Neuropathogenesis of SARS-CoV-2 Infection in Mice

Pratima Kumari

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Neuropathogenesis of SARS-CoV-2 Infection in Mice

by

Pratima Kumari

Under the Direction of Mukesh Kumar, PhD

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2022
ABSTRACT

Although severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection in humans primarily causes respiratory disease; some patients develop symptoms of neurological disease, such as headache, loss of taste and smell, cognitive dysfunction, and impaired consciousness. In this study, we analyzed the tissue tropism, immune response, and pathology in human ACE2-expressing (hACE2) mice after SARS-CoV-2 infection. Intranasal infection of hACE2 mice with SARS-CoV-2 resulted in a lethal disease with high levels of virus replication in the brain tissue. SARS-CoV-2-infected mice exhibited encephalitis hallmarks characterized by production of cytokines and chemokines, leukocyte infiltration, hemorrhage, and neuronal cell death. SARS-CoV-2 was also found to productively infect cells within the nasal turbinate, eye, and olfactory bulb, suggesting SARS-CoV-2 entry into the brain by this route after intranasal infection.

NanoString gene expression analysis was performed to identify differentially expressed genes (DEGs) in the brains of mice following SARS-CoV-2 infection. Genes that were upregulated after infection were mainly associated with toll-like receptor (TLR) signaling, RIG-I like receptor signaling (RLR) and cell death pathways, while down-regulated genes were associated with neurodegeneration and synaptic signaling pathways. Next, we generated primary neuronal cultures from hACE2 mice to investigate the effects of a SARS-CoV-2 infection. Our data show that neuronal cultures obtained from hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. Like the brain, SARS-CoV-2 infection upregulated the expression of genes involved in innate immunity, inflammation, and necroptosis in the neurons.

Because age is a risk factor for COVID-19-related death in humans, we next evaluated the pathogenesis of SARS-CoV-2 infection in young and old mice. Our data show that intranasal
inoculation of SARS-CoV-2 in the older BALB/c mice resulted in severe disease, as evident by rapid loss of body weight, gross and microscopic pathology of lungs, as well as elevated mRNA levels of inflammatory cytokines and chemokines. In addition, our results indicate that a diminished interferon response in the old mice may play a role in severe disease outcome observed in these mice. Together, our data provide new insights into the pathogenesis of SARS-CoV-2 infection in mice.

INDEX WORDS: SARS-CoV-2, COVID-19, K18-hACE2 mice, Neuropathogenesis
Neuroinflammation, Encephalitis
Neuropathogenesis of SARS-CoV-2 Infection in Mice

by

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Office of Graduate Services
College of Arts and Sciences
Georgia State University
August 2022
DEDICATION

This thesis is dedicated to my family: my father, S.R. Prasad and my mother, Kamala Prasad, and my three elder brothers, Jay P. Prasad, Kamlesh Kumar Prasad, and Niranjan Kumar Prasad, for their selfless love, encouragement, sacrifices, and unwavering support. Without their support, I would have never come this far. I am eternally grateful to God for giving me the rock-solid support of my brothers that I cannot ask for anymore. I would especially like to dedicate my Ph.D. thesis to my eldest brother Jay (Bhaiya), for having confidence in me and for all the sacrifices that he has made for us. I always look up to you to obtain the strength to keep going when life seems challenging. I am grateful to my second eldest brother Kamlesh Bhaiya who always remained dedicated to my well-being and helped me achieve this educational career. My third elder brother, Niranjan Bhaiya, remained my greatest teacher for my course subjects when I was in school and my life's journey. He is my champion brother who showed me how to live life and never give up on facing real adversities. You were, you are, and you will always be my source of inspiration and strength. They remain my strongest pillars throughout my life and journey as a Ph.D. student. Their love, motivation, and sacrifices are the backbone of who I am.

This dissertation would be incomplete without expressing my sincere gratitude to my dearest friend Shaligram Sharma - for believing in my goals and being my support system away from home. This five-year Ph.D. journey in science and life would have been much more difficult without your endless support and thoughtful advice. Thank you for helping me with my lab work and experiments, listening to me with so much patience, and sometimes tolerating my endless complaints. I cannot thank you enough for being on my side when I was going through the toughest phase of my life. I consider myself lucky to have a great friend like you, my most profound appreciation for everything you have done for me, Shaligram.
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<tr>
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<td>SARS-CoV-2</td>
</tr>
<tr>
<td>Angiotensin Converting Enzyme-2</td>
<td>ACE-2</td>
</tr>
<tr>
<td>Renin–angiotensin–aldosterone system</td>
<td>RAAS</td>
</tr>
<tr>
<td>Variants of Concern</td>
<td>VOC</td>
</tr>
<tr>
<td>Acute Respiratory Distress Syndrome</td>
<td>ARDS</td>
</tr>
<tr>
<td>Acute Lung Injury</td>
<td>ALI</td>
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<tr>
<td>Retinoic acid-inducible gene I</td>
<td>RIG-1</td>
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<td>Type-I Interferon</td>
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1 INTRODUCTION

The outbreak of novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) during late December 2019 in Wuhan, Hubei Province, China, has become a pandemic of global concern, impacting human life and economic slowdown. The severity of SARS-CoV-2 infection can be ascertained by an increased number of human deaths, specifically in older populations and patients with predisposed cardiovascular disease (CVD) complications [1-4]. As of 12 January 2022, the number of confirmed cases worldwide is over 314 million, with 5.52 million deaths. A few therapies are available to treat COVID-19 in patients; the rapid emergence of SARS-CoV-2 variants of concern (VOC) threatens to diminish their efficacy [3, 5, 6]. The exact origin, location, and natural reservoir of the SARS-CoV-2 are unknown. It is thought to be zoonotic, and bats may be reservoir due to sequence similarity to the bat-CoV. Previous epidemiologic investigations on SARS-CoV-1 and MERS-CoV have found that the bat is the natural reservoir, whereas the palm civet or raccoon dog may be the intermediate (or vulnerable) host for SARS-CoV-1 and the dromedary camel for MERS-CoV [7].

SARS-CoV-2 infection in humans can cause pneumonia, acute respiratory distress syndrome, acute lung injury, cytokine storm syndrome and death [8, 9]. Although SARS-CoV-2 infection primarily causes respiratory disease, some patients develop symptoms of neurological disease, such as headache, loss of taste and smell, ataxia, meningitis, cognitive dysfunction, memory loss, seizures and impaired consciousness [10-16]. SARS-CoV-2 infection also induces long-term neurological sequelae in at least one-third of human cases. Infection with other coronaviruses, such as mouse hepatitis virus (MHV) in mice and SARS-CoV-1 and Middle East Respiratory Syndrome (MERS) virus in humans, has been shown to cause neurological disease [17, 18]. Infection of central nervous system (CNS) cells by other coronaviruses has been reported.
For example, SARS-CoV-1 is proposed to enter the brain via the olfactory bulb resulting in the rapid trans neuronal spread and minimal cellular infiltration [19]. HCoV-OC43 can cause meningoencephalitis and acute disseminated encephalitis [20, 21]. Whereas MERS-CoV has been reported to cause Bickerstaff brainstem encephalitis and disseminated encephalitis [21-23]. However, little is known about the pathophysiology of SARS-CoV-2-associated neurological disease in humans.

SARS-CoV-2 binds to Angiotensin-Converting Enzyme-2 (ACE2) receptors on host cells, followed by its internalization, rapid multiplication, and instigate cytokine storm. CNS cells that express the SARS-CoV-2 receptor angiotensin-converting enzyme 2 (ACE2) include neurons, glial cells and astrocytes [24, 25]. ACE2 is expressed in multiple human brain areas, including the amygdala, cerebral cortex and brainstem, with the highest expression levels found in the pons and medulla oblongata in the brainstem that contain the medullary respiratory centers of the brain [10, 26]. Several human autopsy reports have documented the presence of SARS-CoV-2 RNA in brain tissues [27, 28]. Human iPSC-derived neural progenitor cells (NPCs) have been shown to be permissive to SARS-CoV-2 infection, and both viral proteins and infectious viral particle production were detected in neurospheres and brain organoids infected with SARS-CoV-2 [29, 30]. Human autopsy reports have shown evidence of lymphocytic panencephalitis, meningitis and brainstem perivascular and interstitial inflammatory changes with neuronal loss in COVID-19 patients [31]. These data suggest that SARS-CoV-2 can productively infect human CNS cells [32]. However, the contributions of CNS cell infection and induced neuroinflammation to the pathogenesis of SARS-CoV-2-associated disease are not well understood.
1.1 SARS-CoV-2 Structure and genomic organization

The non-segmented positive-sense RNA genome of Coronaviruses (CoV) is the largest genome among all RNA viruses with approximately 30 Kb in size. Spike-like structures on the outer envelope of a CoV are a characteristic feature of this virus. The virus particle has four structural proteins, namely spike (S), membrane (M), envelope (E) and, nucleocapsid (N) proteins [33]. Functionally, S protein facilitates virus attachment to the host cell surface receptors and internalization of virus inside the host cell. S protein is the most abundant glycoprotein. M protein is required for virus assembly and maintains the shape of the viral envelope. Assembly and release of the virus particle require the interaction between less abundant proteins E and M [34, 35]. According to Stohlman et al., deletion of the E gene attenuates the virus as the E gene encodes a small multifunctional protein with ion channel activity, which plays an essential role in virus-host interaction [36]. N protein is the sole nucleocapsid protein, which has N terminal and C terminal domains. It has been suggested that N protein is heavily phosphorylated, and this triggers a structural change that enhances the viral RNA replication [36, 37].

1.2 COVID-19 Outbreak and treatment options

It is the first time that a coronavirus has caused a pandemic in humans. However, there are reports of previous outbreaks caused by other CoV members, such as SARS-CoV-1 and MERS-CoV. The current pandemic's causative agent is SARS-CoV-2 [8], that affects human circulatory and respiratory systems. The rapid progression of the disease and its higher transmission rate makes it a severe global health concern. Infected patient present symptoms, such as fever, body ache, tiredness, difficulty breathing, and lung infection with pneumonia-like symptoms. Currently, more than 185 countries have contacted the disease outbreak [38]. The major challenge in front of the medical healthcare system and scientists is to contain the disease via social distancing and
utilizing already available drugs approved by the Food and Drug Administration (FDA). There is an urgent need to establish fundamental knowledge and understanding of the host-pathogen interaction to exploit more effective treatment options. Therefore, it is critical to understand the pathogenesis of the virus and its target cells, including the immune response to the virus replication. To date, there are only few approved drugs to treat a coronavirus infection. Several drugs such as Remdesivir, Lopinavir/Ritonavir and Flavipiravir have been shown to be very effective in inhibiting SARS CoV-2 infection [8, 39]. Remdesivir is a nucleotide analog previously used in the treatment of the Ebola outbreak in Africa and is currently used in the treatment of COVID-19. A study conducted by Grein et al., with 61 patients from the United States, Europe, and Japan, were administrated 200 mg Remdesivir through IV on day one followed by 100 mg for the next nine days. At the end of the study, 36 patients out of 53 showed clinical improvements [40]. On May 1, 2020, the FDA issued an Emergency Use Authorization (EUA) for Remdesivir. That means the FDA has not yet approved Remdesivir for treating COVID-19 patients; however, the drug is easily accessible to doctors for the urgent need of COVID-19 hospitalized patients. On June 1, 2020, Gilead pharmaceutical announced Phase 3 clinical trial results in which the Remdesivir is found to improve the condition in moderate COVID-19 patients. However, still more data and extensive studies are required. Proteases are fundamental for virus replication. Protease inhibitors can also inhibit SARS-CoV-2 viral replication by inactivating the proteases. Lopinavir/Ritonavir is currently used in the emergency management plan for COVID-19.

Increased cytokine levels and inflammatory response due to the SARS-CoV-2 infection are among the most critical causes of organ damage. Abnormal release of proinflammatory cytokines, mainly IL-6, TNF-α, and IFN-γ, contributes to cytokine release syndrome. Tocilizumab is a drug
used for the treatment of inflammatory conditions like rheumatoid arthritis. Inflammation is a natural response of our immune system against harmful pathogens. Sometimes due to the overactive immune system, inflammations go haywire, causing cytokine storms in which the immune system works against our own body. IL-6 is a major inflammatory cytokine, and Tocilizumab helps attenuate inflammation by blocking the IL-6 receptor [41, 42]. Corticosteroids are anti-inflammatory drugs that are also used for COVID-19 treatment. Published literature suggests thromboembolic manifestations associated with COVID-19. Activation of the coagulation cascade and endothelial injury are indicated as a cause for the development of a prothrombotic state associated with an exaggerated pro-inflammatory response. The use of anticoagulants such as heparin remains an area of conjecture with no definite guidelines of its usage [43].

Convalescent plasma (CP) is another treatment method in COVID-19 patients in which blood plasma of infected patients is infused in another COVID-19 patient. Patients recovered from COVID-19 carry the SARS-CoV-2 specific antibody in their blood. Therefore, already built antibodies from recovered patients serve as a therapeutic alternative to treat SARS-CoV-2 infected patients [44]. A study of 10 adult patients showed that 200 ml of CP effectively cleared viral load in 7 days. On May 1, 2020, FDA issued an application of Emergency Investigational New Drug (eIND) for CP as the COVID-19 treatment option. However, larger-scale research and random trials are required before making any final conclusion [45].

1.3 ACE-2 Receptor and Function

SARS CoV-2 binds to Angiotensin-Converting Enzyme-2 (ACE2) receptors on host cells, followed by its internalization and replication. ACE2 has a cardioprotective role, and SARS-CoV-2 internalization into cells has been reported to downregulate ACE2 expression [46-50]. In the
normal adult lung, ACE2 is primarily expressed in primary alveolar epithelial type II cells and plays a protective role in the lungs. Surfactant proteins produced by these cells help reduce surface tension and protect alveoli from collapsing [51, 52]. Ang II (Angiotensin II) is the main effector molecule in the RAAS (renin-angiotensin-aldosterone system) pathway, which is upregulated in many diseases and it’s a common target in various cardiovascular disorders [53]. ACE-2 helps inactivate Ang II by converting Ang II to Ang (1-7) [54]. According to a recent study, exogenous administration of recombinant human ACE2 (rhACE2) can prevent SARS-CoV-2 infection by acting as a decoy. hrACE2 effectively reduced the infection in cell culture and human blood vessels organoids and kidney organoids. The protective role of hrACE2 has been reported by different groups in CVDs, rhACE2 could be a promising treatment option for CVD patients with COVID-19 infection [37, 54, 55]. ACE inhibitors/angiotensin receptor blockers (A.C.E.Is/A.R.Bs) are increasingly used in CVD treatments, and according to studies, they help upregulate ACE-2 expression. The fact that ACE-2 expression could correlate with SARS-CoV-2 susceptibility and intake may predispose CVD patients to increased risk of SARS-CoV-2 infection. Therefore, the usage of such drugs should be very carefully evaluated in CVD patients [56].
Figure 1: The SARS CoV-2 binds to ACE2 receptor on endothelial cells by its S protein. ACE2 has a cardioprotective effect. On the contrary, increased ANG II in the absence of ACE2 increases the risk of CVD. SARS CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; S protein, Spike protein; ANG, Angiotensin; AT1R, Angiotensin 1 receptor; NO, nitric oxide; ROS, reactive oxygen species.

1.4 SARS-CoV-2 and Variants of Concern (VOC)

Large genome size and the mutation tendency have resulted in a divergence of coronavirus strains capable of higher infectivity and increased adaptation to new hosts [2]. The lineage B.1.1.7 was first identified in the United Kingdom, lineage B.1.352 was discovered in South Africa, and lineage B.1.617.2 was described in India. These variants have been termed VOC because of the higher risk due to their possible enhanced transmissibility, disease severity, and immune escape [6, 57]. These variants may adapt to new hosts, in part, through mutations on the receptor-binding domain (RBD) of spike (S) protein [58].
The SARS-CoV-2 infection starts with the binding of S protein to the host cell surface receptor. RBD of S protein binds to the angiotensin-converting enzyme (ACE2) receptor on the human cell surface to facilitate the virus entry into the host [4, 59]. The RBD of S protein from the SARS-CoV-2 (Wuhan strain, lineage B.1) does not efficiently bind the mouse ACE-2; wild-type laboratory mice are not suitable for infection with lineage B.1 virus [60-64]. MA10 is a mouse-adapted variant of SARS-CoV-2 with binding affinity to mouse ACE2 that has been obtained after sequential passaging of the virus in mouse lungs tissue [63]. MA10 infection in wild-type BALB/c mice resulted in virus replication in the upper and lower airways [63-65]. The MA10 virus has several mutations, including multiple mutations in the S protein compared with the Wuhan (B.1) reference sequence. These mutations are also present in B.1.1.7 and B.1.351 lineage, which emerged independently of B.1.1.7 lineage. B.1.1.7 variant has a mutation in the RBD region, including N501Y, 69/70 deletion, and P681H near the S1/S2 furin cleavage site. The B.1.351 variant has eight mutations, of which the three most notable mutations are K417N, E484K, and N501Y in the S protein. The B.1.617.2 variant has three unique mutations, E156del/R158G in the N-terminal domain and T478K in RBD of S protein [57, 66-69]. Compared to eight or three mutations in known variants, Omicron; recently identified strain carriers fifty mutations on spike (S)-protein [70]. These mutants are associated with critical public health concerns in the current scenario.
2 DISSERTATION SCOPE

2.1 Background and rationale

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) continues to be a global concern. In addition, several variants of SARS-CoV-2 have been identified that may influence antibody treatment and vaccine efficiency [8, 9, 71, 72]. Neurological complications, including cerebrovascular injury, altered mental status, encephalitis, encephalopathy, dizziness, headache, loss of taste and anosmia have been reported in some patients [10, 11, 17, 32, 73]. Further, human autopsy reports have shown evidence of lymphocytic panencephalitis, meningitis, brainstem perivascular and interstitial inflammatory changes with neuronal loss in COVID-19 patients [31, 32]. As such, viral RNA has been detected in brains of some COVID-19 cases, suggesting the olfactory mucosa could be a port of virus entry [28, 32, 72, 74, 75]. Angiotensin-converting enzyme 2 (ACE2), the entry receptor of SARS-CoV-2, is widely detected in the brain and is highly concentrated in several brain regions, including the substantia nigra, middle temporal gyrus and posterior cingulate cortex [10, 24, 76-79]. However, little is known about the causes of the SARS-CoV-2-induced neurological disease in humans. In addition, there is a lack of robust and practical in vivo and in vitro model systems to investigate the neuropathogenesis of a SARS-CoV-2 infection.

The overall goal of this proposal is to characterize in vivo and in vitro model systems for investigating neuropathogenesis caused by SARS-CoV-2 infection, including neuroinflammation and neuronal cells dysfunction. We hypothesize that SARS-CoV-2 can productively infect and replicate into the neuronal cells, inducing neuroinflammation and neuronal cell dysfunction and death.
2.2 Specific aims

Specific Aim 1: To characterize in vivo and in vitro model systems for investigating SARS-CoV-2-induced neuropathogenesis: It is known that wild-type mice do not support the replication of SARS-CoV-2. As such, the transgenic mice expressing hACE2 under the cytokeratin 18 promoter (K18-hACE2) represent a lethal model of SARS-CoV-2 infection [80-84]. In this aim, we will evaluate the kinetics of SARS-CoV-2 infection, tissue tropism and pathology in K18-hACE2 mice. In addition, we will generate primary cortical neuronal cultures from K18-hACE2 mice to investigate the neurotoxic effects of a SARS-CoV-2 infection in neurons.

Specific Aim 2: To investigate the immunological response to SARS-CoV-2 infection in the mouse brain and primary neuronal cultures.

Sub-Aim 2.1: To examine the global immune response of SARS-CoV-2 infection in the brain: A better understanding of the global gene changes underlying the multi-step progression of pathogenicity during infection could help develop potential therapeutic strategies for SARS-CoV-2. We will use nanostring technology to determine expression levels of various mRNA genes in SARS-CoV-2-infected mouse brain.

Sub-Aim 2.2: To study the effects of a SARS-CoV-2 infection in the primary neurons of human ACE2-expressing mice: Increased mortality has been linked to neuroinvasion and SARS-CoV-2 replication in the CNS of K18-hACE2 mice, although the mechanism remained unclear. We will generate primary neuronal cultures from K18-hACE2 mice to investigate the effects of a SARS-CoV-2 infection in the neurons. We will explore the activation of various immune, inflammatory and cell death pathways in the SARS-CoV-2-infected neurons.

Specific Aim 3: To analyze the pathogenesis of a SARS-CoV-2 infection in the aged mice: The older population is at high risk of coronavirus infections with increased disease severity
and pathogenicity. Inflammaging, a process that favors a constant low-grade pro-inflammatory environment in the older population, may contribute to adverse effects. We will do a comparative study to characterize the pathogenicity of SARS-CoV-2 infection in the different age groups of mice.
3 NEUROINVASION AND ENCEPHALITIS FOLLOWING INTRANASAL INOCULATION OF SARS-COV-2 IN K18-HACE2 MICE

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection can cause neurological disease in humans, but little is known about the pathogenesis of SARS-CoV-2 infection in the central nervous system. Herein, using K18-hACE2 mice, we demonstrate that SARS-CoV-2 neuroinvasion and encephalitis is associated with mortality in these mice. Intranasal infection of K18-hACE2 mice with $10^5$ plaque-forming units of SARS-CoV-2 resulted in 100% mortality by day 6 after infection. The highest virus titers in the lungs were observed at day 3 and declined at days 5 and 6 after infection. In contrast, very high levels of infectious virus were uniformly detected in the brains of all the animals at days 5 and 6. Onset of severe disease in infected mice correlated with peak viral levels in the brain. SARS-CoV-2-infected mice exhibited encephalitis hallmarks characterized by production of cytokines and chemokines, leukocyte infiltration, hemorrhage and neuronal cell death. SARS-CoV-2 was also found to productively infect cells within the nasal turbinate, eye and olfactory bulb, suggesting SARS-CoV-2 entry into the brain by this route after intranasal infection. Our data indicate that direct infection of CNS cells together with the induced inflammatory response in the brain resulted in the severe disease observed in SARS-CoV-2-infected K18-hACE2 mice.

3.1 Introduction
Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection in humans can cause pneumonia, acute respiratory distress syndrome, acute lung injury, cytokine storm syndrome and death [8, 9]. Although SARS-CoV-2 infection primarily causes respiratory disease; some patients develop symptoms of neurological disease, such as headache, loss of taste and smell, ataxia, meningitis, cognitive dysfunction, memory loss, seizures and impaired consciousness [10,
SARS-CoV-2 infection also induces long-term neurological sequelae in at least one-third of human cases. Infection with other coronaviruses, such as mouse hepatitis virus (MHV) in mice, and SARS-CoV-1 and Middle East Respiratory Syndrome (MERS) in humans have been shown to cause neurological disease [17, 91]. However, little is known about the pathophysiology of SARS-CoV-2-associated neurological disease in humans.

Central nervous system (CNS) cells that express the SARS-CoV-2 receptor, angiotensin-converting enzyme 2 (ACE2), include neurons, glial cells and astrocytes [24, 76]. ACE2 is expressed in multiple human brain areas, including the amygdala, cerebral cortex and brainstem with the highest expression levels found in the pons and medulla oblongata in the brainstem that contain the medullary respiratory centers of the brain [10, 77]. Several human autopsy reports have documented the presence of SARS-CoV-2 RNA in brain tissues [28, 72]. Human iPSC derived neural progenitors cells (NPCs) have been shown to be permissive to SARS-CoV-2 infection and both viral proteins and infectious viral particle production were detected in neurospheres and brain organoids infected with SARS-CoV-2 [92, 93]. Human autopsy reports have shown evidence of lymphocytic panencephalitis, meningitis and brainstem perivascular and interstitial inflammatory changes with neuronal loss in COVID-19 patients [31]. These data suggest that SARS-CoV-2 can productively infect human CNS cells [32]. However, the contributions of CNS cell infection and induced neuroinflammation to the pathogenesis of SARS-CoV-2-associated disease are not well understood. Small animal models provide a means for studying the neurological complications associated with SARS-CoV-2 infection. K18-hACE2 is a transgenic mice model which expresses human ACE-2 driven by human cytokeratin 18 promoter. K18-hACE2 mice model was originally developed to study SARS-CoV pathogenesis [94]. It was recently reported that intranasal inoculation with SARS-CoV-2 results in a rapidly fatal disease in K18-hACE2 mice [81-84].
These studies were focused on describing the acute lung injury in SARS-CoV-2 infected K18-hACE2 mice that was associated with high levels of inflammatory cytokines and accumulation of immune cells in the lungs [81-84]. In these published studies, infectious virus or viral RNA was not detected in the olfactory bulbs or brains of the majority of the infected animals, indicating restricted neurotropism of SARS-CoV-2 in K18-hACE2 mice. In the present study, we show that intranasal infection of six-week-old K18-hACE2 mice by SARS-CoV-2 can cause severe neurological disease with the brain being a major target organ for infection by this route of infection and neuroinflammation and neuronal death contributing to the infection-associated morbidity and mortality. The data also suggest that the SARS-CoV-2 can be trafficked to the brain via the olfactory bulb with subsequent transneuronal spread, as has been reported for other coronaviruses [95, 96].

3.2 Materials and Methods

3.2.1 Mice

Hemizygous K-18 hACE2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All the animal experiments were conducted in a certified animal biosafety level-3 (ABSL-3) laboratory at the Georgia State University (GSU). The protocol was approved by the GSU IACUC (Protocol number A20044). Six-week-old hemizygous K-18 hACE2 mice were infected with $10^5$ plaque-forming units (PFU) of SARS-CoV-2 strain USA-WA1/2020 under ABSL-3 containment by intranasal inoculation. SARS-CoV-2 (USA-WA1/2020) was isolated from an oropharyngeal swab from a patient in Washington, USA (BEI NR-52281) [97]. Animals in the control group received equivalent amounts of sterile PBS via the same route. Roughly equal numbers of male and female mice were used. Animals were weighed and their appetite, activity, breathing and neurological signs assessed twice daily [98, 99]. Mice that met the human endpoint criteria were
euthanized to limit suffering. In independent experiments, mice were inoculated with PBS (Mock) or SARS-CoV-2 intranasally, and on days 1, 3, 5 and 6 after infection, animals were anesthetized using isoflurane, perfused with cold PBS and respiratory (nasal turbinate and lung) and other tissues (spleen, heart, liver, kidney, pancreas, eye, olfactory bulb and brain) were collected and flash frozen in 2-methylbutane (Sigma, St. Louis, Missouri, United States) [100-102]. Alternatively, mice were perfused with PBS followed by 4% paraformaldehyde (PFA) and tissues were harvested, cryoprotected in 30% sucrose (Sigma, St. Louis, Missouri, United States), and embedded in optimum cutting temperature (OCT) as described previously [100, 103].

3.2.2 Quantification of the virus load

The virus titers were analyzed in the tissues by plaque assay and quantitative real-time PCR (qRT-PCR) [97, 98]. Briefly, frozen tissues were weighed and homogenized in a bullet blender (Next Advance, Averill Park, New York, United States) using glass or zirconium oxide beads. Virus titers in tissue homogenates were measured by plaque assay using Vero cells. Quantitative RT-PCR was used to measure viral RNA levels using primers and probes specific for the SARS-CoV-2 N gene as described previously [97]. Viral genome copies were determined by comparison to a standard curve generated using a known amount of RNA extracted from previously titrated SARS-CoV-2 samples. Frozen tissues harvested from mock and infected animals were weighed and lysed in RLT buffer (Qiagen) and RNA was extracted using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA). Total RNA extracted from the tissues was quantified, normalized and viral RNA levels per μg of total RNA were calculated.

3.2.3 Measurement of cytokines, chemokines and interferons

The levels of mRNA for select cytokines/chemokines (IL-1β, IL-6, TNF-α, IFN-γ, CCL2 and CCL3) and interferon-α (IFN-α) were determined in total RNA extracted from the lungs and brain
using qRT-PCR. The fold-change in infected tissues compared to mock tissues was calculated after normalizing to the GAPDH gene [100, 103]. The primer sequences and annealing temperatures used for qRT-PCR are listed in Table 1. The protein levels of IFN-α were measured in the lung and brain homogenates using an ELISA kit (PBL Interferon Source, Piscataway, NJ, USA) [98, 99].

Table 1: Primer sequences used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Primer Sequence (5’-3’)</th>
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<tbody>
<tr>
<td></td>
<td>Gene (Accession No.) Primer Sequence (5’-3’)</td>
</tr>
<tr>
<td>IL-1β (NM_000576)</td>
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<td>GGACCAGACATCACCAAGC</td>
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<tr>
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<td>CCCAGGGAGAAGGCAACTG</td>
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<td>Reverse</td>
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<tr>
<td>CCL3 (NM_011337)</td>
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<tr>
<td></td>
<td>ATTCAGTCCAGGTCAGT</td>
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<td></td>
<td>Reverse</td>
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<tr>
<td>IFN-α (NM_010502)</td>
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<td>Forward</td>
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<tr>
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<tr>
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<td>C</td>
</tr>
<tr>
<td>Reverse</td>
<td>TACTACCTGACACATTTC</td>
</tr>
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### 3.2.4 Immunohistochemistry

Sagittal sections (10-µm thick) were cut from the hemi-brain tissues frozen in OCT. Tissue sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation [103, 104]. Additionally, tissue sections were incubated with anti-CD45, anti-NeuN and anti-SARS-CoV-2 spike protein antibodies (Thermo Fisher Scientific, Norcross, GA, USA) overnight at 4°C followed by incubation with Alexa Fluor 546- or Alexa Fluor 488-conjugated secondary antibody.
for 1 hr at room temperature [100, 103]. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was conducted using an in-situ cell death detection kit (Roche, Indianapolis, Indiana, United States) as per the manufacturer’s instructions [100, 103]. Images were acquired using the Invitrogen™ EVOS™ M5000 Cell Imaging System (Thermo Fisher Scientific, Norcross, GA, USA).

3.2.5 Statistical Analysis

An unpaired Student’s t-test was used to calculate p values of significance. Differences with P values of <0.05 were considered significant.

3.3 Results

3.3.1 Characteristics of K18-hACE2 mice following SARS-CoV-2 infection by the intranasal route

Six-week-old K18-hACE2 mice of both sexes were infected intranasally with PBS (mock, n=10 mice) or $10^5$ PFU of SARS-CoV-2 in PBS (n=20 mice). The mock-infected mice remained healthy throughout the observation period. Virus infection resulted in 100% mortality by day 6 after infection (Figure 1A). Infected mice experienced significant weight loss on days 4 through 6 after infection compared to the mock-infected group (Figure 1B). Starting on day 4, all the infected animals began to show signs of disease, such as lethargy, slow movement and labored breathing. Neurological symptoms, such as hunchbacked posture, ruffled fur, tremors and ataxic gait, were also observed in infected mice on days 5 and 6 after infection.
Figure 2: Analysis of survival, body weight and virus titers in K18-hACE2 mice following SARS-CoV-2 infection. K18-hACE2 mice were inoculated intranasally with SARS-CoV-2 (10^5 PFU, n=20) or PBS (Mock, n=10). (A) Percent survival was determined. (B) Percent daily body weight change in the animals. Error bars represent SEM. **p < 0.001. The kinetics and levels of SARS-CoV-2 were determined in the lungs (C and D) and brain (E and F) by plaque assay and qRT-PCR. The data are expressed as PFU/g of tissue or genome copies/μg of RNA. Each data point represents an individual mouse. The solid horizontal lines signify the median.

### 3.3.2 Virus replication in the periphery and brain of K18-hACE2 mice

Six-week-old K18-hACE2 mice of both sexes were infected intranasally with PBS (mock, n=12 mice) or 10^5 PFU of SARS-CoV-2 in PBS (n=20 mice) and groups of 5 mice were used to measure the viral loads in the peripheral organs and brain at early (day 1), middle (day 3) and late (days 5 and 6) stages of infection. High virus levels were observed in the lungs on day 1, reached
peak levels at day 3 and declined at days 5 and 6 after infection (Figures 1C and 1D). In contrast, virus was not detected in the brain on day one but was present by day 3 after infection. Very high levels of viral RNA and infectious virus were detected in the brains of all the animals by days 5 and 6 after infection (Figures 1E and 1F). The onset of neurological symptoms and mortality in infected mice correlated with peak virus titers in the brain.

The virus replication kinetics observed in the nasal turbinates was similar to those in the lungs with the highest viral RNA levels detected during the early stage of infection (days 1 and 3), followed by a decline at later stages of infection (days 5 and 6) (Figure 2). In the olfactory bulbs and eyes, low levels of viral RNA were detected on days 1 and 3 after infection, with very high levels of viral RNA detected in all the animals on days 5 and 6 after infection indicating productive infection within the olfactory system (Figure 2). In contrast, little if any virus was detected in the serum of infected mice at any time after infection tested.

Since the K18 promoter is known to be active in the epithelium of multiple organs of K-18-hACE2 mice [96, 105], we also evaluated viral RNA levels in other peripheral organs. Viral RNA was detected in the heart, kidney, spleen, pancreas and liver on days 1 and 3. There was a slight increase in RNA levels on days 3 and 5 in each of these organs, suggesting limited virus replication at these sites (Figure 2). These data agree with previous reports that also showed the presence of SARS-CoV-2 RNA in these organs [81-84, 96, 105].
Figure 3: Analysis of virus tropism in K18-hACE2 mice. The viral RNA copy number in the nasal turbinates, olfactory bulbs, eye, serum, kidney, spleen, pancreas, heart and liver was determined on days 1, 3, 5 and 6 after infection by qRT-PCR and expressed as genome copies/μg of RNA. Each data point represents an individual mouse. The solid horizontal lines signify the median.

3.3.3 Inflammatory changes in the lungs and brain of SARS-CoV-2-infected mice

IFN signaling has a pivotal role in developing an innate and adaptive immune response to viral infection [106, 107]. Therefore, we measured the mRNA and protein levels of IFN-α in the lungs and brain. In the lungs, an increase in both IFN-α mRNA and protein levels was detected on day 1, peaked at day 3 and then decreased on day 6 after infection (Figures 3A and 3B). In contrast, an IFN response was not detected in the brain on days 1 and 3 after infection. High levels of IFN-
α were detected in the brain only by days 5 and 6 after infection (Figures 3C and 3D). Overall, the induction of IFN-α correlated with the SARS-CoV-2 replication kinetics in the lungs and brain. It is interesting to note that relative IFN-α levels were comparatively higher in the lungs compared to the brains of the infected animals despite higher virus replication in the brain.

![Image of IFN-α mRNA and protein levels](image)

**Figure 4:** Analysis of mRNA and protein levels of IFN-α in the lungs and brain. The mRNA levels of IFN-α were measured in the lungs (A) and brain (C) by qRT-PCR, and the fold change in the infected tissues compared to the corresponding mock-infected controls was calculated after normalizing to the GAPDH gene. The protein levels of IFN-α were measured in the lungs (B) and brain (D) homogenates using ELISA and expressed as pg/g of tissue. Error bars represent SEM (n = 5 mice per group). *p < 0.05; **p < 0.001.

We next examined the mRNA levels of proinflammatory cytokines and chemokines in the lungs and brain of infected mice. SARS-CoV-2 infection resulted in a 10-fold increase on day 1 and a 100-fold increase on day 3 in the IL-6 mRNA expression in the lungs (Figure 4A). The levels
of TNF-α mRNA were elevated ~10-fold in the lungs on day 1 and 3 after infection. The level of IFN-γ mRNA was elevated by 15-fold on day 3. However, the levels of these cytokines had decreased by day 5 after infection. The IL-1β mRNA levels showed no significant increase at any time point after infection. There was a 100-fold increase in the expression of CCL2 on day 3 (Figure 4B). However, the levels of CCL2 mRNA had decreased by day 5 after infection. CCL3 mRNA levels increased slightly on day 1 and were undetectable at days 5 and 6 after infection.

