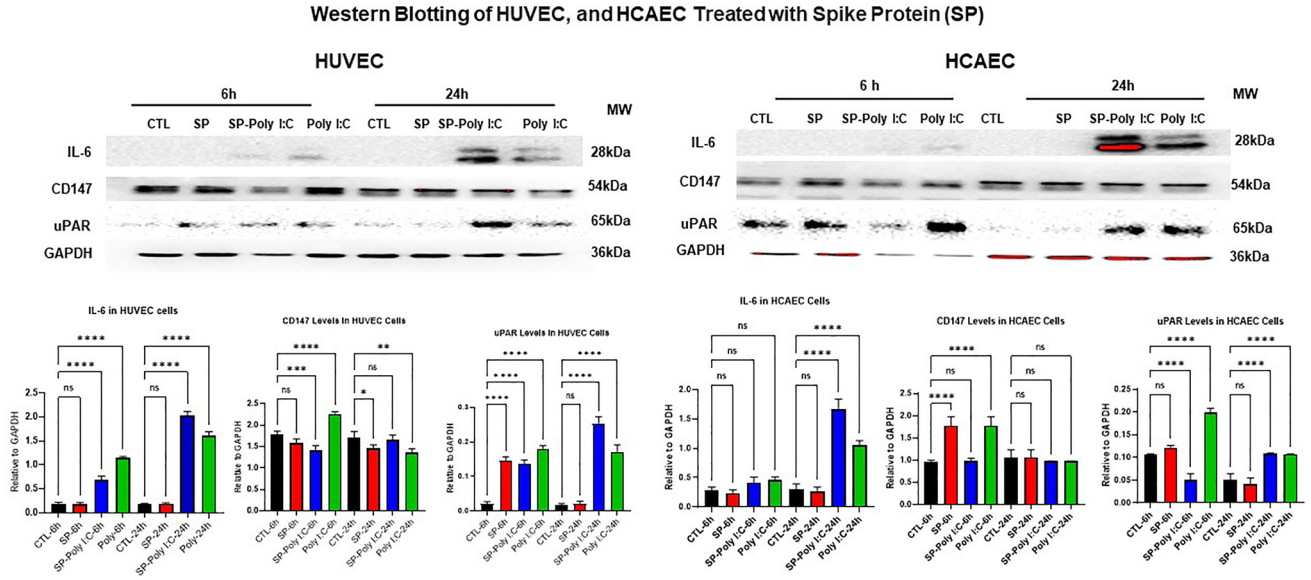

Fig. 6 Western blot analyses of the key target proteins. Supernatants from human primary umbilical vein endothelial cells (HUVEC) at 6- and 24-h post treatment using the control (CTL), SP (spike protein), SP-Poly (spike protein and poly I:C), and Poly (poly I:C). The primary antibodies used were Interleukin-6 (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, $^{*}p < 0.01$, $^{****}p < 0.0001$. Similarly, supernatants from human primary coronary artery endothelial cells (HCAEC) at 6- and 24-h 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, $^{*}p < 0.01$, $^{****}p < 0.0001$

