Early View

Review

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Pathophysiology and Potential Future Therapeutic Targets using Preclinical Models of COVID-19

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Abstract

Severe acute respiratory syndrome–coronavirus 2 (SARS-CoV-2) gains entry into the lung epithelial cells by binding to the surface protein angiotensin-converting enzyme 2. Severe SARS-CoV-2 infection, also known as coronavirus disease 2019 (COVID-19), can lead to death due to acute respiratory distress syndrome mediated by inflammatory immune cells and cytokines. In this review, we discuss the molecular and biochemical bases of the interaction between SARS-CoV-2 and human cells, and in doing so we highlight knowledge gaps currently precluding development of new effective therapies. In particular, discovery of novel treatment targets in COVID-19 will start from understanding pathologic changes based on a large number of autopsy lung tissue samples. Pathogenetic roles of potential molecular targets identified in human lung tissues must be validated in established animal models. Overall, this stepwise approach will enable appropriate selection of candidate therapeutic modalities targeting SARS-CoV2 and the host inflammatory response.
Background

The novel coronavirus disease of 2019 (COVID-19) caused by severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) was first identified in Wuhan, China and quickly spread to become a pandemic. As of September 1, 2020, the World Health Organization (WHO) reported over 25 million confirmed cases of COVID-19 worldwide, resulting in more than 850,000 deaths globally and 180,000 deaths in the United States alone [1].

The clinical symptoms and pathobiology of COVID-19 are similar to those seen in infections caused by another coronavirus, severe acute respiratory syndrome coronavirus (SARS-CoV), which globally resulted in approximately 774 deaths in 2003 [2]. Recent studies have identified that similar to SARS-CoV, SARS-CoV-2 binds a specific human cell surface protein, angiotensin-converting enzyme 2 (ACE2). ACE2 is expressed by multiple cell types including the epithelial cells of the lungs, the intestine, the kidneys, and endothelial cells [3]. Although multiple clinical trials have been underway targeting various aspects of the viral replication cycle and host immune response, COVID-19 continues to spread, resulting in devastating medical and socioeconomic consequences worldwide.

Considering that SARS-CoV-2 in severe cases activates a multitude of inflammatory immune cells leading to cytokine storm, it is not surprising that the strategy of targeting one particular inflammatory signal has not been shown to be highly efficacious in trials [4]. Moreover, long term consequences in recovered patients or asymptomatic carriers remain uncertain. These shortcomings can be addressed by utilizing animal models that recapitulate COVID-19-related pathological characteristics in humans. Recently, multiple animal models such as mouse [5–7], Syrian golden hamster [8], ferret [9], and nonhuman primates [10], have been utilized to evaluate the COVID-19 related pathology. Despite their inherent limitations, these animal models represent powerful tools that will allow us to conduct mechanistic studies of disease
pathogenesis, identify key therapeutic targets, and test efficacies of potential therapeutic interventions. In this review we highlight the overall pulmonary pathophysiology of COVID-19, and provide an overview of animal models well-suited for mechanistic studies and preclinical therapeutic trials.

**Origin of SARS-COV-2 and Mode of Transmission to Humans**

SARS-CoV-2 is the seventh member of *Coronaviruses*, a large family of single-stranded enveloped RNA viruses with sizes ranging from 80 to 120 nanometers in diameter [11]. Comparative phylogenetic analyses revealed a close resemblance between SARS-CoV-2 and SARS-like bat viruses, suggesting that these bat viruses most likely serve as reservoir hosts for SARS-CoV-2 progenitor. Initially critical mutations likely provided the capability to infect an intermediate non-human mammal [12–15]. The virus presumably continued to evolve through natural selection to acquire both the ability to infect humans and the capacity for efficient human-to-human transmission (*Figure 1*) [13]. Based on the ability of SARS-CoV-2 to infect animals in both natural and experimental environments and the lack of evidence demonstrating direct bat to human transmission, it has been suggested that pangolins served as intermediate hosts between bats and humans [16].

**ACE2: A Portal of Entry for SARS-CoV-2**

Infection of mammalian cells occurs through the virus binding to cell surface proteins, with a critical interaction between the spike (S) glycoprotein on SARS-CoV-2 and the mammalian ACE2 surface protein [17]. Once the virus binds to host ACE2, a furin cleavage site on the viral S protein facilitates viral entry into the host cells [13].

ACE2 is ubiquitously expressed on the surface of alveolar epithelial type I and type–II cells [3]. ACE2, like ACE, is a key protein of the renin–angiotensin system (RAS), which contributes to vascular homeostasis; ACE2 dysregulation under pathological conditions leads to multiple
cardiovascular abnormalities [18, 19]. Classic physiological functions of ACE and ACE2 in the RAS are summarized in Figure 2. Briefly, cleavage of angiotensin-I by ACE generates angiotensin-II, a peptide that triggers a multitude of pathologic signaling through the angiotensin II type 1 receptor (AT1 receptor), resulting in vasoconstriction, cell proliferation, inflammation, thrombosis, and vascular remodeling [20, 21]. These pathogenetic effects of ACE/angiotensin II/AT1 axis are counterbalanced by a cardioprotective signaling axis, in which cleavage of angiotensin-II by ACE2 generates angiotensin 1-7, peptides with Mas receptor-mediated anti-inflammatory, anti-apoptotic, anti-thrombotic, and vasodilatory properties.