In the brain, no increase in the mRNAs of the cytokines or chemokines tested was observed on day 1 after infection. Less than a 10-fold increase was observed in the cytokine mRNA levels on day 3 (Figures 4C). There was a 500-fold increase in IL-6 mRNA by day 5 after infection. TNF-α and IFN-γ mRNA levels increased by ~ 750-fold in the brain by day 5 after infection. Similarly, IL-1β mRNA levels increased by 400-fold by day 5 after infection. Both CCL2 and CCL3 mRNA levels were elevated by almost 1,000-fold on days 5 and 6 after infection and consistent with the high level of virus in the brain (Figure 4D). These results indicate that the inflammatory response was more pronounced in the brain than in the lungs at the later stage of infection.
Figure 5: Cytokine and chemokine mRNA level in the lungs and brain. The mRNA levels of various cytokine and chemokine genes were determined in the lungs (A and B) and brain (C and D) using qRT-PCR. Fold change in the infected tissues compared to the corresponding mock controls was calculated after normalizing to GAPDH mRNA in each sample. Error bars represent SEM (n = 5).

3.3.4 SARS-CoV-2 induced neuropathology in K18-hACE2 mice

We next analyzed the brain sections from infected mice for antigen distribution, infiltration of immune cells and cell death. Immunohistochemical staining for the SARS-CoV-2 spike protein detected cell-associated viral antigen throughout the brain at day 6 after infection. Representative data for sections from the cortex, cerebellum and hippocampus regions are shown in figure 5. We also detected virus antigen in sections of the olfactory bulb of infected animals on day 6. H&E
staining of brain sections from the infected mice demonstrated perivascular hemorrhage and neuronal cell death (Figures 6A and 6B). The neurons of infected mice demonstrated shrunken neuron body with light pink cytoplasmic staining representing degenerating neurons (Figure 6B). Enhanced leukocyte infiltration was detected within blood vessel walls and in the perivascular space (Figure 6A). Evidence of leukocyte infiltration was confirmed by direct immunohistochemical analysis of the CD45 antigen, which revealed many CD45-positive cells in the brain parenchyma near neurons (Figure 6C). SARS-CoV-2-induced cell death was evaluated by direct TUNEL staining of brain tissues. On day 6, infected K18-hACE2 mice had elevated numbers of TUNEL-positive cells in the cortex, hippocampus and cerebellum regions, indicating increased cell death (Figure 6D).
Figure 6: Detection of SARS-CoV-2-infected cells in the brains of K18-hACE2 transgenic mice. Brain sections (day 6 after infection) were stained for SARS-CoV-2 spike protein. Representative immunostaining images showing the presence of SARS-CoV-2 spike protein (red) in the cortex, cerebellum, hippocampus and olfactory bulb of infected mice. Nuclei are stained with DAPI (blue). The photomicrographs shown are representative of the images obtained from five animals. Bars, 20 μm.
3.4 Discussion

This study demonstrates a critical role of direct infection of CNS cells and of the inflammatory response in mediating SARS-CoV-2-induced lethal disease in K18-hACE2 mice. Intranasal inoculation of the virus results in a lethal disease with high levels of virus replication in the brain. Virus infection of the CNS was accompanied by an inflammatory response as indicated by the production of cytokines/chemokines, infiltration of leukocytes into the perivascular space and parenchyma and CNS cell death. Our data also indicate that following infection by the intranasal
route, the virus enters the brain by traversing the cribriform plate and infecting neuronal processes located near the site of intranasal inoculation.

Some animal coronaviruses, such as MHV readily infect the neurons and cause lethal encephalitis in mice [91, 108]. SARS-CoV infection also induces severe neurological disease after intranasal administration in K18-hACE2 mice [96]. Similarly, in our study, SARS-CoV-2 virus antigen was detected throughout the brain, including the cortex, cerebellum and hippocampus. The onset of severe disease in SARS-CoV-2 infected mice correlated with peak viral levels in the brain and immune cell infiltration and CNS cell death. Peak virus titers in the brains were approximately 1,000 times higher than the peak titers in the lungs, suggesting a high replicative potential of SARS-CoV-2 in the brain. The relative up-regulation of cytokine and chemokine mRNAs was approximately 10 to 50 times higher in the brain compared to the lungs, strongly suggesting that extensive neuroinflammation contributed to clinical disease in mice.

It was recently reported that SARS-CoV-2 infection of K18-hACE2 mice causes severe pulmonary disease with high virus levels detected in the lungs of these mice and that mortality was due to the lung infection [81-84]. In these studies, viral RNA was undetectable in the brains of the majority of the infected animals, indicating a limited role of brain infection in disease induction. An important distinction between our study and others is that we detected high infectious virus titers in the olfactory system and brains of 100% of the infected K18-hACE2 mice. This phenotype was not consistently observed in the aforementioned K18-hACE2 mouse studies [81-84]. Moreover, none of the published studies evaluated the extent of neuroinflammation and neuropathology at the later stages of infection. Our results showed that the inflammatory response was more pronounced in the brain than in the lungs on days 5 and 6 after infection. Although both our study and the previous studies infected mice via the intranasal route, the other studies used
older (7- to 9-week-old) K18-hACE2 and a lower viral dose (10^4 PFU) and in one study, only analyzed samples at 3 days after infection [84]. In our study, six-week-old K18- hACE2 mice were infected with 10^5 PFU. However, unpublished data from our laboratory demonstrate that six-week-old K18- hACE2 mice infected with a lower viral dose (10^3 PFU) also exhibit a similar phenotype, suggesting that the brain is a major site of infection following infection by the intranasal route regardless of the virus dose used. Additional studies are needed to clarify the parameters that differentially affect tissue tropism, routes of virus dissemination, and mechanisms of lung and brain injuries in K18-hACE2 mice following SARS-CoV-2 infection. Recent studies have suggested that humans have a higher chance of developing a brain infection if they are infected intranasally with a high dose of virus [109].

Alterations in smell and taste are features of COVID-19 disease in humans [10, 73]. Pathological analyses of human COVID-19 autopsy tissues detected the presence of SARS-CoV-2 proteins in endothelial cells within the olfactory bulb [73, 75]. Our data indicate that SARS-CoV-2 can productively infect cells within the nasal turbinate, eye and olfactory bulb in intranasally infected K18- hACE2 mice. Virus infection of cells in these tissues in humans may explain the loss of smell associated with some COVID-19 cases [73]. The detection of virus replication in these tissues suggests that SARS-CoV-2 can access the brain by first infecting the olfactory bulb and then spreading into the brain by infecting connecting brain neuron axons. This hypothesis is consistent with previously published reports that neurotropic coronaviruses infect olfactory neurons and are transmitted to the brain via axonal transportation [10, 95, 96, 110]. Many viruses, such as HSV-1, Nipah virus, rabies virus, Hendra virus and influenza A virus, have also been shown to enter the CNS via olfactory sensory neurons [111-114]. Another route by which a virus can gain access to the brain is via the disruption of the blood-brain barrier (BBB). However,
we could not detect any virus in the serum of the infected mice at any time after infection tested, suggesting a limited role of BBB disruption in SARS-CoV-2 neuroinvasion. This finding is in agreement with previously published studies that detected little or no virus in the blood of K18-hACE2 mice after infection with SARS-CoV-1 or SARS-CoV-2 [81-84, 96, 105].

In summary, we found that intranasal Infection of K18-hACE2 mice by SARS-CoV-2 causes severe neurological disease. Our data demonstrate that the CNS is the major target of SARS-CoV-2 infection in K18-hACE2 mice under the conditions used, and that brain infection leads to immune cell infiltration, inflammation and cell death.
4 SARS-COV-2 INFECTS PRIMARY NEURONS FROM HUMAN ACE2 EXPRESSING MICE AND UPREGULATES GENES INVOLVED IN THE INFLAMMATORY AND NECROPTOTIC PATHWAYS

Transgenic mice expressing human angiotensin-converting enzyme 2 under the cytokeratin 18 promoter (K18-hACE2) have been extensively used to investigate the pathogenesis and tissue tropism of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. Neuroinvasion and replication of SARS-CoV-2 within the central nervous system (CNS) of K18-hACE2 mice is associated with increased mortality, although the mechanisms by which this occurs remain unclear. In this study, we generated primary neuronal cultures from K18-hACE2 mice to investigate the effects of a SARS-CoV-2 infection. We also evaluated the immunological response to SARS-CoV-2 infection in the CNS of K18-hACE2 mice and mouse neuronal cultures. Our data show that neuronal cultures obtained from K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. SARS-CoV-2 infection upregulated the expression of genes involved in innate immunity and inflammation, including IFN-α, ISG-15, CXCL10, CCL2, IL-6 and TNF-α, in the neurons and mouse brains. In addition, we found that SARS-CoV-2 infection of neurons and mouse brains activates the ZBP1/pMLKL-regulated necroptosis pathway. Together, our data provide insights into the neuropathogenesis of SARS-CoV-2 infection in K18-hACE2 mice.

4.1 Introduction

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) continues to be a global concern. In addition, several variants of SARS-CoV-2 have been identified that may influence antibody treatment and vaccine efficiency [8, 9, 27, 71]. Neurological complications, such as brain fog, loss of taste and smell, changed
mental status and anosmia have been reported in some COVID-19 patients [10, 11, 17, 32, 115]. Studies have shown presence of meningitis, encephalitis, leukocytes infiltration and neuronal death in COVID-19 patients [31, 32]. Evidence of SARS-CoV-2 neuroinvasion in COVID-19 patient brain autopsies has been demonstrated and the olfactory mucosa has been suggested as a route of entry [27, 28, 32, 109, 116]. Several studies have also reported that neurologic symptoms may result from the exacerbated systemic pro-inflammatory responses without direct infection of the brain cells [115, 116]. Angiotensin-converting enzyme 2 (ACE2), the entry receptor of SARS-CoV-2, has recently been demonstrated to be present on neurons and glial cells of different brain regions[10, 24-26, 78, 79].Studies using brain organoids derived from human pluripotent stem cell (hPSC)-derived have shown the presence of virus in neuronal cells [29, 30, 117, 118]. In addition, anti-ACE2 antibodies can inhibit the SARS-CoV-2 infection of neuronal cells[117] [19].

The K18-hACE2 mouse model is commonly used to study pathogenesis of SARS-CoV-2 infection and to test the efficacy of anti-viral compounds and vaccines. These mice express human ACE2, the entry receptor of SARS-CoV-2 [81-84].We previously reported that infection of K18-hACE2 mice with SARS-CoV-2 results in a lethal disease associated with viral neuroinvasion and severe neuronal damage [119]. However, the molecular mechanism by which SARS-CoV-2 infection of neurons leads to acute encephalitis in K18-hACE2 mice remain unclear. The present study was undertaken to i) investigate the permissiveness neurons to SARS-CoV-2 infection, and ii) evaluate the immunological response to SARS-CoV-2 infection in the CNS of K18-hACE2 animals and mouse neuronal cultures. Our data show that neuronal cultures obtained from K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. In response to infection, genes involved in the innate immune response, inflammation and cell death were upregulated in the neurons. In addition, SARS-CoV-2 infection of mouse brains also
resulted in increased expression of genes associated with the inflammatory and cell death pathways.

4.2 Materials and Methods

4.2.1 Neuronal cultures and SARS-CoV-2 infection

Hemizygous K18-hACE2 mice and non-hACE2-carrier (NC) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). One-day-old pups were obtained from established breeding pairs of K18-hACE2 and NC mice as previously described [98, 120, 121]. The neurons were plated for 24 hours onto poly-D-lysine-coated plates in serum Neurobasal A medium. Neurons were then cultured in serum-free Neurobasal A medium containing B27 for seven days to allow differentiation. The neurons isolated from each pup were plated separately and genotyped to identify hACE2-expressing and NC neurons. Neuronal cultures were infected with SARS-CoV-2 (USA-WA1/2020) or mock-infected at a multiplicity of infection of 0.1. At various time points after infection, supernatants and cell lysates were collected[39, 122-124].

4.2.2 Animal infection experiments

The in vivo animal experiments with SARS-CoV-2 were conducted in an Animal Biosafety Level 3 (ABSL-3) laboratory. Georgia State University Institutional Animal Care and Use Committee approved the experimental protocol of this study (Protocol number A20044). Hemizygous K18-hACE2 mice aged eight-weeks were inoculated with PBS or \(10^4\) PFU of SARS-CoV-2 via the intranasal route [119, 124]. On various days after the infection, animals were anesthetized, perfused with PBS and brain tissues were collected.

4.2.3 Quantification of the viral titers

The levels of infectious virus in cell culture supernatants and brain tissues were determined by using plaque formation assay. Quantitative RT-PCR was used to measure intracellular viral
RNA levels using SARS-CoV-2 N gene primers [39, 80]. Total RNA was extracted from cell pellets and brain tissues using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA). Viral genome copies per ug of total RNA were calculated using a standard curve of known amount of viral RNA [39, 99, 119].

4.2.4 Immunostaining

Neuronal cells were grown on coverslips in 12-well plates and infected with SARS-CoV-2 or PBS at a MOI of 0.1 for 48 hours[123]. Cells were washed with PBS and fixed in 4% paraformaldehyde for 1 hour at room temperature. The cells were permeabilized and incubated with anti-MAP2 (Catalog # PA5-17646) and anti-dsRNA (MABE1134) antibodies overnight at 4 C (Thermo Fisher Scientific, Norcross, GA, USA). Next day, cells were incubated with Alexa Fluor 546- or Alexa Fluor 488-conjugated secondary antibody for 1 hour at room temperature[119, 123, 125]. The Invitrogen EVOS™ M5000 Cell Imaging System. was used to capture the images.

4.2.5 Western blot analysis

Protein extracted from neuronal cultures and mouse brains were separated on SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with primary antibodies against ZBP1 (Cat #703166), pMLKL (Thermo Fisher Scientific, Norcross, GA, USA) and β-actin. To visualize the protein bands, the membranes were incubated with secondary antibody conjugated with IRDye 800 and IRDye 680 (Li-Cor Biosciences). The membranes were scanned using the Odyssey infrared imager (Li-Cor Biosciences)[125, 126].

4.2.6 ELISA

ELISA was used to measure the protein levels of IL-6 (Invitrogen, Catalog # 50-246-676) and IFN-β (PBL Assay Science, Catalog # 12405-1) and CXCL10 (Invitrogen, Catalog # 50-182-
92) in the cell culture supernatants, according to the manufacturer’s instructions. The plates were analyzed using a Victor 3 microtiter reader as previously described [80].

4.2.7 qRT-PCR

A Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA) was used to extract total RNA from cell pellets and brains. cDNA was synthesized from RNA using an iScript™ cDNA Synthesis Kit (Bio-Rad). qRT-PCR was used to determine the expression levels of multiple host genes [2]. The fold-change in infected samples compared to control samples was calculated after normalizing to the housekeeping GAPDH gene [39, 119, 123, 124]. The primer sequences used for qRT-PCR are listed in Table 1.

Table 2: Primer sequences used for qRT-PCR for gene expression.

<table>
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<tr>
<th>Gene (Accession No.)</th>
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<td>IL-1β (NM_000576)</td>
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</tr>
<tr>
<td></td>
<td>GGACCAGACATCACCAAGC</td>
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<td>IL-6 (NM_000600)</td>
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<tr>
<td></td>
<td>CCCAGGGAGAAGGCAACTG</td>
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<tr>
<td>CCL3 (NM_011337)</td>
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<tr>
<td>-------------</td>
<td>-----------------</td>
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<tr>
<td>TNF-α (NM_013693)</td>
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<td>Caspase 8 (NM_009812)</td>
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</tbody>
</table>
4.2.8 Statistical Analysis

Unpaired student t-tests using GraphPad Prism 5.0 were used to calculate the p values. Differences of p < 0.05 were considered significant.

4.3 Results

4.3.1 SARS-CoV-2 infection of primary mouse cortical neurons

Primary neuronal cultures were established from one-day-old K18-hACE2 (hACE2 neurons) and non-hACE2-carrier (NC neurons) pups and cultured for seven days to allow differentiation to occur. The neuronal cultures were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.1. Plaque assay, qRT-PCR and immunofluorescence were used to determine the kinetics of SARS-CoV-2 replication at various time points after infection. Productive SARS-CoV-2 replication, as indicated by the release of virions was detected at 24 hours after infection of the hACE2 neurons. Viral titers peaked at 48 hours after infection (log 5–6 PFU/mL) followed by a slight decrease in the virus titers at 72 hours (Figure 1A). We next measured intracellular viral RNA levels using qRT-PCR. High SARS-CoV-2 RNA levels were detected in the hACE2 neurons at 48 and 72 hours after infection (log 6-7 genome copies/ug RNA). Neurons derived from NC mice were relatively resistant to infection compared to the hACE2
neurons. There was a slight increase in virus and RNA levels at 48 and 72 hours, suggesting limited virus replication in these cells (Figure 1B). Immunofluorescence assay of SARS-CoV-2-infected hACE2 neurons showed strong dsRNA staining. dsRNA was detected in both the neuronal bodies and axons of the MAP2-positive cells at 48 hours after infection (Figure 1C). dsRNA detection is considered as evidence of viral RNA replication. Approximately 40% of hACE2 neurons were positive for dsRNA at 48 hours after infection. Overall, these findings indicate that neurons derived from K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication.

Figure 8: SARS-CoV-2 infection of mouse neuronal cultures. K18-hACE2 (hACE2 neurons) and non-hACE2-carrier (NC neurons) were prepared from one-day-old pups and cultured for seven days for differentiation. (A) hACE2 (blue bars) and NC neurons (red bars) were infected with SARS-CoV-2 at a MOI of 0.1. Virus infectivity titers in the supernatants were measured by
plaque formation assay and are expressed as plaque forming units (PFU)/mL. (B) Intracellular viral RNA copies were determined by qRT-PCR. The data are expressed as genome copies/ug of RNA. Values are the mean ± SEM of three independent infection experiments conducted in duplicate. Each data point represents an independent experiment. (C) hACE2 neurons grown coverslips were fixed at 48 hours after infection and stained with anti-MAP2 (red), dsRNA (green) and DAPI (blue) antibodies. In the bottom row of panels, the boxed areas from the first row are expanded. The images shown are representative of three independent infection experiments. 20× magnification.

### 4.3.2 Host immune responses in SARS-CoV-2-infected neurons and mouse brains

We next investigated the effect of SARS-CoV-2 infection on the mRNA expression of key innate immune and inflammation genes in the neurons. Changes in gene expression levels in hACE2 neurons infected with SARS-CoV-2 for 48 hours compared to mock-infected controls were analyzed by qRT-PCR. Interferon stimulated gene (ISG)-15 mRNA expression increased by >100-fold after SARS-CoV-2 infection (Figure 2A). The levels of interferon (IFN)-α and IFN-β were elevated more than 10-fold. The mRNA levels of the chemokine pathway-associated genes, chemokine (C-C motif) ligand-2 (CCL2) and chemokine (C-X-C motif) ligand-10 (CXCL10), were upregulated by more than 50-fold in infected neuronal cultures (Figure 2B). Interleukin-6 (IL-6), IL-1β and tumor necrosis factor (TNF)-α mRNA expression levels were upregulated >10-fold by a SARS-CoV-2 infection. CCL3 mRNA levels were also increased compared to the mock-infected controls (Figure 2B).

Next, we examined the mRNA levels of innate immune and inflammatory genes in the brains of infected mice. K18-hACE2 mice infected with PBS or 10^4 PFU of SARS-CoV-2 via the intranasal route [80]. The mice were sacrificed at days 1, 3 and 6 after infection, and the brains were harvested. Plaque assay was conducted to determine infectious virus titers in the brain homogenates. No infectious virus was detected in the brains on day one but virus infectivity titers were very high at day 3 (log 3-4 PFU/gram of brain tissue) and day 6 after infection (log 7-8...
PFU/gram of brain tissue) [28]. IL-6 and TNF-α mRNA levels increased by >5-fold on day 3 after SARS-CoV-2 infection (Figure 2C). By the sixth day after infection, IL-6 and TNF-mRNA levels in the brain had increased by 300-fold (Figure 2C). There was also a 100-fold increase in the IL-1β mRNA levels on day 6. There was a slight upregulation in the levels of IFN-α mRNA (Figure 2C). At day 6 after infection, expression levels of chemokines, including CXCL10 and CCL2 and CCL-3 were elevated by more than 300-fold (Figure 2D).

As these pro-inflammatory cytokines are secreted proteins, their release in the culture media of mock- and SARS-CoV-2-infected hACE2 neurons was detected using ELISA. In controls, basal levels of IL-6 and IFN-β in cell culture media were very low. On the other hand, significant amounts of soluble IL-6 and IFN-β were detected in supernatant from infected cells at 48 hours after infection (Figure 3). Basal level of CXCL10 was relatively high, but it also increased significantly after SARS-CoV-2 infection. These results indicate that SARS-CoV-2 infection upregulates the expression of innate immune and inflammatory genes in neuron cultures and mouse brains.
Figure 9: Analysis of upregulation of the expression of immune genes involved in innate immunity and inflammation in primary mouse neurons and mouse brains. (A and B) hACE2 neurons were infected with SARS-CoV-2 or mock-infected at a MOI of 0.1. At 48 hours after infection, cell pellets were collected, and total RNA was extracted. qRT-PCR was conducted to determine the fold-change of (A) ISG15, IFN-β and IFN-α, and (B) CXCL10, CCL2, IL-6, IL-1β, TNF-α and CCL3 mRNA levels. Data for each sample was normalized to the value for GAPDH and expressed as the relative fold increase compared to mock-infected controls. Data represent the mean ± SEM of three independent infection experiments conducted in duplicate. Each data point represents an independent experiment. (C and D) Eight-week-old hemizygous K18-hACE2 mice were infected with SARS-CoV-2 (10^4 PFU, n = 12) or PBS (Mock, n = 9). Brains were harvested after extensive perfusion with PBS at days 1, 3 and 6 after infection and RNA was extracted. The mRNA levels of (C) IL-6, IL-1β, TNF-α and IFNα, and (D) CXCL10, CCL2 and CCL3 were determined by qRT-PCR. Each data point represents an individual mouse. Data represent the mean ± SEM.
Supernatant collected from hACE2 neurons infected with SARS-CoV-2 or mock-infected for 48 hours was used to determine the levels of IL-6 and IFN-β and CXCL10 using ELISA. The data expressed are the mean concentration (pg/ml) ± SEM of the amount of IL-6 and IFN-β and CXCL10 secreted in the supernatant and is representative of three independent experiments. **p < 0.001.

4.3.3 SARS-CoV-2 infection activates the ZBP1/MLKL pathway in neurons and mouse brains

We examined the mRNA and protein levels of genes involved in cell death pathways in neurons after SARS-CoV-2 infection. qRT-PCR was used to analyze the changes in the gene mRNA levels. Key genes involved in the necroptotic pathway were highly upregulated in hACE2 neurons infected with SARS-CoV-2 for 48 hours. The levels of Z-DNA binding protein 1 (ZBP1) and mixed lineage kinase domain-like (MLKL) mRNA were elevated ~50-fold after SARS-CoV-2 infection. mRNA expression levels of caspase-8 and receptor-interacting kinase-3 (RIPK3) were upregulated >10-fold after infection (Figure 4A). Pyroptotic gene caspase-1 was upregulated by 10-fold while the apoptotic genes, caspase-3 and caspase-7 showed no significant increase after SARS-CoV-2 infection (Figure 4B). To verify the activation of the necroptotic pathway, protein
levels of ZBP1 and phosphorylated MLKL (pMLKL) were measured by immunoblotting. The levels of ZBP1 increased at 24 and 48 hours after infection. We detected a modest increase in the protein levels of pMLKL at 24 and 48 hours after infection. However, there was a significant increase in the levels of pMLKL protein at 72 hours (Figure 4C).

Next, we evaluated the activation of the necroptotic pathway in mouse brains infected with SARS-CoV-2. mRNA expressions of ZBP1 and MLKL increased gradually in the brains from days 1 to 6 after SARS-CoV-2 infection. By the sixth day after infection, IL-6 and TNF-mRNA levels were upregulated ~50-fold in the brains (Figure 4D). The mRNA levels of RIPK3, RIPK1, Caspase 8 and Caspase 1 were also elevated by day 6 (Figure 4D and E). However, there was no significant increase in the levels of caspase-3 and caspase-7 mRNA in infected brains. Western blot data showed an increase in the protein levels of ZBP1 and pMLKL in the infected brains in a time-dependent manner (Figure 4F). The increase in the mRNA and protein levels of ZBP1 and MLKL correlate with the increase in the infectious virus titers in the brains[119]. Together, these results indicate that a SARS-CoV-2 infection in neurons and mouse brains activates the ZBP1/MLKL-regulated necroptosis pathway.
Figure 11: mRNA and protein levels of genes involved in cell death pathways in primary mouse neurons and mouse brains. (A and B) hACE2 neurons were infected with SARS-CoV-2 or mock-infected at a MOI of 0.1. At 48 hours after infection, cell pellets were collected, and total RNA was extracted. qRT-PCR was conducted to determine the fold-change in (A) ZBP1, MLKL, RIPK3 and RIPK1, and (B) Caspase 8, Caspase 1, Caspase 7 and Caspase 8 mRNA levels. Data for each sample was normalized to the value for GAPDH and expressed as the relative fold increase compared to mock-infected controls. Data represent the mean ± SEM of three independent infection experiments conducted in duplicate. Each data point represents an independent experiment. (C) hACE2 neurons were infected with SARS-CoV-2 or mock-infected at a MOI of 0.1. At 24, 48 and 72 hours, cell pellets were collected, and total protein was extracted. Protein was blotted with ZBP1, pMLKL or β-actin antibodies. Data are representative of three independent experiments. (D and E) K18-hACE2 mice were inoculated with SARS-CoV-2 or PBS via the intranasal route. Brains were harvested after extensive perfusion at days 1, 3 and 6 after infection and RNA was extracted. qRT-PCR was used to determine the mRNA levels of (D) ZBP1, MLKL, RIPK3 and RIPK1, and (E) Caspase 8, Caspase 1, Caspase 7 and Caspase 8. After normalizing individual sample to GAPDH level, the fold change in infected tissues compared to mock-infected controls was determined. Each data point represents an individual mouse (n = 4). Values are the mean ± SEM. (F) Protein extracted from mock- and SARS-CoV-2-infected brain tissues were blotted with ZBP1, pMLKL or β-actin antibodies. Data are representative of four mice per time point.
4.4 Discussion

In this study, we show that SARS-CoV-2 establishes a productive infection in neuronal cultures obtained from hACE2-expressing mice. In response to infection, the expression of innate immune and inflammatory genes was upregulated in the neurons as well as in mouse brains. In addition, we found that SARS-CoV-2 infection of neurons and mouse brains upregulated genes involved in the necroptotic pathway (ZBP1, MLKL RIPK3 and caspase-8), suggesting that necroptosis may play a role in the pathogenesis of SARS-CoV-2 infection in the CNS.

It is known that some animal (mouse hepatitis virus) and human (HCoV-OC43) coronaviruses productively infect neuronal cells [80, 127]. SARS-CoV-2 infection has been detected in the brains of some COVID-19 patients [27, 28, 32, 109, 116]. SARS-CoV-2 has also been shown to replicate and induce cell death in human neural progenitor cells and brain organoids [29, 30, 117, 118, 128]. Transgenic K18-hACE2 mice represent a lethal model of SARS-CoV-2 infection [80-84]. Neuroinvasion and replication of SARS-CoV-2 within the CNS is associated with mortality in these mice. In the present study, we show that neurons derived from one-day-old K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. dsRNA was detected in the neuronal bodies and axons infected with SARS-CoV-2. In comparison, virus replication was limited in the non-hACE2-expressing mouse neurons.

A cytokine storm is one of the pathological hallmarks of severe outcomes resulting from SARS-CoV-2 infection [129, 130]. Several studies have reported that increased TNF-α and IL-6 levels correlate with severe disease outcomes [131, 132]. In the present study, we show that SARS-CoV-2 infection in K18-hACE2 mouse brains is also characterized by upregulation of innate immune and inflammatory genes, including TNF-α and IL-6 [119]. Similarly, a significant increase in the expression of IL-6, TNF-α, CXCL10 and CCL2 was observed in SARS-CoV-2-
infected neuron cultures. These inflammatory genes may activate downstream cell death signaling pathways in the neurons, leading to neuronal death, and/or stimulate glial cells, exacerbating neuroinflammation [132-134]. TNF-α has been reported as a potent inducer of neuronal injury in several neurodegenerative diseases, such as cerebral ischemia, spinal cord injury, multiple sclerosis and viral infections including HIV-associated dementia [33,36]. CXCL10 and CCL2 are important chemokines involved in the infiltration of leukocytes into the CNS after virus infection [134].

ZBP1 is one of the cytoplasmic sensors that regulate cell death and inflammation [122, 135]. ZBP1 initiates RHIM-dependent activation of RIPK3-dependent necroptosis during virus infections. Necroptosis is an inflammatory cell death caused by RIPK3 phosphorylation, which activates the pseudo-kinase MLKL, which oligomerizes and ruptures the plasma membrane, resulting in cell death. Necroptosis can eradicate virus-infected cells and activate innate and adaptive immunity to limit virus replication. This process can also trigger the release of inflammatory cytokines and damage-associated molecular patterns, resulting in robust inflammation [135, 136]. In the present study, we found significant upregulation of the necroptotic genes, ZBP1, MLKL, and RIPK3, in neuronal cells and mouse brains after SARS-CoV-2 infection. Previous studies have demonstrated that infection with beta coronaviruses can induce necroptosis in certain cell types. Human coronavirus, HCoV-OC43, induces necroptosis in human neural cells [127] and mouse hepatitis virus infection induces necroptosis in murine bone-marrow-derived macrophages by phosphorylation of MLKL [137]. We previously reported that ZBP1 restricts replication of West Nile virus and Zika virus in primary mouse cortical neurons [98] Future studies are warranted to understand the role of ZBP1 in SARS-CoV-2 pathogenesis.
Together, our results demonstrate that SARS-CoV-2 robustly replicates in neuronal cultures obtained from K18-hACE2 mice. Like the SARS-CoV-2-infected K18-hACE2 mouse brains, virus infection of neuronal cultures induces up-regulation of genes involved in the innate immune response, inflammation and cell death.
5 THE GLOBAL IMMUNE RESPONSE OF SARS-COV-2 INFECTION IN THE MOUSE BRAIN

5.1 Introduction

Since the first outbreak in China in 2019, coronavirus disease 2019 (COVID-19) has spread rapidly and globally with a mortality rate of 2% resulting in the ongoing pandemic. The lack of highly efficacious antiviral drugs that can manage this ongoing global emergency gives urgency to establishing a comprehensive understanding of the molecular pathogenesis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Typical clinical presentation of COVID-19 can be characterized by upper and lower respiratory tract infections that are often associated with fever and cough. Although most infections remain mild or asymptomatic, some patients experience more severe disease and develop systemic inflammation, tissue damage, acute respiratory distress syndrome, thromboembolic complications, cardiac injury, and/or cytokine storm [41]. Furthermore, SARS-CoV-2 infection is also associated with a wide variety of neurological manifestations, such as headache, loss of taste and smell, ataxia, meningitis, cognitive dysfunction, memory loss, seizures and impaired consciousness, as well as long-term neurological problems in more than 30% of adults [27, 31]. However, the mechanism by which SARS-CoV-2 infection causes neurological diseases remains unclear.

A better understanding of the global gene changes underlying the multi-step progression of pathogenicity during infection could help develop potential therapeutic strategies for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Neuroinflammation is a prominent neuropathological signature in many neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Frontal Temporal Dementia, Amyotrophic Lateral Sclerosis, and prion diseases. Using a Nanostring Neuroinflammation panel, we looked at the gene expression in the
brains of K18-hACE2 mice on days 1, 3, and 6 after SARS-CoV-2 infection. This panel examines several critical processes and pathways to present a comprehensive picture of the immunological and inflammatory responses in the nervous system. Assessment of neurotransmission, innate immunity, inflammation, and cell death pathways are all important areas of neuropathology study. Without the need for enzymatic target amplification, the nCounter technology allows for high-throughput, sensitive, quantitative, and repeatable gene expression analysis. Our data indicate that the SARS-CoV-2 infection caused changes in multiple mRNAs in the brain.

We previously showed that SARS-CoV-2 intranasal infection of K18-hACE2 mice resulted in brain encephalitis characterized by secretion of cytokines and chemokines, leukocyte infiltration, hemorrhage, and neuronal cell death [119]. In the follow up study, we demonstrate that neuronal cultures obtained from K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. Furthermore, SARS-CoV-2 infection upregulated the expression of genes involved in antiviral immunity and inflammation in the brain [138].

Furthermore, we identified several significantly dysregulated and functionally relevant genes associated with neuroinflammation during SARS-CoV-2 infection that are of value for further studies.

5.2 Materials and Methods

5.2.1 SARS-CoV-2 Infection in Mice

As previously described, SARS-CoV-2 (USA-WA1/2020), was isolated from an oropharyngeal swab from a patient in Washington, USA (BEI NR-52281)[97]. Virus titration was performed using VeroE6 cells to further infection as previously described [80, 97]. Hemizygous K18-hACE2 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). To infect mice with SARS-CoV-2, we performed all the animal experiments in the Animal Biosafety Level 3
laboratory (ABSL3) at Georgia State University (GSU) following the protocol A20044 that was accepted by the GSU Institutional Animal Care and Use Committee [80].

### 5.2.2 RNA Extraction and Evaluation

For RNA extraction, 6-week-old K18-hACE2 mice were intranasally inoculated with $10^5$ PFU of SARS-CoV-2 (USA-WA1/2020) in ABSL3, whereas mock control group was inoculated with equivalent amounts of PBS [80]. Approximately similar numbers of male and female mice were used. During experiments, mice were checked for body weight, appetite, activity, and neurological signs every 2 days following SARS-CoV-2 infection. On days 1-, 3-, and 6- after inoculation, the mice were euthanized to limit suffering when met the human endpoint. The mock- or SARS-CoV-2-infected mice were anesthetized using isoflurane and perfused with PBS and RNA was collected from the brain.

RNeasy MiniKit (Qiagen, Hilden, Germany) was used to isolate total RNA from the brain tissues by using the manufacturer’s instructions [139]. To measure the purity and quantity of total RNA, Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) were used and the following criteria: wavelength absorbance ratio A260/280 ~2.0, and A260/230 ~2.0), the percentage of RNA fragments ≥300 nucleotides DV300 ≥ 50%, or integrity RIN > 4.

### 5.2.3 NanoString nCounter® Gene Expression

A commercially available NanoString nCounter® Mouse Neuroinflammation Panel to count 770 immune-related genes (NanoString, Cat: XT-CSO-MIP1-12) were used following the NanoString guidelines. A set of housekeeping genes were used for normalization for gene expression by using the nSolver Analysis Software (NanoString) as previously described [120].
5.2.4 Gene Ontology (GO) and pathway enrichment analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG)

GO (https://david.ncifcrf.gov/) and KEGG (www.genome.jp/kegg/pathway.html) pathway analysis was conducted to identify DEGs at the biologically functional level [140]. Among the GO classification, three categories of biological process, cellular component, molecular function, and the number of involved genes were represented.

5.2.5 Ingenuity Pathway Analysis (IPA)

NanoString data were analyzed using IPA (QIAGEN, Redwood City, USA) as described previously [120, 134, 141]. Briefly, graphical abstracts, comparative analysis, and individual analysis were analyzed using the identified list of DEGs by NanoString and IPA Knowledge Base as described previously. The Ingenuity Knowledge Base is the largest database of manually curated and experimentally validated physical, transcriptional, and enzymatic molecular interactions. Statistical comparisons between pathways and network data, p values (Fisher’s exact test), and activation z-score, were calculated by IPA. p < 0.05 is considered significant.