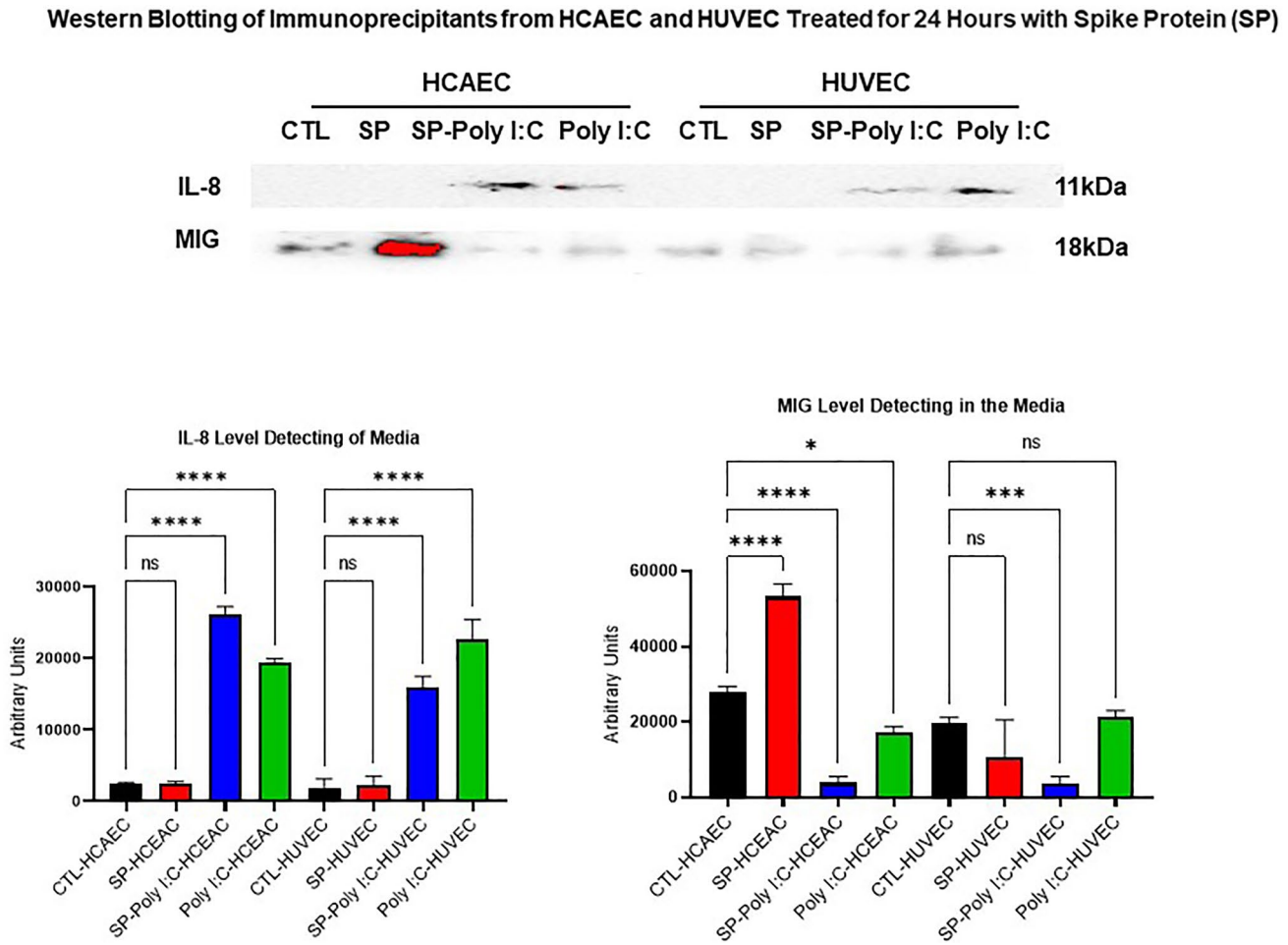

Fig. 7 Western blot analysis of the key target proteins employing the immuno-precipitate. Immunoprecipitants from the human primary coronary artery endothelial cells (HCAEC) and human primary umbilical vein endothelial cells (HUVEC) post 24 h treatment were used from the samples: control (CTL), SP (spike 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, $*p < 0.01$, $****p < 0.0001$

as potential biomarkers in COVID-19 patients and probably for COVID-19 disease prognosis also.

Visceral organ observation, and histopathological investigation

When mice visceral organs were collected, and observed for their appearance, it became abundantly clear that there was significant change in their appearance most likely because of the blood clots that have been often shown in patients suffering from COVID-19. More importantly, thrombi in the vasculature have also been reported in patients. The vital organs in our SP treated mice looked very dark in color (Fig. 8). In addition to gross observation of the organs, histological study on these vital organs employing hematoxylin and eosin (H&E) staining revealed a significant inflammatory phenotype more in the lung, and kidney than heart signifying extensive infiltrations of immune cells, e.g., neutrophils in the SP treated mice in comparison to the untreated control mice. Kidney, in fact, exhibited extensive tissue damage in the SP treated mice than the non-treated control mice (Fig. 9).