This normally protective enzyme ACE2 is exploited by SARS-CoV-2, serving as a portal of viral entry into mammalian cells. A schematic of the series of events underlying the viral infection of host cells is shown in Figure 3. Both SARS-CoV and SARS-CoV-2 belong to the β-genus of Coronavirus and share ~80% homology [22]. Despite substantial genetic and structural similarities between the two coronaviruses, the S protein of SARS-CoV-2 has a stronger binding affinity for ACE2, which may underlie the greater transmissibility of SARS-CoV-2 and the more pronounced clinical impact of COVID-19 [23, 24].

The S glycoprotein protruding out from the SARS-CoV-2 transmembrane surface forms homotrimers. Each S glycoprotein is comprised of two functional subunits, S1 and S2. The distal S1 subunit contains a receptor binding domain (RBD) that exclusively facilitates binding of the viral envelope to the transmembrane ACE2 protein on the host cells [25]. The membrane-anchored S2 subunit allows fusion of the viral and host cellular membranes, resulting in delivery of the viral nucleocapsid into the cytoplasm of the target cell.

The binding affinity of SARS-CoV-2 for ACE2 and its entry into host cells are modulated by multiple proteases expressed by host cells, including cathepsins L and B, trypsin, factor X,
elastase, furin, and transmembrane protease serine 2 (TMPRSS2) [11, 26–29]. TMPRSS2 cleaves the viral S2 subunit at a site immediately upstream of the peptide sequence that facilitates virus-host cell fusion [11]. This cleavage results in an irreversible conformational change of the S2 glycoprotein, ultimately resulting in more efficient viral entry into the host cell [11, 30]. A recent study showed that blockade of TMPRSS2 activity attenuates SARS-CoV-2 entry into host cells, suggesting inhibitors of TMPRSS2 may serve as therapeutic approaches [31].

Recent studies have reported a higher prevalence of hypertension and diabetes in COVID-19 patients [32–34], which could partially reflect their altered ACE2 expression. While ACE2 protects against the potentially detrimental effects of unopposed RAS by limiting substrate availability for the ACE/angiotensin II/AT1 receptor axis, there is compensatory increased expression of ACE2 following the use of pharmacologic ACE inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). These medications cause an approximately 3 to 5-fold increase in ACE2 expression [35]. This increased expression of the host viral entry protein could hypothetically augment ACE2-mediated viral infection. However, the true clinical impact of these medications in the context of COVID-19 remains uncertain, and whether ACE inhibitors or ARBs should be stopped in those infected with SARS-CoV-2, or potentially even started as an antiviral adjunctive therapy, is currently hotly debated [36–38]. The potential benefit of these medications is that ACEI-mediated inhibition of ACE could through a negative feedback loop limit local tissue accumulation of angiotensin-II, thereby decreasing the overall severity of inflammation [39, 40]. For example, multiple studies have shown that RAS inhibitors effectively suppress the symptoms of acute severe pneumonia in other settings, and thus could be beneficial in patients with COVID-19 [40–43]. These conflicting theoretical risks and benefits, and the lack of robust clinical data on their overall effect in COVID-19 patients with underlying
cardiovascular comorbidities preclude drawing a firm conclusion regarding the clinical use of ACEIs and ARBs in the context of COVID-19.

As discussed below, the pathogenesis of COVID-19 involves a number of pathophysiologic processes including dysregulated innate and adaptive immune responses and upregulated expression of inflammatory cytokines, culminating as acute respiratory distress syndrome (ARDS). It is worthwhile to note that ACE2 has shown to be implicated in regulating all these processes [41, 44, 45]. Studies have shown higher risk of mortality in COVID-19 patients with cardiovascular comorbidities, but with no correlation between the use of ACEIs/ARBs and patient morbidity and mortality [46]. Therefore, in the absence of data to the contrary, it seems logical to continue RAS inhibitors in hypertensive patients, as was also suggested by another recent review [47]. In summary, the current consensus is that previously prescribed RAS inhibitors should be continued in hypertensive patients with known or suspected COVID-19.

**Role of Animal Models in Elucidating the Pathogenesis of COVID-19**

Preclinical models are critical to facilitate the selection of candidate therapeutic approaches for clinical trials. One approach relies on *in vitro* model systems, such as pseudoviral infection assays and direct examination of cells and tissues harvested from COVID-19 patients, which can take place in biosafety level-2 settings, or biosafety level-3 when dealing directly with viral samples. In this section, we focus on another important investigational tool in COVID-19 research: utilization of *in vivo* animal models that can recapitulate key clinical or pathological characteristics of COVID-19. A major challenge in COVID-19 research is the currently limited understanding of the series of events that link the initial upper respiratory tract infection to the subsequent development of lower respiratory tract infection and ARDS. Establishment of robust and reproducible COVID-19 animal models may thus elucidate pathogenetic mechanisms leading to development of effective therapeutic targets.
Historically, mouse models have been extensively used to explore molecular and pathological mechanisms involved in various infectious as well as non-infectious diseases. Specifically, mouse models were previously utilized to investigate diseases caused by other coronaviruses, for example severe acute respiratory syndrome (SARS caused by SARS-CoV-1) and Middle East respiratory syndrome (MERS) [48–50]. These former studies strongly support the promising role of mouse models in COVID-19 research. In addition to the relatively fast reproductive rates and low maintenance costs, another key advantage of using mouse models is the accessibility to numerous inbred and transgenic lines harboring genetic changes that can be inducible and cell-type specific. Examples of currently available mice well-suited for SARS-CoV-2 investigation include knockout models of the following genes: Ace2−/−, Tmprss2−/−, IL-6−/−, and INF-γ−/−. Inducible and cell compartment specific deletion can be used to determine the precise roles of lung epithelial and endothelial cells underlying the crosstalk between the capillary endothelial cells and the alveolar epithelium in SARS-CoV-2 infection in COVID-19. Simultaneously, transgenic mice lacking the TMPRSS2 protein might be helpful in understanding the effect of different therapeutic approaches against SARS-CoV-2 alone or in combination with TMPRSS2 blockade [51]. Aged or inbred mice with chronic underlying disease phenotypes such as hypertension or diabetes can be used to understand potential drivers of age and co-morbid conditions on higher mortality rates in COVID-19. Of note, these mouse models have been used for many years to understand the molecular or immune pathobiology of other pulmonary pathologies, including ARDS, thrombosis, fibrosis, and vasculopathy, all aspects of COVID-19 disease. Key features of mouse models used in studies of coronavirus infections, including SARS-CoV-2, are summarized in Table 1.