5.3 Results

5.3.1 Identification of differentially expressed upregulated and downregulated genes in mouse brain

Using cutting-edge nCounter technology, the distribution of mRNA in K18-hACE 2 mice brain infected with SARS-CoV-2 was examined. Six genes were found to be downregulated on day 1 out of over 800 genes analyzed. On day 3, 14 genes were downregulated, and 6 were upregulated. On day 6, 89 genes were downregulated, whereas 104 were upregulated. The highest number of genes were either up or downregulated on day 6, with three genes being downregulated
at all three-time points and five genes being downregulated on both days 3 and 6. 6 genes were identified to be elevated on both days 3 and 6.

![Diagram of differentially expressed genes on days 1, 3, and 6 after infection with SARS-CoV-2.](image)

**Figure 12**: Diagram of differentially expressed genes on days 1, 3, and 6 after infection with SARS-CoV-2. (A) The number of up (red) and down (blue) regulated genes in each group. (B) Venn diagrams depict the number of genes differentially expressed in the brain on different days after infection. (C) RT-PCR data for N gene showing viral load in the brain post-infection with SARS-CoV-2.

5.3.2 **Analysis of Cell death pathway associated genes expression**

Neurologic symptoms may result from enhanced systemic pro-inflammatory reactions without a direct infection of brain cells. Our data indicate that SARS-CoV-2 infection increased the expression of genes implicated in innate immunity, inflammation, and cell death pathways such as apoptosis, necroptosis, and NLR signaling. Several genes in NLR signaling pathways were upregulated at day 6 with 3 downregulated genes.
Figure 13: Heat map showing the two-fold change in cell death-associated genes.

Genes of cell death pathways are upregulated, including apoptosis and necroptosis pathways at day 6 brain post-SARS-CoV-2 infection.

The inflammasome's activation is a crucial step in the inflammatory immune response that leads to pyroptotic cell death. Members of the NLR family are inflammasome-initiating sensors [75, 142]. TLR signaling, which upregulates NLPR3 and pro-IL-1 gene expression, is required for Nlrp3 inflammasome activation in macrophages. Signals, which culminate in the activation of NF-kB, carefully govern this process [143]. Furthermore, type I IFN signaling through STAT1 reduce
the activity of the Nlrp3 inflammasome, which causes caspase-1 to process the IL1-beta precursor in response to a wide range of intracellular PAMPs [134].

5.3.3 **Analysis of genes associated with major Inflammatory pathways**

Most of the genes of NF-kB, TLR, and TNF signaling pathways were upregulated on day 6, whereas only a few were downregulated on days 1, 3, and 6. In JAK/STAT pathways, the genes were all upregulated in the day 6 brain. Cytokines and receptor interaction genes were highly upregulated in the mouse brain after SARS-CoV-2 infection at day 6, with only two genes in the pathways that were downregulated on days 1, 3, and 6. Major genes that play a role in COVID-19 infection were upregulated, with only three genes down-regulated at day 6 post-SARS-CoV-2 infection.

![Heat map showing the two-fold change in inflammatory and cytokines and chemokines gene expression.](image)
5.3.4 Pathway enrichment analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG)

The enrichment factor is calculated by dividing the number of DEGs by the total number of genes in each enrichment pathway. Our data indicate that the genes involved in cytokine/chemokine upregulation, inflammatory pathways, and leading programmed cell death interact in the brain of SARS-CoV-2 infected mice, leading to the severity of the brain infection.

Table 3: Top 20 up and down DEGs in D1, D3, and D6

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<td>6.29</td>
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</tr>
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<td>6.11</td>
<td>Ccl5</td>
<td>328.46</td>
</tr>
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<td>5.89</td>
<td>Rsad2</td>
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<td>5.83</td>
<td>Ccl9</td>
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<td>167.77</td>
<td>Gbp2</td>
<td>134.63</td>
</tr>
<tr>
<td>Gbp2</td>
<td>126.66</td>
<td>Ccl3</td>
<td>99.06</td>
</tr>
<tr>
<td>Zbp1</td>
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<td>Dlx1</td>
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<td>Dlx1</td>
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<td>Olig1</td>
<td>-2.07</td>
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<tr>
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<td>55.60</td>
<td>Bdnf</td>
<td>-2.05</td>
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<td>Grm2</td>
<td>-2.01</td>
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<td>Bcl2a1a</td>
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<td></td>
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<tr>
<td>Sell</td>
<td>39.69</td>
<td>Stmn1</td>
<td>-3.30</td>
</tr>
<tr>
<td>Casp4</td>
<td>35.66</td>
<td>Ago4</td>
<td>-3.25</td>
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<tr>
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<td>35.53</td>
<td>Gria4</td>
<td>-3.12</td>
</tr>
<tr>
<td>Sifn8</td>
<td>34.66</td>
<td>Reln</td>
<td>-3.09</td>
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The above table shows the top 20 upregulated and downregulated DEGs on days 1, 3, and 6 mice brains after infection with SARS-CoV-2. Genes associated with inflammatory response, cytokine and chemokine pathways, and cell death pathways were upregulated.
Table 4: KEGG pathway enrichment analysis of DEGs in the SARS-CoV-2 infected mouse brain.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Entry</th>
<th>KEGG pathway</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>mmu05210</td>
<td>Colorectal cancer</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>mmu01522</td>
<td>Endocrine resistance</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>mmu05224</td>
<td>Breast cancer</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>mmu05161</td>
<td>Hepatitis B</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>mmu04921</td>
<td>Oxytocin signaling pathway</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>mmu05200</td>
<td>Pathways in cancer</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>mmu05167</td>
<td>Kaposi sarcoma-associated herpesvirus infection</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>mmu05166</td>
<td>Human T-cell leukemia virus 1 infection</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>mmu05031</td>
<td>Amphetamine addiction</td>
<td>2</td>
</tr>
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<td></td>
<td>mmu04928</td>
<td>Parathyroid hormone synthesis, secretion and action</td>
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<td>IL-17 signaling pathway</td>
<td>4</td>
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<td>mmu05164</td>
<td>Influenza A</td>
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<tr>
<td></td>
<td>mmu05171</td>
<td>Coronavirus disease - COVID-19</td>
<td>3</td>
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<tr>
<td></td>
<td>mmu04623</td>
<td>Cytosolic DNA-sensing pathway</td>
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<td>RIG-I-like receptor signaling pathway</td>
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<td></td>
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<td>TNF signaling pathway</td>
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<td>MAPK signaling pathway</td>
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<td></td>
<td>mmu05417</td>
<td>Lipid and atherosclerosis</td>
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<tr>
<td></td>
<td>mmu05161</td>
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<td>3</td>
</tr>
<tr>
<td>Day 6</td>
<td>mmu04056</td>
<td>Cytokine-cytokine receptor interaction</td>
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<td>Toll-like receptor signaling pathway</td>
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<td>Influenza A</td>
<td>20</td>
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<td></td>
<td>mmu05417</td>
<td>Lipid and atherosclerosis</td>
<td>19</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>mmu04380</td>
<td>Osteoclast differentiation</td>
<td>18</td>
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</table>

5.4 Discussion

Previously, we have shown that genes and proteins of innate immunity and inflammatory pathways were upregulated in neurons and mice brain in response to SARS-CoV-2 infection. Furthermore, we discovered that SARS-CoV-2 infection of neurons and mouse brains upregulated genes such as ZBP1, MLKL, RIPK3, and caspase-8, which are involved in the necroptotic pathways implying that necroptosis may play a role in SARS-CoV-2 infection pathogenesis in the CNS [123].
Cytokine storm is involved in the pathogenesis of severe COVID-19 cases. The levels of many cytokines and chemokines have been found to be increased after SARS-CoV-2 infection[27, 31]. Our Nanostring data show increased expression of several genes, including the innate immune system and inflammatory genes such as TNF, IL-1 beta, CCL2, CCL3, CXCL10, and TLR. These genes were also upregulated in the neuron cells of human ACE-2 expressing transgenic mice after infection [123]. These inflammatory genes may cause neuronal death and exacerbation of neuroinflammation in the brain by activating downstream cell-death signaling pathways. TNF is known to be a potent inducer of neuronal injury in a variety of neurodegenerative illnesses, including cerebral ischemia, spinal cord injury, multiple sclerosis, and viral infections, such as HIV-associated dementia. CXCL10 and CCL2 are key chemokines involved in leukocyte infiltration into the CNS following viral infection [39, 119, 138].

At both days 3 and 6, we discovered that ZBP1, CCL2, and CXCL10 were three genes that were consistently elevated. ZBP1 is a crucial innate sensor that detects and binds Z-RNA structures produced by various viruses, including herpesvirus, orthomyxovirus, and flavivirus, and causes various forms of cell death [139]. ZBP1 is a key activator of necroptosis, a programmed cell death process in response to death-inducing TNF-alpha family members. ZBP1 interacts with and promotes the RIPK3 kinase, which phosphorylates and activates MLKL, causing programmed necrosis to occur. We found highly upregulated ZBP1 expression in the brain of infected mice on day 6, indicating ZBP1-dependent necroptosis in the mouse brain. Infection with beta coronaviruses has been shown to cause necroptosis in some cell types in previous research. Necroptosis is induced in human neural cells by the human coronavirus HCoV-OC43.
To summarize, the nanostring data corresponds to the mRNA and protein levels of genes implicated in necroptosis and inflammation reported in primary neurons isolated from K18-hACE-2 animals, as previously demonstrated.
6 PATHOGENESIS OF A SARS-COV-2 INFECTION IN THE AGED MICE

The aged population is at increased risk of severe illness from Coronavirus disease 2019 (COVID-19). Because age is a risk factor for COVID-19-related morbidity and death in humans, we evaluated the course of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection in younger adults and older BALB/c mice. In this study, BALB/c mice aged six and twenty months were injected intranasally with $10^5$ Plague Forming Units (PFU) of SARS-CoV-2 mouse adapted (MA10) virus. The survival rate, weight loss, viral load, cytokine and chemokine levels, and lung pathology were used to correlate disease severity and pathogenic index. Our data show that the MA10 virus-infected and replicated in both younger and older BALB/c mice. On day 3 post-infection, the mice lost a significant amount of body weight and reached the human endpoint. On day 3, plaque assay and qRT-PCR revealed a considerably high virus titer in the lungs and elevated inflammatory cytokines and chemokines. Gross pathology with multifocal lung lesions was seen in 20-months-old, infected mice, and an H&E section of the lungs revealed extensive leucocyte infiltrations. Interestingly, IFN-α expression was found to be upregulated in younger adults compared to older mice at day 3 post-infection. Our data show that older BALB/c mice facilitated rapid MA10 viral replication in an age-dependent manner, as evidenced by rapid body weight loss, gross and microscopic pulmonary pathology, and mRNA levels of inflammatory cytokines and chemokines. Overall, our results suggest that elderly hosts have a higher risk of infection.

6.1 Introduction

Due to immunological changes that occur as people age, they are more susceptible to numerous illnesses. Immunosenescence refers to a set of alterations that include decreased innate and adaptive immune responses and increased production of inflammatory cytokines. The United
States and Brazil are among the countries with the most cases and deaths due to the Covid-19 pandemic. COVID-19 is responsible for about 51% of SARS cases in Brazil, with 73 percent of deaths occurring in those over 60 years of age [144]. It has been discovered that older adults have a more severe form of the disease and constitute the leading COVID-19 risk group [145]. SARS-CoV-2 infection in experimental models confirmed this observation, with infected old Syrian hamsters developing alveolar and perivascular edema [146]. SARS-CoV-1 and MERS have both been linked to a higher severity in older people [147].

Moreover, Obesity, hypertension, and metabolic disorders are also risk factors for COVID-19 [148]. SARS-CoV-2 virus causes worse outcomes and higher mortality in older adults and those with comorbidities like hypertension, cardiovascular disease, diabetes, chronic respiratory disease, and chronic kidney disease (CKD). These conditions affect many older Americans, putting them at risk of severe infection. Many people with hypertension, diabetes, and CKD are also given Angiotensin-Converting Enzyme (ACE) inhibitors and angiotensin II receptor blockers. The ACE2 receptor, which the virus uses to enter host cells, is upregulated by these drugs (5).

Animal models are essential for the development and validation of prophylactics and treatments, as well as the identification of the mechanisms underlying COVID-19 pathogenesis. Because the elderly is more prone to severe SARS-CoV-2 infection, older mice may also be more sensitive than younger mice. In this study, we evaluated the pathogenicity of the SARS-CoV-2 in younger adults (six months) and aged (Twenty months) old BALB/c mice. Our data show that intranasal inoculation of SARS-CoV-2 in the older BALB/c mice resulted in severe disease, as evident by rapid loss of body weight, gross and microscopic pathology of lungs, as well as elevated mRNA levels of inflammatory cytokines and chemokines.
It is thought that cytokine storm in the lungs is one of the immunological components implicated in COVID-19 pathophysiology in the elderly. Although it has been suggested that alveolar macrophages from older people have an anti-inflammatory profile, they can develop higher and uncontrolled responses of cellular activation and cytokine production in response to a pathogen insult, as well as a lower ability to control tissue damage due to infection, putting the lungs in a debilitated state [149-151]. Indeed, levels of complement and surfactant proteins, as well as pro-inflammatory cytokines, are higher in the lungs of older people at baseline [150, 151]. Surprisingly, a cytokine storm occurs in half of all fatal COVID-19 cases, with 82 percent of those over 60 years of age [152]. This situation of immune failure and its link with disease development in the elderly has been extensively researched, particularly in potentially lethal infections, such as influenza and, more recently, COVID-19. Understanding the infection dynamics in this scenario can benefit a better comprehension of these mechanisms.

6.2 Materials and Methods

6.2.1 Animal Infection Experiments

BALB/c mice were obtained from the Jackson laboratory, USA. All the animal experiments were conducted in a certified Animal Biosafety Level 3 (ABSL-3) laboratory at Georgia State University (GSU). The protocol was approved by the GSU Institutional Animal Care and Use Committee (Protocol number A20044). Six- and 20- months old BALB/c mice were inoculated intranasally with PBS (mock) or $10^5$ plaque-forming units (PFU) of MA10 as described previously[119]. Animals were weighed, and their appetite, activity, breathing, and neurological signs were accessed twice daily. In an independent experiment, mice were inoculated with PBS (mock) or SARS-CoV-2 variants intranasally, and on day 3 after infection, animals were anesthetized using isoflurane and perfused with cold PBS. The lungs and other tissues were
collected and flash-frozen in 2-methyl butane (Sigma, St. Louis, MO, USA) for further analysis as described below [125, 153, 154].

6.2.2 **Quantification of the virus load**

Tissues harvested from mock and virus-inoculated animals were weighed and homogenized in a bullet blender (Next Advance, Averill Park, NY, USA) using stainless steel beads, followed by centrifugation and titration. Virus titers in tissue homogenates were measured by plaque assay using Vero E6 cells. To titer the infectious virus, tissue homogenates were 10-fold serially diluted with DMEM and applied to monolayered Vero E6 cells for 1 hour. After inoculation, cells were washed once before overlaid with 1% low-melting agarose. Cells were further incubated for 72 hours and stained with neutral red to visualize plaque formation.

6.2.3 **Quantitative real time-polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from tissues using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA). One microgram (ug) of RNA was reverse transcribed to make cDNA using iScript TM cDNA Synthesis Kit (Bio-Rad, Des Plaines, IL, USA) [39, 80]. qRT-PCR was conducted on tissue lysates from mock and MA10 virus-infected samples to determine virus N gene expression and expression of various pro-inflammatory cytokines and chemokine. The mRNA levels of multiple host genes were determined using qRT-PCR, and the fold change in infected samples compared to controls was calculated after normalizing to the GAPDH gene. Primer sequences are listed in Table 1.
Table 5: Primer sequences used for Quantitative real time-polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward</th>
<th>Reverse</th>
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<td>IL-6 (NM_000600)</td>
<td>CCAGGAGCCCAGCTATGAAC</td>
<td>CCCAGGGAGAAGGCAACTG</td>
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<tr>
<td>TNF-α (NM_013693)</td>
<td>CCAGTCTGTATCCTTCTAA</td>
<td>TCTTGTGTTTCTGAGTAGT</td>
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<tr>
<td>CCL2 (NM_011333)</td>
<td>TCACCTGCTGCTACTCATTCAACA</td>
<td>TACAGCTTCTTTGGGACACCTGCT</td>
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<tr>
<td>IFN-α (NM_010502)</td>
<td>CTCTGTGCTTTTCTCTGATG</td>
<td>CTGAGGTTATGAGTCTGAG</td>
</tr>
</tbody>
</table>

6.2.4 Histopathological Analysis

Lung sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation[104, 126]. Additionally, tissue sections were incubated with anti-SARS-CoV-2 nucleocapsid antibody (Thermo Fisher Scientific, Norcross, GA, USA) overnight at 4 °C, followed
by incubation with Alexa Fluor 555-conjugated secondary antibody (Invitrogen) for 1 hour at room temperature. Images were acquired using the Invitrogen™ EVOS™ M500 Cell Imaging System (Thermo Fisher Scientific, Norcross, GA, USA).

### 6.2.5 Statistical Analysis

Mann–Whitney tests and unpaired student t-tests using GraphPad Prism 5.0 were used to calculate the \( p \) values of the difference between viral titers and immune responses, respectively. Differences of \( p < 0.05 \) were considered significant.

### 6.3 Results

#### 6.3.1 MA10 virus replication in younger adult and aged BALB/c mice

The six months old younger adult and 20 months old aged BALB/c mice were infected intranasally with PBS (mock, \( n=10 \) mice) or \( 10^5 \) PFU of MA10 in PBS (\( n=10 \) mice) to evaluate the pathogenicity of the virus. Mice were monitored for clinical signs and changes in body weight. The mock-infected mice remained healthy throughout the observation period. Infected mice experienced significant weight loss by day 3 pi. We observed approximately 18-20% loss in body weight of infected mice of both age groups but not mock (Figure 1A). All the infected animals were euthanized at day 3 because they reached the humane endpoint. Lungs of 20- months old mice had gross lesions with focal or multifocal discoloration in the lung lobes compared to younger adults or mock-infected mice (Figure 1B). In 20-month-old mice, gross pathological examinations indicated macroscopically evident discoloration of lung tissues on day 3 pi.
6.3.2 Viral load in the lungs of BALB/c mice after SARS-CoV-2 infection

Six months and 20 months old BALB/c mice were infected intranasally with PBS (mock, n= 10) or log^5 PFU of MA10 in PBS (n=10), and groups of 5 mice were used to measure the viral loads in the lungs at day 3 pi. We used plaque assay and qRT-PCR to quantify viral titers in the lungs of infected mice. Our data show that the MA10 was able to infect and replicate in both the age group, younger adult and older mice. High virus titer levels were observed in the lungs at day
3. The virus titer was approximately log 7 in 20 months old whereas the six months old virus titer was one log value less and it was log 6. Six months old younger adults and 20 months old-aged mice had significantly higher viral titers; however, the virus titer in the older mice was comparably higher.

**Figure 16: Replication of MA10 virus in the lungs of the infected mice.** (A) 6- and 20-months old mice were inoculated intranasally with log⁶ PFU of MA10. A Group of 4-5 mice was euthanized on Day 3 after infection, and lung tissues were collected. Virus titers were analyzed in the lungs by (A) plaque assay and (B) qRT-PCR.

6.3.3 Cytokines and chemokines expression levels in the MA10 virus-infected mice lungs

Interferons (IFNs) are the body's first line of antiviral defense, inhibiting virus replication at several levels by triggering the expression of hundreds of IFN-stimulated genes, several of which have antiviral properties. IFN signaling interacts with pathways that control apoptosis, inflammation, and cellular stress response programs to establish the cell's overall antiviral state [80, 155]. The level of IFN-α mRNA expression in day 3 lungs was investigated. When compared to the mock group, IFN-α expression was ~ 25 times higher in 6-month-old younger adults. In
comparison to the mock, there was only a negligible increase in IFN-α expression in 20-month-old mice.

Next the mRNA levels of inflammatory cytokines and cytokines in the day 3 lungs were examined. MA10 infection caused ~50-fold increase in IL-6 gene expression in the lungs of 6-months old mice, but an ~80 fold increase in the lungs of 20-month-old mice. TNF-α levels were somewhat elevated ~5 fold in the lungs of younger adults and ~7 fold in the lungs of older mice when compared to mock. When compared to mock, CCL-2 mRNA expression was enhanced approximately 50-fold in younger mice and around 120-fold in older mice. It's worth noting that relative IFN-α levels were higher in younger adults aged 6 months compared to mice aged 20 months. On contrary, the expression of inflammatory genes, IL-6, TNF-α, and CCL-2 was increased in 20-month-old mice than in 6-month-old mice. When compared to 20-month-old mice, our findings suggest that younger mice produced a better IFN-α response. This finding is consistent with prior research that found IFN signaling was critical in SARS-CoV-2 MA viral replication attenuation [156].
6.3.4 MA10 infection-induced pathological features in the infected mice lungs

We analyzed gross and histopathological changes in the infected mice lungs. We analyzed the lung sections from 20 months old, infected mice for infiltration of immune cells and antigen distribution. The H&E staining of lung sections from 20 months old, infected mice demonstrated abundant leukocyte infiltration in alveolar spaces, interstitial thickening, and extravasated blood in day 3 pi.
Figure 18: Histopathological analysis of MA10 infected lungs in older mice.

Hematoxylin and eosin (H&E) staining of lung sections from mock- and MA10 infected mice on day 3 after infection. Lesions with significant leukocyte infiltration, hemorrhages, and interstitial thickening were seen in lung sections of infected mice. The photomicrographs shown are representative of the images obtained from five animals.

Lung sections from mock and infected mice were also stained for SARS-CoV-2 nucleocapsid protein. The immunofluorescence images show the presence of SARS-CoV-2 N protein (red) in the infected mice. Nuclei are stained with DAPI (blue). This shows the virus replication in the lungs at D3.

CD45 is a major transmembrane glycoprotein expressed in all leucocytes. It has been established that the expression of CD45 is essential for activating T cells via the T cell receptor (TCR). Evidence of leucocyte infiltration was confirmed by immunofluorescence staining analysis of the CD45 antigen, which revealed many CD45 positive cells in these lungs.
Figure 19: Detection of infected cells in the lungs of MA10-infected mice. Lung sections from mock- and MA10-infected mice (day 3 after infection) were stained for (A) SARS-CoV-2 nucleocapsid protein and (B) leucocyte marker CD45. Representative immunostaining images show SARS-CoV-2 nucleocapsid protein (red) in the MA10-infected mice. Nuclei are stained with DAPI (blue). The photomicrographs shown are representative of the images obtained from five animals.

6.4 Discussion

Aging affects many characteristics of innate and adaptive immune cells, impairing or compromising their function and response. Several variables can also disrupt intracellular homeostasis as people age, increasing the production of inflammatory cytokines and chemokines [144]. To investigate the pathogenesis of SARS-CoV-2 and explore possible antiviral treatments
and vaccines, appropriate animal models are required. An ideal animal model would be able to emulate viral replication and clinical consequences in COVID-19 patients [157]. Although several SARS-CoV-2 mouse models have been developed, their utility for studying alveolar disease etiology may be restricted. The COVID-19 syndrome is captured by the SARS-CoV-2 MA10 model, which includes a spectrum of morbidity and mortality determined by host genetics and increasing age, as well as severe clinical characteristics of Acute Lung Injury/Acute Respiratory Distress Syndrome (ALI/ARDS) and related lung function deficits. The lung pathology for SARS-CoV-2 MA10 was established to quantify ALI and ARDS pathological aspects in SARS-CoV and MERS-CoV [157] mouse models. Our data show MA10 replicated rapidly and caused interstitial pneumonia like symptoms with robust infiltration of leucocytes in aged BALB/c mice. Clinical features of COVID-19 in people were identical to those reported in these animals. Testing of vaccines and treatments against SARS-CoV-2 has been delayed due to the limited availability of transgenic mouse models that can be infected with the SARS-CoV-2 virus [157]. Because older people are more susceptible to SARS-CoV-2, using aged BALB/c or C57BL/6J mice provides a simple and reliable way to assess COVID-19 vaccination efficacy.

Furthermore, telomeric disturbances and oxidative stress are prevalent in senescent cells, activating signaling pathways such as nuclear factor B (NF-kB) and enhancing the production of cytokines and chemokines [158]. In addition, pathogenic processes can exacerbate the inflammatory response by releasing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) as people age [159]. Inflammatory mediators such as prostaglandin E2, IL-6, and TNF- are released during cytomegalovirus (CMV) infection, affecting 40–100% of the world's population, demonstrating the pathogen's crucial role in
inflammation. Additionally, a strong interaction of viral S protein of SRAS-CoV-2 and TLR4 suggests that SARS-CoV-2 can directly activate proinflammatory pathways [160]. The accumulation of NLRP3 puncta was found in monocytes infected in vitro with SARS-CoV-2, and the same was seen in mononuclear cells recovered from COVID-19 patients, showing activation of the inflammasome pathway.

Our data demonstrate a significantly high viral load in day 3 lungs of six-month and 20-month-old mice. Both age groups showed increased virus titer, a little bit higher at 20 months; however, not much difference between the two groups. Aged mice lungs showed abundant leucocyte infiltration and hemorrhage at day 3 pi. The inflammatory genes IL-6, TNF-a, and CCL-2 expression were comparatively higher in 20 months old than six months old. Interestingly the mRNA expression of IFN-a was contrary as it was upregulated in six months old than 20 months old mice. It has been reported that SARS-CoV-1 N protein interacts with TRIM25 to prevent IFN-1 generation via RIG-I [161]. A balanced production of IFN-I has also been documented in COVID-19 patients, and it appears to be related to disease severity [162, 163]. Furthermore, SARS-CoV-2 infection causes a decrease in IFN-I and interferon-stimulated gene expression [130]. Due to poor expression of TLR7 and TLR9 in old people, the population of Plasmacytoid Dendritic Cells (pDCs), which is one of the key strategies for fighting viral infections, is diminished and has less potential for IFN-secretion when stimulated with influenza virus. Unpublished data from our lab showed upregulated genes associated with TLR, RIG-I and cell death pathways. In addition, due to defective IFN transcription, elderly human monocytes produce an imbalanced amount of IFN-I and IFN-III in response to influenza infection. These findings imply that a diminished IFN-I response in advanced age may play a role in COVID-19 clinical outcomes due to its diminished aid in virus clearance.
The natural killer (NK) cell response is weakened in the elderly. In the absence of stimulation, NK cells produced by older persons produce less IFN-a, which helps to explain why they are more susceptible to viral infections at this age [164]. An animal model of influenza infection reported a decrease in NK cells in the lungs, with decreased potential for IFN-a production and degranulation [165]. Similarly, following SARS-CoV-2 infection, clinical observations of individuals with COVID-19 demonstrated a dramatic decline in this cell type. Furthermore, NK cells have been demonstrated to move to the lungs in a BALB/c senescent mouse model of SARS-CoV-1 infection [166], indicating that these cells may play a role in coronavirus infection pathogenesis.

These data suggest that innate immune cell malfunction connected to immunosenescence may play a role in COVID-19 pathogenesis in the elderly, either by promoting a less effective infection-fighting response and/or favoring an enhanced inflammatory response.
7 OTHER SIGNIFICANT CONTRIBUTIONS

7.1 The FDA-approved gold drug auranofin inhibits novel coronavirus (SARS-COV-2) replication and attenuates inflammation in human cells

Abstract: SARS-COV-2 has recently emerged as a new public health threat. Herein, we report that the FDA-approved drug, auranofin, inhibits SARS-COV-2 replication in human cells at low micro molar concentration. Treatment of cells with auranofin resulted in a 95% reduction in the viral RNA at 48 h after infection. Auranofin treatment dramatically reduced the expression of SARS-COV-2-induced cytokines in human cells. These data indicate that auranofin could be a useful drug to limit SARS-CoV-2 infection and associated lung injury due to its antiviral, anti-inflammatory and anti-reactive oxygen species (ROS) properties. Further animal studies are warranted to evaluate the safety and efficacy of auranofin for the management of SARS-COV-2 associated disease.

7.2 SARS-CoV-2 Variants of Concern Infect the Respiratory Tract and Induce Inflammatory Response in Wild-Type Laboratory Mice

Abstract: The emergence of new severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) variants of concern pose a major threat to public health, due to possible enhanced virulence, transmissibility and immune escape. These variants may also adapt to new hosts, in part through mutations in the spike protein. In this study, we evaluated the infectivity and pathogenicity of SARS-CoV-2 variants of concern in wild-type C57BL/6 mice. Six-week-old mice were inoculated intranasally with a representative virus from the original B.1 lineage, or the emerging B.1.1.7 and B.1.351 lineages. We also infected a group of mice with a mouse-adapted SARS-CoV-2 (MA10). Viral load and mRNA levels of multiple cytokines and chemokines were analyzed in the lung tissues on day 3 after infection. Our data show that unlike the B.1 virus, the B.1.1.7 and
B.1.351 viruses are capable of infecting C57BL/6 mice and replicating at high concentrations in the lungs. The B.1.351 virus replicated to higher titers in the lungs compared with the B.1.1.7 and MA10 viruses. The levels of cytokines (IL-6, TNF-α, IL-1β) and chemokine (CCL2) were upregulated in response to the B.1.1.7 and B.1.351 infection in the lungs. In addition, robust expression of viral nucleocapsid protein and histopathological changes were detected in the lungs of B.1.351-infected mice. Overall, these data indicate a greater potential for infectivity and adaptation to new hosts by emerging SARS-CoV-2 variants.

7.3 Differential Pathogenesis of SARS-CoV-2 Variants of Concern in Human ACE2-Expressing Mice

Abstract: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the current pandemic, resulting in millions of deaths worldwide. Increasingly contagious variants of concern (VoC) have fueled recurring global infection waves. A major question is the relative severity of the disease caused by previous and currently circulating variants of SARS-CoV-2. In this study, we evaluated the pathogenesis of SARS-CoV-2 variants in human ACE-2-expressing (K18-hACE2) mice. Eight-week-old K18-hACE2 mice were inoculated intranasally with a representative virus from the original B.1 lineage or from the emerging B.1.1.7 (alpha), B.1.351 (beta), B.1.617.2 (delta), or B.1.1.529 (omicron) lineages. We also infected a group of mice with the mouse-adapted SARS-CoV-2 (MA10). Our results demonstrate that B.1.1.7, B.1.351 and B.1.617.2 viruses are significantly more lethal than the B.1 strain in K18-hACE2 mice. Infection with the B.1.1.7, B.1.351, and B.1.617.2 variants resulted in significantly higher virus titers in the lungs and brain of mice compared with the B.1 virus. Interestingly, mice infected with the B.1.1.529 variant exhibited less severe clinical signs and a high survival rate. We found that B.1.1.529 replication was significantly lower in the lungs and brain of infected mice in comparison
with other VoC. The transcription levels of cytokines and chemokines in the lungs of B.1- and B.1.1.529-infected mice were significantly less when compared with those challenged with other VoC. Together, our data provide insights into the pathogenesis of previous and circulating SARS-CoV-2 VoC in mice.

7.4 **Influenza virus-like particle-based hybrid vaccine containing RBD induces immunity against influenza and SARS-CoV-2 viruses**

Abstract: Several approaches have produced an effective vaccine against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). However, the influence of immune responses induced by other vaccinations on the durability and efficacy of the immune response to SARS-CoV-2 vaccine is still unknown. We have developed a hybrid vaccine for SARS-CoV-2 and influenza viruses using influenza virus-like particles (VLP) incorporated by protein transfer with glycosylphosphatidylinositol (GPI)-anchored SARS-CoV-2 S1 RBD fused to GM-CSF as an adjuvant. GPI-RBD-GM-CSF fusion protein was expressed in CHO-S cells, purified and incorporated onto influenza VLPs to develop the hybrid vaccine. Our results show that the hybrid vaccine induced a strong antibody response and protected mice from both influenza virus and mouse-adapted SARS-CoV-2 challenges, with vaccinated mice having significantly lower lung viral titers compared to naive mice. These results suggest that the hybrid vaccine strategy is a promising approach for developing multivalent vaccines to prevent influenza A and SARS-CoV-2 infections.
8 SUMMARY

8.1.1 SARS-CoV-2 infection of mouse brain

Our study shows that direct infection of CNS cells and the inflammatory response are important in triggering SARS-CoV-2-induced lethality in K18-hACE2 mice. The induction of cytokines/chemokines, infiltration of leukocytes into the perivascular space and parenchyma, and cell death are characteristics of a virus infection of the CNS. In our analysis, SARS-CoV-2 viral antigen was identified throughout the brain, including the cortex, cerebellum, and hippocampus. Peak viral levels in the brain, immune cell infiltration, and CNS cell death were correlated with the onset of severe disease in SARS-CoV-2-infected animals. The highest virus titers in the brains were 1000 times greater than in the lungs, showing that SARS-CoV-2 has a large replicative ability in the brain. The brain showed a 10- to 50-fold higher levels of cytokine and chemokine mRNAs than the lungs, indicating that neuroinflammation had a role in clinical disease in these mice. We found significant infectious virus titers in the olfactory system and brains of all the mouse mice. On days 5 and 6, the inflammatory reaction in the brain was more prominent than in the lungs. In humans, COVID-19 illness is characterized by changes in smell and taste [8,41]. SARS-CoV-2 proteins were found in endothelial cells inside the olfactory bulb by pathological analysis of human COVID-19 autopsy tissues [41,42]. The presence of virus replication in these tissues shows that SARS-CoV-2 can infect the olfactory bulb and subsequently propagate throughout the brain by infecting connecting brain neuron axons. This hypothesis is supported by earlier research showing that neurotropic coronaviruses infect olfactory neurons and then go to the brain via axonal transport [8,43].

Many viruses have been found to enter the CNS through olfactory sensory neurons, including HSV-1, Nipah virus, rabies virus, Hendra virus, and influenza A virus [44,45].
Disruption of the blood–brain barrier is another way for a virus to get entry to the brain (BBB). However, no virus was found in the serum of infected mice at any point after infection, implying that BBB disruption plays only a minor role in SARS-CoV-2 neuroinvasion. After infection with SARS-CoV-1 or SARS-CoV-2, little or no virus was identified in the blood of K18-hACE2 mice [22,23]. In conclusion, we discovered that intranasal SARS-CoV-2 infection induces severe neurological illness in K18-hACE2 mice. Under the conditions utilized, our findings show that the CNS is the primary target of SARS-CoV-2 infection in K18-hACE2 mice, and that brain infection results in immune cell infiltration, inflammation, and cell death.

**8.1.2 SARS-CoV-2 infection of mouse neurons**

We showed that SARS-CoV-2 can infect neuronal cells from hACE2-K18 mice and cause infection. Inflammatory and immunological genes were increased in neurons and mouse brain in response to infection. Furthermore, we discovered that SARS-CoV-2 infection increased genes involved in the necroptotic pathway (ZBP1, MLKL, RIPK3, and caspase-8) in neurons and mouse brain, implying that necroptosis may have an important role in the pathophysiology of SARS-CoV-2 infection in the CNS. Some COVID-19 patients have had SARS-CoV-2 infection detected in their brains [12,13]. In human neural progenitor cells and brain organoids, SARS-CoV-2 has also been shown to proliferate and cause cell death [19,20]. In addition, dsRNA was found in SARS-CoV-2-infected neuronal bodies and axons. In non-hACE2-expressing mouse neurons, however, virus replication was restricted. One of the pathophysiological markers of the severe consequences associated with SARS-CoV-2 infection is a cytokine storm [30,31]. Increased TNF- and IL-6 levels have been linked to poor illness outcomes in several studies [32,33]. We show that SARS-CoV-2 infection causes elevation of innate immune and inflammatory genes such as TNF- and IL-6 in K18-hACE2 mouse brains [28]. Similarly, SARS-CoV-2-infected neuron cells showed
a considerable rise in the production of IL-6, TNF-, CXCL10, and CCL2. These inflammatory
genes may excite glial cells, increasing neuroinflammation, and/or trigger subsequent cell-death
signaling pathways in neurons, resulting in neuronal death [34,35]. TNF- is known to be a
significant inducer of neuronal injury in a variety of neurodegenerative illnesses, including
cerebral ischemia, spinal cord injury, multiple sclerosis, and viral infections, such as HIV-
associated dementia [33,36]. Both CXCL10 and CCL2 are significant chemokines that have a role
in leukocyte infiltration into the CNS following viral infection [35]. During virus infections, ZBP1
is one of the cytoplasmic sensors that regulates cell death and inflammation [37,38,39,40], and it
begins RHIM-dependent activation of RIPK3-dependent necroptosis. Necroptosis is an
inflammatory cell death triggered by phosphorylation of RIPK3, which activates the pseudo-kinase
MLKL, which oligomerizes and ruptures the plasma membrane, causing cell death [41,42].
Necroptosis inhibits virus multiplication by eradicating virus-infected cells and activating innate
and adaptive immunity. Inflammatory cytokines and damage-associated molecular patterns are
released as a result of this process, resulting in widespread inflammation [38,39,43]. After SARS-
CoV-2 infection, we discovered considerable overexpression of the necroptotic genes ZBP1,
MLKL, and RIPK3 in neuronal cells and animal brains. To explain the role of ZBP1 in SARS-
CoV-2 pathogenesis, more research is needed. SARS-CoV-2 replicates robustly in neuronal
preparations produced from K18-hACE2 mice, according to our findings. The virus infection of
neuronal cultures causes up-regulation of genes implicated in the innate immune response,
inflammation, and cell death, much as it does in SARS-CoV-2-infected K18-hACE2 mouse brains.