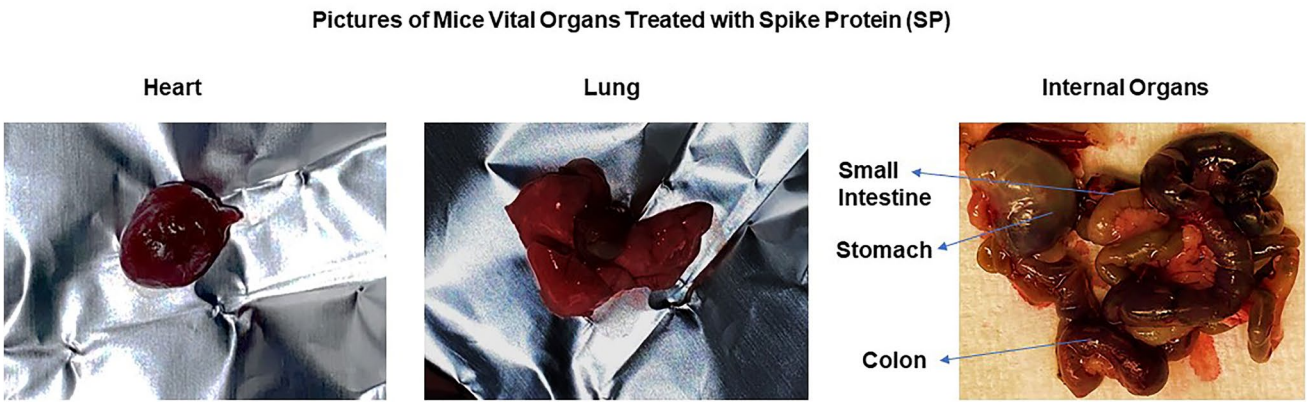

Fig. 8 Representative pictures of the mice heart, lung, and internal visceral organs. Heart, and intestine show most likely the evidence of blood clots, and thrombi formation. The vital organs look very dark in color indeed, n = 3–5 mice/group

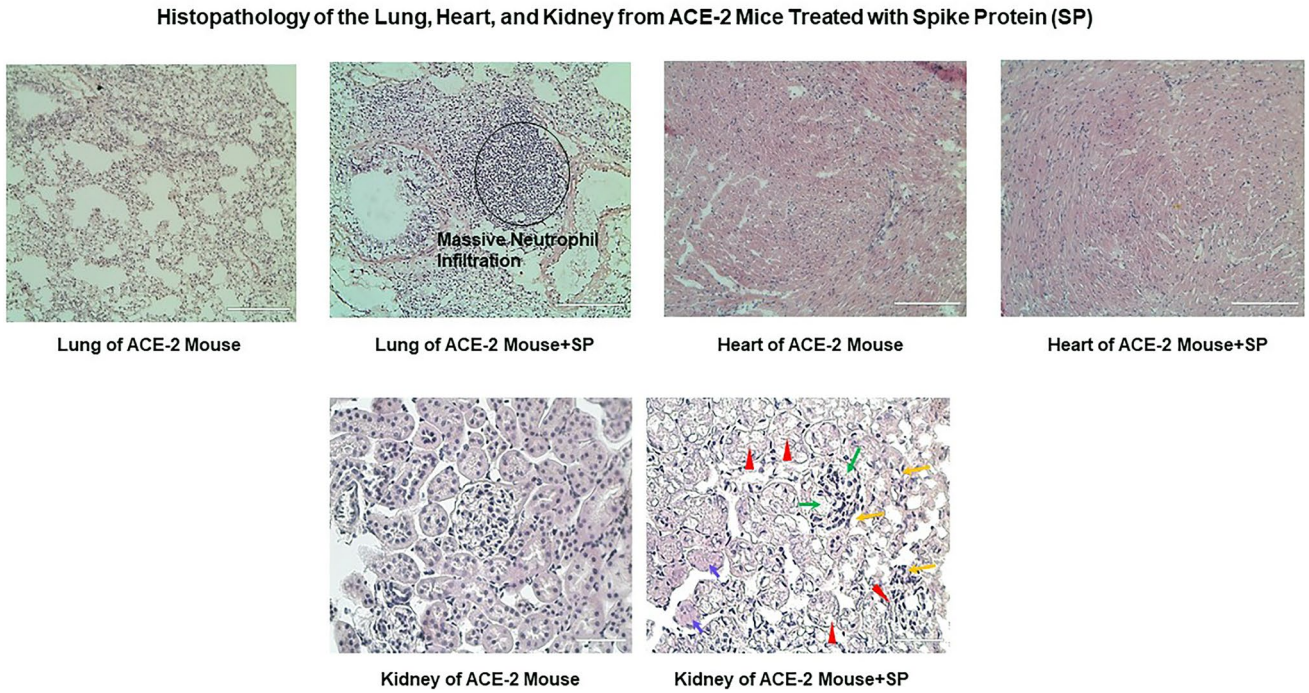

Fig. 9 Hematoxylin and eosin (H&E) staining of the lung, heart, and kidney samples from the humanized ACE-2 (B6. Cp-Tg) and ACE2 (B6.Cp-Tg) + Spike protein. Black circle clearly depicts a cluster of infiltrated immune cell populations while heart Sect. (5 µm thickness) shows diffused inflammatory cells throughout the parenchyma, magnification × 20, scale bar—50 µm $n = 3–5$ mice/group. Kidney Sects. (5 µm thickness) showing representative image of control kidney and spike protein (SP) treated mice. Pictures depict 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 × 60, scale bar—50 µm. The control mice received saline/PBS, $n = 3–5$ mice/group