While mouse models are valuable tools in uncovering pathobiological mechanisms of SARS-CoV-2 infection, mouse models are also characterized by important limitations. One key
limitation of mouse models of COVID-19 is their relatively mild disease phenotype owing to the inability of SARS-CoV-2 to utilize the mouse orthologue of the human angiotensin-converting enzyme 2 (hACE2) [52]. In response, the hACE2 transgenic mouse model was previously developed to study SARS-CoV, which has now been recently repurposed for investigating the pathogenesis of SARS-CoV-2 infection [6, 7]. Critically, the hACE2 mouse model upon SARS-CoV-2 infection recapitulates the lung pathologies observed in COVID-19 patients, such as interstitial pneumonia with significant infiltration of macrophages and lymphocytes into the alveolar interstitium, with a phenotype of diffuse alveolar damage and ARDS [6]: major drivers of death in COVID-19 [41, 53, 54]. Recently another reliable mouse model incorporating adeno-associated virus (AAV)–mediated hACE2 expression was developed [5]. Another major limitation of mouse models is the inherent different between the immune systems of mice and those of humans. This shortcoming can in part be overcome by utilizing humanized mice (e.g., mice with human peripheral blood mononuclear cells, or huPBMC-NOG), which represents a useful approach to studying the contribution of various immune cells to COVID-19.

Hamsters and ferrets demonstrate disease phenotypes closer to those of humans without requiring transgenic modification. Hamsters infected with SARS-CoV-2 recapitulate key elements of the human lung pathology seen in severe cases of COVID-19, including inflammation, diffuse alveolar damage, peripheral lymphopenia, and marked activation of the innate immune response including high levels of chemokines and other cytokines [55]. Interestingly, conferring passive immunity by transferring sera from recovered hamsters to recipient hamsters newly infected with SARS-CoV-2 led to attenuated viral replications but without a significant reduction in lung pathology [55]—data supportive of the clinical use of convalescent plasma.
Ferrets can also be infected with SARS-CoV-2, resulting in fever and relatively mild lung disease. Ferret-to-ferret SARS-CoV-2 transmission has been reported, suggesting ferrets may be well-suited for studying prophylactic treatments. One major limitation of hamster and ferret models is that all animals uniformly recover following SARS-CoV-2 infection, precluding their clinical relevance to more severe forms of COVID-19 clinical disease characterized by severe ARDS resulting in death. Therefore, both hamster and ferret SARS-CoV-2 models may be most applicable to humans with mild clinical disease or asymptomatic carriers.

Pathologic changes in nonhuman primates typically phenocopy those in human diseases, and for this reason nonhuman primates are considered the gold standard for testing vaccines and therapeutic strategies. In a prior study of Chinese rhesus monkeys (Macaca mulatta), SARS-CoV infection led to the development of neutralizing antibodies against the SARS S glycoprotein; paradoxically, the anti-S neutralizing antibody response occurred significantly faster in monkeys that ultimately died when compared to those that recovered [56, 57]. These data suggest that anti–S–IgG may contribute to a more severe ARDS phenotype, such as by downregulating an anti-inflammatory response [56].

Nonhuman primate models previously used to study SARS-CoV infection have now been repurposed to study SARS-CoV-2 [58–60]. One study showed more pronounced viral shedding in the upper respiratory tract of aged animals as compared to younger animals following intranasal SARS-CoV-2 inoculation [10]. Of note, a similar association between viral shedding and age has been observed in humans with both SARS-CoV and SARS-CoV-2 infections [61, 62]. Although the higher maintenance cost, longer reproductive period, more rigorous ethical and regulatory oversight, and the lack of readily available transgenic variants limit the ability to widely use nonhuman primates to study SARS-CoV-2, their close resemblance to humans and
specifically their similar immune system makes them a powerful model system, potentially in particular for vaccine development [63].

**The Pulmonary Pathophysiology of COVID-19**

SARS-CoV-2 infection involves both the upper and lower respiratory tracts. Approximately 80% of patients with COVID-19 do not require hospitalization, as their symptoms are relatively mild, and their immune systems effectively contain the virus within the upper respiratory tract [64, 65]. In the remaining 20% of patients, the viral infection progresses to involve the lower respiratory tract, resulting in pneumonia [65]. Approximately 6% of COVID patients with pneumonia develop respiratory failure requiring admission to the intensive care unit (ICU) for support due to acute respiratory distress syndrome (ARDS) [66, 67]. The time from disease onset to death in fatal cases ranges from 15 to 52 days [68].