8.1.3 **SARS-CoV-2 infection of wild-type mice**

Our study also shows that SARS-CoV-2 variants can infect wild-type laboratory mice. The
B.1 virus did not infect C57BL/6 mice, but the B.1.351 and B.1.1.7 viruses did. Increased cytokine
and chemokine levels, as well as leukocyte infiltration in the lungs, were seen in B.1.351- and B.1.1.7-infected animals. Wild-type mice are not vulnerable to SARS-CoV-2 infection because the first SARS-CoV-2 strains did not use murine ACE-2 as a receptor [8,9]. The mouse-adapted strain of SARS-CoV-2 (MA10) produces infection, inflammation, and pneumonia in BALB/c mice [11]. When compared to the Wuhan reference sequence, MA10 includes many alterations, including the N501Y mutation in the RBD of the spike protein, which is likewise found in the B.1.351 and B.1.1.7 variations [6,7]. These alterations in the spike protein's RBD may have increased the binding affinity for the natural mouse ACE-2 receptor, allowing the variations to reproduce more effectively in mice. Despite the fact that both B.1.351 and B.1.1.7 viruses were capable of replicating in the lungs of mice, inoculation with the B.1.351 virus resulted in a much larger viral load in the lungs than inoculation with the B.1.1.7 virus. Aside from the N501Y mutation, many amino acid alterations have been hypothesized to be important for SARS-CoV-2 adaptation in mouse species, including K417N, E484K, Q493H/K, and Q498H. The occurrence of E484K and K417N mutations in B.1.351 has been demonstrated to boost infectivity, and E484K has also been identified as an immunological escape mutation that develops during antibody exposure [28]. This corresponds to our finding that the B.1.351 virus multiplied at a greater rate than the B.1.1.7 virus. It's probable that mice with co-morbid illnesses including old age, diabetes, and hypertension will have more severe symptoms. The capacity of SARS-CoV-2 variations to reproduce and cause inflammation in wild-type mice will aid investigations employing transgenic mouse strains to test therapeutic treatments and pathogenesis. These findings suggest that emergent SARS-CoV-2 variants may be able to adapt to new animal species.
8.1.4 Differential pathogenesis of SARS-CoV-2 infection in mice

SARS-CoV-2 evolves rapidly as the viral genome accumulates mutations, resulting in several variants of concern (VoC) [4,5,10]. Most K18-hACE2 mouse research have used the original SARS-CoV-2 virus strains, and just a few investigations have used emergent VoC. Our findings show that the pathogenicity of SARS-CoV-2 in K18-hACE2 mice is VoC-dependent, with the alpha, beta, and delta variants having the highest pathogenicity. In comparison to the B.1 lineage, we identified considerably greater virus titers in the lungs and brains of mice infected with the B.1.1.7, B.1.351, and B.1.617.2 variations. In mice, however, the omicron form replicated much less efficiently than the other SARS-CoV-2 variants. The modifications in the spike protein's RBD may have increased the ACE2 receptor's binding affinity, allowing the variations to reproduce more rapidly in mice. Indeed, our findings show that SARS-CoV-2 variants B.1.1.7 and B.1.351, which contain the N501Y and E484K mutations, cause significant pathogenicity in K18-hACE2 mice. In comparison to other SARS-CoV-2 variations, mice infected with the omicron variant showed minimal illness despite having the highest number of mutations that could allow for more efficient binding to ACE2. Epidemiological evidence also suggests that the omicron virus infects humans less severely than the original strains and other VoC [29]. Overall, our findings show that SARS-CoV-2 pathogenicity in K18-hACE2 mice is VoC-dependent, with the alpha, beta, and delta variants having the highest pathogenicity and the omicron variant having the lowest.

8.1.5 Efficacy of Influenza and SARS-CoV-2 hybrid vaccine in mice

The combination of COVID-19 and seasonal influenza will produce high morbidity and mortality, placing a pressure on the health system. Due to the logistical challenges of immunizing with two vaccines each year, the notion of a combination vaccine is very appealing [167]. We've shown that a hybrid vaccination based on influenza VLPs can provide effective protection against
SARS-CoV-2 and influenza viruses. The antigen carrying VLPs are connected to cytokine adjuvants in our vaccine platform (7). Antigens and biological adjuvants are delivered to the immune system in a particulate form in this method. The hybrid vaccination elicited similar levels of antibody response when administered subcutaneously or intramuscularly. Even 6 months after vaccination, neutralizing antibody titers against inactivated influenza A/PR8 (H1N1) remain robust, showing the longevity of the anti-influenza immune response induced by hybrid vaccine. A comparison analysis of GPI-RBD-GM-CSF and GPI-RBD was not performed in this study to demonstrate the contribution of GM-CSF as an adjuvant in VLP vaccination. Furthermore, our research only looked at the RBD domain of the SARS-CoV-2 S protein, rather than the entire S protein, which could limit the scope of the protective immune response. Finally, our findings suggest that influenza VLP-based delivery of SARS-CoV-2 RBD protein combined with cytokine adjuvants can be used to develop multivalent vaccines that target the various virus strains currently found in the ongoing SARS-CoV-2 pandemic.

8.1.6 SARS-CoV-2 infection in aged mice

To investigate acute lung injury in the elderly, we used old BALB/c mice to imitate the disease caused by SARS-CoV-2. Our findings suggest that acute lung injury and inflammatory immune responses in MA10 infected old BALB/c mice lungs were similar to the clinical symptoms of human illnesses. It has been suggested that a cytokine storm is involved in the pathogenesis of severe COVID-19 cases. The levels of many cytokines and chemokines have been found to be increased after SARS-CoV-2 infection [129, 130], and increased IL-6 level have been correlated with severe disease outcomes [131, 132].

In aged human population a low-grade pro-inflammatory state, with a rise in serum inflammatory mediators such as IL-6, IL-1RA, TNF-, IL-1, and C-reactive protein, is a prominent
hallmark of the immunosenescence process [168, 169]. During the aging process, a low-grade inflammatory condition is known as "inflammaging" is linked to a reduced ability to mount effective immune responses [169].

The SARS-CoV-1 N protein has been found to interact with TRIM25, preventing IFN-I production via RIG-I [161]. In COVID-19 patients, imbalanced IFN-I production has been observed, and it appears to be linked to disease severity [162, 163]. Furthermore, in response to influenza infection, older human monocytes produce an imbalanced amount of IFN-I and IFN-III due to faulty IFN transcription. These findings suggest that in advanced age, a reduced IFN-I response may have a role in COVID-19 clinical outcomes due to its reduced ability to help virus clearance.
9 REFERENCES


10 APPENDICES


Neuroinvasion and Encephalitis Following Intranasal Inoculation of SARS-CoV-2 in K18-hACE2 Mice

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Abstract: Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection can cause neurological disease in humans, but little is known about the pathogenesis of SARS-CoV-2 infection in the central nervous system (CNS). Herein, using K18-hACE2 mice, we demonstrate that SARS-CoV-2 neuroinvasion and encephalitis is associated with mortality in mice. Intranasal infection of K18-hACE2 mice with 10^6 plaque-forming units of SARS-CoV-2 resulted in 100% mortality by day 6 after infection. The highest virus titers in the lungs were observed on day 3 and declined on days 5 and 6 after infection. By contrast, very high levels of infectious virus were uniformly detected in the brains of all animals on days 5 and 6. Onset of severe disease in infected mice correlated with peak viral levels in the brain. SARS-CoV-2-infected mice exhibited encephalitis hallmarks characterized by production of cytokines and chemokines, leukocyte infiltration, hemorrhage and neuronal cell death. SARS-CoV-2 was also found to productively infect cells within the nasal turbinate, eye and olfactory bulbs, suggesting SARS-CoV-2 entry into the brain by this route after intranasal infection. Our data indicate that direct infection of CNS cells together with the induced inflammatory response in the brain resulted in the severe disease observed in SARS-CoV-2-infected K18-hACE2 mice.

Keywords: SARS-CoV-2; COVID-19; K18-hACE2 mice; neuroinvasion; neuroinflammation; encephalitis

1. Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection in humans can cause pneumonia, acute respiratory distress syndrome, acute lung injury, cytokine storm syndrome and death [1,2]. Although SARS-CoV-2 infection primarily causes respiratory disease, some patients develop symptoms of neurological disease, such as headache, loss of taste and smell, ataxia, meningo, cognitive dysfunction, memory loss, seizures and impaired consciousness [3-10]. SARS-CoV-2 infection also induces long-term neurological sequelae in at least one-third of human cases. Infection with other coronaviruses, such as mouse hepatitis virus (MHV) in mice and SARS-CoV-1 and Middle East Respiratory Syndrome (MERS) virus in humans, has been shown to cause neurological disease [11,12]. However, little is known about the pathophysiology of SARS-CoV-2-associated neurological disease in humans.

Central nervous system (CNS) cells that express the SARS-CoV-2 receptor angiotensin-converting enzyme 2 (ACE2) include neurons, glial cells and astrocytes [13,14]. ACE2 is expressed in multiple human brain areas, including the amygdala, cerebral cortex and brainstem, with the highest expression levels found in the pons and medulla oblongata in the brainstem that contain the medullary respiratory centers of the brain [6,15]. Several human autopsy reports have documented the presence of SARS-CoV-2 RNA in brain tissues [16,17]. Human iPSC-derived neural progenitor cells (NPCs) have been shown to be permissive to SARS-CoV-2 infection, and both viral proteins and infectious viral
particle production were detected in neurospheres and brain organoids infected with SARS-CoV-2 [18,19]. Human autopsy reports have shown evidence of lymphocytic panencephalitis, meningitis and brainstem perivascular and interstitial inflammatory changes with neuronal loss in COVID-19 patients [20]. These data suggest that SARS-CoV-2 can productively infect human CNS cells [21]. However, the contributions of CNS cell infection and induced neuroinflammation to the pathogenesis of SARS-CoV-2-associated disease are not well understood.

Small animal models provide a means for studying the neurological complications associated with SARS-CoV-2 infection. K18-hACE2-transgenic mice were originally developed for the study of SARS-CoV-1 pathogenesis. hACE2 expression in K18-hACE2 transgenic mice is driven by the human cytokeratin 18 (K18) promoter. It was recently reported that intranasal inoculation with SARS-CoV-2 results in a rapidly fatal disease in K18-hACE2 mice [22–25]. These studies were focused on describing the acute lung injury in SARS-CoV-2-infected K18-hACE2 mice that was associated with high levels of inflammatory cytokines and accumulation of immune cells in the lungs [22–25]. In these published studies, neither infectious virus nor viral RNA was detected in the olfactory bulbs or brains of the majority of the infected animals, indicating restricted neurotropism of SARS-CoV-2 in K18-hACE2 mice. In the present study, we show that intranasal infection of six-week-old K18-hACE2 mice by SARS-CoV-2 can cause severe neurological disease, with the brain being a major target organ for infection by this route of infection, and neuroinflammation and neuronal death contributing to the infection-associated morbidity and mortality. The data also suggest that SARS-CoV-2 can be trafficked to the brain via the olfactory bulb with subsequent transneuronal spread, as has been reported for other coronaviruses [26,27].

2. Materials and Methods

2.1. Mice

Hemizygous K18-hACE2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All the animal experiments were conducted in a certified Animal Biosafety Level 3 (ABSL-3) laboratory at Georgia State University (GSU). The protocol was approved by the GSU Institutional Animal Care and Use Committee (Protocol number A20044). Six-week-old hemizygous K18-hACE2 mice were infected with 10^7 plaque-forming units (PFU) of SARS-CoV-2 strain USA-WA1/2020 under ABSL-3 containment by intranasal inoculation. SARS-CoV-2 (USA-WA1/2020) was isolated from an oropharyngeal swab from a patient in Washington, USA (BEI NR-52281) [28]. Animals in the control group received equivalent amounts of sterile PBS via the same route. Roughly equal numbers of male and female mice were used. Animals were weighed and their appetite, activity, breathing and neurological signs assessed twice daily [29,30]. Mice that met the human endpoint criteria were euthanized to limit suffering. In independent experiments, mice were inoculated with PBS (Mock) or SARS-CoV-2 intranasally, and on days 1, 3, 5 and 6 after infection, animals were anesthetized using isoflurane and perfused with cold PBS; then, respiratory (nasal turbinate and lung) and other tissues (spleen, heart, liver, kidney, pancreas, eye, olfactory bulb and brain) were collected and flash frozen in 2-methylbutane (Sigma, St. Louis, MO, USA) [31–33]. Alternatively, mice were perfused with PBS followed by 4% paraformaldehyde (PFA), and tissues were harvested and cryoprotected in 30% sucrose (Sigma, St. Louis, MO, USA), and embedded in optimum cutting temperature (OCT) as described previously [31,34].

2.2. Quantification of the Virus Load

The viral titers were analyzed in the tissues by plaque assay and quantitative real-time PCR (qRT-PCR) [28,29]. Briefly, frozen tissues were weighed and homogenized in a bullet blender (Next Advance, Averill Park, NY, USA) using glass or zirconium oxide beads. Virus titers in tissue homogenates were measured by plaque assay using Vero cells. Quantitative RT-PCR was used to measure viral RNA levels with primers and probes specific for the SARS-CoV-2 N gene as described previously [28]. Viral genome copies were determined
by comparison to a standard curve generated using a known amount of RNA extracted from previously titrated SARS-CoV-2 samples. Frozen tissues harvested from mock and infected animals were weighed and lysed in RLT buffer (Qiagen), and RNA was extracted using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA). Total RNA extracted from the tissues was quantified and normalized, and viral RNA levels per μg of total RNA were calculated.

2.3. Measurement of Cytokines, Chemokines and Interferons

The levels of mRNA for select cytokines/chemokines (IL-1β, IL-6, TNF-α, IFN-γ, CCL2 and CCL3) and interferon-α (IFN-α) were determined in total RNA extracted from the lungs and brain using qRT-PCR. The fold change in infected tissues compared to mock tissues was calculated after normalizing to the GAPDH gene [31,34]. The primer sequences used for qRT-PCR are listed in Table 1. The protein levels of IFN-α were measured in the lung and brain homogenates using an ELISA kit (PBL Interferon Source, Piscataway, NJ, USA) [29,30].

Table 1. Primer sequences used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>IL-1β (NM_000576)</td>
<td>AGCACCTCTTCTCCCTTCATC</td>
</tr>
<tr>
<td></td>
<td>GGACCAGACATCAGCCAAGC</td>
</tr>
<tr>
<td>IL-6 (NM_000600)</td>
<td>CCAGGAGGAGCGGATGACAC</td>
</tr>
<tr>
<td></td>
<td>CCCAGGGAGGAAGCGCAGTG</td>
</tr>
<tr>
<td>CCL3 (NM_011337)</td>
<td>ATTCCACGGCAATTCATC</td>
</tr>
<tr>
<td></td>
<td>ATTCAGTTCCAGGT CTAG</td>
</tr>
<tr>
<td>IFN-α (NM_010502)</td>
<td>CTCTGTGCTTCTGCTGAG</td>
</tr>
<tr>
<td></td>
<td>CTGAGGGATATGAGCTGAG</td>
</tr>
<tr>
<td>TNF-α (NM_013695)</td>
<td>CCACTGTGATCCCTCCTAA</td>
</tr>
<tr>
<td></td>
<td>TCTGTGTTTCTGAGTAGT</td>
</tr>
<tr>
<td>CCL2 (NM_011333)</td>
<td>TACACGTGTGCTAATCACTTC</td>
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<tr>
<td></td>
<td>TACACGTGTGCTAATCACTTC</td>
</tr>
<tr>
<td>IFN-γ (NM_008337)</td>
<td>TTCAGCTGACAAATAAGAAC</td>
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<tr>
<td></td>
<td>TACACGTGACACATCG</td>
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2.4. Immunohistochemistry

Sagittal sections (10-μm thick) were cut from the hemibrain tissues frozen in OCT. Tissue sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation [34,35]. Additionally, tissue sections were incubated with anti-CD45, anti-NeuN and anti-SARS-CoV-2 spike protein antibodies (Thermo Fisher Scientific, Nercross, GA, USA) overnight at 4 °C, followed by incubation with Alexa Fluor 546- or Alexa Fluor 488-conjugated secondary antibody for 1 h at room temperature [31,34]. Terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL) staining was conducted...
using an in situ cell death detection kit (Roche, Indianapolis, Indiana, United States) as per the manufacturer's instructions [31,34]. Images were acquired using the Invitrogen™

2.5. Statistical Analysis

An unpaired Student's t-test was used to calculate p values of significance. Differences with p values of <0.05 were considered significant.

3. Results

3.1. Characteristics of K18-hACE2 Mice Following SARS-CoV-2 Infection by the Intranasal Route

Six-week-old K18-hACE2 mice of both sexes were infected intranasally with PBS (mock, n = 10 mice) or 10⁷ PFU of SARS-CoV-2 in PBS (n = 20 mice). The mock-infected mice remained healthy throughout the observation period. Virus infection resulted in 100% mortality by day 6 after infection (Figure 1A). Infected mice experienced significant weight loss on days 4 through 6 after infection compared to the mock-infected group (Figure 1B). Starting on day 4, all the infected animals began to show signs of disease, such as lethargy, slow movement and labored breathing. Neurological symptoms such as hunchbacked posture, ruffled fur, tremors and atactic gait were also observed in infected mice on days 5 and 6 after infection.

![Figure 1](image_url)

Figure 1. Analysis of survival, body weight and virus titers in K18-hACE2 mice following SARS-CoV-2 infection. K18-hACE2 mice were inoculated intranasally with SARS-CoV-2 (10⁷ plaque-forming units (PFU), n = 20) or PBS (Mock, n = 10). (A) Percentage of survival was determined. (B) Percentage of daily body weight change in the animals. Error bars represent SEM. ** p < 0.001. The kinetics and levels of SARS-CoV-2 were determined in the lungs (C,D) and brain (E,F) by plaque assay and qRT-PCR. The data are expressed as PFU/g of tissue or genome copies/µg of RNA. Each data point represents an individual mouse. The solid horizontal lines signify the median value.
3.2. Virus Replication in the Periphery and Brains of K18-hACE2 Mice

Six-week-old K18-hACE2 mice of both sexes were infected intranasally with PBS (mock, n = 12 mice) or $10^6$ PFU of SARS-CoV-2 in PBS (n = 20 mice), and groups of 5 mice were used to measure the viral loads in the peripheral organs and brain at early (day 1), middle (day 3) and late (days 5 and 6) stages of infection. High virus levels were observed in the lungs on day 1, reached peak levels on day 3 and declined on days 5 and 6 after infection (Figure 1C, D). In contrast, virus was not detected in the brain on day one after infection, but was present by day 3. Very high levels of viral RNA and infectious virus were detected in the brains of all the animals by days 5 and 6 after infection (Figure 1E, F). The onset of neurological symptoms and mortality in infected mice correlated with peak virus titers in the brain.

The virus replication kinetics observed in the nasal turbinates was similar to those in the lungs, with the highest viral RNA levels detected during the early stage of infection (days 1 and 3), followed by a decline at later stages of infection (days 5 and 6) (Figure 2). In the olfactory bulbs and eyes, low levels of viral RNA were detected on days 1 and 3 after infection, with very high levels of viral RNA detected in all the animals on days 5 and 6 after infection, indicating productive infection within the olfactory system (Figure 2). By contrast, little if any virus was detected in the serum of infected mice at any time after infection.

![Graphs showing viral load in various tissues](image)

Figure 2. Analysis of virus tropism in K18-hACE2 mice. The viral RNA copy number in the nasal turbinates, olfactory bulbs, eye, serum, kidney, spleen, pancreas, heart and liver was determined on days 1, 3, 5 and 6 after infection by qRT-PCR and expressed as genome copies/μg of RNA. Each data point represents an individual mouse. The solid horizontal lines signify the median value.
Since the K18 promoter is known to be active in the epithelium of multiple organs of K18-hACE2 mice [27,36], we also evaluated viral RNA levels in other peripheral organs. Viral RNA was detected in the heart, kidney, spleen, pancreas and liver on day 1. There was a slight increase in RNA levels on day 3 and/or 5 in each of these organs, suggesting limited virus replication at these sites (Figure 2). These data agree with previous reports that also showed the presence of SARS-CoV-2 RNA in these organs [22–25,27,36].

3.3. Inflammatory Changes in the Lungs and Brain of SARS-CoV-2-Infected Mice

IFN signaling has a pivotal role in developing an innate and adaptive immune response to viral infection [37,38]. Therefore, we measured the mRNA and protein levels of IFN-α in the lungs and brain. In the lungs, an increase in both IFN-α mRNA and protein levels was detected on day 1; levels peaked at day 3 and then decreased on day 6 after infection (Figure 3A,B). By contrast, an IFN response was not detected in the brain on days 1 and 3 after infection. High levels of IFN-α were only detected in the brain on days 5 and 6 after infection (Figure 3C,D). Overall, the induction of IFN-α correlated with the SARS-CoV-2 replication kinetics in the lungs and brain. It is interesting to note that relative IFN-α levels were higher in the lungs compared to the brains of the infected animals despite higher virus replication in the brain.

**Figure 3.** Analysis of mRNA and protein levels of interferon-α (IFN-α) in the lungs and brain. The mRNA levels of IFN-α were measured in the lungs (A) and brain (C) by qRT-PCR, and the fold change in the infected tissues compared to the corresponding mock-infected controls was calculated after normalizing to GAPDH mRNA. The protein levels of IFN-α were measured in the lungs (B) and brain (D) homogenates using ELISA and expressed as pg/g of tissue. Error bars represent SEM (n = 5 mice per group). * p < 0.05; ** p < 0.001.
We next examined the mRNA levels of proinflammatory cytokines and chemokines in the lungs and brain of infected mice. SARS-CoV-2 infection resulted in a 10-fold increase on day 1 and a 100-fold increase on day 3 in the interleukin (IL)-6 mRNA expression in the lungs (Figure 4A). The levels of TNF-α mRNA were elevated 10-fold in the lungs on days 1 and 3 after infection. The level of IFN-γ mRNA was elevated by 15-fold on day 3. However, the levels of these cytokines had decreased by day 5 after infection. The IL-1β mRNA levels showed no significant increase at any time point after infection. There was a 100-fold increase in the expression of CCL2 on day 3 (Figure 4B). However, the levels of CCL2 mRNA had decreased by day 5 after infection. CCL3 mRNA levels increased slightly on day 1 and were undetectable on days 5 and 6 after infection.

![Figure 4. Cytokine and chemokine mRNA levels in the lungs and brain. The mRNA levels of various cytokine and chemokine genes were determined in the lungs (A,B) and brain (C,D) using qRT-PCR. Fold change in the infected tissues compared to the corresponding mock controls was calculated after normalizing to GAPDH mRNA in each sample. Error bars represent SEM (n = 5 mice per group).](image)

In the brain, no increase in the mRNAs of the cytokines or chemokines tested was observed on day 1 after infection. Less than a 10-fold increase was observed in the cytokine mRNA levels on day 3 (Figure 4C). There was a 500-fold increase in IL-6 mRNA by day 5 after infection. TNF-α and IFN-γ mRNA levels increased by ~750-fold in the brain by day 5 after infection. Similarly, IL-1β mRNA levels increased by 400-fold by day 5 after infection. Both CCL2 and CCL3 mRNA levels were elevated by almost 1000-fold on days 5 and 6 after infection consistent with the high level of virus in the brain (Figure 4D). These results indicate that the inflammatory response was more pronounced in the brain than in the lungs at the later stage of infection.

3.4. SARS-CoV-2-Induced Neuropathology in K18-hACE2 Mice

We next analyzed the brain sections from infected mice for antigen distribution, infiltration of immune cells and cell death. Immunohistochemical staining for the SARS-CoV-2 spike protein detected cell-associated viral antigen throughout the brain on day 6 after infection. Representative data for sections from the cortex, cerebellum and hippocampus
regions are shown in Figure 5. We also detected virus antigen in sections of the olfactory bulb of infected animals on day 6. H&E staining of brain sections from the infected mice demonstrated perivascular hemorrhage and neuronal cell death (Figure 6A,B). The neurons of infected mice demonstrated a shrunken neuron body with light pink cytoplasmic staining, representing degenerating neurons (Figure 6B). Enhanced leukocyte infiltration was detected within blood vessel walls and in the perivascular space (Figure 6A). Evidence of leukocyte infiltration was confirmed by direct immunohistochemical analysis of the CD45 antigen, which revealed many CD45-positive cells in the brain parenchyma near neurons (Figure 6C). SARS-CoV-2-induced cell death was evaluated by direct TUNEL staining of brain tissues. On day 6, infected K18-hACE2 mice had elevated numbers of TUNEL-positive cells in the cortex, hippocampus and cerebellum regions, indicating increased cell death (Figure 6D).

![Image of brain sections stained for SARS-CoV-2 spike protein](image)

**Figure 5.** Detection of SARS-CoV-2-infected cells in the brains of K18-hACE2 transgenic mice. Brain sections (day 6 after infection) were stained for SARS-CoV-2 spike protein. Representative immunostaining images showing the presence of SARS-CoV-2 spike protein (red) in the cortex, cerebellum, hippocampus and olfactory bulb of infected mice. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). The photomicrographs shown are representative of the images obtained from five animals. Bars, 20 μm.
Figure 6. Histopathological analysis of SARS-CoV-2-infected brains. Hematoxylin and eosin (H&E) staining of brain sections from mock and SARS-CoV-2-infected mice on day 6 after infection. (A, B) Brain sections show perivascular hemorrhage, enhanced leukocyte infiltration (blue arrows) and neuronal cell death (red arrows). (C) Brain sections are stained for CD45 (red, leukocyte marker) and NeuN (green, neuronal cell marker). Nuclei are stained with DAPI (blue). (D) A terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay was conducted on brain sections from mock and SARS-CoV-2-infected mice on day 6 after infection to detect apoptotic cells. The boxed areas in the second row of panels are enlarged in the bottom row of panels. The photomicrographs shown are representative of the images obtained from five animals. Bars, 20 μm.

4. Discussion

This study demonstrates a critical role of direct infection of CNS cells and of the inflammatory response in mediating SARS-CoV-2-induced lethal disease in K18-hACE2 mice. Intranasal inoculation of the virus results in a lethal disease with high levels of virus replication in the brain. Virus infection of the CNS was accompanied by an inflammatory response as indicated by the production of cytokines/chemokines, infiltration of leukocytes into the perivascular space and parenchyma and CNS cell death. Our data also indicate that following infection by the intranasal route, the virus enters the brain by traversing the cribriform plate and infecting neuronal processes located near the site of intranasal inoculation.

Some animal coronaviruses, such as MHV readily infect neurons and cause lethal encephalitis in mice [11,39]. SARS-CoV-1 infection also induces severe neurological disease after intranasal administration in K18-hACE2 mice [27]. Similarly, in our study, SARS-CoV-2 virus antigen was detected throughout the brain, including the cortex, cerebellum and hippocampus. The onset of severe disease in SARS-CoV-2-infected mice correlated with peak viral levels in the brain, immune cell infiltration and CNS cell death. Peak virus titers in the brains were approximately 1000 times higher than the peak titers in the lungs, suggesting a high replicative potential of SARS-CoV-2 in the brain. The relative upregulation of cytokine and chemokine mRNAs was approximately 10 to 50 times higher in the brain compared to the lungs, strongly suggesting that extensive neuroinflammation contributed to clinical disease in these mice.

It was recently reported that SARS-CoV-2 infection of K18-hACE2 mice causes severe pulmonary disease with high virus levels detected in the lungs of these mice and that mortality was due to the lung infection [22–25]. In these studies, viral RNA was undetectable in the brains of the majority of the infected animals, indicating a limited role of brain infection in disease induction. An important distinction between our study and
others is that we detected high infectious virus titers in the olfactory system and brains of 100% of the infected K18-hACE2 mice. This phenotype was not consistently observed in the aforementioned K18-hACE2 mouse studies [22–25]. Moreover, none of the published studies evaluated the extent of neuroinflammation and neuropathology at the later stages of infection. Our results showed that the inflammatory response was more pronounced in the brain than in the lungs on days 5 and 6 after infection. Although both our study and the previous studies infected mice via the intranasal route, the other studies used older (7-to-9-week-old) K18-hACE2 mice and a lower viral dose (10^4 PFU), and in one study, only analyzed samples 3 days after infection [22]. In our study, six-week-old K18-hACE2 mice were infected with 10^9 PFU. However, unpublished data from our laboratory demonstrated that six-week-old K18-hACE2 mice infected with a lower viral dose (10^5 PFU) also exhibit a similar phenotype, suggesting that the brain is a major site of infection following infection by the intranasal route, regardless of the virus dose used. Additional studies are needed to clarify the parameters that differentially affect tissue tropism, routes of virus dissemination and mechanisms of lung and brain injuries in K18-hACE2 mice following SARS-CoV-2 infection. Recent studies have suggested that humans have a higher chance of developing a brain infection if they are infected intranasally with a high dose of virus [40].

Alterations in smell and taste are features of COVID-19 disease in humans [8,41]. Pathological analyses of human COVID-19 autopsy tissues detected the presence of SARS-CoV-2 proteins in endothelial cells within the olfactory bulb [41,42]. Our data indicate that SARS-CoV-2 can productively infect cells within the nasal turbinates and olfactory bulb in intranasally infected K18-hACE2 mice. Virus infection of cells in these tissues in humans may explain the loss of smell associated with some COVID-19 cases [41]. The detection of virus replication in these tissues suggests that SARS-CoV-2 can access the brain by first infecting the olfactory bulb and then spreading into the brain by infecting connecting brain neuron axons. This hypothesis is consistent with previously published reports that neurotropic coronaviruses infect olfactory neurons and are transmitted to the brain via axonal transportation [8,26,27,43]. Many viruses, such as HSV-1, Nipah virus, rabies virus, Hendra virus and influenza A virus, have also been shown to enter the CNS via olfactory sensory neurons [44–47]. Another route by which a virus can gain access to the brain is via the disruption of the blood–brain barrier (BBB). However, we could not detect any virus in the serum of the infected mice at any time after infection, suggesting a limited role of BBB disruption in SARS-CoV-2 neuroinvasion. This finding is in agreement with previously published studies that detected little or no virus in the brain of K18-hACE2 mice after infection with SARS-CoV-1 or SARS-CoV-2 [22–25,27,36].

In summary, we found that intranasal infection of K18-hACE2 mice by SARS-CoV-2 causes severe neurological disease. Our data demonstrate that the CNS is the major target of SARS-CoV-2 infection in K18-hACE2 mice under the conditions used, and that brain infection leads to immune cell infiltration, inflammation and cell death.


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SARS-CoV-2 Infects Primary Neurons from Human ACE2 Expressing Mice and Upregulates Genes Involved in the Inflammatory and Necroptotic Pathways

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Abstract: Transgenic mice expressing human angiotensin-converting enzyme 2 under the cytokeratin 18 promoter (K18-hACE2) have been extensively used to investigate the pathogenesis and tissue tropism of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. Neuroinvasion and the replication of SARS-CoV-2 within the central nervous system (CNS) of K18-hACE2 mice is associated with increased mortality; although, the mechanisms by which this occurs remain unclear. In this study, we generated primary neuronal cultures from K18-hACE2 mice to investigate the effects of a SARS-CoV-2 infection. We also evaluated the immunological response to SARS-CoV-2 infection in the CNS of K18-hACE2 mice and mouse neuronal cultures. Our data show that neuronal cultures obtained from K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. Furthermore, SARS-CoV-2 infection upregulated the expression of genes involved in innate immunity and inflammation, including IFN-α, ISG-15, CXCL10, CCL2, IL-6 and TNF-α, in the neurons and mouse brains. In addition, we found that SARS-CoV-2 infection of neurons and mouse brains activates the ZBP1/pMLKL-regulated necroptosis pathway. Together, our data provide insights into the neuropathogenesis of SARS-CoV-2 infection in K18-hACE2 mice.

Keywords: COVID-19; SARS-CoV-2; K18-hACE2 mice; neurons; neuropathogenesis; inflammation; necroptosis

1. Introduction
Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), continues to be a global concern. In addition, several variants of SARS-CoV-2 have been identified that may influence antibody treatment and vaccine efficiency [1–4]. Neurological complications, such as brain fog, loss of taste and smell, changed mental status and anosmia have been reported in some COVID-19 patients [5–9]. Studies have shown presence of meningitis, encephalitis, leukocytosis infiltration and neuronal death in COVID-19 patients [6,10]. Evidence of SARS-CoV-2 neuroinvasion in COVID-19 patients’ brain autopsies has been demonstrated and the olfactory mucosa has been suggested as a route of entry [4,6,11–13]. Several studies have also reported that neurologic symptoms may result from the exacerbated systemic pro-inflammatory responses without a direct infection of the brain cells [9,13]. Angiotensin-converting enzyme 2 (ACE2), the entry receptor of SARS-CoV-2, has recently been demonstrated to be present in neurons and glial cells of different brain regions [5,14–18]. Studies using brain organoids derived from human pluripotent stem cell (hPSC) have shown the presence of the virus in neuronal cells [19–23]. In addition, anti-ACE2 antibodies can inhibit the SARS-CoV-2 infection of neuronal cells [19].
The K18-hACE2 mouse model is commonly used to study the pathogenesis of SARS-CoV-2 infection and to test the efficacy of anti-viral compounds and vaccines. These mice express human ACE2, the entry receptor of SARS-CoV-2 [24–27]. We previously reported that the infection of K18-hACE2 mice with SARS-CoV-2 results in a lethal disease associated with viral neuroinvasion and severe neuronal damage [28]. However, the molecular mechanism by which SARS-CoV-2 infection of neurons leads to acute encephalitis in K18-hACE2 mice remains unclear. The present study was undertaken to (i) investigate the permissiveness of neurons to SARS-CoV-2 infection, and (ii) evaluate the immunological response to SARS-CoV-2 infection in the CNS of K18-hACE2 animals and mouse neuronal cultures. Our data show that neuronal cultures obtained from K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. In response to infection, genes involved in the innate immune response, inflammation and cell death were upregulated in the neurons. In addition, SARS-CoV-2 infection of mouse brains also resulted in increased expression of genes associated with the inflammatory and cell death pathways.