Discussion

In this study we show that upon SARS-CoV-2 virion spike protein (SP) treatment of the genetically engineered mice expressing the human ACE-2 receptor, and human cells led to the hyper-inflammatory state/phenotype relative to the untreated/control mice or human cells. The SP elicited secretome from inflamed targets (organs/cells), that is, from the in vivo (mice) or in vitro (human cells) systems causing an increased expression of the important proteins/targets such as cytokines/chemokines most likely mimicking the "cytokine storm" that is commonly observed in COVID-19 humans. It is well documented that excessive

production of pro-inflammatory cytokines/chemokines is a severe clinical syndrome known to develop as a serious complication of infectious or inflammatory diseases such as during SARS-CoV-2 infection responsible for COVID-19. Evidence from clinical cases suggests that the occurrence of cytokine storm in severe acute respiratory syndrome secondary to SARS-CoV-2 infection is closely associated with a rapid deterioration of human health and high mortality in severe cases [86]. In our work, a significant increase in the levels of cytokines/chemokines or alterations in mice organs relative to untreated/control cells or mice confirm our hypothesis that biding of the SP to host cell is associated with downstream cellular processes/events that are highly detriment to host or its cells/organs. Such pathological events/processes are akin to the observations in the COVID-19 patients during SARS-CoV-2 viral pathogen replication in the target host cells. [87–90]. In people who recover from acute COVID-19 disease the pathology is still characterized and associated with mild form of cytokine storm that may or may not lead to long-term endothelial inflammation, microvascular thrombosis, and organ dysfunction but post COVID-19 related implications may still haunt some susceptible individuals for a foreseeable future [91–94].

Our findings support the hypothesis and corroborates some of the clinical observations of targeting SP as a COVID-19 preventing strategy for safeguarding human health against this deadly disease in susceptible human population. The same is true for the fact that therapeutically targeting the SP 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 patients with alleviation of both the morbidity and mortality. Our results from the preclinical mouse model also suggest 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 but also in obese or numerous chronic diseases that affect individuals. In short, animal