ARDS is a common complication of infectious pneumonias, including those caused by the pathogenic coronaviruses SARS-CoV (SARS), SARS-CoV-2 (COVID-19) and MERS-CoV (MERS) [66, 69, 70]. Autopsy findings in patients who die of COVID-19 include diffuse alveolar damage, bronchogenic pneumonia, alveolar hemorrhage with capillary damage, and microvascular thrombosis, all of which are similarly seen in ARDS secondary to other etiologies [67, 69, 71–75]. Although excessive host inflammatory response has been thought to drive ARDS and result in multi-organ failure [76], precise immunological features and molecular mechanisms underlying severe cases of COVID-19 are not completely understood. *In vivo* and cell-based studies indicated that lung injury in SARS-CoV infection is initially triggered by the viral spike protein [25]. For example, intraperitoneal administration of Spike-Fc fusion protein was sufficient to cause lung injury in mice, and Spike-Fc treatment exacerbated the severity of lung injury in acid-challenged mice. In contrast, Spike-Fc protein administration did not affect lung disease severity in ACE2 knockout mice, supportive of the concept that ACE2 plays a
critical role in the initial stages of SARS-CoV-2 infection [53]. Using a non-human primate model of SARS-CoV, another group of investigators showed early administration of anti–spike IgG antibody resulted in more pronounced production of inflammatory cytokines by recruited macrophages and severe lung injury [56].

Pathologic changes of the pulmonary vasculature and the lung alveoli result in impaired gas exchange. Lungs in COVID-19 are characterized by aberrant vasodilation, arteritis, macro- and microvascular thromboses, and endothelial dysfunction which individually and collectively exacerbate ventilation-perfusion mismatch [77–80]. Lung tissues in fatal cases of both SARS-CoV and SARS-CoV-2 infections share similar molecular, immunological, and pathological signatures [81, 67, 82, 83]. As illustrated in Figure 4, autopsy studies of lungs from COVID-19 patients reveal findings of diffuse alveolar damage, characterized by widespread type II pneumocyte hyperplasia, epithelial necrosis, fibrin deposition, hyaline membrane formation and inflammation [68, 71, 84].

There is strong evidence for a role of CD4+ T (helper, or Th) cells in promoting lung injury in COVID-19. Immunohistochemistry of autopsy specimens revealed increased infiltration of both CD4+ and CD8+ (cytotoxic) T lymphocytes within the alveolar septa, with relatively few CD68+ macrophages [71, 85]. The lung parenchymal pathology is associated with highly increased production of Th1 inflammatory cytokines, including INF-γ, IL-1β, and IL-6. In addition, peripheral blood lymphopenia and a reduction in circulating CD4+ and CD8 T cells are observed in COVID-19, consistent with these cells exiting the circulation as they move into the lung tissue [68, 71, 85]. In both ICU and non-ICU patients with COVID-19, Th1 cytokine concentrations positively correlated with viral loads and the severity of clinical lung injury [67]. These studies indicate that the Th1 cytokines may serve as biomarkers for predicting disease severity [67, 86] and potentially as therapeutic targets which exacerbate the disease pathology.
It has been hypothesized that the lung physiology in COVID-19 might differ from those seen in other forms of ARDS, as it has been observed that lung compliance in patients with COVID-19 may be preserved relative to the degree of hypoxemia, as compared to other ARDS etiologies [87]. One possible explanation for this observation is that the COVID-19 pathophysiology might predominantly involve the pulmonary vascular endothelium rather than the alveolar epithelium. While not unique to COVID-19-triggered ARDS, this hypothesis is supported by many clinical and pathological observations: small-vessel inflammation and thrombosis on histopathology [75, 88]; higher rates of clinical venous thromboembolism; abnormal coagulation profiles as indicated by elevated D-dimer concentrations [80, 89] and thromboelastogram data [90]; endothelial ACE2 expression [35]; and endothelial SARS-CoV-2 infection [80]. Furthermore, cardiovascular comorbidities characterized by endothelial dysfunction, such as hypertension, diabetes and obesity, are also risk factors for increased mortality in COVID-19. Of note, autopsy reports have also demonstrated the presence of SARS-CoV-2 antigens in extra-pulmonary organs, including the kidneys, liver, spleen, neurons, and the gastrointestinal tract. It is plausible that ACE2 in the arterial and venous endothelial cells of these organs might serve as conduits for the systemic inflammatory response seen in COVID-19 [91]. Therefore, targeting endothelial ACE2 could be a potential therapeutic strategy in SARS-CoV-2 infection.

Potential Molecular and Biochemical Therapeutic Targets in the Host

Given the data discussed above regarding the components of the host which facilitate viral entry such as ACE2 and contribute to an over-exuberant immune response such as CD4 T cells, there are many potential candidate therapeutic targets which could be found to be effective in COVID-19. Additionally there are many targets within the virus itself, review of which has been recently published elsewhere [92] and is outside the scope of the present review, but the combination of anti-viral and host-modulating therapeutics may prove to be especially powerful.
The RAS pathway

As depicted in Figure 1, physiologic effects of ACE inhibitors and ARBs can be complex, and the overall outcome of such interventions in the context of COVID-19 is unpredictable. ACE2 facilitates viral cell entry, but ACE2 can also have protective effects through the conversion of angiotensin II into cardioprotective angiotensin 1-7, thereby attenuating AT1 receptor-induced pathologic downstream effects [93]. Recently, it was reported that circulating angiotensin II levels were markedly elevated in a cohort of COVID-19 patients compared to healthy controls [42, 94], suggesting that upregulation of ACE2-mediated angiotensin 1-7 production could reduce RAS-derived multi-organ injury in these patients. It is noteworthy that clinically used ACEIs do not affect the ACE2 isoform, the substrate binding site of which demonstrates amino acid substitutions when compared to that of the ACE isoform (Figure 1).