2. Results
2.1. SARS-CoV-2 Infection of Primary Mouse Cortical Neurons

Primary neuronal cultures were established from one-day-old K18-hACE2 (hACE2 neurons) and non-hACE2-carrier (NC neurons) pups and cultured for seven days to allow differentiation to occur. The neuronal cultures were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.1. Plaque assay, qRT-PCR and immunofluorescence were used to determine the kinetics of SARS-CoV-2 replication at various time points after infection. Productive SARS-CoV-2 replication, as indicated by the release of virions, was detected at 24 h after infection of the hACE2 neurons. Viral titers peaked at 48 h after infection (log 5–6 PFU/mL), followed by a slight decrease in the virus titers at 72 h (Figure 1A). We next measured the intracellular viral RNA levels using qRT-PCR. High SARS-CoV-2 RNA levels were detected in the hACE2 neurons at 48 and 72 h after infection (log 6–7 genome copies/µg RNA). Neurons derived from NC mice were relatively resistant to infection compared to the hACE2 neurons. There was a slight increase in virus and RNA levels at 48 and 72 h, suggesting limited virus replication in these cells (Figure 1B). An immunofluorescence assay of SARS-CoV-2-infected hACE2 neurons showed strong dsRNA staining. Additionally, dsRNA was detected in both the neuronal bodies and axons of the MAP2-positive cells at 48 h after infection (Figure 1C). dsRNA detection is considered as evidence of viral RNA replication. Approximately 40% of hACE2 neurons were positive for dsRNA at 48 h after infection. Overall, these findings indicate that neurons derived from K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication.

2.2. Host Immune Responses in SARS-CoV-2-Infected Neurons and Mouse Brains

We next investigated the effect of SARS-CoV-2 infection on the mRNA expression of key innate immune and inflammation genes in the neurons. Changes in gene expression levels in hACE2 neurons infected with SARS-CoV-2 for 48 h compared to mock-infected controls were analyzed by qRT-PCR. Interferon stimulated gene (ISG)-15 mRNA expression increased by >100-fold after SARS-CoV-2 infection (Figure 2A). The levels of interferon (IFN)-α and IFN-β were elevated more than 10-fold. The mRNA levels of the chemokine pathway-associated genes, chemokine (C-C motif) ligand-2 (CCL2) and chemokine (C-X-C motif) ligand-10 (CXCL10), were upregulated by more than 50-fold in infected neuronal cultures (Figure 2B). Furthermore, interleukin-6 (IL-6), IL-1β and tumor necrosis factor (TNF)-α mRNA expression levels were upregulated >10-fold by a SARS-CoV-2 infection. The CCL3 mRNA levels were also increased compared to the mock-infected controls (Figure 2B).
Figure 1. SARS-CoV-2 infection of mouse neuronal cultures. K18-hACE2 (hACE2 neurons) and non-hACE2-carrier (NC neurons) were prepared from one-day-old pups and cultured for seven days for differentiation. (A) hACE2 (blue bars) and NC neurons (red bars) were infected with SARS-CoV-2 at a MOI of 0.1. Virus infectivity titers in the supernatants were measured using a plaque formation assay and are expressed as plaque-forming units (PFU)/mL. (B) Intracellular viral RNA copies were determined by qRT-PCR. The data are expressed as genome copies/ug of RNA. Values are the mean ± SEM of three independent infection experiments conducted in duplicate. Each data point represents an independent experiment. (C) The hACE2 neurons grown on coverslips were fixed at 48 h after infection and stained with anti-MAP2 (red), dsRNA (green) and DAPI (blue) antibodies. In the bottom row of panels, the boxed areas from the first row are expanded. The images shown are representative of three independent infection experiments, with 20× magnification.

Next, we examined the mRNA levels of innate immune and inflammatory genes in the brains of infected mice. K18-hACE2 mice infected with PBS or 10³ PFU of SARS-CoV-2 via the intranasal route [28]. The mice were sacrificed at days 1, 3 and 6 after infection, and the brains were harvested. A plaque assay was conducted to determine infectious virus titers in the brain homogenates. No infectious virus was detected in the brains on day one, but virus infectivity titers were very high at day 3 (log 3-4 PFU/gram of brain tissue) and day 6 after infection (log 7-8 PFU/gram of brain tissue) [28]. Both the IL-6 and TNF-α mRNA levels increased by >5-fold on day 3 after SARS-CoV-2 infection (Figure 2C). By the sixth day after infection, IL-6 and TNF-mRNA levels in the brain had increased by 300-fold (Figure 2C). There was also a 100-fold increase in the IL-1β mRNA levels on day 6. The was a slight upregulation in the levels of IFN-α mRNA (Figure 2C). At day 6 after infection, expression levels of chemokines, including CXCL10 and CCL2 and CCL-3 were elevated by more than 300-fold (Figure 2D).

As these pro-inflammatory cytokines are secreted proteins, their release in the culture media of mock- and SARS-CoV-2-infected hACE2 neurons was detected using ELISA. In the controls, basal levels of IL-6 and IFN-β in cell culture media were very low. On the other
hand, significant amounts of soluble IL-6 and IFN-β were detected in the supernatant from infected cells at 48 h after infection (Figure 3). The basal level of CXCL10 was relatively high, but it also increased significantly after SARS-CoV-2 infection. These results indicate that SARS-CoV-2 infection upregulates the expression of innate immune and inflammatory genes in neuron cultures and mouse brains.

![Graphs and diagrams](image)

Figure 2. Analysis of upregulation of the expression of immune genes involved in innate immunity and inflammation in primary mouse neurons and mouse brains. (A, B) The hACE2 neurons were infected with SARS-CoV-2 or mock-infected at a MOI of 0.1. At 48 h after infection, cell pellets were collected, and total RNA was extracted. qRT-PCR was conducted to determine the fold-change of (A) ISG15, IFN-β and IFN-α, and (B) CXCL10, CCL2, IL-6, IL-1β, TNF-α and CCL3 mRNA levels. Data for each sample was normalized to the value for GAPDH and expressed as the relative fold increase compared to mock-infected controls. Data represent the mean ± SEM of three independent infection experiments conducted in duplicate. Each data point represents an independent experiment. (C, D) Eight-week-old hemizygous KI8-hACE2 mice were infected with SARS-CoV-2 (10^4 PFU, n = 12) or PBS (Mock, n = 9). Brains were harvested after extensive perfusion with PBS at days 1, 3 and 6 after infection and RNA was extracted. The mRNA levels of (C) IL-6, IL-1β, TNF-α and IFNα, and (D) CXCL10, CCL2 and CCL3 were determined by qRT-PCR. Each data point represents an individual mouse. Data represent the mean ± SEM.
2.3. SARS-CoV-2 Infection Activates the ZBP1/MLKL Pathway in Neurons and Mouse Brains

We examined the mRNA and protein levels of genes involved in cell-death pathways in neurons after SARS-CoV-2 infection. The qRT-PCR was used to analyze the changes in the gene mRNA levels. The key genes involved in the necroptotic pathway were highly upregulated in hACE2 neurons infected with SARS-CoV-2 for 48 h. The levels of Z-DNA binding protein 1 (ZBP1) and mixed lineage kinase domain-like (MLKL) mRNA were elevated -50-fold after SARS-CoV-2 infection. mRNA expression levels of caspase-8 and receptor-interacting kinase-3 (RIPK3) were upregulated >10-fold after infection (Figure 4A). Pyroptotic gene caspase-1 was upregulated by 10-fold while the apoptotic genes, caspase-3 and caspase-7 showed no significant increase after SARS-CoV-2 infection (Figure 4B). To verify the activation of the necroptotic pathway, protein levels of ZBP1 and phosphorylated MLKL (pMLKL) were measured by immunoblotting. The levels of ZBP1 increased at 24 and 48 h after infection. We detected a modest increase in the protein levels of pMLKL at 24 and 48 h after infection. However, there was a significant increase in the levels of pMLKL protein at 72 h (Figure 4C).

Next, we evaluated the activation of the necroptotic pathway in mouse brains infected with SARS-CoV-2. The mRNA expressions of ZBP1 and MLKL increased gradually in the brains from days 1 to 6 after SARS-CoV-2 infection. By day 6 after infection, ZBP1 and MLKL mRNA levels were upregulated -50-fold in the brains (Figure 4D). The mRNA levels of RIPK3, RIPK1, Caspase 8 and Caspase 1 were also elevated by day 6 (Figure 4D,E). However, there was no significant increase in the levels of caspase-3 and caspase-7 mRNA in infected brains. Western blot data showed an increase in the protein levels of ZBP1 and pMLKL in the infected brains in a time-dependent manner (Figure 4F). The increase in the mRNA and protein levels of ZBP1 and MLKL correlate with the increase in the infectious virus titers in the brains [28]. Together, these results indicate that SARS-CoV-2 infection in neurons and mouse brains activates the ZBP1/MLKL-regulated necroptosis pathway.
Figure 4. mRNA and protein levels of genes involved in cell-death pathways in primary mouse neurons and mouse brains. (A, B) The hACE2 neurons were infected with SARS-CoV-2 or mock-infected at a MOI of 0.1. At 48 h after infection, cell pellets were collected, and total RNA was extracted. qRT-PCR was conducted to determine the fold-change in (A) ZBP1, MLKL, RIPK3 and RIPK1, and (B) Caspase 8, Caspase 1, Caspase 7 and Caspase 3 mRNA levels. Data for each sample was normalized to the value for GAPDH and expressed as the relative fold increase compared to mock-infected controls. Data represent the mean ± SEM of three independent infection experiments conducted in duplicate. Each data point represents an independent experiment. (C) The hACE2 neurons were infected with SARS-CoV-2 or mock-infected at a MOI of 0.1. At 24, 48 and 72 h, cell pellets were collected, and total protein was extracted. Protein was blotted with ZBP1, pMLKL, or β-actin antibodies. Data are representative of three independent experiments. (D, E) K18-hACE2 mice were inoculated with SARS-CoV-2 or PBS via the intranasal route. Brains were harvested after extensive perfusion at days 1, 3 and 6 after infection and RNA was extracted. qRT-PCR was used to determine the mRNA levels of (D) ZBP1, MLKL, RIPK3 and RIPK1, and (E) Caspase 8, Caspase 1, Caspase 7 and Caspase 8. After normalizing individual sample to GAPDH level, the fold change in infected tissues compared to mock-infected controls was determined. Each data point represents an individual mouse (n = 4). Values are the mean ± SEM. (F) Protein extracted from mock- and SARS-CoV-2-infected brain tissues were blotted with ZBP1, pMLKL or β-actin antibodies. Data are representative of four mice per time point.

3. Discussion

In this study, we demonstrated that SARS-CoV-2 establishes a productive infection in neuronal cultures obtained from hACE2-expressing mice. In response to infection, the expression of innate immune and inflammatory genes was upregulated in the neurons as well as in mouse brains. In addition, we found that the SARS-CoV-2 infection of neurons and mouse brains upregulated genes involved in the necrotic pathway (ZBP1, MLKL, RIPK3 and caspase-8), suggesting that necroptosis may play a role in the pathogenesis of SARS-CoV-2 infection in the CNS.

It is known that some animal (mouse hepatitis virus) and human (HCoV-OC43) coronaviruses productively infect neuronal cells [28,29]. The SARS-CoV-2 infection has been detected in the brains of some COVID-19 patients [4,6,11–13]. Furthermore, SARS-CoV-2 has also been shown to replicate and induce cell death in human neural progenitor cells and brain organoids [19–23]. Transgenic K18-hACE2 mice represent a lethal model of
SARS-CoV-2 infection [24–28]. Neuroinvasion and the replication of SARS-CoV-2 within the CNS is associated with mortality in these mice. In the present study, we found that neurons derived from one-day-old K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. Additionally, dsRNA was detected in the neuronal bodies and axons infected with SARS-CoV-2. In comparison, virus replication was limited in the non-hACE2-expressing mouse neurons.

A cytokine storm is one of the pathological hallmarks of the severe outcomes resulting from SARS-CoV-2 infection [30,31]. Several studies have reported that increased TNF-α and IL-6 levels correlate with severe disease outcomes [32,33]. In the present study, we show that SARS-CoV-2 infection in K18-hACE2 mouse brains is also characterized by the upregulation of innate immune and inflammatory genes, including TNF-α and IL-6 [28]. Similarly, a significant increase in the expression of IL-6, TNF-α, CXCL10 and CCL2 was observed in SARS-CoV-2-infected neuron cultures. These inflammatory genes may activate downstream cell-death signaling pathways in the neurons, leading to neuronal death, and/or stimulate glial cells, exacerbating neuroinflammation [33–35]. As previously reported, TNF-α is thought to be a potent inducer of neuronal injury in several neurodegenerative diseases, such as cerebral ischemia, spinal cord injury, multiple sclerosis and viral infections including HIV-associated dementia [33,36]. Both CXCL10 and CCL2 are important chemokines involved in the infiltration of leukocytes into the CNS after virus infection [35].

ZBP1 is one of the cytoplasmic sensors that regulate cell death and inflammation [37–40] and initiates the RIPK3-dependent activation of RIPK3-dependent necroptosis during virus infections. Necroptosis is an inflammatory cell death caused by RIPK3 phosphorylation, which activates the pseudo-kinase MLKL, which oligomerizes and ruptures the plasma membrane, resulting in cell death [41,42]. Necroptosis can eradicate virus-infected cells and activate innate and adaptive immunity to limit virus replication. This process can also trigger the release of inflammatory cytokines and damage-associated molecular patterns, resulting in robust inflammation [38,39,43]. In the present study, we found significant upregulation of the necrotic genes, ZBP1, MLKL, and RIPK3, in neuronal cells and mouse brains after SARS-CoV-2 infection. Previous studies have demonstrated that infection with beta coronaviruses can induce necroptosis in certain cell types. Human coronavirus, HCoV-OC43, induces necroptosis in human neural cells [29] and mouse hepatitis virus infection induces necroptosis in murine bone-marrow-derived macrophages by phosphorylation of MLKL [44]. We previously reported that ZBP1 restricts replication of West Nile virus and Zika virus in primary mouse cortical neurons [37]. Future studies are warranted to understand the role of ZBP1 in SARS-CoV-2 pathogenesis.

Together, our results demonstrate that SARS-CoV-2 robustly replicates in neuronal cultures obtained from K18-hACE2 mice. In the same way as the SARS-CoV-2-infected K18-hACE2 mouse brains, the virus infection of neuronal cultures induces the up-regulation of genes involved in the innate immune response, inflammation and cell death.

4. Materials and Methods

4.1. Neuronal Cultures and SARS-CoV-2 Infection

Hemizygous K18-hACE2 mice and non-hACE2-carrier (NC) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). One-day-old pups were obtained from established breeding pairs of K18-hACE2 and NC mice as previously described [37,45,46]. The neurons were plated for 24 h onto poly-D-lysine-coated plates in serum Neurobasal A medium. The neurons were then cultured in serum-free Neurobasal A medium containing B27 for seven days to allow differentiation. The neurons isolated from each pup were plated separately and genotyped to identify hACE2-expressing and NC neurons. Neuronal cultures were infected with SARS-CoV-2 (USA-WA1/2020) or mock-infected at a multiplicity of infection of 0.1. At various time points after infection, supernatants and cell lysates were collected [37,45,47,48].
4.2. Animal Infection Experiments

The in vivo animal experiments with SARS-CoV-2 were conducted in an Animal Biosafety Level 3 (ABSL-3) laboratory. Georgia State University Institutional Animal Care and Use Committee approved the experimental protocol of this study (Protocol number A20044). Hemizygous K18-hACE2 mice aged eight-weeks were inoculated with PBS or 10^4 PFU of SARS-CoV-2 via the intranasal route [28, 48]. On various days after the infection, animals were anesthetized, perfused with PBS, and brain tissues were collected.

4.3. Quantification of the Viral Titers

The levels of infectious virus in cell culture supernatants and brain tissues were determined by using a plaque formation assay. Quantitative RT-PCR was used to measure the intracellular viral RNA levels using SARS-CoV-2 N gene primers [28, 47]. Total RNA was extracted from cell pellets and brain tissues using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA). Viral genome copies per ug of total RNA were calculated using a standard curve of the known amount of viral RNA [28, 47, 48].

4.4. Immunostaining

Neuronal cells were grown on coverslips in 12-well plates and infected with SARS-CoV-2 or PBS at a MOI of 0.1 for 48 h [43]. Cells were washed with PBS and fixed in 4% paraformaldehyde for 1 h at room temperature. The cells were permeabilized and incubated with anti-MAP2 (Catalog # PA5-17646) and anti-dsRNA (MABE1134) antibodies overnight at 4°C (Thermo Fisher Scientific, Norcross, GA, USA). The next day, cells were incubated with Alexa Fluor 546- or Alexa Fluor 488-conjugated secondary antibody for 1 h at room temperature [28, 45, 49]. The Invitrogen EVOS™ MS5000 Cell Imaging System was used to capture the images.

4.5. Western Blot Analysis

Protein extracted from neuronal cultures and mouse brains were separated on SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with primary antibodies against ZBP1 (Cat #703166), pMLKL (Thermo Fisher Scientific, Norcross, GA, USA) and β-actin [49, 50]. To visualize the protein bands, the membranes were incubated with a secondary antibody conjugated with IRDye 800 or IRDye 680 (Li-Cor Biosciences). The membranes were scanned using the Odyssey infrared imager (Li-Cor Biosciences) [49, 50].

4.6. ELISA

ELISA was used to measure the protein levels of IL-6 (Invitrogen, Catalog # 50-246-676), IFN-β (PBL Assay Science, Catalog # 12405-1) and CXCL10 (Invitrogen, Catalog # 50-152-92) in the cell culture supernatants, according to the manufacturer’s instructions. The plates were analyzed using a Victor 3 microtiter reader as previously described [28].

4.7. qRT-PCR

A Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA) was used to extract total RNA from cell pellets and brains. The cDNA was synthesized from RNA using an iScript™ cDNA Synthesis Kit (Bio-Rad). The qRT-PCR was used to determine the expression levels of multiple host genes [51]. The fold-change in infected samples compared to control samples was calculated after normalizing to the housekeeping GAPDH gene [28, 45, 47, 48]. The primer sequences used for qRT-PCR are listed in Table 1.
Table 1. Primer sequences used for qRT-PCR.

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<tr>
<th>Gene (Accession No.)</th>
<th>Primer Sequence (5’–3’)</th>
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4.8. Statistical Analysis

Unpaired student t-test using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used to calculate the p values. Differences of p < 0.05 were considered significant.


Funding: This work was supported by a grant (R21OD024896) from the Office of the Director, National Institutes of Health, and Institutional funds.

Institutional Review Board Statement: The animal study protocol was approved by the Georgia State University Institutional Animal Care and Use Committee (Protocol number A2004).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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The FDA-approved gold drug auranofin inhibits novel coronavirus (SARS-CoV-2) replication and attenuates inflammation in human cells

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ABSTRACT
SARS-CoV-2 has recently emerged as a new public health threat. Herein, we report that the FDA-approved drug, auranofin, inhibits SARS-CoV-2 replication in human cells at low micromolar concentration. Treatment of cells with auranofin resulted in a 50% reduction in the viral RNA at 48 h after infection. Auranofin treatment dramatically reduced the expression of SARS-CoV-2-induced cytokines in human cells. These data indicate that auranofin could be a useful drug to limit SARS-CoV-2 infection and associated lung injury due to its antiviral, anti-inflammatory and anti-oxidative oxygen species (ROS) properties. Further animal studies are warranted to evaluate the safety and efficacy of auranofin for the management of SARS-CoV-2 associated disease.

Gold-based compounds have shown promising activity against a wide range of clinical conditions and microorganisms infections. Auranofin, a gold-containing triethyl phosphine, is an FDA-approved drug for the treatment of rheumatoid arthritis since 1985 (Roder and Thomson, 2015). It has been investigated for potential therapeutic application in a number of other diseases including cancer, neurodegenerative disorders, HIV/AIDS, parasitic infections and bacterial infections (Roder and Thomson, 2015; Harbut et al., 2015). Auranofin was approved by FDA for phase II clinical trials for cancer therapy (Hoa et al., 2018; Oh et al., 2017; Rigobello et al., 2009). Oral auranofin was effective in rodent models of various parasitic infections (Letisch, 2017; Capusselli et al., 2017). A preclinical study showed that auranofin significantly reduces HIV load in combination with antiretroviral therapy (Lewis et al., 2011). A clinical trial is ongoing to develop auranofin as a drug candidate to reduce the latent viral load in patients with HIV infection utilizing the role of auranofin in redox-sensitive cell death pathways (Diaz et al., 2015; Chiurlo et al., 2015).

The mechanism of action of auranofin involves the inhibition of redox enzymes such as thioredoxin reductase, induction of endoplasmic reticulum (ER) stress and subsequent activation of the unfolded protein response (UPR) (Harbut et al., 2018; May et al., 2018; Wiederhold et al., 2017; Thangamani et al., 2016). Inhibition of these redox enzymes leads to cellular oxidative stress and intrinsic apoptosis (Logea et al., 2017; Hetz, 2012). In addition, auranofin is an anti-inflammatory drug that reduces cytokines production and stimulate cell-mediated immunity (Witz et al., 1983). It has been reported that auranofin interferes with the Interleukin 6 (IL-6) signaling by inhibiting phosphorylation of JAK1 and STAT3 (Han et al., 2008; Kim et al., 2009). The dual inhibition of inflammatory pathways and thiol redox enzymes by auranofin makes it an attractive candidate for cancer therapy and treating microbial infections.

Coronaviruses are a family of enveloped viruses with positive sense, single-stranded RNA genomes (Rothan and Byrarrd, 2020). SARS-CoV-2, the causative agent of COVID-19, is closely related to severe acute respiratory syndrome coronavirus (SARS-CoV-1) (Rothan and Byrarrd, 2020; Mehta et al., 2020). It is known that ER stress and UPR activation contribute significantly to the viral replication and pathogenesis during a coronavirus infection (Fang and Liu, 2014). Infection with SARS-CoV-1 increases the expression of the ER protein folding chaperones GRP78, GRP94 and other ER stress related genes to maintain protein folding (Fang and Liu, 2014). Cells overexpressing the SARS-CoV spike protein and other viral proteins exhibit high levels of UPR activation (Shi et al., 2014; Sung et al., 2020). Thus, inhibition of redox enzymes such as thioredoxin reductase and induction of ER stress by auranofin could significantly affect SARS-CoV-2 protein synthesis (Rothan and Kumari, 2019).

In addition, SARS-CoV-2 infection causes acute inflammation and neutrophilia that leads to a cytokine storm with over expression of IL-6, TNF-alpha, monocyte chemoattractant protein (MCP-1) and reactive oxygen species (ROS) (Mehta et al., 2020). The severe COVID-19 illness...
Fig. 1. Auranofin inhibits replication of SARS-CoV-2 in human cells. HeLa cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 1 for 2 h and treated with 4 μM of auranofin or with 0.1% DMSO. Cell pellets and culture supernatants were collected at 24 and 48 h after infection and viral RNA levels were measured by RT-PCR using primers and probe targeting the SARS-CoV-2 N1 region and the SARS-CoV-2 N2 region. The cellular RNA extracted from infected cells was quantified, normalized and viral RNA levels per μg of total cellular RNA were calculated. The results were identical for both sets of primers showing dramatic reduction in viral RNA at both 24 and 48 h. SARS-CoV-2 infectivity titers were measured in cell culture supernatants at 48 h after infection by plaque assay. Data represent the mean ± SEM, representing two independent experiments conducted in duplicate, t-test p < 0.001.

represents a devastating inflammatory lung disorder due to cytokine storm that is associated with multiple-organ dysfunction leading to high mortality (Mehta et al., 2020; Sarzi-Puttal et al., 2020). Taken together, these studies suggest that auranofin could mitigate SARS-CoV-2 infection and associated lung damage due to its anti-viral, anti-inflammatory and anti-ROS properties.

We investigated the anti-viral activity of auranofin against SARS-CoV-2 and its effect on virus-induced inflammation in human cells. We infected HeLa cells with SARS-CoV-2 (USA-WA1/2020) at a multiplicity of infection (MOI) of 1 for 2 h, followed by the addition of 4 μM of auranofin. DMSO (0.1%) was used as control (the solvent was used to prepare drug stock). We used HeLa cells in this study as these cells are highly permissive for SARS-CoV-2 replication. Cell culture supernatants and cell lysates were collected at 24 and 48 h after infection. Virus RNA copies were measured by RT-PCR using two separate primers specific for the viral N1 region and N2 region (Roohan et al., 2015; Kumar et al., 2020). As depicted in Fig. 1, treatment of cells with auranofin resulted in a 70% reduction in the viral RNA in the supernatants compared to the DMSO at 24 h after infection. At 48 h, there was an 85% reduction in the viral RNA in the supernatants compared to the DMSO. Similarly, the levels of intracellular viral RNA decreased by 85% at 24 h and 95% at 48 h in auranofin-treated cells compared to the DMSO-treated cells. Both sets of primers showed nearly identical results. We next assayed virus titer in cell culture supernatants by plaque assay. Treatment with auranofin significantly reduced SARS-CoV-2 infectivity titers in cell culture supernatants at 48 h after infection (Fig. 1).

To determine the effective concentration of auranofin that inhibits 50% of viral replication (IC50), we treated SARS-CoV-2 infected HeLa cells with serial dilutions of auranofin. Supernatants and cell lysates were collected at 48 h after infection and viral RNA was quantified by RT-PCR. The data were plotted in graphs using non-linear regression
model (GraphPad software). At 48 h, there was a dose-dependent reduction in viral RNA levels in the auranofin-treated cells. Fig. 2 represents the EC50 values of auranofin treatment against SARS-CoV-2 infected Huh7 cells. Auranofin inhibited virus replication in the infected cells at EC50 of approximately 1.4 μM. It is important to note that in this study, we used 20 to 100 times more virus dose (MOI of 1) to infect the cells compared to the recently published reports on anti-viral activities of chloroquine, hydroxychloroquine and remdesivir against SARS-CoV-2 in vitro (Wang et al., 2020; Liu et al., 2020).

To assess the effect of auranofin on inflammatory response during SARS-CoV-2 infection, we measured the levels of key cytokines in auranofin and DMSO-treated cells at 24 and 48 h after infection (Natochar et al., 2019). SARS-CoV-2 infection induces a strong up-regulation of IL-6, IL-1β, TNFα, and NF-κB in Huh7 cells (Fig. 3). Treatment with auranofin dramatically reduced the expression of SARS-CoV-2-induced cytokines in Huh7 cells. SARS-CoV-2 infection resulted in a 100-fold increase in the mRNA expression of IL-6 at 48 h after infection compared to corresponding mock-infected cells. In contrast, there was only a 2-fold increase in expression of IL-6 in auranofin-treated cells. TNFα levels increased by 60-fold in the DMSO-treated cells at 48 h after infection, but this increase was absent in the auranofin-treated cells. Similarly, no increase in the expression of IL-1β and NF-κB was observed in the auranofin-treated cells.

Taken together, these results demonstrate that auranofin inhibits replication of SARS-CoV-2 in human cells at low micromolar concentration. We also demonstrate that auranofin treatment resulted in significant reduction in the expression of cytokines induced by virus infection. These data indicate that auranofin could be a useful drug to limit SARS-CoV-2 infection and associated lung injury. Further animal studies are warranted to evaluate the safety and efficacy of auranofin for the management of SARS-CoV-2-associated disease.

1. Methods

1.1. SARS-CoV-2 infection and drug treatment

In this study, we used a novel SARS-CoV-2 (USA WA1/2020) isolated from an oropharyngeal swab from a patient in Washington, USA (BEI NR-52258). Virus strain was amplified once in Vero E6 cells and had titers of $5 \times 10^6$ plaque-forming units (PFU)/mL. Huh7 cells (human liver cell line) were grown in DMEM (Gibco) supplemented with 5% heat-inactivated fetal bovine serum. Cells were infected with SARS-CoV-2 or PBS (Mock) at a multiplicity of infection (MOI) of 1 for 2 h (Natochar et al., 2019; Anour et al., 2019; Esm et al., 2019; Krause et al., 2019). Cells were washed twice with PBS and media containing different concentrations of auranofin (0.1–10 μM, Sigma) or DMSO (0.1%, Sigma) was added to cells. Supernatants and cell lysates were harvested at 24 and 48 h after infection. The cytopathicity of auranofin in Huh7 cells was measured using trypan blue dye method as described previously (Varghese and Rauschberg, 2014). Briefly, Huh7 cells were treated with different concentrations of auranofin (0.1–10 μM) for 48 h and percentage cell numbers were quantified using trypan blue.

1.2. Virus quantification

Virus infectivity titers were measured in cell culture supernatants by plaque formation assay using Vero cells as we described previously (Natochar et al., 2019). Virus RNA levels were analyzed in the supernatant and cell lysates by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA from cell culture supernatants was extracted using a Viral RNA Mini Kit (Qiagen) and RNA from cell lysate was extracted using a RNeasy Mini Kit (Qiagen) as described previously (Natochar et al., 2019). The cellular RNA extracted from infected cells was quantified, normalized and viral RNA levels per ng of total cellular RNA were calculated. qRT-PCR was used to measure viral RNA levels using previously published primers and probes specific for the SARS-CoV-2. Forward (5’-GACCACCAAAACTCAGGGAAT-3’), reverse (5’-CTCTCTTACTGACCATGCTC-3’), probe (5’-FAM-ACC CGGATACAGTGGGATACCC-3’), targeting the SARS-CoV-2 N1 region and Forward (5’-TTTAAAGTGTGAGACGTTC-3’), reverse (5’-GGGGAAGATGAGGACCACA-3’), probe (5’-FAM-AAGATGAGGACCACA-3’), targeting the SARS-CoV-2 N2 region (Integrated DNA Technologies). Viral RNA copies were determined after comparison with a standard curve produced using serial 10-fold dilutions of SARS-CoV-2 RNA (Kumar et al., 2017; Kim et al., 2018).
Table 1

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<th>Gene (Accession No.)</th>
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1.3. Cytokine analysis

For mRNA analysis of IL-6, IL-1β, TNFa, and NF-kB, cDNA was prepared from RNA isolated from the cell lysates using a Script™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), and qRT-PCR was conducted as described previously (Nairkar et al., 2019). The fold change in infected cells compared to corresponding controls was calculated after normalizing to the GAPDH gene. The primer sequences used for qRT-PCR are listed in Table 1.

Declaration of competing interest

Authors declare no conflict of interest.

Acknowledgements

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Hsu, S., Kim, K., Kim, K., Kwon, J., Lee, Y.H., Lee, C.K., Song, Y., Lee, S.J., Han, N., Kim, S., 2016. Auranofin Treatment Dramatically Reduced the Expression of SASB-CGO-2-induced Cytokines in Human Cells. mRNAs, levels of IL-6, IL-1β, TNFα, and NF-kB were determined using qRT-PCR at 24 and 48 h after infection. The fold change in infected cells compared to corresponding controls was calculated after normalizing to the GAPDH gene. Data represent the mean ± SdM, representing two independent experiments conducted in duplicate.
SARS-CoV-2 Variants of Concern Infect the Respiratory Tract and Induce Inflammatory Response in Wild-Type Laboratory Mice

Shannon Stone, Hussin Alwan Rothan, Janhavi Prasad Natekar, Pratima Kumari, Shaligram Sharma, Heather Pathak, Komal Arora, Tabassum Tasnim Auroni, and Mukes Kumar

Abstract: The emergence of new severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) variants of concern pose a major threat to public health, due to possible enhanced virulence, transmissibility, and immune escape. These variants may also adapt to new hosts, in part through mutations in the spike protein. In this study, we evaluated the infectivity and pathogenicity of SARS-CoV-2 variants of concern in wild-type C57BL/6 mice. Six-week-old mice were inoculated intranasally with a representative virus from the original B.1 lineage, or the emerging B.1.1.7 and B.1.351 lineages. We also infected a group of mice with a mouse-adapted SARS-CoV-2 (MA10). Viral load and mRNA levels of multiple cytokines and chemokines were analyzed in the lung tissues on day 3 after infection. Our data show that unlike the B.1 virus, the B.1.1.7 and B.1.351 viruses are capable of infecting C57BL/6 mice and replicating at high concentrations in the lungs. The B.1.351 virus replicated to higher titers in the lungs compared with the B.1.1.7 and MA10 viruses. The levels of cytokines (IL-6, TNF-α, IL-1β) and chemokine (CCL2) were upregulated in response to the B.1.1.7 and B.1.351 infection in the lungs. In addition, robust expression of viral nucleocapsid protein and histopathological changes were detected in the lungs of B.1.351-infected mice. Overall, these data indicate a greater potential for infectivity and adaptation to new hosts by emerging SARS-CoV-2 variants.

Keywords: COVID-19; SARS-CoV-2 variants; C57BL/6 mice; host-range; inflammation

1. Introduction

Coronaviruses are a family of positive-sense single-strand RNA viruses. Their large genomes and propensity for mutation have resulted in a diversity of coronavirus strains that are capable of adapting to new hosts. COVID-19, the disease caused by the new beta coronavirus, SARS-CoV-2, has caused significant human and economic burden [1–3]. As of 10 December 2021, the number of confirmed cases worldwide is over 269 million, with 5.29 million deaths. Few therapies are available to treat COVID-19 in humans, and the rapid evolution of SARS-CoV-2 variants threatens to diminish their efficacy [2,4]. The lineage B.1.1.7, first identified in the United Kingdom, and lineage B.1.351, first described in South Africa, have been termed variants of concern because of the greater risk they pose due to their possible enhanced transmissibility, disease severity and immune escape [4–7]. These variants may also adapt to new hosts, in part, through mutations on the receptor-binding domain (RBD) of the spike protein [6,7].

SARS-CoV-2 infection begins with the viral particles binding to the receptors on the host cell surface. The RBD of the spike protein binds to angiotensin-converting enzyme 2 (ACE-2), present on the host cellular surfaces [3,8]. The RBD of the spike protein from the SARS-CoV-2 strain (Wuhan strain, lineage B.1) that started the pandemic does not
efficiently bind mouse ACE2, and therefore wild-type laboratory mice are not susceptible to infection with lineage B1 virus [8–12]. A mouse-adapted SARS-CoV-2 variant (MA10) with binding affinity to mouse ACE2 has been obtained, after the sequential passaging of the virus in mouse lung tissue [11]. Infection of wild-type BALB/c mice with the MA10 virus resulted in replication in both the upper and lower airways [11,13]. The MA10 virus has several mutations, including multiple mutations in the spike protein compared with the Wuhan reference sequence. These mutations are also present in the B.1.1.7 lineage that first emerged in the UK, and the B.1.351 lineage that emerged independently of B.1.1.7 in South Africa. The B.1.1.7 variant has a mutation in the RBD of the spike protein, including N501Y, E69/70 deletion and P681H near the S1/S2 furin cleavage site. The B.1.351 variant has eight mutations, most notable are the three mutations (K417N, E484K and N501Y) in the RBD of spike protein [6,7,11,14,15]. This is associated with critical public health importance, since these mutations have been implicated in an increased recognition of mouse ACE2, hinting that the naturally emerging SARS-CoV-2 variants, including B.1.1.7 and B.1.351 may have evolved to infect rodents. However, the infectivity and pathogenicity of these emerging variants in mice have not yet been determined.

In this study, we evaluated the replication and pathogenicity of the original B.1 lineage and emerging SARS-CoV-2 lineages, B.1.1.7 and B.1.351, in wild-type C57BL/6 mice. We also used a mouse-adapted SARS-CoV-2 variant (MA10) that causes disease in the wild-type mice [11]. Our data show that the B.1.1.7 and B.1.351 viruses are capable of infecting wild-type C57BL/6 mice and replicating at high concentrations in the lungs. The B.1.351 virus replicated to higher titers in the lungs compared with the B.1.1.7 and MA10 viruses. We found that infection with the B.1.351, B.1.1.7 and MA10 viruses trigger an inflammatory response in the lungs characterized by upregulation of inflammatory cytokines and chemokines, and infiltration of leukocytes.