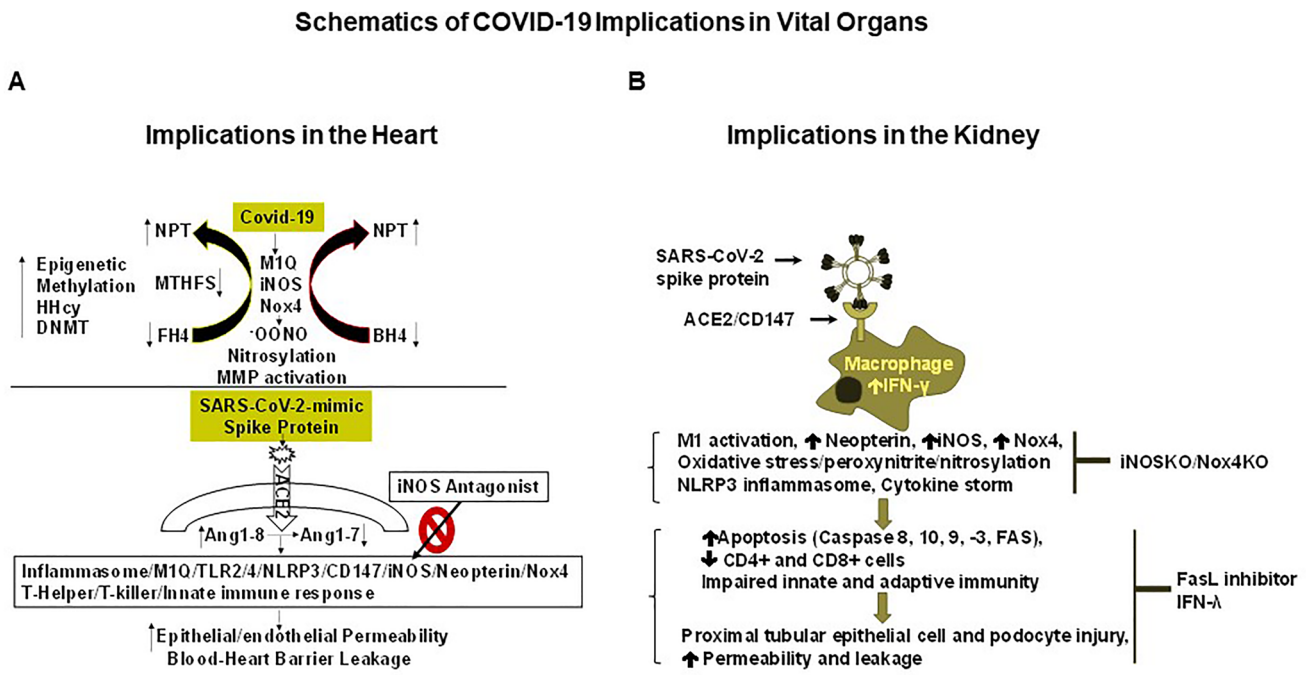

Fig. 10 Schematics of plausible hypothesis regarding SARS-CoV-2 induced visceral organ damage. The binding of SARS-CoV-2 spike protein (SP) with ACE-2 receptor mimics SARS-CoV-2 infection, and causes the accumulation of Ang1-8, activation of inflammasome, and M1Q macrophages via the "TLR4/NLRP3/CD147/Nox4/iNOS/neopterin" axis in the heart. This cascade of events leads to endothelial blood-heart barrier (BHB) leakage; however, the iNOSKO/Nox4KO and iNOS antagonists may help 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 protein (SP) to ACE-2/CD147 on macrophages can cause M1Q activation by IFN-γ toward 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; tetrahydrobiopterin, FH4; tetrahydrofolate

models such as genetically engineered ones may play important role(s) in studying "in-depth" disease mechanism(s) toward developing lifesaving therapeutics, and effective 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 (ACE-2) receptor, it causes a robust surge of inflammatory markers, epithelial barrier dysfunction, and multi-organ damage and congestive (cardio-pulmonary) heart failure (CHF) [36, 95]. Interestingly, the ACE-2 receptor 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 (Fig. 10). 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 acute kidney injury (AKI).

We strongly believe that pro-inflammatory macrophage (M1Q) activation leads to oxidative stress, and peroxynitrite/nitrosylation in cells/organs during COVID-19. Further, the resultant NLRP3 inflammasome formation may potentially activate the apoptosis pathway(s) 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. 10). It is known that the COVID-19 activates innate immune system causing AKI as reported in 27–40% of the ICU admissions [95–109]. More importantly, the humanized ACE-2 engineered mouse model can also be used to identify potential safety issues that may be associated with COVID-19 inhibitors that are being developed by pharmaceutical industry. We further hypothesize that newer version(s) of the modified approaches such as delivering beneficial molecules to the engineered mouse models or even to the cultured host cells via employing the protein transduction technology might reveal new disease target(s) in the coming future [110]. In the light of new emerging SARS-CoV-2 variants/sub-variants, it is somewhat difficult to predict whether we are going to have a peaceful future, COVID-wise, but it is certain that only the robust 'cutting-edge' tools, and technology might navigate us out of this deadly pandemic.

Limitation regarding extrapolation of mice experimental findings to human clinical observations: We do recognize that our work has limitations such as: (1) we did not use the actual infectious virus particles (virions) in our experiments, and (2) although we did use human cells in conducting in vitro experiments with SP alone or in combination with poly I:C, and a genetically engineered mouse model expressing the human angiotensin converting enzyme 2 (ACE-2) receptor to obtain the experimental data; however, despite above shortcomings, we were able to demonstrate many important features that seem to be similar, if not identical, to that of human COVID-19 as seen in real clinical settings.

In conclusion, we present a set of interesting evidence that interaction between the SARS-CoV-2 virion's spike protein (SP) with that of the human angiotensin converting enzyme 2 (ACE-2) receptor leads to a robust cellular signaling cascade of 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 ongoing 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 > than ~ 2 years since the start of the pandemic [79, 111–122]. To dissect out further the physiological, and pathological implications of the SARS-CoV-2 induced changes, we carried out this important study to capture some of the initial/beginning phase of the intimate interaction(s) between the host cell receptor with the SARS-CoV-2 spike protein (SP) employing a genetically engineered mouse model expressing the human angiotensin converting enzyme 2 (ACE-2) receptor and the recombinant SARS-CoV-2 spike protein (SP) that was delivered via the intranasal route [123]. The SARS-CoV-2 spike protein (SP) binding to ACE-2 receptor did seem to amplify the susceptibility to COVID-19 virion-induced inflammation in various mice organs along with occurrence of the cytokine storm as elaborated in this study.

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

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

Declarations

Conflict of interest The authors declare that they have no conflict of interest, financial or otherwise.

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

Consent to participate Not applicable.

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