One potential therapeutic strategy targeting RAS is blocking the interaction between ACE2 and SARS-CoV-2, for example through the small molecule APN01 (Aperion Biologics, Vienna, Austria), which is a recombinant human ACE2 protein. By mimicking endogenous human ACE2 and binding SARS-CoV-2, APN01 can block viral cell entry. In addition, it can also lessen the AT1 receptor-mediated injurious inflammatory responses in the lungs, protecting from ARDS and other lung damages. APN01 was well-tolerated in patients with pulmonary arterial hypertension and ARDS, as well as in healthy volunteers in phase I and phase II clinical trials. APN01 is currently being studied in a phase II clinical trial (NCT04335136) in COVID-19 patients.

Another promising approach to inhibit the virus-host cell interaction is targeting the receptor-binding domain (RBD) of the SARS-CoV-2 S-protein, thereby blocking its association with pulmonary cell surface receptors like ACE2. Recently, the human monoclonal antibody 47D11
was shown to neutralize both SARS-CoV and SARS-CoV-2 and prevent their host cell entry by binding to a conserved epitope of the S protein RBD in cell culture [95]. Another recent study suggested an essential role of CD147, another cell-surface protein, in facilitating SARS-CoV-2 invasion of the host cells [96]. Based on this observation, efficacy of CD147-binding meplazumab in COVID-19 is being tested in a clinical trial [97]. One limitation of these approaches is that mutation of the viral protein from evolutionary pressure may result in resistance to any one compound—something that will need to be carefully monitored during clinical studies and with clinical use if these medications are approved.

**The Complement System in COVID-19**

The complement system is an essential component of the innate immune system [62, 98–100]. The complement system is comprised of more than 30 soluble and cell surface-associated proteins, which are activated through three interconnected pathways: classical, alternative, and lectin [101]. Proteolytic cleavage of C3 by C3 convertase represents the final common pathway of the three pathways, resulting in the generation of anaphylatoxins, including C3a, C4a, C3b, and C4b. Nominally these complement fragments contribute to the elimination of pathogens through multiple biological processes, including opsonization, myeloid cell activation, and B and T cell activation. The binding of C3b to C3 convertase generates C5 convertase, which in turn cleaves C5 to generate the terminal anaphylatoxins, C5a and C5b [102]. These anaphylatoxins exacerbate inflammation resulting in cell injury [103, 104]. In prior work on SARS-CoV infection, complement activation promoted systemic inflammation, rather than suppressing viral replication. Dysregulated complement activation has been previously associated with acute lung injury induced by other viral infections.

Similar to SARS-CoV infection, complement activation appears to be a characteristic feature of COVID-19 [105, 106]. Recent reports of COVID-19 patients documented systemic complement
activation involving multiple organs, including the lungs and the kidneys [79, 107–110]. Lung tissue from severe COVID-19 patients revealed C3a generation and C3-fragment deposition, accompanied by elevated serum C5a levels [111]. In addition, deposition of membrane attack complexes composed of complement factors C5b-9 has been seen in the renal tubules of COVID-19 patients [108]. The SARS-CoV-2 nuclear (N) protein can activate the lectin pathway, and deposited mannose-binding lectin serine protease 2 and C4 were observed in the lungs of COVID-19 patients [110, 111].

The likely contribution of complement proteins to tissue injury in COVID-19 has led to therapeutic studies targeting multiple checkpoints in the complement cascade. The therapeutic potential of manipulating the complement system was previously suggested by studies of SARS-CoV and MERS. For example, SARS-CoV-infected C3 knockout mice, when compared to wildtype mice, demonstrated fewer neutrophils and inflammatory monocytes in the lungs along with lower levels of lung and serum cytokines [99]. Importantly, C3 deletion itself did not affect the viral load in the lungs. Similarly, a potential benefit of blocking complement signaling has been demonstrated in animal models of SARS-CoV-2 infection. Blockade of the C5a-C5aR axis abrogated proinflammatory cytokine expression and the pulmonary infiltration of macrophages, neutrophils, and lymphocytes [111]. There are also early reports of a potential benefit from administration of anti-C5a monoclonal antibody in severe COVID-19 patients [111, 112]. Based on these data, there are ongoing studies (NCT04369469 and NCT04371367) of the C5 inhibitor ravulizumab and the anti-C5a receptor antibody avdoralimab in COVID-19 patients [113–115]. Overall, it is conceivable that targeting more proximate complement pathway targets in the upstream activation cascades (e.g., C3 or C4) may lead to more deleterious off-target consequences by attenuating the virus-eliminating effects of the complement system, while intervening at more terminal anaphylatoxins like C5a-C5aR may result in a more favorable and effective treatment strategy.
Targeting Inflammatory Cells and Cytokines in COVID-19

Immune dysregulation and excessive inflammatory cytokine production are implicated in COVID-19-induced ARDS and multi-organ injury [76]. Biopsy reports of severe COVID-19 patients have shown T cell and myeloid cell infiltration in the lungs and other organs such as the kidneys, heart, spleen and lymph nodes [85, 89, 108, 116]. There is also excessive activation of pro-inflammatory airway macrophages results into increased proinflammatory cytokines including IL-6 and TNFα [117]. Higher levels of serum chemokines including CCL2 and CCL3 further points to recruitment of bone marrow-derived macrophages to the lungs [117]. The exact molecular mechanisms underlying pathologic immune cell activation and cytokine production in COVID-19, however, are not well understood.