2. Materials and Methods

2.1. Animal Infection Experiments

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All the animal experiments were conducted in a certified Animal Biosafety Level 3 (ABSL-3) laboratory at Georgia State University (GSU). The protocol was approved by the GSU Institutional Animal Care and Use Committee (Protocol number A20044). Six-week-old C57BL/6 mice were inoculated intranasally with PBS (mock) or 10^7 plaque-forming units (PFU) of SARS-CoV-2 as described previously [16]. We used B.1 Wuhan virus (BEI# NR-52281), B.1.1.7 virus (BEI# NR-54000), B.1.351 virus (BEI# NR-54008) and MA10 virus (BEI# NR-55329). Roughly equal numbers of male and female mice were used. Animals were weighed and their appetite, activity, breathing and neurological signs were assessed twice daily. In independent experiments, mice were inoculated with PBS (Mock) or SARS-CoV-2 intranasally, and on day 3 after infection, animals were anesthetized using isoflurane and perfused with cold PBS. The lungs were collected and flash-frozen in 2-methylbutane (Sigma, St. Louis, MO, USA) for further analysis, as described below [16,17]. Alternatively, mice were perfused with PBS, followed by 4% paraformaldehyde (PFA), and tissues were harvested, cryoprotected in 30% sucrose (Sigma, St. Louis, MO, USA), and embedded in optimum cutting temperature (OCT), as described previously [16,17].

2.2. Quantification of the Virus Load

The virus titers were analyzed in the lungs by plaque assay and quantitative real-time PCR (qRT-PCR). Briefly, frozen tissues were weighed and homogenized in a bullet blender (Next Advance, Averill Park, NY, USA) using stainless steel beads. Virus titers in tissue homogenates were measured by plaque assay using Vero cells [16,17]. Quantitative RT-PCR was used to measure viral RNA levels with primers and probes specific for the SARS-CoV-2 N gene as described previously [18]. Viral genome copies were determined by comparison to a standard curve generated using a known amount of RNA extracted from previously titrated SARS-CoV-2 samples [18]. Frozen tissues harvested from mock and
infected animals were weighed and lysed in RLT buffer (Qiagen), and RNA was extracted using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA) [17,19]. Total RNA extracted from the tissues was quantified and normalized, and viral RNA levels per μg of total RNA were calculated.

2.3. Analysis of Cytokines and Chemokines

Total RNA was extracted from the lungs using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA). cDNA samples were prepared using an iScript™ cDNA Synthesis Kit (Bio-Rad). The expression levels of multiple host genes were determined using qRT-PCR, and the fold change in infected lungs compared to the mock-infected controls was calculated after normalizing each sample to the level of the endogenous GAPDH gene mRNA [16,17,19]. The primer sequences used for qRT-PCR are listed in Table 1.

Table 1. Primer sequences used for qRT-PCR.

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2.4. Histopathological Analysis

Lung sections were stained with hematoxylin and eosin (HE) for histopathological evaluation [16,17]. Additionally, tissue sections were incubated with anti-SARS-CoV-2 nucleocapsid antibody (Thermo Fisher: Scientific, Norcross, GA, USA) overnight at 4°C, followed by incubation with Alexa Fluor 555-conjugated secondary antibody (Invitrogen) for 1 h at room temperature. Images were acquired using the Invitrogen™ EVOS™ MS5000 Cell Imaging System (Thermo Fisher Scientific, Norcross, GA, USA).

2.5. Immunoblot Analysis

Total cellular protein was extracted from mouse lungs and separated by SDS-PAGE, transferred onto PVDF membranes and incubated overnight with polyclonal antibody against IL-6 (Thermo Fisher Scientific, Norcross, GA, USA) [16,17]. Membranes were stripped and re-probed with antibodies against GAPDH (Protein-tech). Following incubation with the secondary antibodies conjugated with IRDye 800 and IRDye 680 (Li-Cor Biosciences, Lincoln, NE, USA), the membranes were scanned using the Odyssey infrared imager (Li-Cor Biosciences) [16,17].

2.6. Statistical Analysis

Mann-Whitney tests and unpaired student t-tests using GraphPad Prism 5.0 were used to calculate the p values of the difference between viral titers and immune responses, respectively. Differences of p < 0.05 were considered significant.

3. Results

3.1. B.1.1.7 and B.1.351 Variants Replicate in the Lungs of C57BL/6 Mice

We inoculated six-week-old C57BL/6 mice with 10⁵ PFU of SARS-CoV-2 or PBS (mock) via the intranasal route. We used a representative virus from the original B.1 lineage, or
the emerging B.1.1.7 and B.1.351 lineages. We also infected a group of mice with a mouse-adapted SARS-CoV-2 variant (MA10) [11]. Mice were monitored daily for weight loss. Mice infected with B.1 virus exhibited no changes in body weight. However, B.1.1.7-, B.1.351- and MA10-infected mice had approximately 10% body-weight loss at days 2–4 after infection (Figure 1). To evaluate virus replication in the lungs, groups of 5–8 mice were euthanized at 3 days after infection, and the lungs were collected. Viral infectivity titers in the lungs were measured by plaque assay. The virus was not detected in the mock-infected lungs. High levels of infectious virus were detected in the lungs of all the MA10-infected mice (10⁶ PFU/gram) (Figure 2A). By contrast, little, if any, infectious virus was detected in the lungs of the B.1-infected mice suggesting limited virus replication. High levels of infectious virus were detected in the lungs of both B.1.1.7- (10⁴ PFU/gram) and B.1.351-infected mice (10⁶ PFU/gram). The viral load was significantly lower for the B.1.1.7 compared with the B.1.351 virus. Virus titers in the lungs of the B.1.351-infected mice were higher than the MA10-infected mice, but the difference was not statistically significant. Intracellular SARS-CoV-2 RNA levels assessed by qRT-PCR followed a similar pattern. The viral RNA levels in the B.1.1.7-, B.1.351- and MA10-infected groups were significantly higher than those in the B.1-infected group (Figure 2B). These data indicate that MA10, B.1.1.7 and B.1.351 viruses replicated efficiently in the lungs of the infected animals, and the B.1.351 virus replicated to high titers in the lungs of mice compared with the other groups.

Body Weight

![Body Weight Graph](image)

**Figure 1.** Analysis of body weight in C57BL/6 mice following SARS-CoV-2 infection. Six-week-old C57BL/6 mice were inoculated intranasally with 10⁶ plaque-forming units (PFU) of SARS-CoV-2 variants (n = 8–10 mice per group). Percentage of initial weight for SARS-CoV-2-infected mice over 6 days. Values are the mean ± SEM.

A

![Graph A](image)

B

![Graph B](image)

**Figure 2.** Replication of SARS-CoV-2 variants in the lungs. Six-week-old C57BL/6 mice were inoculated intranasally with PBS (mock) or 10⁵ plaque-forming units (PFU) of SARS-CoV-2 variants. Groups of 5–8 mice were euthanized at 3 days after infection and lungs were collected. Virus titers were analyzed in the lungs by (A) plaque assay and (B) qRT-PCR. The data are expressed as PFU/g of tissue or genome copies/µg of RNA. Each data point represents an individual mouse. *, p < 0.05; **, p < 0.001.
3.2. B.1.1.7 and B.1.351 Variants Induce an Inflammatory Response in the Lungs

The excessive inflammatory host response to SARS-CoV-2 infection contributes to pulmonary pathology and the development of respiratory distress in some COVID-19 patients [20,21]. To compare the host responses in SARS-CoV-2-infected mice, we investigated changes in the mRNA levels of IL-6, TNF-α, IL-1β and CCL2 in the lungs of mice infected with SARS-CoV-2. Gene expression changes in the lungs of infected mice, compared with the mock-infected controls, were analyzed by qRT-PCR. No significant increase in the mRNAs of the cytokines or chemokines tested was observed in the B.1-infected mice. MA10 infection resulted in a 20-fold increase in IL-6 and CCL2 mRNA expression (Figure 3). IL-1β and TNF-α mRNA levels increased by 4–6-fold in the lungs of the MA10-infected mice. The B.1.351 infection resulted in a >25-fold increase in IL-6 and CCL2 mRNA expression. Similarly, TNF-α mRNA levels increased by 10-fold in the B.1.351-infected mice. There was also a 6-fold increase in IL-1β mRNA in the B.1.351-infected mice. In the B.1.1.7-infected mice, the mRNA levels of the IL-6 and CCL2 were elevated by 10-fold. Although the expression of IL-1β mRNA did not increase, a modest 3-fold increase in the TNF-α mRNA level was observed in the lungs of the B.1.1.7-infected mice. Inflammatory response observed in the B.1.351-infected group was significantly higher than in the B.1.1.7- and B.1-infected group. We also measured the protein levels of IL-6 in mock- and B.1.351-infected mice at day 3 after infection. Immunoblotting data showed an increase in the protein levels of IL-6 in the B.1.351-infected lungs, compared with the mock-infected controls (Figure 4). These results indicate that infection with B.1.351 and B.1.1.7 variants upregulates the expression of inflammatory genes in the lungs.

Figure 3. Analysis of inflammatory cytokine and chemokine levels in the lungs of SARS-CoV-2-infected mice. The mRNA levels of IL-6, CCL2, TNF-α and IL-1β genes were determined by qRT-PCR. The fold change in the infected tissues compared to the corresponding mock-infected controls was calculated after normalizing individual samples to GAPDH levels. Values are the mean ± SEM (n = 5–8 mice per group). *, p < 0.05; **, p < 0.001.
Figure 4. Analysis of protein levels of IL-6 in the lungs of B.1.351-infected mice. (A) Protein extracted from mock- and B.1.351-infected lung tissues (day 3 after infection) were immunoblotted with antibodies against IL-6 or GAPDH. Data are representative of five animals per group. (B) Quantitative analysis of Western blot results represented as fold-change compared to GAPDH. **, p < 0.001 as compared to mock controls (n = 5 mice per group).

We next analyzed lung sections from mock- and B.1.351-infected mice for antigen distribution, infiltration of immune cells and other pathological changes. Immunostaining for the SARS-CoV-2 nucleocapsid revealed intense staining at day 3 after infection, consistent with the lung viral titer data (Figure 5). We also stained the lung sections with hematoxylin and eosin (HE) for histopathological evaluation. The histopathological features of the lungs infected with B.1.351 virus showed multifocal lesions with abundant leukocyte infiltration, hemorrhages and interstitial thickening. Representative images of lung sections from mock- or B.1.351-infected lungs on day 3 after infection are shown in Figure 6.

Figure 5. Detection of SARS-CoV-2-infected cells in the lungs of B.1.351-infected mice. Lung sections from mock- and B.1.351-infected mice (day 3 after infection) were stained for SARS-CoV-2 nucleocapsid protein. Representative immunostaining images showing the presence of SARS-CoV-2 nucleocapsid protein (red) in the B.1.351-infected mice. Nuclei are stained with DAPI (blue). The boxed areas in the top row of panels (20×) are enlarged in the bottom row of panels (40×). The photomicrographs shown are representative of the images obtained from five animals.
4. Discussion

This study demonstrates that wild-type laboratory mice are susceptible to infection with the emerging SARS-CoV-2 variants. While the B.1 virus was unable to infect C57BL/6 mice, the B.1.351 and B.1.1.7 viruses efficiently infected the C57BL/6 mice. Enhanced virus replication in the B.1.351- and B.1.1.7-infected mice was accompanied by elevated cytokine and chemokine levels and infiltration of leukocytes in the lungs.

Mice are a useful small animal model for the evaluation of vaccines, immunotherapies, and antiviral drugs [8,12]. As the initial SARS-CoV-2 strains did not utilize murine ACE-2 as a receptor, wild-type mice are not susceptible to SARS-CoV-2 infection [8–10,12]. Transgenic mice that express human ACE-2 often develop severe and fatal disease upon intranasal inoculation of virus [16,22]. The initially available SARS-CoV-2 isolates require adaptation to use the mouse ACE-2 entry receptor, and to productively infect the cells of the murine respiratory tract. The mouse-adapted strain of SARS-CoV-2 (MA10) causes infection, inflammation and pneumonia in BALB/c mice after intranasal inoculation [11,13]. MA10 has several mutations, including the N501Y mutation in the RBD of the spike protein, compared with the Wuhan reference sequence, which also appears in the B.1.351 and B.1.1.7 variants [6,7,11,23]. These mutations in the RBD of the spike protein may have enhanced the binding affinity for the endogenous mouse ACE-2 receptor, thereby allowing the variants to replicate efficiently in mice. Indeed, studies have shown that SARS-CoV-2 variants containing N501Y and E484K mutations display a substantially enhanced infection of mouse cells. Experimental studies using S glycoprotein and pseudo type viruses have shown that SARS-CoV-2 variants are significantly more infective toward cells expressing murine ACE2 [23–26].

Although both B.1.351 and B.1.1.7 viruses were able to replicate in the mice lungs, B.1.351 virus inoculation yielded significantly higher viral load in the lungs than the B.1.1.7 virus. Both B.1.1.7 and B.1.351 variants shared the N501Y mutation that has been suggested to be associated with mouse adaptation [27]. Other differences between virus lineages
might also play a role in the resulting phenotype in mice. Apart from the N501Y mutation, several amino acid substitutions including, K417N, E484K, Q493H/K, and Q498H were also suggested to be critical for SARS-CoV-2 adaptation in murine species. It has been shown that the presence of E484K and K417N mutations in B.1.351 increase the infectivity of B.1.351, and E484K has also been identified as an immune escape mutation that emerges during exposure to antibodies [28]. This is congruous with our observation that B.1.351 virus replicated to a higher level than B.1.1.7 virus.

It has been suggested that a cytokine storm is involved in the pathogenesis of severe COVID-19 cases [20,21]. The levels of many cytokines and chemokines have been found to be increased after SARS-CoV-2 infection in humans. In the present study, we show that B.1.351 and B.1.1.7 infection induced significantly higher levels of cytokines and chemokine expression in the lungs. Inflammatory response observed in the B.1.351-infected group was higher compared with the other groups. In this study, we did not conduct a comprehensive transcriptome analysis of mice lungs that were infected with the B.1.351 and B.1.1.7 viruses. However, other recent studies have shown that intranasal infection of K18-hACE2 mice with B.1.1.7 and B.1.351 variants resulted in distinct arrays of cytokine response than those induced by early SARS-CoV-2 strains. Several myeloid cell chemoattractants showed enhanced pulmonary secretions in K18-hACE2 mice following exposures to B.1.1.7 or B.1.351 variant [29,30]. These findings are consistent with previous studies in humans, which established a consistent link between the mutations exhibited by the B.1.351 lineage and a greater potential for infectivity and immune escape [4,6,24]. More studies are needed to characterize the pathological consequences of infection with these variants in different mouse strains, and mice with co-morbid conditions. It is possible that more severe conditions could be observed in mice with co-morbid conditions such as old age, diabetes and hypertension. The ability of SARS-CoV-2 variants to replicate and induce inflammation in wild-type mice will facilitate studies to evaluate therapeutic interventions and pathogenesis studies using transgenic mouse strains. These data indicate the possibility of adaptation to new animal species by emerging SARS-CoV-2 variants.


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References


Differential Pathogenesis of SARS-CoV-2 Variants of Concern in Human ACE2-Expressing Mice

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Abstract: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the current pandemic, resulting in millions of deaths worldwide. Increasingly contagious variants of concern (VoC) have fueled recurring global infection waves. A major question is the relative severity of the disease caused by previous and currently circulating variants of SARS-CoV-2. In this study, we evaluated the pathogenesis of SARS-CoV-2 variants in human ACE2-expressing (K18-hACE2) mice. Eight-week-old K18-hACE2 mice were inoculated intranasally with a representative virus from the original B.1 lineage or from the emerging B.1.1.7 (alpha), B.1.351 (beta), B.1.617.2 (delta), or B.1.529 (omicron) lineages. We also infected a group of mice with the mouse-adapted SARS-CoV-2 (MA10). Our results demonstrate that B.1.1.7, B.1.351 and B.1.617.2 viruses are significantly more lethal than the B.1 strain in K18-hACE2 mice. Infection with the B.1.1.7, B.1.351, and B.1.617.2 variants resulted in significantly higher virus titers in the lungs and brain of mice compared with the B.1 virus. Interestingly, mice infected with the B.1.529 variant exhibited less severe clinical signs and a high survival rate. We found that B.1.529 replication was significantly lower in the lungs and brain of infected mice in comparison with other VoC. The transcription levels of cytokines and chemokines in the lungs of B.1- and B.1.529-infected mice were significantly less when compared with those challenged with other VoC. Together, our data provide insights into the pathogenesis of previous and circulating SARS-CoV-2 VoC in mice.

Keywords: COVID-19; SARS-CoV-2 variants; omicron; ACE2-expressing mice; inflammation

1. Introduction

SARS-CoV-2 is a positive-sense, single-stranded RNA virus belonging to the Betacoronavirus family [1,2]. Since the emergence of SARS-CoV-2 in late 2019, several new variants of concern (VoC), alpha (B.1.1.7 lineage), beta (B.1.351 lineage), gamma (P.1 lineage), delta (B.1.617.2 lineage), and omicron (B.1.529 lineage), have fueled recurring global infection waves. These variants have been termed VoC because of the higher risk due to their possible enhanced transmissibility, disease severity, immune escape, and increased adaptation to new hosts [3–8]. Mutations occurring in the spike protein are of major concern due to the role of this glycoprotein in mediating virus entry and as the major target of neutralizing antibodies [3,9–11]. The lineage B.1.1.7 was first identified in the United Kingdom, lineage B.1.351 was discovered in South Africa, and lineage B.1.617.2 was first described in India. Most recently, the omicron (B.1.529) VoC that emerged in South Africa was estimated to have been responsible for the majority of infections worldwide. The B.1.1.7 variant has mutations in the receptor-binding domain (RBD) region, including N501Y, 69/70 deletion, and P681H near the S1/S2 furin cleavage site [7,12–14]. The B.1.351 variant has eight mutations, of which the three most notable mutations are K417N, E484K, and N501Y in the
spike protein [3,7,9,15]. The B.1.617.2 variant has three unique mutations: E156del/R158G in the N-terminal domain and T478K in RBD of the spike protein. The B.1.1.529 variant has an unusually large number of mutations in the spike protein, including 30 amino acid substitutions, 3 short deletions, and one insertion [16–18].

The main goal of this study was to compare the replication and pathogenesis of SARS-CoV-2 variants in K18-hACE2 mice. K18-hACE2 is a transgenic mouse model that expresses human ACE2 driven by the human cytokeratin 18 promoter. K18-hACE2 mice is a well-established model for SARS-CoV-2 studies that supports virus replication in the respiratory and central nervous systems, resulting in elevated chemokine and cytokine levels, and significant tissue pathologies [19–21]. Our results demonstrate that the B.1.1.7 (alpha), B.1.351 (beta), and B.1.617.2 (delta) variants are more virulent than the original SARS-CoV-2 B.1. Wuhan strain in K18-hACE2 mice. Infection with the B.1.1.7, B.1.351, and B.1.617.2 variants resulted in significantly high virus titers in the lungs and brain of mice compared with the B.1 virus. Interestingly, the replication capacity of the omicron variant was significantly lower than other VoC. Mice infected with the B.1.529 virus exhibited high survival rate and had a lower virus load in the lungs and brain compared with mice infected with the B.1.1.7, B.1.351, and B.1.617.2 viruses. In addition, B.1- and B.1.529-infected mice had significantly attenuated inflammation in the lungs compared with those inoculated with other VoC.

2. Materials and Methods
2.1. In Vivo Mouse Challenge Experiments

In vivo mouse experiments involving infectious SARS-CoV-2 were performed in Animal Biosafety Level 3 laboratory and strictly followed the approved standard operation procedures. The protocol was approved by the Georgia State University Institutional Animal Care and Use Committee (Protocol number A20044). Hemizygous K18-hACE2 mice (286.Cg-Tg (K18-ACE2)2Primm /J) were obtained from The Jackson Laboratory. Eight-week-old hemizygous K18-hACE2 mice were inoculated intranasally with PB5 (mock) or 106 plaque-forming units (PFU) of SARS-CoV-2, as described previously [8,21,22]. We used the B.1 Wuhan virus (BE# NR-52281), B.1.1.7 virus (BE# NR-54000), B.1.351 virus (BE# NR-54008), B.1.617.2 virus (Northwestern Reference laboratory, Clinical isolate #2333067), B.1.1.529 virus (BE# NR-56461), and MA10 virus (BE# NR-55329). Roughly equal numbers of male and female mice were used. The animals were weighed, and their appetite, activity, breathing, and neurological signs were assessed twice daily. In independent experiments, mice were inoculated with PB5 (Mock) or SARS-CoV-2 intranasally, on days 3 and 5–7 after infection, the animals were anesthetized using isoflurane and perfused with cold PB5. The lungs and brain were collected and flash-frozen in 2-methylbutane (Sigma, St. Louis, MO, USA) for further analysis, as described below [8,23].

2.2. Infectious Virus Titration by Plaque Assay

Tissues harvested from virus-infected animals were weighed and homogenized in a bullet blender (Next Advance, Averill Park, NY, USA) using stainless steel or zirconium beads, followed by centrifugation and titration. Virus titers in tissue homogenates were measured by plaque assay using Vero E6 cells [8]. To titrate the infectious virus, tissue homogenates were 10-fold serially diluted with DMEM and applied to monolayered Vero E6 cells for 1 h. After inoculation, the cells were washed once before overlayed with 1% low-melting agarose. The cells were further incubated for 48 h and stained with neutral red for visualizing plaque formation [8,24].

2.3. RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from the lungs using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA). The cDNA was synthesized from RNA using an iScript™ cDNA Synthesis Kit (Bio-Rad). The qRT-PCR was used to determine the expression levels of IL-6 and CCL-2, as described previously [22,24]. The fold-change in infected samples
compared with control samples was calculated after normalizing to the housekeeping GAPDH gene [22,23]. The primer sequences used for qRT-PCR are listed in Table 1.

Table 1. Primer sequences used for qRT-PCR.

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2.4. Statistical Analysis

GraphPad Prism 8.0 was used to perform a Kaplan–Meier log-rank test to compare survival curves. For body weight changes, two-way analysis of variance (ANOVA) with the post hoc Bonferroni test was used to calculate the p values. The Mann–Whitney test and unpaired student t-test were used to calculate the p values of the difference between viral titer and immune responses, respectively. Differences of p < 0.05 were considered significant.

3. Results

3.1. Clinical Disease Progression of K18-hACE2 Mice Infected with SARS-CoV-2 VoC

To evaluate the pathogenicity of the original B.1 lineage and emerging SARS-CoV-2 VoC derived from the B.1 lineage, K18-hACE2 mice were infected intranasally with a representative virus from the original B.1 lineage or the emerging B.1.1.7 (alpha), B.1.351 (beta), B.1.617.2 (delta), and B.1.1.529 (omicron) lineages. We also infected a group of mice with the mouse-adapted SARS-CoV-2 (MA10) [25]. The animals were monitored for clinical signs and survival. The mock-infected mice remained healthy throughout the study period. While infectious doses of 10⁸ plaque-forming units (PFU) of B.1 virus resulted in 75% mortality, mortality in the B.1.1.7-, B.1.351-, and B.1.617.2-infected mice was 100% (Figure 1A). The median survival times of mice infected with the alpha, beta, and delta variants were also shorter than those in the B.1-infected mice. Statistically, mouse survival for the B.1 virus was significantly higher than the alpha, beta, and delta variants. K18-hACE2 mice infected with the MA10 virus showed a faster disease progression and severity after infection compared with all SARS-CoV-2 clinical isolates. Interestingly, infection with the B.1.1.529 (omicron) virus resulted in only 50% mortality with extended survival time in mice (Figure 1A). There was a significant difference between the survival of the B.1.1.529 challenged mice compared with the other VoC at the same dose.

As early as 3 days after infection, mice inoculated with the B.1.1.7 or B.1.351 virus began to lose body weight and showed signs of infection. By 6 days, all mice infected with the B.1.1.7 or B.1.351 virus died after losing 20% body weight and experiencing severe symptoms (Figure 1B). Mice infected with the B.1.617.2 also lost significant body weight, and all succumbed to death by day 7 after infection. Statistically, body weight loss for the B.1.1.7, B.1.351, and B.1.617.2 viruses was significantly higher than that for the B.1 virus infections. Compared with the other VoC, a body weight loss of mice infected by the B.1.1.529 virus was significantly milder with onset time at a later stage during the infection (Figure 1B).

3.2. Viral Load in K18-hACE2 Mice Infected with SARS-CoV-2 VoC

To evaluate virus replication in the tissues, groups of 3–7 mice were euthanized at 3- and 5-7 days after infection, and the lungs and brain were collected. Viral infectivity titer in the tissues were measured by a plaque assay. A median infectious virus titer of
$5 \times 10^5$ PFU/g was detected at day 3 after infection in the lungs from the animals infected with the B.1 virus. Compared with the B.1 virus, infections with B.1.1.7, B.1.351, and B.1.617.2 viruses resulted in significantly higher levels of infectious virus in the lungs at day 3 after infection (Figure 2A). On days 5–7 after infection, mice infected with the B.1.1.7, B.1.351, and B.1.617.2 viruses sustained significantly high levels of viral load in the lungs compared with the B.1 virus (Figure 2B). In contrast, the replication of the B.1.1.529 virus was dramatically reduced in comparison with those of B.1.1.7, B.1.351, and B.1.617.2 viruses, despite using the same inoculation titer for virus challenge. The level of infectious virus in the lungs of B.1.1.529-infected mice was approximately 100-fold lower than in the animals infected with other VoC at both 3 and 5–7 days after infection (Figure 2A, B).

**Figure 1.** Analysis of survival and body weight in K18-hACE2 mice following infection with SARS-CoV-2 VoC. Eight-week-old K18-hACE2 mice were inoculated intranasally with PBS (mock) or $10^4$ PFU of B.1 and individual variants ($n = 10–15$ mice per group). (A) Survival curve. (B) Body weight change in percentage. Values are the mean ± SEM.
Figure 2. Replication of SARS-CoV-2 VoC in the lungs and brain. Eight-week-old K18-hACE2 mice were inoculated intranasally with 10^9 PFU of SARS-CoV-2 or variants. Groups of 3–7 mice were euthanized on day 3 and days 5–7 after infection, and tissues were collected. Virus titers were analyzed in the lungs and brain by plaque assay. The data are expressed as PFU/g of tissue. (A) Virus titer in day 3 lungs, (B) virus titer in day 5–7 lungs, (C) virus titer in day 3 brain, and (D) virus titer in day 5–7 brain. Each data point represents an individual mouse. *, p < 0.05.

In the brain, mice infected with the B.1 virus exhibited significantly lower levels of infectious virus than B.1.1.7, B.1.351, and B.1.617.2-infected animals at day 3 after infection (Figure 2C). At 5–7 days after infection, the viral loads were similar in animals infected with the B.1, B.1.1.7, B.1.351, and B.1.617.2 viruses (Figure 2D). Consistent with the lung data, the infectious virus in the brain harvested from the B.1.1.529-infected mice was also
significantly reduced compared with that of the other groups. We did not detect any infectious virus in the B.1.1.529-infected mice at day 3 after infection. On 5–7 days after infection, the viral load was approximately 1000-fold higher in the B.1.1.7-, B.1.351-, and B.1.617.2-infected mice than the B.1.1.529-infected mice (Figure 2C,D). We did not detect any infectious virus in the lungs and brain of B.1 and B.1.1.529-infected mice that survived the infection. A relative decrease in viral replication of the B.1 and B.1.1.529 lineages when comparing the data from lungs and brain after 3 days and 5–7 days of infection (Figure 2) was correlated with the observed decline in clinical and pathological severity and recovery seen in infected mice in these groups (Figure 1).

3.3. Inflammation in the Lungs following Infection with SARS-CoV-2 VoC

The excessive inflammatory host response to SARS-CoV-2 infection contributes to pulmonary pathology and the development of respiratory distress in patients infected with COVID-19 [26,27]. We next quantified the gene expression of IL-6 and CCL-2 in the lungs of K18-hACE2 mice at day 3 after infection. Gene expression changes in the lungs of infected mice, compared with the mock-infected controls, were analyzed after normalizing each sample to the level of the endogenous GAPDH gene. We did not find any significant change in the expression level of the GAPDH gene as a consequence of viral inflammation. As shown in Figure 3, infections with the B.1.1.7, B.1.351, B.1.617.2, and MA10 viruses resulted in >100-fold increases in IL-6 and CCL-2 mRNA expression. In contrast, the IL-6 and CCL-2 mRNA levels increased by 60–75-fold in the lungs of the B.1-infected mice. Mice inoculated with the omicron variant had the lowest mRNA levels of IL-6 and CCL-2 (10–20-fold) compared with those inoculated with other VoC and the B.1 virus, suggesting attenuated inflammation (Figure 3).

![Graphs of IL-6 and CCL-2 mRNA expression](image)

Figure 3. Analysis of inflammatory response in the lungs. Eight-week-old K18-hACE2 mice were inoculated intranasally with PBS (mock) or 10^6 PFU of SARS-CoV-2 or variants (n = 5–7 mice per group). Lungs and brain were harvested after extensive perfusion with PBS and RNA was extracted. The mRNA levels of (A) IL-6 and (B) CCL-2 were determined by qRT-PCR. Values are the mean ± SD.

* p < 0.05, ** p < 0.001.

4. Discussion

To date, most K18-hACE2 mouse studies have utilized the original viral strains of SARS-CoV-2 and few studies have been performed with emergent VoC. In this study, we investigated the pathogenicity of emerging SARS-CoV-2 VoC derived from the B.1 lineage...
in K18-hACE2 mice. Our results demonstrate that the pathogenicity of SARS-CoV-2 in K18-hACE2 mice is VoC-dependent and the highest for the alpha, beta, and delta variants. We found significantly higher virus titers in the lungs and brain of mice infected with the B.1.1.7, B.1.351, and B.1.617.2 variants compared with the B.1 lineage. In contrast, the omicron variant replicated significantly less efficiently than other SARS-CoV-2 variants in mice. In comparison with the alpha, beta, and delta variants, the omicron variant results in less body weight loss and a lower mortality rate. This is also reflected by less extensive inflammatory processes in the lungs of the B.1.1.529-infected mice.

SARS-CoV-2 evolves rapidly with the accumulation of mutations in the viral genome, giving rise to multiple variants of concern (VoC) [4,5,10]. Among all mutations, N501Y is the most critical because it involves amino acid residues that account for the tight binding of RBD of the SARS-CoV-2 and ACE2 receptors on the host cell surface [7,10,11]. The initial strains of SARS-CoV-2 had spike proteins unable to utilize mouse ACE2 and infect standard laboratory mice. To overcome this barrier, a mouse-adapted SARS-CoV-2 variant (MA10) with binding affinity to mouse ACE-2 was obtained after sequential passaging of virus in mouse lung tissues [25,28]. The MA10 virus causes infection, inflammation, and pneumonia in BALB/c mice after intranasal inoculation. MA10 has several mutations, including the N501Y mutation in the RBD of the spike protein compared with the Wuhan reference sequence [25,28]. B.1.1.7 and B.1.351 lineages contain N501Y in RBD in addition to widespread D614G in a spike protein, while the B.1.351 variant also has two additional mutations: K417N and E484K [10,12,13,15]. Both N501Y and D614G have been shown to increase RBD binding to ACE2 and to promote virus entry and replication in humans and animal models [37–11]. These mutations in the RBD of the spike protein may have enhanced the binding affinity for the ACE2 receptor, thereby allowing the variants to replicate more efficiently in mice. Indeed, our results demonstrate that SARS-CoV-2 variants B.1.1.7 and B.1.351 containing N501Y and E484K mutations display a substantially severe pathogenicity in K18-hACE2 mice. B.1.1.7 and B.1.351 caused lethal disease in K18-hACE2 mice accompanied with higher-titer viral burden in the lungs and brain. The B.1.617.2 (delta) lineage does not harbor the N501Y substitution in the spike protein but has additional mutations within the spike protein that diverge from other VoC. In the B.1.617.2 lineage, two modifications, namely L452R and T478K in the RBD, increase the interaction with ACE2 with the highest binding affinity [29,30], which may contribute to an increase in pathogenicity observed in mice.

Our results corroborated the recent findings that the alpha, beta, and delta variants were able to cause enhanced disease in K18-hACE2 mice [6,14,31,32]. In the present study, we did not conduct a comprehensive transcriptome analysis of mice lungs and brain that were infected with SARS-CoV-2 variants. However, other recent studies have shown that intranasal infection of K18-hACE2 mice with B.1.1.7 and B.1.351 variants result in distinct tissue-specific proinflammatory cytokine signatures and a lack of extensive pulmonary hypoxia signaling before death. Several myeloid cell chemo-attractants showed enhanced pulmonary secretions in K18-hACE2 mice following exposure to the B.1.1.7 or B.1.351 variants [6,31]. It remains unclear whether these distinct cytokine profiles are due to enhanced replication of the B.1.1.7 and B.1.351 viruses in mice or are caused by specific mutations in these variants. Recent studies have also shown reduced leukocyte infiltration to the lungs during B.1.617.2 infection, suggesting that this variant may have a novel mechanism to evade immune response [32].

Despite carrying the highest number of mutations that might allow for more efficient binding to ACE2, we observed mild disease in mice infected with the omicron variant compared with other SARS-CoV-2 variants. Epidemiological data also suggest that the omicron virus causes a less severe pathology in humans than the ancestral strains and the other VoC [29]. Our results agree with recent studies that demonstrated attenuated replication of the omicron variant in mice and hamsters [16,18]. Recently, in vitro studies have shown that the omicron variant is inefficient in transmembrane serine protease 2 (TMPRSS2) usage in comparison with that of previous variants [33,34]. It is possible
that the attenuated replication of the omicron variant in human cells and mice is due to a reduced efficiency in host protease cleavage by changes in the spike protein. However, other species-specific factors may play a role in attenuated pathogenicity of the omicron variant observed in hACE2 mice. One possibility is that the omicron variant uses alternative (ACE2-independent) infection routes and that hACE2-expressing mice are a less effective model for this variant. Overall, our study demonstrates that SARS-CoV-2 pathogenicity in K18-hACE2 mice is VγC-dependent; is the highest for the alpha, beta, and delta variants; and is the lowest for the omicron variant. These results will be valuable for understanding the pathogenesis of emerging SARS-CoV-2 variants.


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Influenza Virus-like Particle-Based Hybrid Vaccine Containing RBD Induces Immunity against Influenza and SARS-CoV-2 Viruses


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Abstract: Several approaches have produced an effective vaccine against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Since millions of people are exposed to influenza virus and SARS-CoV-2, it is of great interest to develop a two-in-one vaccine that will be able to protect against infection of both viruses. We have developed a hybrid vaccine for SARS-CoV-2 and influenza viruses using influenza virus-like particles (VLP) incorporated by protein transfer with glycosylphosphatidylinositol (GPI)-anchored SARS-CoV-2 RBD fused to GM-CSF as an adjuvant. GPI-RBD-GM-CSF fusion protein was expressed in CHO-S cells, purified and incorporated onto influenza VLPs to develop the hybrid vaccine. Our results show that the hybrid vaccine induced a strong antibody response and protected mice from both influenza virus and mouse-adapted SARS-CoV-2 challenges, with vaccinated mice having significantly lower lung viral titers compared to naive mice. These results suggest that a hybrid vaccine strategy is a promising approach for developing multivalent vaccines to prevent influenza A and SARS-CoV-2 infections.

Keywords: influenza; virus-like particles; SARS-CoV-2; GM-CSF; RBD; mice; IL-12; antibodies

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) first appeared in late 2019 in China before beginning its rapid spread across the globe [1]. The disease, named coronavirus disease of 2019 (COVID-19), presents a severe respiratory disease course and a high fatality rate in the elderly and immunocompromised [2]. The spike (S) protein of the virus binds to the human angiotensin-converting enzyme-2 (ACE2) protein for entry into epithelial cells of the respiratory tract [1,3]. This S protein, specifically the conserved ACE2 receptor-binding domain (RBD), is a proven target for vaccine design. Antibodies
and vaccines directed to the S protein and the RBD are effective in preventing SARS-CoV-2 infection [4]. Most of the recent vaccine strategies for SARS-CoV-2 target the full-length S protein (Pfizer/BioNTech, Moderna, Johnson & Johnson, AstraZeneca/Oxford, Novavax, Innovio, Curevac and others) [5,6] and some use inactivated whole virus vaccines (COVAXIN/BBV152 by Bharath Biotech, Sinovac/BBBIPI-CoV from Sinopharm) [7,8].

Vaccines for influenza and SARS-CoV-2 have been developed using various platforms and approved by FDA for the general population. Live attenuated viruses, virus-like particles, subunit vaccines, DNA and mRNA vaccines have been tested in the past, but the COVID pandemic created the urgency to develop the mRNA vaccine using lipid nanoparticles for millions of people within a short time. However, the need for affordable vaccines that protect against more than one virus prompted us to develop a two-in-one vaccine since millions of people are exposed to influenza virus in addition to SARS-CoV-2. Preclinical studies evaluating the efficacy of SARS-CoV-2 vaccine (Picovacc, Sinovac Biotech Ltd., Beijing, China) and flu vaccine (Sinovac Biotech Ltd.) in human ACE2 transgenic mice demonstrated that co-administration of two vaccines protected mice from SARS-CoV-2 and influenza virus challenge [9]. Intranasal administration of live attenuated influenza A (LAIV) expressing RBD of SARS-CoV-2 has been shown to prevent SARS-CoV-2 infection in BALB/c mice [10]. However, its efficacy against influenza virus infection was not assessed. Novavax tested a co-administration approach of their SARS-CoV-2 vaccine (NVX-CoV2373) and influenza vaccines in a phase 3 trial and found that most people responded to both vaccines as measured by antibody response and hemagglutination inhibition assay [11]. These studies suggested that it is feasible to develop a vaccine for multiple viruses which can be administered simultaneously.

The present study used a two-in-one hybrid vaccine for SARS-CoV-2 and influenza using a protein transfer method to incorporate cytokine adjuvants and RBD antigen into influenza VLPs. The protein transfer approach achieves a higher level of incorporation of antigen and cytokine adjuvants in the VLP vaccine [12]. Simultaneous delivery of cytokines and protein antigens by the VLP to antigen-presenting cells (APCs) may enhance the efficient presentation of the antigens to the immune system. Cytokines are known to increase the efficacy of vaccines by attracting and activating key immune cells [13–15].

Two cytokines under evaluation for their potential as biological adjuvants are granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-12 (IL-12). In addition, targeting antigens to APCs via a GM-CSF receptor enhances cross-priming [16,17]. GM-CSF potentiates a strong immune response primarily through the maturation and differentiation of dendritic cells [17–20]. The FDA approved a prostate cancer vaccine, Provenge®, by Dendreon, which uses an antigen fused to GM-CSF that delivers antigens effectively to the immune system [21,22]. Adjuvants that have a tolerable safety profile and generate a Th1 immune response are of high importance. Moreover, proinflammatory cytokines produced by activated dendritic cells (DCs) have been shown to play an important role in inducing a robust immune response [23,24]. IL-12 has been well documented to induce a Th1 response, with a promising clinical benefit in cancer patients [13]. IL-12, a heterodimeric cytokine (p35 and p40 subunits), activates DCs, T lymphocytes and natural killer (NK) cells to release IFN-γ, TNF-α, etc. [25–27]. IL-12 also induces T-cell precursors to differentiate toward a Th1 lineage, which also promotes the development of a robust CTL response [28]. Preclinical and clinical trials demonstrated the potential of recombinant soluble IL-12 as an adjuvant in treating several cancers and viral hepatitis, resulting in enhanced immune response [14,29–33]; however, it also resulted in unfavorable side effects and systemic toxicity [34,35].

Despite this, delivering IL-12 in a membrane-anchored form has been proved a successful approach in achieving the desired adjuvant effect with minimal toxicity [36–38]. We engineered the membrane-bound form of cytokines by attaching a GPI-anchor [39]. The GPI-anchor permits the incorporation of purified GPI-anchored proteins into the lipid bilayer of influenza VLPs or any amphiphilic micro/nanoparticles by a simple protein transfer technique [12,40]. By introducing the membrane incorporated GPI-cytokines into
VLPs, viral antigens can be presented to the immune system to mount a robust immune response. In addition, the administration of VLP vaccines containing membrane-anchored cytokines will localize the cytokines to the area of injection, thereby reducing the systemic effects associated with soluble cytokines [12]. The VLP vaccine prepared by our protein transfer approach requires only a low amount of GPI-GM-CSF (25 ng/μg of VLP) for the optimum antiviral response in mice. Moreover, the physical linkage of adjuvant and antigen sources leads to simultaneous adjuvant and antigen delivery to immune cells, resulting in enhanced immune reactivity and increased vaccine efficacy when compared to an unconjugated antigen and adjuvant mixture [41,42]. In the present study, we demonstrate that a hybrid vaccine developed using influenza VLP vaccine incorporated with GPI-RBD-GM-CSF fusion protein and GPI-IL-12 protected mice from influenza virus challenge and also induced a robust, durable antibody response in BALB/c mice as well as decreased viral load and less weight loss when challenged with mouse-adapted SARS-CoV-2.

2. Materials and Methods

2.1. Antibodies and Proteins

Purified anti-mouse GM-CSF (clone M1-22E9) and anti-mouse IL-12 (clone C17.8) mAbs were obtained from BioXcell and used for affinity chromatography purification of GPI-RBD-GM-CSF fusion protein and GPI-IL-12, respectively. Anti-RBD mAb (clone MM57) was obtained from Sino Biologicals (Cat#40592). FITC-conjugated goat secondary antibody against mouse IgG/IgM was purchased from BD Pharmingen (Cat#55988). Peroxidase (HRP)-conjugated goat anti-mouse IgG Fab’2 specific antibody was from ThermoFisher Scientific/Pierce (Cat#31436). The antibody isotyping kit was purchased from Southern Biotech (Cat#5300-05). FITC-conjugated streptavidin was purchased from BD Biosciences (Cat#554060). Human COVID-19 convalescent serum samples were purchased from Ray Biotech (Atlanta, GA, USA). HRP-conjugated donkey anti-human IgG antibody was obtained from Jackson Immunoresearch (Cat#709-036-098). Biotinylated human ACE2 from ACRO Biosystems (Cat#AC2-H82F9) and purified RBD protein from Ray Biotech (Cat#230-30162) were purchased.

2.2. Mice

BALB/c mice (JAX Labs, Bar Harbor, ME or Taconic Biosciences Inc., Germantown, NY, USA) 2-3 months age (female) were purchased and housed in the Emory University Division of Animal Resources (DAR) facility and used according to the University IACUC guidelines.

2.3. Construction and Expression of GPI-Anchored SARS-CoV-2 RBD-GM-CSF Fusion Protein

We constructed a fusion protein gene by joining the DNA sequences of RBD (amino acids 319-541), mouse GM-CSF and human CD59 GPI-anchor signal sequence as in Figure 1A. This construct was cloned into the pCHO 1.0 vector using the AvrII and BstI/Z171 sites (Invitrogen). The DNA construct was then transfected into CHO-S cells (Invitrogen) and selected with puromycin and methotrexate. Expression of both the RBD and GM-CSF on the surface of transfected CHO-S cells was confirmed by flow cytometry using fluorophore-conjugated mAbs against RBD, Clone MM57 (Sino Biologicals) and GM-CSF, Clone M1-22E9 (BioLegend). To confirm whether the fusion protein binds to its cognate receptor ACE2, flow cytometry analysis using biotinylated human ACE2 (ACRO Biosystems) was used and verification that the RBD-GM-CSF fusion protein retains its ability to bind to its cognate receptor was confirmed. CHO-S cell clones transfected with the fusion protein were grown in large quantities using a 5L bioreactor and used for purification by mAb-affinity chromatography.
Figure 1. Design, expression and characterization of GPI-RBD-GM-CSF fusion protein. (A) Design of GPI-RBD-GM-CSF fusion protein gene. (B) GPI-RBD-GM-CSF fusion protein binds both anti-RBD mAb and anti-GM-CSF mAb on CHO-S cell transfectants. (C) PIPLC treatment of CHO-S cells expressing GPI-RBD-GM-CSF reduced the level of expression and (D) flow cytometry analysis showed binding of human ACE2 to GPI-RBD-GM-CSF fusion protein expressed in CHO-S cells.

2.4. PIPLC Treatment of CHO-S Cells

To test the ability of phosphatidylinositol-specific phospholipase C (PIPLC) to cleave the GPI-RBD-GM-CSF fusion protein from the CHO-S cells, 0.25 × 10⁶ cells were treated with 0.2 U PIPLC (Sigma cat# S5446) in 250 µL HBSS containing 0.1% BSA for 2 h at 37 °C. After treatment, cells were washed twice with FACS buffer before staining using PE anti-mouse GM-CSF antibody (BD cat# 554406) or PE Rat IgG2a,κ isotype control (BioLegend cat# 400508).

2.5. Purification of GPI-RBD-GM-CSF and GPI-IL-12

Frozen CHO-S cell transfectants stably expressing GPI-IL-12 [43] or GPI-RBD-GM-CSF fusion protein were lysed for 1 h at 4 °C in lysis buffer (50 mM Tris, 20 mM Iodoacetamide, 5 mM EDTA, 0.2% Tween 20, 2 mM PMSF and protease inhibitor cocktail, pH 8.0) and membranes containing the GPI-proteins were collected by centrifugation at 17,000 × g for 1 h at 4 °C. The membranes were lysed with octyl glucoside [40], and GPI-RBD-GM-CSF and GPI-IL-12 present in the lysates were purified using anti-mouse GM-CSF antibody (Clone MP1-22E9, BioXCell) and anti-mouse IL-12 antibody (clone C17.8) coupled to NHS-Sepharose affinity (Cytiva) columns, respectively.

2.6. SDS-PAGE and Western Blot

Proteins were separated on a NuPAGE™ 12% Bis-Tris Gel, 1.0 mm × 10 well (Cat# NP0341BOX), and stained with Invitrogen’s Colloidal Blue Staining Kit, “Stain NuPAGE, Novex Bis-Tris Gel” (catalog # 46-7015, 46-7016), following manufacturer’s instructions. For Western blotting, proteins separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing condition were transferred onto a nitrocellulose membrane using semi-dry transfer apparatus via an electrical current. After
transfer, the membranes with the proteins were blocked for 1 h at room temperature with 5% milk in phosphate-buffered saline and 0.2% Tween 20 (PBS-T) and incubated with a primary antibody (anti-RBD, anti-mouse GM-CSF or anti-mouse IL-12) overnight at 4 °C in PBS-T with on a shaker at low speed. The next day, membranes were washed three times with PBS-T and then incubated with an appropriate secondary antibody conjugated with alkaline phosphatase that provides a visual color change upon the addition of the chromogenic substrate (mixture of BCIP (S-bromo-4-chloro-3-indoly) phosphate- catalog# 34040) and NBT (nitro-blue tetrazolium chloride, catalog# 34035 from Thermo Scientific).

2.7. Hybrid Vaccine Preparation by Protein Transfer of GPI-RBD-GM-CSF Fusion Protein and GPI-IL-12 onto VLP

Influenza VLPs containing codon-optimized hemagglutinin (H1 HA) and matrix M1 proteins derived from A/Puerto Rico/8/1934 (PR8) were produced in insect cells (Sf9) as described in [44] and purified by tangential flow diafiltration and anion exchange (Capto Q) chromatography by Medigen (Frederick, MD, USA). We incorporated the purified GPI-RBD-GM-CSF fusion protein along with GPI-IL-12 into PR8 influenza VLPs by protein transfer to prepare our VLP-RBD-GM-CSF-IL-12 vaccine. Protein transfer was performed by incubating 1 mg of enveloped influenza VLPs with 200 μg of purified GPI-RBD-GM-CSF and 25 μg of purified GPI-IL-12 at 37 °C for 1 h. Unincorporated GPI-cytokines were washed out by ultracentrifugation at 210,000 × g at 4 °C, and the resulting pellet was resuspended in DPBS. Protein incorporation was detected by Western blot and flow cytometry analysis using anti-RBD mAb, clone MM57 (Sino Biologicals), anti-mouse GM-CSF (clone MP1-22E9, BioLegend, San Diego, CA, USA) and anti-mouse IL-12 (clone C17.8, Invitrogen, Rochester, NY, USA) antibodies.

2.8. Bone Marrow-Derived Dendritic Cell Stimulation Assay

BMDCs were generated according to established protocols [45]. Briefly, femurs of female BALB/c mice were removed and cleaned from surrounding muscle tissue. Bone marrow was flushed using RPMI-1640 medium with a 22 G needle and syringe. Red blood cells (RBC) were lysed using RBC lysis buffer (MilliporeSigma, Burlington, MA, USA), and the resulting cells were cultured in a complete RPMI-1640 medium containing various concentrations of recombinant murine GM-CSF (rGM-CSF, Biolegend, San Diego, CA, USA), GPI-GM-CSF, GPI-RBD-GM-CSF or VLPs incorporated with GPI-RBD-GM-CSF at a density of 2 × 10⁵ cells/mL. After 5 days of culture, 2,3-bis-(2-methoxy-4-nitro-5-sulfo phenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay reagent was added, and absorbance was measured after 3 h at 480 nm and 660 nm for background reference.

2.9. Immunization with VLP Vaccine

BALB/c mice were administered with VLP vaccine, control VLP or PBS subcutaneously (100 μL volume per mouse) or intramuscularly (50 μL per mouse in the hind leg). A booster dose was administered after 2 or 4 weeks. VLP vaccines were diluted in sterile PBS before administering to the mice.

2.10. Enzyme-Linked Immunosorbent Assay (ELISA)

SARS-CoV-2 S protein RBD specific antibodies of different subtypes (IgG, IgG1, IgG2a) were determined in sera by enzyme-linked immunosorbent assay (ELISA) using an approach similar to one previously described using PR8 or WSN as targets [46,47]. Briefly, 96-well ELISA plates were coated with 100 μL of 3 μg/mL GPI-RBD-GM-CSF in coating buffer (BioLegend) overnight at 4 °C. In some experiments, RBD of spike protein from commercial sources (RayBiotech, Atlanta, GA, USA or Sino Biologicals, Wayne, PA, USA) was used as indicated in the figure legends. Plates were washed using a plate washer and washing buffer (PBS with 0.05% Tween20). Plates were blocked with assay diluent (PBS with 3% BSA) for 2 h at room temperature on a rocker. Plates were washed again as described above, and diluted serum samples were added and incubated for
another 2 h at room temperature. Plates were washed, and diluted secondary antibody (HRP-conjugated) against mouse total IgG or immunoglobulin isotypes (IgG1, IgG2a) was added and incubated for 30 min at room temperature. The plates were washed, and TMB substrate solution (Cat# 555214, BD Biosciences) was added to the wells for color development. The reaction was stopped by adding 2N H$_2$SO$_4$ and reading the absorbance at 450 nm.

To measure influenza antigen-specific antibody levels in immune sera, inactivated A/PR8 H3N2 virus (200 ng/well) was coated onto ELISA plates, followed by the addition of diluted immune sera. IgG isotypes were measured using goat anti-mouse immunoglobulin (Ig) G, IgG1 and IgG2a and horse-radish peroxidase (HRP)-conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL, USA). Color reactions were developed with tetramethylbenzidine substrates (TMB, Invitrogen). Antibody levels are presented as optical density absorbance values at 450 nm (BioTek ELISA plate reader, Winusky, VT, USA).

2.11. Flow Cytometry Analysis of Antibody Response

For the detection of RBD-GM-CSF fusion protein on CHO-S cells, CHO-S cells (2–3 × 10$^6$/mL) were incubated with anti-mouse GM-CSF antibody, anti-RBD (clone MM57, Sino Biological) in FACS buffer (PBS containing 2% BCS, 5 mM EDTA and 0.05% sodium azide), for 30–60 min on ice. For mouse sera, CHO-S cells were incubated with diluted serum samples (100–100,000 times diluted in FACS buffer). FITC-conjugated goat anti-mouse IgG/IgM was added as a secondary antibody after washing off the unbound primary antibody. After incubation with secondary antibody, cells were washed with FACS buffer and resuspended in FACS buffer and acquired in a FACSCalibur (BD Biosciences) flow cytometer. Data were analyzed using FlowJo software (BD Biosciences, Ashland, OR, USA).

2.12. Focus Reduction Neutralization Assay

Live-virus SARS-CoV-2 neutralization antibodies were assessed using a full-length mNeonGreen SARS-CoV-2 (2019-nCoV/USA_WA1/2020), generated as previously described [48]. FRNT-mNG assays were performed as previously described [49]. Briefly, samples were diluted at 3-fold in 8 serial dilutions using DMEM (VWR, #45000-304) in duplicates with an initial dilution of 1:10 in a total volume of 60 μL. Serially diluted samples were incubated with an equal volume of SARS-CoV-2-mNG (100–200 foci per well) at 37 °C for 1 h in a round-bottomed 96-well culture plate. The antibody-virus mixture was then added to Vero cells and incubated at 37 °C for 1 h. Post-incubation, the antibody-virus mixture was removed, and 100 μL of prewarmed 0.85% methylcellulose (Sigma-Aldrich, #Mo512-250G) overlay were added to each well. Plates were incubated at 37 °C for 24 h. After 24 h, the methylcellulose overlay was removed, and cells were washed three times with PBS. Cells were then fixed with 2% paraformaldehyde in PBS (Electron Microscopy Sciences) for 30 min. Following fixation, plates were washed twice with PBS and fixed for visualization on a fluorescence ELIspot reader (C1L ImmunoSpot 56 Universal Analyzer) and counted using Viridot [50]. The neutralization titer were calculated as follows: 1–ratio of the mean number of foci in the presence of sera and foci at the highest dilution of the respective sera sample. Each specimen was tested in duplicate. The FRNT-mNG50 titers were interpolated using 4-parameter nonlinear regression in GraphPad Prism 8.4.3.

2.13. Plaque Reduction Neutralization Test (PRNT)

The titers of anti-SARS-CoV-2 neutralizing antibodies were measured in the serum of BALB/c mice using a PRNT assay as described previously [51]. Serum was diluted serially from 1:4 to 1:1024, and PRNT was conducted using the Wuhan strain of SARS-CoV-2 (Bel NR-52251). The highest dilution of serum resulting in a 50% reduction in the number of plaques compared to the growth of the virus control was determined.
2.14. Hemagglutination Inhibition (HAI) Assay

To assess the ability of immune sera to inhibit HA activity, we performed an HAI assay using immune sera. The immune sera from each group were treated with receptor destroying enzymes (RDE, Sigma-Aldrich, St. Louis, MO, USA) for 18 h at 37 °C. Sera were incubated at 56 °C for 30 min for the inactivation of complement, followed by 10-fold serial dilutions in PBS. The serially diluted sera were incubated with 4 HA units of the A/PR8 H1N1 virus for 30 min at room temperature, and then 0.5% chicken red blood cells (Lampire Biological Laboratories) were added to determine HAI titers. HAI titers were determined as the highest dilution factor inhibiting the formation of haemagglutination with 0.5% chicken red blood cells.

2.15. SARS-CoV-2 Virus Challenge

Female BALB/c mice (10-week-old) were purchased from Taconic Biosciences and housed in the Emory University DAR facility. Mice were immunized with the VLP or VLP vaccine with cytokine adjuvants containing RBD and administered a booster dose 33 days after the first dose (n = 10 mice per treatment). Blood was collected 2 weeks after the booster dose for the antibody titer. Mice were transferred 3 months after the booster dose to ABSL-2 (n = 5 for each treatment) for challenge with influenza A virus or ABSL-3 (n = 5 for each treatment) for challenge with mouse-adapted SARS-CoV-2 virus MA10 at the Georgia State University (GSU). All the animal infection experiments using the SARS-CoV-2 virus were conducted in a certified Animal Biosafety Level 3 (ABSL-3) laboratory at GSU. The protocol was approved by the GSU Institutional Animal Care and Use Committee (Protocol number A20044). MA10 virus is not 100% lethal in young BALB/c mice. Around 50% of animals clear the virus and survive the infection. Peak virus titers in the lungs are detected between days 2 and 4 after the infection. No virus is detected in the animals that survive the infection. Therefore, we euthanized all the animals on day 3 to compare the viral load in the lungs. Mice were inoculated intranasally with 10^6 plaque-forming units (FFU) of mouse-adapted SARS-CoV-2 MA10. Animals were weighed, and their appetite, activity, breathing and neurological signs were assessed twice daily. On day 3 after infection, animals were anesthetized with isoflurane and perfused with cold PBS. Lungs were collected and flash-frozen in 2-methylbutane (Sigma, St. Louis, MO, USA).

2.16. Quantification of the SARS-CoV-2 Virus Load in the Lungs

Quantitative RT-PCR was used to measure viral RNA levels with primers and probes specific for the SARS-CoV-2 N gene as described previously [51]. Viral genome copies were determined by comparison to a standard curve generated using a known amount of RNA extracted from previously titrated SARS-CoV-2 samples. Frozen tissues harvested from mock and infected animals were weighed and lysed in RLT buffer (Qiagen), and RNA was extracted using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA). Total RNA extracted from the tissues was quantified and normalized, and viral RNA levels per µg of total RNA were calculated.

2.17. Influenza A/PR8 H1N1 Virus Challenge

Three months after the booster dose, BALB/c mice were challenged with a lethal dose of influenza A/PR8 H1N1 virus (10 × LD50). After challenge, the mice were monitored for 14 days to record body weight changes and survival rates. To determine the protective efficacy and T cell responses after A/PR8 H1N1 infection, an additional set of immunized BALB/c mice was euthanized on day 5 post-infection, and lung tissues were collected for further analysis.

2.18. Influenza Virus Titration in the Lung

The lungs of the immunized BALB/c mice were harvested on day 5 after A/PR8 H1N1 infection and ground mechanically in 1.5 mL of PBS per lung. The lung extracts and lung cells were separated after centrifugation. Embryonated chicken eggs (Hy-Line North
America, LLC, Wilton, IA, USA) were incubated at 37 °C for 9–12 days to be inoculated with 10-fold serially diluted lung extracts. The virus titers were determined by hemagglutination assay of the allantoic fluids collected after 3 days of incubation. Virus titers as 50% egg inoculation dose (EID50)/mL were evaluated according to the Reed and Muench method [54].

2.19. Statistical Analysis

GraphPad Prism (version 9.1.0) was used to generate the graphs and analysis of the statistical significance as indicated in the figure legends. A p-value < 0.05 was considered significant.

3. Results

3.1. Characterization of GPI-RBD-GM-CSF Fusion Protein

We constructed a fusion protein gene by joining the DNA sequences specific to the SARS-CoV-2 S protein RBD domain, mouse GM-CSF and the GPI-anchor signal from human CD59 (Figure 1A) and expressed the gene in CHO-S cells. Flow cytometry analysis demonstrated the expression of the RBD-GM-CSF fusion protein on the surface of transfected CHO-S cells (Figure 1B). To verify whether the GPI-RBD-GM-CSF fusion protein contained the GPI tail, CHO-S cells expressing the fusion protein were incubated with phosphatidylinositol-specific phospholipase C (PIPLC), which is known to cleave the GPI moiety from the proteins and release them from the cell membranes as described in Methods. The flow cytometry data showed that nearly 76% of the fusion protein was released from the cell surface, suggesting that the fusion protein is anchored to the cell membrane via the GPI tail (Figure 1C). To determine whether the fusion protein on the CHO-S cell surface binds to its cognate receptor ACE2, flow cytometry analysis was used to detect the binding of biotinylated human ACE2 (ACRO Biosystems) to the CHO-S cells. The results verified that the RBD in the fusion protein retained its ACE2 binding activity (Figure 1D). RBD-specific neutralizing antibody (clone MM57, Sino Biologicals) was able to bind to GPI-RBD-GM-CSF on CHO-S cell transfectants, which was blocked by pre-incubation with purified GPI-RBD-GM-CSF and commercially available RBD (RayBiotech), confirming that the fusion protein retains the RBD conformation (Figure S1). Transfected cells were cloned, and CHO-S clone 3C3 was used for further studies.

3.2. GPI-RBD-GM-CSF Fusion Protein Retains Functional Activity

CHO-S cell-expressed GPI-RBD-GM-CSF fusion protein was affinity purified using NHS-Sepharose coupled to an anti-mouse GM-CSF antibody. The purified fusion protein was run on 12% SDS-PAGE under non-reducing conditions and either stained with Colloidal blue (Figure 2A, lane 1) or detected with Western blots using an anti-RBD antibody (Figure 2A, lanes 3 and 4) and anti-GM-CSF (Figure 2A, lanes 6 and 7). Affinity purified GPI-RBD-GM-CSF fusion protein runs as a smear ranging from 50 kDa (size of non-glycosylated fusion protein) to 250 kDa with several distinct bands with sizes 55 kDa, 110 kDa and 220 kDa. Western blot analysis was performed for identity and size comparison of the fusion protein to wild-type mouse GPI-GM-CSF (Figure 2A, lane 5). Most of the colloidal blue-stained bands were detected by anti-mouse GM-CSF and anti-RBD antibodies, suggesting that they are multimeric forms of the fusion protein.

To test whether the anti-RBD antibodies in the convalescent sera from SARS-CoV-2 infected patients (RayBiotech) recognize the RBD in the purified GPI-RBD-GM-CSF fusion protein, we performed a direct ELISA. The data show that antibodies from COVID-19 patients’ sera bind to the GPI-RBD-GM-CSF (Figure 2B). Next, we tested whether the purified fusion protein has a dual function as GM-CSF and also binds human ACE2. To test whether GM-CSF in the fusion protein retains its function, we cultured mouse bone marrow-derived dendritic cells (BMDC) in vitro with purified GPI-RBD-GM-CSF and measured BMDC proliferation using an XTT assay. The results show that GPI-RBD-GM-CSF is capable of stimulating BMDC proliferation (Figure S2). An ELISA using biotinylated human ACE2 confirmed that the RBD in purified GPI-RBD-GM-CSF fusion
protein retains its ACE2 receptor binding activity (Figure 2C left panel). RBD-specific neutralizing antibody MM57 was used as a positive control (Figure 2C right panel).

Figure 2. Purified GPI-RBD-GM-CSF fusion protein retains functional activity. (A) Colloidal blue (lane 1) and Western blot (lanes 2–7) of the immunoaffinity column purified fusion protein from CHO-S cells probed with anti-RBD antibody (lanes 3 and 4) or anti-GM-CSF mAb (lanes 6 and 7). Lane 2 is control RBD probed with anti-RBD Ab, and lane 5 is control GM-CSF probed with anti-GM-CSF antibody. (B) ELISA for GPI-RBD-GM-CSF binding to antibodies in the human COVID-19 patients’ sera, and (C) ELISA of purified GPI-RBD-GM-CSF binding to ACE2 and RBD specific MM57 mAb. (D) FACS analysis of VLPs incorporated with GPI-IL-12 and GPI-RBD-GM-CSF fusion protein by protein transfer.

To develop the VLP hybrid vaccine, we incorporated influenza VLPs with GPI-IL-12 and GPI-RBD-GM-CSF by protein transfer. Influenza VLPs contain a lipid bilayer which is amenable to protein transfer mediated incorporation of GPI-proteins. Flow cytometry was performed using fluorochrome-conjugated anti-IL-12 and anti-GM-CSF antibodies to confirm the dual incorporation of the GPI-RBD-GM-CSF and GPI-IL-12 onto VLPs (Figure 2D). To test whether VLP-incorporated GM-CSF retains its function, we cultured mouse bone marrow-derived dendritic cells (BMDC) in vitro with soluble GPI-RBD-GM-CSF or VLPs incorporated with GPI-RBD-GM-CSF and measured BMDC proliferation using an XTT assay. The results show that GPI-RBD-GM-CSF incorporated in the VLP vaccine is capable of stimulating BMDC proliferation (Figure S2).

3.3. Hybrid Vaccine Induces Durable Antibody Response

To test whether the GPI-RBD-GM-CSF fusion protein induces antibody response, we immunized BALB/c (2–3 months old) mice with GPI-RBD-GM-CSF fusion protein (0.1, 1.0, 2.0 and 5.0 μg) or VLPs (1.0, 2.0, 5.0 and 10 μg) incorporated with the fusion protein and GPI-IL-12 (hybrid vaccine). Controls included VLP without cytokines, commercially available RBD (RBD-His from Ray Biotech) or PBS. A booster dose was administered 2–4 weeks after the first dose. The route of administration was either subcutaneous (s.c.) or intramuscular (i.m.) (Figure S3).
Blood was collected every 2 to 4 weeks for antibody titer, ACE2 binding inhibition and virus neutralization assays. The VLP hybrid vaccine induced a robust antibody response after the booster dose (Figure S4). The antibody response against GPI-RBD-GM-CSF fusion protein was comparable in both i.m. and s.c. routes of vaccine administration (Figure S5). The antibodies bind to CHO-S cells expressing the GPI-RBD-GM-CSF fusion protein but not untransfected CHO-S cells (Figure S6), confirming the specificity of the antibody response to GPI-RBD-GM-CSF fusion protein.

3.4. Hybrid Vaccine Induces SARS-CoV-2 Neutralizing Antibodies

To test whether antibodies generated by the hybrid vaccine block the binding of RBD to human ACE2, we performed an inhibition assay using biotinylated ACE2 and CHO-S cells expressing GPI-RBD-GM-CSF fusion protein. Our results show that antibodies induced by both purified fusion protein and hybrid vaccine blocked ACE2 binding to RBD on CHO-S cells (Figure S7). We used ACE2 blocking anti-RBD (clone MM57) mAb (2µg and 10 µg) as a positive control. For live virus neutralization, Vero.E6 cells and the WA1 strain of SARS-CoV-2 were used in a modified FRNT assay as described in a previously published study [49]. While the purified GPI-RBD-GM-CSF induced levels of antibody response similar to that of the VLP hybrid vaccine (Figure S8A), neutralizing antibody titers were very low in the mice that received only the purified fusion protein GPI-RBD-GM-CSF without VLP. The data suggest that VLP incorporated fusion protein induced stronger neutralizing antibodies than the soluble fusion protein (Figure S8B).

3.5. Hybrid Vaccine Induces IgG2a Antibody Response against RBD

We observed that purified GPI-RBD-GM-CSF fusion protein by itself or incorporated onto VLPs induced a strong antibody response (Figure 3A). However, the recombinant RBD-His tag failed to induce an antibody response, suggesting that the GM-CSF in our fusion protein is acting as an adjuvant (Figure S9). To determine the isotype of antibodies induced by the hybrid vaccine, we performed an antibody isotyping ELISA as described in the Section 2. While the purified GPI-RBD-GM-CSF fusion protein alone induced an antibody response, which is mostly Th2 type IgG1 (blue circles, Figure 3B), the hybrid VLP vaccine induced both IgG2a (a Th1-induced response) and IgG1 (red symbols, Figure 3B). However, the addition of GPI-IL-12 to the VLP vaccine did not further enhance the IgG2a response (green versus red bars).

![Figure 3](image_url)

Figure 3. Hybrid vaccine induces antibody response against RBD in mice. ELISA plates were coated with GPI-RBD-GM-CSF, and serum samples from various groups of mice (n = 5) immunized with GPI-RBD-GM-CSF, VLP vaccine containing the GPI-RBD-GM-CSF or hybrid vaccine (VLP vaccine containing the GPI-RBD-GM-CSF + GPI-IL-12) were diluted and added to the wells after blocking the plates. (A) Total IgG levels 6 weeks after booster dose, and (B) IgG isotype in the sera 10 weeks after the booster dose. Anti-mouse IgG (A) or isotype-specific anti-mouse IgG-HRP conjugate (B) was used to detect the bound antibody.
3.6. Hybrid Vaccine Induces Anti-Influenza Virus IgG1 and IgG2a Antibody Response

To test whether the hybrid vaccine-induced antibodies against influenza virus antigens, we analyzed the sera for antibodies and the isotype of the antibodies. The VLP vaccine incorporating the cytokines (purple and red symbols) induced higher levels of antibodies compared to the VLP without cytokines (blue symbols, Figure 4A). The antibody response is a mixed Th1 and Th2 type (IgG1 and IgG2a) against influenza A/PR8 antigens in inactivated virus (Figure 4A–C). Interestingly, we observed that the VLP vaccine without RBD-GM-CSF that was incorporated with GPI-GM-CSF instead (purple symbols) induced significantly higher levels of IgG2a (Figure 4C) but not IgG1 (Figure 4B) compared to the VLP administered group suggesting that GPI-IL-12 and GPI-GM-CSF in the VLP vaccine augment a Th1-type IgG2a antibody response. The incorporation of GPI-RBD-GM-CSF instead of GPI-GM-CSF onto the VLP vaccine diminished the level of total anti-influenza antibody response (red symbols in Figure 4C). This may be because GPI-GM-CSF is more active than GPI-RBD-GM-CSF or may be due to the difference in the level of incorporation onto VLP or both, which requires further investigation. However, the hemagglutination inhibition (HAI) antibody titer induced by the VLP vaccine with RBD and VLP vaccine without RBD vaccines are comparable (Figure 4D), suggesting the differences in IgG2a antibody are probably due to non-neutralizing antibodies induced against influenza A/PR8 VLPs. Our results also show that the VLP vaccine with GPI-RBD-GM-CSF with and without GPI-IL-12 induced equally potent antibody responses against inactivated influenza A/PR8 virus (Figure S10).

![Figure 4](image-url)

**Figure 4.** Hybrid vaccine induces influenza A/PR8 virus-specific antibody response. (A–C) ELISA plates were coated with inactivated influenza A/PR8 H1N1. Serum samples from mice vaccinated with VLP, VLP with GPI-GM-CSF and GPI-IL-12 or hybrid vaccine were serially diluted (5-fold) and added to the wells after blocking the plates. (D) Hemagglutination inhibition (HAI) titer in the sera of mice. Isotype specific anti-mouse Ig-HRP conjugate was used to detect the isotype of the antibody bound. * p < 0.05.
(Figure 6A). Mice were challenged with 10 LD_{50} of influenza A/PR8 and monitored for their body weight changes and survival rates. Since the mice administered with VLP or VLP vaccines were protected from weight loss compared to control infected mice (data not shown), we euthanized three mice from each group after 5 days of infection and measured A/PR8 H1N1 titers in the lungs. We observed that the viral loads were significantly reduced up to a level of detection limit in mice that received VLP; hybrid vaccine or hybrid vaccine without IL-12 (Figure 6B) compared to the PBS control. The virus was undetectable in mice administered with a hybrid vaccine, but VLP alone was also equally protective against influenza virus. Consistent with the viral titers, the mice vaccinated with VLP or VLP with cytokine adjuvants survived the lethal challenge (Figure 6C).