One possible mechanism could be impaired type I interferon (IFN) production in severe COVID-19 [9, 118]. IFNs are expressed by multiple immune cells, and in SARS and MERS they negatively regulate the activation and infiltration of alveolar and monocyte-derived macrophages into the lungs [119–121]. Reduced systemic IFN production has been observed in more severe cases of COVID-19 [118]. Therefore, an early intervention which augments IFN signaling, such as by administration of recombinant IFN, might be useful in mitigating the virus-mediated inflammatory response. Blockade of IFN receptor in a SARS-CoV-2 mouse model did not attenuate viral replication [5], however, suggesting the ability of the SARS-CoV-2 virus to evade immune cell-mediated opsonization by suppressing IFN-mediated signaling pathways through currently unknown mechanisms.

Multiple ongoing trials are focusing on blocking inflammatory cytokines including using small molecules, antibodies, or cell-based approaches to reduce endothelial cell activation and injury. These approaches may focus on many pathways simultaneously, or be precisely focused on
single molecules. As in other inflammatory diseases, multiple immune pathways are simultaneously activated in COVID-19, and therefore therapeutically targeting one particular pathway may or may not produce a clinically desirable benefit.

One broad approach is the administration of mesenchymal stem cells (MSCs), which are well-known immuno-modulators that have been shown to alleviate lung injury and enhance lung repair in preclinical models of ARDS, and in early clinical trials of patients with inflammatory lung diseases [122, 123]. In a phase II clinical trial (NCT02097641), a single dose of intravenous bone-marrow-derived MSC administration was both safe and efficacious in patients with moderate to severe non-COVID-19 ARDS [124]. This therapy could be similarly effective in COVID-19 patients as demonstrated in a recent case series of severe COVID-19 patients [125, 126]. This approach has led to the now-ongoing STAT trial of MSCs in ARDS (NCT03818854), which while not focused on COVID-19 a priori is presently enrolling many COVID-19 subjects due to the current preponderance of this disease.

Just as important as uncovering individual therapeutic targets is testing the efficacy of combination therapies, which simultaneously target multiple arms of the immune system or combine anti-viral with host modulating treatments. One example is a clinical trial (NCT04409262) studying the concurrent administration of the anti-viral remdesivir with the IL6 receptor inhibitor tocilizumab—targeting the virus and the host immune response together. Other examples are the combinations of lopinavir/ritonavir, ribavirin and interferon beta-1b, or of interferon beta-1a and remdesivir are being tested (NCT04276688, NCT04492475).

Ongoing pre-clinical studies and the results of these clinical trials will help address important questions regarding the role of immune cells in COVID-19 pathogenesis: Which subset(s) of myeloid cells take up SARS-CoV2 antigens? Which antigen-presenting cells are responsible for
T cell antigen recognition in the lymph nodes? Differentiation into which subsets of T cells is induced by antigen presentation? Which cytokines trigger bone marrow production of inflammatory monocytes and what are the mechanisms underlying their recruitment to the lungs and other organs? How do these immune cells trigger injury of the lungs and other organs in COVID-19? As these questions are answered through mechanistic studies utilizing animal models of SARS-CoV-2 infection and clinical trials, therapeutic approaches will be refined and promising combination therapies will be identified.

*Balancing Type 1 and Type 2 immunity in COVID-19*

It is well established that during viral infections, cells of the innate immune system recognize viral replication intermediates and secrete pro-inflammatory cytokines, contributing to tissue damage [127]. Virus-derived antigens are taken up by antigen-presenting cells and carried to local draining lymph nodes [127]. Depending on the local cytokine milieu in the draining lymph node, CD4+ T cell stimulation by antigen presenting cells induce different types of Th adaptive cell responses. There is a critical balance between an anti-viral innate response crucial to eliminate the invading virus, versus a robust and persistent immune response damaging host tissues. Related to this is an unbalanced adaptive immune response, marked by lower percentages and absolute counts of CD3+, CD4+, and CD8+ lymphocyte populations associated with worse organ injury and COVID-19 related mortality [128]. The exact contributions of Th1 versus Th2 immunity to viral clearance or host tissue injury is not clear in COVID-19. Severe COVID-19 is characterized by a “cytokine storm” which has features predominantly of Th1, including elevated concentrations of IL-2, IL-7, IL-12, G-CSF, IP10, MCP-1, MIP-1α, TNFα, and IFN-γ [55, 68, 71, 85, 118, 129]. Interestingly, similar patterns have been observed in other respiratory viral infections, with predominant features of Th1 cell and cytokines, accompanied by suppression of Th2 immunity [130–133].
Considering that there is a mutually antagonistic balance between Th1 and Th2, with viral induced Th1 immunity blunting Th2 immunity, it may be that promoting a Th2 immune response either prior to or during early infection might suppress the robust and potentially excessive Th1 derived inflammatory response triggered by SARS-CoV-2. This approach has worked with other viral infections such as rhinovirus, respiratory syncytial virus, or influenza A virus, when animals which have biased immune systems that promote a Type 2 immune response were found to have less severe disease than wildtype or Type 1-biased animals. [133–139].

In COVID-19, the equivalent natural experiment will be to observe the outcomes in patients who have chronic, comorbid conditions which drive Th2 immunity, such as Type 2 asthma or concurrent parasitic infections. For example, it may be observed that patients with preexisting Type 2 inflammatory conditions are more susceptible to the initial stages of viral replication due to blunted anti-viral type 1 immunity, but may be relatively protected from later excessive inflammatory complications of COVID-19 such as severe ARDS. Importantly, recent studies found that asthma does not appear to be a significant comorbidity for patients hospitalized with severe COVID-19, and asthma does not increase risk for mechanical ventilation [140, 141]. It would be interested to see how individuals with Th2-dominated parasitic infections like schistosomiasis [142, 143] respond following SARS-CoV-2 infection.