![Graph A](image1.png)

**A**

A/PR8 H1N1

<table>
<thead>
<tr>
<th>Serum HAI titer (Log_{10})</th>
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<td>VLP</td>
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![Graph B](image2.png)

**B**

A/PR8 H1N1

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![Graph C](image3.png)

**C**

A/PR8 H1N1

<table>
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![Graph D](image4.png)

**Figure 6.** Hybrid vaccine protects against influenza A virus challenge. BALB/c mice were administered with a hybrid vaccine (10 μg/dose) or a hybrid vaccine without GPI-IL-12, as described in Figure 5. A booster dose was given after 33 days of the first dose. (A) HAI titer in the blood 2 weeks after the booster dose (day 48). Lung viral titer (B) and survival (C) of mice challenged with influenza A/PR8 H1N1 virus 3 months after the booster dose. The inoculation dose was 10 times LD_{50}. (C) All three groups (VLP; hybrid vaccine and hybrid vaccine without IL-12) survived from PR8 challenge; therefore, the lines are superimposed, and only the red symbol is visible. Hybrid vaccine: VLP incorporated with GPI-RBD-GM-CSF and GPI-IL-12. Statistical significance was calculated by one-way ANOVA and Dunnett’s post-multiple comparison tests. Error bars indicate the mean ± standard errors of the mean (SEM). ***; p < 0.001, ns; not significant.
3.7. Hybrid Vaccine Protects Mice from SARS-CoV-2 and Influenza Virus Challenges

To test whether the vaccine administration induces protective response, mice were immunized with unmodified VLP, hybrid vaccine without IL-12 and hybrid vaccine. A booster dose was administered 33 days after the first dose. Blood was collected 2 weeks after the booster dose, and anti-RBD antibody response against RBD and influenza virus antigens and neutralizing antibody titers using live SARS-CoV-2 (Wuhan strain) were performed as described in the Section 2. Our results indicate that the hybrid vaccine and hybrid vaccine without IL-12 induced strong antibody responses against RBD (Figure S11A) and influenza VLP antigens (Figure S11B) but did not bind to recombinant GM-CSF (Figure S11C). The antibodies were able to neutralize live virus infection in a plaque reduction neutralization titer (PRNT) assay (Figure 5A). Those mice were transferred to the ABSL3 facility for challenging with mouse-adapted SARS-CoV-2 virus (MA10). Mice were challenged with 10^5 plaque-forming units of the SARS-CoV-2 virus MA10. We observed acute weight loss in control VLP administered mice, but the mice that were administered either the hybrid vaccine or hybrid vaccine without IL-12 were protected from weight loss (Figure 5B). Since this virus is not lethal for mice, mice were euthanized for quantification of lung viral titer after 3 days of challenge. Virus titer estimates revealed that the hybrid vaccine decreased virus replication significantly compared to the mice that received VLP. The hybrid vaccine that contained IL-12 in addition to RBD-GM-CSF fusion protein was more effective in controlling lung viral loads than the hybrid vaccine without IL-12 (Figure 5C).

Figure 5. Hybrid vaccine protects against SARS-CoV-2 challenge. BALB/c mice (n = 10; 2–3 months old) were administered with a hybrid vaccine, a hybrid vaccine without IL-12 or VLP in 50 μl volume via an intramuscular route. Control mice received either PBS or influenza VLP. A booster dose was given on day 33 and blood was collected 2 weeks later (week 7). Mice were challenged with mouse-adapted SARS-CoV2 (n = 5) 16–18 weeks after the first dose. (A) Neutralizing antibody titers in the serum of BALB/c mice (n = 5–6 per group). Serum collected from BALB/c mice 2 weeks after the booster dose was serially diluted from 1:4 to 1:1024, and PRNT was conducted against SARS-CoV-2 (Wuhan virus). (B) BALB/c mice were inoculated intranasally with mouse-adapted SARS-CoV-2 (10^5 plaque-forming units) 3 months after the booster dose. Percentage of daily body weight change in the animals. (C) The RNA levels of SARS-CoV-2 were determined in the lungs by qRT-PCR (n = 4–5 per group). Error bars represent SEM. The data are expressed as genome copies/μg of RNA. Each data point represents an individual mouse. Data are expressed as mean log_{10} titer. For body weight changes, a two-way analysis of variance (ANOVA) with the post hoc Bonferroni test was used to calculate values of p. Mann-Whitney test was used to calculate the p values of the difference between viral titers. Differences of p < 0.05 were considered significant. * p < 0.05; ** p < 0.01 *** p < 0.001. Hybrid vaccine: VLP incorporated with GPI-RBD-GM-CSF and GPI-IL-12.

Another cohort of mice from the same treatment groups was transferred to the ABSL2 facility for influenza A/PR8 virus challenge. Hemagglutination inhibition titers against the A/PR8 virus were determined before challenge. The results showed that VLP and hybrid vaccine administration induced high titers of hemagglutination inhibiting antibodies.
(Figure 6A). Mice were challenged with 10 LD$_{50}$ of influenza A/PR8 and monitored for their body weight changes and survival rates. Since the mice administered with VLP or VLP vaccines were protected from weight loss compared to control infected mice (data not shown), we euthanized three mice from each group after 5 days of infection and measured A/PR8 H1N1 titers in the lungs. We observed that the viral loads were significantly reduced up to a level of detection limit in mice that received VLP, hybrid vaccine or hybrid vaccine without IL-12 (Figure 6B) compared to the PBS control. The virus was undetectable in mice administered with a hybrid vaccine, but VLP alone was also equally protective against influenza virus. Consistent with the viral titers, the mice vaccinated with VLP or VLP with cytokine adjuvants survived the lethal challenge (Figure 6C).

Figure 6. Hybrid vaccine protects against influenza A virus challenge. BALB/c mice were administered with a hybrid vaccine (10 μg/dose) or a hybrid vaccine without GPI-IL-12, as described in Figure 5. A booster dose was given after 33 days of the first dose. (A) HAI titer in the blood 2 weeks after the booster dose (day 48). Lung viral titer (B) and survival (C) of mice challenged with influenza A/PR8 H1N1 virus 3 months after the booster dose. The inoculation dose was 10 times LD$_{50}$. (C) All three groups (VLP, hybrid vaccine and hybrid vaccine without IL-12) survived from PR8 challenge; therefore, the lines are superimposed, and only the red symbol is visible. Hybrid vaccine: VLP incorporated with GPI-RBD-GM-CSF and GPI-IL-12. Statistical significance was calculated by one-way ANOVA and Dunnett’s post-multiple comparison tests. Error bars indicate the mean ± standard errors of the mean (SEM). ***, p < 0.001, ns; not significant.
4. Discussion

Developing vaccines using nanomaterials such as lipid nanoparticles (LNPs) to increase the efficacy and stability of mRNA vaccines was a breakthrough in recent times. LNPs not only enhance the stability of the viral mRNA but also act as adjuvants [55,56]. Virus-like particles (VLPs) are also considered nanomaterials because of their size (10–200 nm) [57]. These are successfully used to deliver multiple antigens as vaccines [58,59]. Influenza VLPs are lipid enveloped nanoparticles and therefore amenable to the protein transfer mediated incorporation of GPI-anchored antigens and cytokines [12,40,60]. Protein transfer allows us to incorporate multiple GPI-anchored antigens and adjuvants into lipid enveloped VLPs. The novelty of our vaccine is that the GPI-anchored antigen is fused with cytokine adjuvant and anchored to VLPs, which deliver antigen and cytokine adjuvant to antigen-presenting cells simultaneously for a better immune response.

We demonstrated that a hybrid vaccine based on influenza VLPs induces effective immunity against SARS-CoV-2 and influenza viruses. Our vaccine platform is based on cytokine adjuvants linked to the VLPs that carry the antigens [12]. This approach delivers both antigens and biological adjuvants to the immune system simultaneously in a particulate form. We generated CHO-S cells expressing GPI-anchored RBD-GM-CSF fusion protein and GPH-L12, purified the proteins and incorporated them onto influenza VLPs to develop the hybrid vaccine. Administration of the hybrid vaccine via either subcutaneous or intramuscular routes induced comparable levels of antibody response. Since soluble GPI-RBD-GM-CSF induced antibody response, but not the recombinant RBD-His tag, our results suggest that GM-CSF in the fusion protein acts as an adjuvant. Our approach to using cytokine adjuvants targets the antigen to APCs via their receptors and also allows the cytokines to enhance APC maturation. GM-CSF alone acting as an adjuvant in our GPI-RBD-GM-CSF fusion protein can induce a robust antibody response. Interestingly, the antibody response induced by the purified fusion protein is primarily the non-neutralizing IgG1 isotype, whereas fusion protein delivered using VLPs induced a neutralizing IgG2a and IgG1 mixed isotype antibody response. Our results are consistent with the recent report by the Bjorkman laboratory [44] and our previous studies on tumor antigens [40], demonstrating the use of VLP as a delivery vehicle for antigens to induce a protective immune response. The hybrid vaccine also induced neutralizing antibodies against influenza A/PR8 (H1N1), suggesting that this approach of delivering RBD on an influenza VLP along with cytokine adjuvants confers dual protection against both influenza A H1N1 and SARS-CoV-2 viruses.

Mice challenged with H1N1 live virus 3 months after the booster dose were still well protected, suggesting that anti-influenza antibody and T cell responses induced by hybrid vaccine are long-lasting. Neutralizing antibody titers against inactivated influenza A/PR8 (H1N1) are high even after 6 months of vaccination, also confirming the durability of the anti-influenza immune response induced by the hybrid vaccine. Mouse-adapted SARS-CoV-2 virus infection causes body weight loss but does not cause lethality in BALB/c mice [51]. This finding was observed within 3 days of infection in control mice that were vaccinated with VLP, and the hybrid vaccine prevented mice from losing weight. Lung virus titers were significantly decreased in mice that were vaccinated with a hybrid vaccine compared to plain VLP. This suggests that the hybrid vaccine containing GPI-RBD-GM-CSF with cytokine adjuvants confers protection from severe disease caused by SARS-CoV-2 infection. Administration of purified GPI-RBD-GM-CSF fusion protein without VLP also induced antibody response. However, the antibodies were not able to neutralize the live virus even though they were able to block ACE2 binding to RBD. The antibodies are mostly IgG1 in mice vaccinated with purified GPI-RBD-GM-CSF fusion protein alone, whereas a hybrid vaccine (VLP with GPI-RBD-GM-CSF fusion protein and GPH-L12) induced both IgG1 and IgG2a isotypes, and blocked SARS-CoV-2 virus infection. This suggests that the Th1 type response induced by the hybrid vaccine is more protective than the Th2 type response induced by the purified GPI-RBD-GM-CSF.
The protein transfer approach used here to prepare a hybrid vaccine allows the anchoring of these cytokines to the surface of the VLPs, which limits the systemic toxicity of the cytokines by acting as a depot at the site of vaccination. The physical linkage of adjuvant and antigen sources results in the presentation of the adjuvant and antigen simultaneously to the immune cells, leading to enhanced immune reactivity and increased vaccine efficacy. Such a physical linkage of antigen and adjuvants is more effective than an unconjugated antigen and adjuvant mixture [1,41]. In addition, IL-12 and GM-CSF target antigen-presenting cells, such as dendritic cells, by binding to IL-12 and GM-CSF receptors and enhancing antigen uptake and presentation, thereby enhancing subsequent T cell responses.

The limitation of the current study is that a comparative analysis of GPI-RBD-GM-CSF and GPI-RBD was not carried out to demonstrate the contribution of GM-CSF as an adjuvant in the VLP vaccine. Attempts to develop GPI-RBD in CHO S cells were not successful. However, the comparison of antibody response induced by purified GPI-RBD-GM-CSF molecule with RBD-His-Tag suggests that GM-CSF stimulated antibody production against RBD. Our studies suggest that it is possible to develop a two-in-one hybrid vaccine for influenza and SARS-CoV-2 viruses. In the future, when new variants of concern for SARS-CoV-2 arise, our hybrid vaccine can be modified to incorporate the RBD of new variants.

5. Conclusions

In summary, our results demonstrate that influenza VLP-based delivery of SARS-CoV-2 RBD protein in combination with cytokine adjuvants can be used as a platform to develop multivalent vaccines targeting the variant strains of viruses which are currently observed in the ongoing SARS-CoV-2 pandemic. Our fusion protein vaccine design also allows for the creation of fusion proteins with new variant sequences and quickly purify them using anti-GM-CSF mAb affinity chromatography. Further, the use of immobilized cytokines as adjuvants will provide a safer way to induce anti-viral immunity with minimal side effects.

6. Patents

Title: Composition and methods for detecting and treating a SARS-CoV-2 infection (patent filed).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/10.3390/vaccines10060944/s1, Figure S1. Purified GPI-RBD-GM-CSF fusion protein blocks binding of MM57 mAb to CHO-S cells expressing GPI-RBD-GM-CSF. Figure S2. Purified GPI-RBD-GM-CSF fusion protein and VLP vaccine incorporated with GPI-RBD-GM-CSF induce BMDC proliferation. Figure S3. Design for VLP vaccination in BALB/c mice. Figure S4. Booster dose induces high titers of anti-RBD antibody. Figure S5. VLP vaccine induces comparable levels of antibody against RBD. Figure S6. Hybrid vaccine induces RBD specific antibody response. Figure S7. VLP vaccine-induced antibody blocks ACE-2 binding to RBD. Figure S8. Hybrid vaccine but not the purified RBD-GM-CSF induces SARS-CoV-2 neutralizing antibody. Figure S9. GPI-RBD-GM-CSF fusion protein but not RBD induces antibody response against RBD. Figure S10. Hybrid vaccine induces antibody response against influenza VLP antigens. Figure S11. Hybrid vaccine induces antibody response against RBD-GM-CSF and influenza VLP antigens.


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Committee (IACUC). IACUC protocol # 2017-00-504 (Emory University), Protocol number A20044 (Georgia State University).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are available in the main text or supplementary materials.

Conflicts of Interest: PS. and S.J.C.R. are the co-founders of the Metacclipse Therapeutics Corporation (MTC) and hold equity and stock options. The corresponding author (PS.) holds shares in Metacclipse Therapeutics Corporation, a company that is planning to use CPI-anchored molecules to develop a VLP-based vaccine in the future, as suggested in the current manuscript. C.D.P. and S.R. declare competing financial interests in the form of stock ownership and paid employment by Metacclipse Therapeutics Corporation. K.M.I., S.-H.N., A.N.B., L.J., T.V.G. and C.N.W. declare competing financial interests in the form of paid employment by Metacclipse Therapeutics Corporation. One or more embodiments of one or more patents and patent applications filed by Metacclipse Therapeutics Corporation and Emory University may encompass the methods, reagents, and data disclosed in this manuscript. All other authors have no competing interests to declare.

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ACE2: A Double-Edged Sword Against SARS
CoV-2 Associated Cardiovascular Complications
and Endothelial Dysfunction

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Abstract: The outbreak of novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) during late December 2019 in Wuhan, Hubei Province, China, has become a pandemic of global concern in a very short time, impacting human life and economic slowdown. The severity of SARS-CoV-2 infection can be accentuated by an increased number of human deaths, specifically in older populations and patients with pre-existing cardiovascular disease (CVD) complications. SARS-CoV-2 binds to Angiotensin-Converting Enzyme-2 (ACE2) receptors on host cells, followed by its internalization, rapid multiplication, and instigating cytokine storm. This review aims to decipher the role of ACE2 in SARS-CoV-2-infected patients with pre-existing CVD conditions. While in CVD patients, stimulation of ACE2 expression protects against CVD-associated complications through antagonizing the detrimental effects of Angiotensin II (Ang II) to maintain vascular homeostasis and production of nitric oxides in blood vessels. It is still unclear why CVD patients are at higher risk of SARS-CoV-2 infection and have a higher mortality rate. Endothelial Cells (ECs) are monolayers of cells covering the inner wall of blood vessels and all major organs in our body. They play an essential role in maintaining normal vasculature; therefore, ECs’ dysfunction has been considered the CVD hallmark. Improvement in CVD is related to the restoration of ECs’ function. Exploring the role of ECs dysfunction concerning the SARS-CoV2-CVD molecular axis could help decipher why CVD patients are at increased risk of novel coronavirus-related fatalities.

Keywords: Atherosclerosis, Cardiovascular disease, SARS-CoV-2, ACE2 receptors, Endothelial dysfunction, Blood vessels

I. INTRODUCTION

CVD, including atherosclerosis, is the most common cause of death worldwide, posing severe health and economic burden [1]. Family history of coronary heart disease, high level of Low-density lipids (LDL) or lower level of high-density lipid (HDL), obesity, diabetes, and cigarette smoking are the most common risk factors associated with CVD [2, 3]. Pathobiology of atherosclerosis involves injury to endothelial lining, accumulation of lipid into the subendothelial lining in vascular smooth muscle cells, and inflammation. ECs are monolayers of cells covering the inner wall of blood vessels and are responsible for maintaining vascular homeostasis through the secretion of vasoactive substances such as NO to maintain vasculature integrity. Therefore, dysfunction of endothelial cells has been considered a prominent hallmark of cardiovascular events for decades. ECs respond to various stimuli by releasing vasoactive substances like nitric oxide (NO), carbon mono oxide (CO), endothelin, and superoxide. NO is a small gaseous molecule that diffuses into Vascular Smooth Muscle Cells (VSMCs) in blood vessels and regulates cyclic guanosine monophosphate (cGMP) production, which is required for vasodilation and subsequent relaxation of vessels [4-7]. The presence of membrane-bound receptors for growth factors, metabolites, as endothelin-1 and hormones, and surface receptors for cell-cell and cell-matrix interaction, including ACE2, make ECs a critical barrier of the vasculature. EC line all major organs in the body, such as kidneys, heart, lungs, gut, and brain, and it also expresses ACE2 cell surface receptors that the virus binds to. As a hallmark of atherosclerosis and CVD, ECs dysfunction draws our attention as a target that could be linked with COVID-19 severity. Restoration of ECs function may play an essential role in minimizing viral burden, given its critical role in the vasculature.

Virus Structure and genomic organization

The non-segmented positive-sense RNA genome of Coronaviruses (CoV) is the largest genome among all RNA viruses with approximately 30 Kb in size. Spike-like structures on the outer envelope of CoV are a characteristic feature of the enveloped virus. The virus particle has four structural proteins, namely spike (S), membrane (M), envelope (E) and, nucleocapsid (N) proteins (Fehr and Perlman 2015). Functionally, S protein facilitates virus attachment to the host cell surface receptors and internalization of virus inside the host cell. S protein is the most abundant glycoprotein. M protein is required for virus assembly and maintains the shape of the viral envelope. Assembly and release of the virus particle require the interaction between less abundant protein E and M [8, 9]. According to Stohlman et al., deletion of the E gene attenuates the virus as the E gene encodes a small multifunctional protein with ion channel activity, which plays an essential role in virus-host interaction [10]. N
It is the sole nucleoside protein, which has N terminal and C terminal domains. It has been suggested that N protein is heavily phosphorylated, and this triggers a structural change that enhances the viral RNA [10].

**COVID-19 Outbreak and treatment options**

It is the first time that coronavirus has caused a pandemic in humans. However, there are reports on previous outbreaks caused by other CoV members, such as SARS-CoV and MERS. The current pandemic's causative agent is SARS-CoV-2 [11], affecting the hematologic circulatory and respiratory systems. The rapid progression of the disease and its higher transmission rate makes it a severe global health concern. During the early outbreak, a pattern was observed in the infected population, such as fever, body ache, tiredness, difficulty breathing, and lung infection with pneumonia-like symptoms. Currently, more than 185 countries have contacted the disease outbreak [12]. The major challenge in front of the medical healthcare system and scientists is to contain the disease via social distancing and utilizing already available drugs approved by the Food and Drug Administration (FDA), such as Hydroxychloroquine and Remdesivir. There is an urgent need to establish fundamental knowledge and understanding of the host-pathogen interaction to exploit more effective treatment options. Therefore, it is of grave need to understand the pathlogy of the virus and its target cells, including the immune response to the virus replication and infection. To date, there is no specific approved oral drug to treat novel coronavirus infection. However, a few alternative medicines effectively treat COVID-19 patients, such as Remdesivir, Hydroxychloroquine, chloroquine, Azithromycin, convalescent plasma Tocilizumab, Lopinavir/Ritonavir, Tamiflu, Flavivirapin, Colchicine, Ivermectin, and ACE2 inhibitors, etc. Recent research has shown that Auranofin, an FDA-approved drug for Arthritis treatment, is also very effective in SARS CoV-2 infection in human cells [11,13].

A study conducted by Grein et al., with 61 patients from the United States, Europe, and Japan, were administrated 200mg Remdesivir through IV on day one followed by 100mg for the next nine days. At the end of the study, on March 20, 2020, 36 patients out of 53 showed clinical improvements [14].

On May 1, 2020, the FDA issued an Emergency Use Authorization (EUA) for Remdesivir. That means the FDA has not yet approved Remdesivir for treating COVID-19 patients; however, the drug is easily accessible to doctors for the urgent need of COVID-19 hospitalized patients. On June 1, 2020, Gilead pharmaceutical announced Phase 3 clinical trial results in which the Remdesivir is found to improve the condition in moderate COVID-19 patients. However, still no data and extensive studies are required.

Hydroxychloroquine and chloroquine are the approved drugs used to treat malaria and autoimmune conditions such as Arthritis and Lupus. A very small French population with COVID19 was recruited to establish the efficacy of Hydroxychloroquine in COVID19 patients. The patients were administrated with 600mg (three times a day with 200mg dosage each time) of oral hydroxychloroquine sulfate for 10days. On Day 6, the viral load decreased significantly among the infected group as compared to the control. Subsequently, on March 28, 2020, Hydroxychloroquine is put on the FDA's EUA list [15].

Convalescent plasma (CP) is another treatment method in COVID-19 patients in which blood plasma of infected patients is infused in another COVID-19 patient. A study of 10 adult patients showed that 200ml of CP effectively cleared viral load in 7 days. However, larger-scale research and random trials are required before making any final conclusion [16]. Additionally, combination therapy is also being employed to find the best combination. On May 1, 2020, FDA issued an application of Emergency Investigational New Drug (eIND) for CP as the COVID-19 treatment option. Tocilizumab is a drug used for the treatment of inflammatory conditions like rheumatoid arthritis. Inflammation is a natural response of our immune system against harmful pathogens. Sometimes due to the overactive immune system, inflammations go haywire, causing cytokine storms in which the immune system works against our own body. IL-6 is a major inflammatory cytokine, and Tocilizumab help attenuate inflammation by blocking the IL-6 receptor [17,18].

Proteases are fundamental for virus replication. Protease inhibitors are used to inhibit SARS-CoV-2 viral replication by inactivating the proteases. Lopinavir/Ritonavir is used in the emergency management plan for COVID-19. Remdesivir is a nucleotide analog previously used in the treatment of the Ebola outbreak in Africa and is currently used in the treatment of COVID-19. Convalescent plasma therapy is also used as a treatment strategy against SARS-CoV-2. Patients recovered from COVID-19 carry the SARS-CoV-2 specific antibody in their blood. Therefore already built antibodies from recovered patients serve as a therapeutic alternative to treat SARS-CoV-2 infected patients [19].

Increased cytokine levels and inflammatory response due to the SARS-CoV-2 infection are among the most critical causes of organ damage. Abnormal release of proinflammatory cytokines, mainly IL-6, TNFα, and IFNγ, contributes to cytokine release syndrome. Tocilizumab is a monoclonal antibody against the IL-6 receptor is used as a treatment option in severe COVID-19 patients. Tocilizumab is a monoclonal antibody against the IL-6 receptor is used as a treatment option in severe COVID-19 patients. Published literature suggests thromboembolic manifestations associated with COVID-19. Activation of the coagulation cascade and endothelial injury are indicated as a cause for the development of a prothrombotic state associated with an exaggerated pro-inflammatory response. The use of anticoagulants such as heparin remains an area of conjecture with no definite guidelines of its usage [20].

As of June 17, 2021, according to NIH...
(www.covid19treatmentguidelines.nih.gov), the antiviral drugs that are approved or under consideration for COVID-19 are listed in table 1.

<table>
<thead>
<tr>
<th>Treatment category</th>
<th>Treatment options</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiviral drugs</td>
<td>Remdesivir, Chloroquine or Hydroxychloroquine with or Without Azithromycin, Ivermectin, Lopinavir/Ritonavir, or other HIV-1 Protease inhibitors</td>
</tr>
<tr>
<td>Antibody therapy</td>
<td>Anti-SARS-CoV-2 Monoclonal Antibodies, Convalescent Plasma, SARS-CoV-2 Specific Immunoglobulins, IVIG-SARS-CoV-2</td>
</tr>
<tr>
<td>Cell-Based Therapy Under Evaluation</td>
<td>Mesenchymal stem cells could reduce acute lung injury and inhibit the cell-mediated inflammatory response induced by SARS-CoV-2.</td>
</tr>
<tr>
<td>Immune-modulators</td>
<td>Colchicine, Corticosteroids, Flavonoids, Interleukins-1 and 6 inhibitors, Kinase inhibitors</td>
</tr>
<tr>
<td>Anti-thrombotic therapy</td>
<td>Vitamin C, Vitamin D, and Zinc supplements</td>
</tr>
</tbody>
</table>

CVD is associated with low-grade chronic inflammation; whether the chronic inflammation synergistically facilitates the virus infection leading to organ failure remains unknown. Secondary organ failure is a major concern in SARS-CoV-2 infected CVD patients. Direct infection of ECs by SARS-CoV-2 has been reported and is associated with a microvascular injury that may be deleterious in prevalent CVD patients [21-23]. In addition to social distancing and isolation measures, careful monitoring of COVID-19 symptoms and frequent evaluation of cardiac and inflammatory biomarkers to identify early signs of cardiac injury can be helpful. Re-optimizing CVD treatments after infection and closer monitoring for post-COVID symptoms can also be useful [24].

ACE-2 has a cardioprotective role, and SARS-CoV-2 internalization into cells has been reported to downregulate ACE-2 expressions [25-29]. In the normal adult lung, ACE-2 is primarily expressed in primary alveolar epithelial type II cells and plays a protective role in the lungs. Surfactant proteins produced by these cells help reduce surface tension and protect alveoli from collapsing [30, 31]. Ang II is the main effector molecule in the RAAS pathway, which is upregulated in many diseases and it’s a common target in various cardiovascular disorders [32]. ACE-2 helps inactivate Ang II by converting Ang II to Ang (1-7) [33]. According to a recent study, exogenously administered recombinant human ACE-2 (rhACE-2) can prevent SARS-CoV-2 infection by acting as a decoy. rhACE-2 effectively reduced the infection in cell culture and human blood vessels organoids and kidney organoids. The protective role of rhACE-2 has been reported by different groups in CVDs, rhACE-2 could be a promising treatment option for CVD patients with COVID-19 infection [34-36]. ACE inhibitors/angiotensin receptor blockers (ACEIs/ARBs) are increasingly used in CVD treatments, and according to studies, they help upregulate ACE-2 expression. The fact that ACE-2 expression could correlate with SARS-CoV-2 susceptibility and intake may predispose CVD patients to increased risk of SARS-CoV-2 infection. Therefore, the usage of such drugs should be very carefully evaluated in CVD patients [37].

Cardiovascular complications in COVID-19 patients and the role of Endothelial Cells

ACE2 plays an essential role in inhibiting the pathogenic effect of AngII. AngII is a potent inducer of ECs dysfunction, cardiovascular-associated disease, the proliferation of VSMCs, hypertension, and diabetes (Ferrario, Jessup et al. 2005, Tikellis, Bernardi et al. 2011). Functionally, ACE2 promotes the degradation of AngII to Ang-(1-7) and promotes ACEI and AT1R to increase circulating Ang-(1-7) [38]. Ferrario, et al. 2005 and others have established that inhibition of AngII synthesis or its activity by ACEIs (lisinopril) and/or ARBs (losartan) can significantly enhance ACE2 activity [39]. Following the footsteps, a clinical trial is underway to evaluate its effect against COVID-19 (NCT04340557).

At basal conditions, endothelial function in arterial vasculature plays a crucial role in the maintenance of vascular tone by regulating key mechanisms such as adhesion of circulating blood cells, vascular smooth muscle cells (VSMC) growth, and proliferation and inflammation, and immune response. Under normal physiological conditions, ECs maintain basal perfusion determined by cardiac output. Vascular contraction and relaxation in local blood flow are balanced by EC-derived vaso-dilative and vaso-constrictive factors. Nitric oxide (NO) is one of the most critical signaling molecules required to maintain a healthy vasculature. NO is a potent vasodilator released by EC due to shear stress. NO production results from endothelial nitric oxide synthase (eNOS), in which L-arginine is used as a substrate to produce intracellular cyclic GMP [40]. Compromised NO production in Endothelial dysfunction in the vasculature is profoundly implicated in the pathogenesis of cardiovascular diseases.

A plethora of research has demonstrated that ECs dysfunction is characterized by altered vascular tone, increased inflammatory molecules, and redox imbalance within the blood vessels [41, 42]. Impairment of EC-dependent vasodilation is the hallmark of endothelial dysfunction, responsible for various types of CVD, including diabetes mellitus, hypertension, atherosclerosis, and heart failure [43]. Activation of EC refers to increased expressions of cytokines, chemokines, and adhesion molecules leading to the pro-inflammatory and prothrombotic microenvironment in the blood vessels.

Underlying CVD complications in COVID-19 is very alarming, especially considering the high number of CVD patients worldwide and in the United States. It is not well understood why CVD enhances COVID-19 infection and severity in infected patients. It is well researched that ECs are essential in maintaining vascular homeostasis and that they play a vital role in the development of CVD. It is still not well known if ECs are also involved in cardiovascular complications in COVID-19 patients.
According to Varga et al., 2020, a kidney transplant 71 years old male patient with coronary artery disease and arterial hypertension died on day eight after COVID-19 infection [44]. Postmortem of the patient's transplanted kidney's electron microscopy showed virus inclusion bodies in ECs of the organ. Histological analysis also showed ECs associated with inflammatory cells and apoptotic bodies in the heart, small intestine, and lungs. Infiltration of mononuclear cells and small congealed vessels in the lungs were observed [44]. In another case, a 58-year-old female COVID-19 patient with pre-existing diabetes, arterial hypertension, and obesity developed respiratory failure due to SARS-CoV-2 infection. The complications led to multi-organ failure and required renal replacement therapy [44].

Another example is a 69-year-old male patient with hypertension who contracted respiratory failure due to COVID-19 and was put on a mechanical ventilator. Echocardiography showed reduced left ventricular ejection fraction. The patient survived, while histological analysis showed prominent endothelial of submucosal vessels and apoptotic bodies. This piece of work indicates that SARS-CoV-2 binds to the ACE2 expressed by ECs on the host cell membrane, followed by its internalization into the lung, heart, kidneys, brain, and the gut as these organs predominantly express the ACE2 receptor [44, 45].

Reactive oxygen species (ROS) act as a double-edged sword in CVD and have been implicated in atherosclerosis [46]. Though ROS is required for vascular homeostasis, uncontrolled production has detrimental effects, and ECs maintain this balance of ROS production and oxidative stress (Figure 1). According to Green et al., 2020, the lungs' respiratory distress caused by COVID-19 quickly extends to the vascular system in the heart, gut, brain, and kidneys in association with ECs dysfunction and fatal blood clotting events [46]. Guan et al., 2020 reported that the mortality rate of patients with CVD-associated diseases such as hypertension was 14.9%, diabetes 7.4%, and coronary heart disease was 2.5%, while hospital stay was approximately 12 days among 1099 patients [47, 48]. Additionally, a large pool of 44672 COVID-19 patients were screened for CVD-associated disorders. About 4.2% of them had CVD, 12.8% were hypertensive, and 4.7% were critically ill. 4.2% of CVD made around 22.7% of all fatalities reported, with a mean fatal rate being 10.5%. Another study conducted by Huang and Chen et al., 2020 confirms that patients with underlying CVD primes for severe COVID-19 infection in association with severe cytokine storm [17, 18, 48, 49].

ACE2: An advantage or disadvantage in CVD associated SARS-CoV-2 infection

Since host ACE2 is the prime target for SARS-CoV-2 infection (the spike protein) and has a stronger binding affinity to human ACE2 [17, 45, 50] so, manipulating the point of interaction between ACE2 and SARS-CoV-2, or inhibition of ACE2 activity, has gained serious attention and prompted newer dimensions to therapeutic approach (Clinical trials - NCT04405999, NCT03535096, NCT03435786, NCT0340557, and NCT04325195). Mechanistically, ACE2 degrade Ang II into Ang-(1-7), while Ang I is degraded to Ang-(1-7) via endopeptidase (NEP). Ang-(1-7) then binds with Mas receptor (Mas-R), facilitating anti-inflammatory, anti-fibrotic response, generate NO and maintain blood pressure to antagonize the detrimental effect of Ang II [45]. During viral infection, ACE2 binds with SARS-CoV-2, disrupting the traditional pathway responsible for maintaining vascular homeostasis. Internalization of the virus particle in ECs increases viral load to promote cardiovascular complications. Complications associated with CVD patients are administering drugs to inhibit the renin-angiotensin-aldosterone system (RAAS or RAS) or the status [45]. Inhibition of RAAS in hypertensive patients shifts the pathway towards ACE2 mediated Ang-(1-7) generation and further maintained vascular homeostasis. On the other hand, suppression of ACE2 results in the accumulation of Ang II, which stimulates angiotension II type 1a receptor to increase pulmonary vascular permeability, thus explains the pathology associated with decreased ACE2. While in the case of SARS-CoV-2 infection, ACE2 inhibition could be a treatment option, but this option could be unfavorable in CVD patients and those taking ACE2 activators. Atherosclerosis is the most prominent form of CVD and is associated with an increased level of Ang II. A study by Tesarovici et al., 2010, establishes that Ang-(1-7) infusion in ApoE−/− mice fed with a high-fat diet can significantly improve ECs function, which confers atheroprotection via restoration of available NO, while inhibition of RAAS can prevent atherosclerosis in ApoE/Ace2 doubleKO mice [51, 52]. A combination of ACE inhibitors (ACEis) to enhance ACE2 activity may help cardiovascular patients suffering from COVID-19. Owing to the critical role played by the ACE2 receptor, various clinical trials are focused on finding the alternative to overcome the ACE 2 pathway [17, 49]. Some of the critical clinical trials are Bromhexine Hydrochloride and valsartan; some recombinant proteins used such as • Recombinant human angiotensin-converting enzyme 2 (rHACE2) • APN01, Recombinant Bacterial ACE2 receptors -like enzyme of B38-CAP.

II. DISCUSSION

Researchers worldwide explore various avenues to establish the cure, decrease mortality rate, and minimize hospital stay due to SARS-CoV-2. Having pre-existing CVD in COVID-19 patients results in severe complications. It makes it more difficult for clinicians to establish a fine-tune between available drugs and treatment methodology to save a patient's life. Maintaining a proper clinical record and standard procedure utilization is crucial to establish a generalized treatment strategy that holds the key to a successful drug trial. The discrepancy in research methodology and negligence to recognize genetic and geographical variations have been a righthand to achieving consensus for the COVID19 treatment strategy.
Approximately 735,000 Americans suffer a heart attack every year, 525,000 undergo a first heart attack, and 210,000 have already had a heart attack [39]. ECs dysfunction or injury was first proposed more than 20 years ago, and then several scientific works of literature came out in support of this concept [28]. There is extensive evidence that ECs dysfunction is of primary importance in the pathogenesis of atherosclerosis and lesion formation [53]. EC is the first line of defense against various risk factors and vascular disease. EC dysfunction is the starting stage of atherosclerosis and an important prognostic marker of CVD. Since ECs are in direct contact with blood flow and serves as a barrier in the vascular system, it is susceptible to any change in the blood vessels. EC is the first line of defense against velous risks factors and vascular disease. Endothelial cells in the brain are known as brain endothelial microvascular cells. A recent study showed that SARS-CoV-2 could infect the central nervous system in mice brain and cause neuroinflammation and encephalitis [54]. As the number of research concerning SARS-CoV-2 grows, it is expected to unfold many more unclued factors. To better understand the cause and effect of COVID-19 infection, we need to explore cardiovascular complications in specific conditions, along with the identification of disease models. Mesenchymal stem cells are multipotent adult stem cells found in most human tissues. These cells can regenerate and differentiate into multiple tissue types. Therefore, Mesenchymal stem cells could have an enormous scope in regenerative medicine. Moreover, they lack ACE2 receptors to which SARS-CoV-2 binds for entry into cells; these cells are resistant to infection making them a promising target against SARS-CoV-2 [55, 56].

Figure 1: The SARS CoV-2 binds to ACE2 receptor on endothelial cells by its S protein. ACE2 has a cardioprotective effect. On the contrary, increased ANG II in the absence of ACE2 increases the risk of CVD. SARS CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; S protein, Spike protein; ANG, Angiotensin; AT1R, Angiotensin I receptor; NO, nitric oxide; ROS, reactive oxygen species.

REFERENCE


[51] Tesanovic, S. et al., Paspargynic and atheroprotective effects of...