Promoting Type 2 immunity such as administering recombinant Type 2 cytokines could be a therapeutic approach. For example, IL-13 has been shown to downregulate ACE2 expression in airway epithelial cells [144], which may reduce viral entry and excessive production of Th1 cytokines that are detrimental to the cells. An alternative approach would be administration of non-infectious compounds which promote Type 2 immunity, or even auto-transfusion of CD4 T cells that were biased ex vivo to a Th2 phenotype, such as through sensitization to ovalbumin or
Schistosoma egg antigen—approaches which could deliver the benefits of type 2 inflammation without long-term complications associated with chronic asthma or parasitic infections.

**Genetic Association Studies Can Help Guide Therapeutic Development**

The heterogeneity of COVID-19 pathology suggests genetic polymorphisms may contribute to specific immune phenotypes and ultimately the severity and outcome of SARS-CoV-2 infection [145]. Of note, several single nucleotide polymorphisms (SNPs) within genes in the RAS pathway, including the ACE2 gene, have been previously linked to cardiovascular diseases [93, 146–148]. RAS polymorphisms are also associated with pharmacodynamic differences between individuals treated with ACE inhibitors or ARBs [149]. As examples, synonymous and non-synonymous SNPs in pro-inflammatory genes including CXCL10/IP-10, heme oxygenase 1, IL-1α, IL-18, FGL2, and leukocyte antigen class I and II have been associated with either infection, severity or nasopharyngeal SARS-CoV viral shedding [150–154]. Genetic studies which identify risk factors for SARS-CoV-2 infection or COVID-19 pathology in relation to host–pathogen interactions and inter-individual disease phenotypes will help identify at-risk populations, host factors which can be targeted to modulate the disease phenotype, and even potentially novel therapeutic approaches which may be personalized to patients with specific genotypes.

**Conclusions:**

Effective treatments for COVID-19 are urgently needed as respiratory SARS-CoV-2 infection is a devastating condition which is not yet effectively treated. This viral infection represents a unique challenge to the host immune system, but at the same time is a unique opportunity to identify precise therapeutic approaches to this infection and host pathology resulting from a single agent. Three major challenges to developing effective treatments against COVID-19 we have discussed here are 1) the incomplete understanding of the disease pathogenesis, 2) the versatile functions of the virus receptor ACE2, and 3) the delicate balance between the virus-
eliminating and the lung-injuring effects of the host inflammatory response. Discovery of new, effective and safe treatments will follow selection of appropriate therapeutic targets based on human lung histopathology and conduct of mechanistic studies utilizing animal models, followed by appropriate clinical trials (Figure 5).

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**Conflicts of Interest:** None declared.
Figures and Legends:

**Figure 1. Life cycle of severe acute respiratory syndrome (SARS)-coronavirus (CoV)-2.**

Based on the phylogenetic study transmission of the SARS-CoV-2 evolves from the natural reservoir of bats (non-human host) to current pathogenic state of human outbreak through natural selection or through another mammals, probably pangolin [15, 16] as a career of a non-pathogenic version of the SARS-CoV-2 and later jumped from into humans with the acquired capacity to current pandemic outbreak through human-to-human transmission [12, 155]. The viral ability to infect small and large animals under laboratory settings point to animal as an intermediate natural host [17, 64]. Based on the CDC report, a very small number of pets, including dogs and cats, outside the United States reported to be infected with the virus that causes COVID-19 after close contact with people with COVID-19. However, a tiger at a zoo and two pet cats in New York have also tested positive for the SARS-CoV-2 [64].

**Figure 2. Schematic illustration of enzymatic component the renin–angiotensin system (RAS).** The ACE2–Ang-(1–7)–Mas axis (right side) counterbalances the harmful effects of the ACE1–Ang II–AT1 axis (left side). Angiotensinogen (AGT) gets converted into angiotensin-I through enzymatic action of Renin. ACE2 degrades angiotensin II and generates angiotensin-(1-7) which antagonizes the effects of angiotensin II. Moreover, clinical evidence suggests that RAS blockade by ACE inhibitors or AT1 receptor blockers and mineralocorticoid antagonists enhance ACE2 level that is ultimately beneficial to the patients with cardiovascular diseases [156–159] but deleterious for COVID-19 patients [17, 30, 160]. The ACE inhibitor and angiotensin receptor blockers (ARBs) as shown in red blockers antagonizes the angiotensin II/AT1R axis.
Figure 3. Biological mechanism of SARS-CoV-2 infection. Once the SARS-CoV-2 approaches the cell membrane, basal S1 subunit of viral spike (S) glycoprotein binds to a membrane-bound molecule of ACE-2. As more S1 subunits binds to membrane-bound molecules of ACE-2, the membrane starts to form an envelope around the virus (an endosome). A cell membrane-bound serine protease, TMPRSS2, cleaves the S1 subunits of SARS-CoV-2 from its S2 subunits that mediated endosome entry into the cells (endocytosis). Inside the cell, viral genetic material is released by either acidification or by proteolysis (cathepsin). Viral replication and translation forms new virion that cleaves out from cells by exocytosis. Of note, ACE2 mediated cardiovascular protection is lost following endocytosis of ACE2 with SARS-CoV-2 viral particles. The endocytosis triggers ADAM-17 mediated ectodomain shedding of tissue ACE2 [161, 162], which through Integrin pathway induces pathologic intracellular signaling [163]. Lack of ACE2 availability, increases Angiotensin II levels that results into detrimental effect due to increased activity of angiotensin 1 receptors (AT1R) at the expense of ACE2/Angiotensin 1–7 driven protective pathways [94]. Viral infection also results into activation of circulatory inflammatory cytokines, antibody response and immune cells; these may damage airways epithelia.

Figure 4. Overall lung pathophysiology, immune cells activation and cytokines production. SARS-CoV-2 infects upper respiratory tract. Most of the patients (~80%) recovers with mild to moderate upper respiratory symptoms. In remaining patients, the virus reaches to lower respiratory track triggers pathologic immune response. Around 6% of the patients shows very severe symptoms ARDS and require ICU admission. The autopsy of patients with COVID-19 showed clusters of severe respiratory illness including associated features of diffuse alveolar damage (DAD) such as diffuse type II pneumocyte hyperplasia, epithelial necrosis, fibrin deposition and hyaline membrane formation [68, 71, 84]. Most patients who died of SARS-CoV develop acute respiratory distress syndrome (ARDS) with interstitial mononuclear inflammatory
infiltrates [42, 62, 67, 68, 71, 85]. In addition, several nonspecific histologic observations have also been observed that includes edema, fibrinous/proteinaceous exudates, hyperplastic pneumocytes, patchy interstitial chronic inflammation, and multinucleated giant cells with dysregulated immune system that results into very high amount of inflammatory cytokines [89]. PD1, programmed cell death protein-1; TIM3, T cell immunoglobulin domain and mucin domain-3; and NKG2A, killer cell lectin-like receptor subfamily C member 1; granulocyte-colony stimulating factor (G-CSF), GM-CSF, granulocyte macrophage-colony stimulating factor; IP10, interferon inducible protein-10; MCP1, monocyte chemotactic protein 1.

**Figure 5. A schematic summary of the potential therapeutic targets.** Recapitulation of COVID-19 pathological conditions in global or cell specific knockouts in the hACE2 mouse model will enable investigators to dissect the inflammatory immune cascades that are involved in disease pathology. As shown in figure, blockade of cell specific receptors, Th1 and/or Th2 cytokines, complement activation, RAS pathway activity, administration of MSCs, and antithrombotic treatments could all be useful as therapeutic targets in COVID-19. hACE2: humanized ACE2; Th1 and Th2: type 1 and type 2 CD4 T helper cell phenotypes; RAS: renin-angiotensin system; MSCs: mesenchymal stem cells.
References:


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Airway Hyperresponsiveness, M2 Muscarinic Receptor Dysfunction, and Antiviral Effects. 


ACE2 Mediated Counter Regulation of RAS

- Renin → AGT
- ACE-1
- ACE-2

Angiotensin I (Decapeptide)

- Angiotensin II (Octapeptide)

- Angiotensin (1-9) (Nonapeptide)

- Angiotensin (1-7) (Heptapeptide)

- Agonist
- Antagonist

AT1R

Vasoconstriction
Proliferation
Inflammation
Thrombosis
Vascular remodelling

Vasodilator
Anti-proliferative
Anti-inflammatory
Anti-thrombotic
Anti-hypertrophic

Detrimental
Beneficial

MAS-R

COVID-19
Lymphopenia ↑
(CD4+ T, CD8+ T, NK, and B cell number) ↓

**Lymphocyte activation and dysfunction**
Cytokine Production, TNF-α, INF-γ, IL-2

**T cells exhaustion markers**
(PD-1, TIM3, NKG2A) ↑

**Granulocytes**
Neutrophil ↑ Eosinophil ↓ Basophil ↓

**Monocytes**

**Cytokine Storm**
Inflammatory cytokines, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10; G-CSF, GM-CSF, IP10, MCP1, IFN-γ, and TNF-α

**Complement Activation**
(C3a, C5a, C5b-9) ↑

**Antibodies**
(IgM and IgG) ↑
COVID-19 phenotypes in Mouse model

- Hyperactive Immune cells
- Cytokine Storm
- Inflammation
- Endothelial dysfunction
- Coagulopathy, Thrombosis
- **MSCs, antithrombin, thrombomodulin supplementation**

hACE2 mouse

- Hyperactive Immune cells
- Cytokine Storm
- Inflammation
- Endothelial dysfunction
- Coagulopathy, Thrombosis
- **(Cell specific receptors including ACE2)**
- **(Th1 and Th2 cytokines)**
- **(Complement pathways)**
- **(RAS pathway)**
Table 1:

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Disease model</th>
<th>Key findings</th>
<th>References</th>
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<tbody>
<tr>
<td>Wildtype</td>
<td>SARS</td>
<td>SARS-CoV infections resulted into shedding of large amounts of infectious virus with the development of lung injury due to lowering of ACE2 expression.</td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td>SARS</td>
<td>SARS-CoV-infected C3−/− mice exhibited significantly less weight loss and less respiratory dysfunction with reduced lung pathology and lower cytokine and chemokine levels in both the lungs and the sera.</td>
<td>[99]</td>
</tr>
<tr>
<td>Ace2−/−</td>
<td>SARS</td>
<td>SARS-CoV infections resulted into less shedding infectious virus with mild lung pathological changes due to reduced amount of spike RNA.</td>
<td>[53]</td>
</tr>
<tr>
<td>ARDS/SARS</td>
<td></td>
<td>ACE2 blockade in mice resulted into enhanced vascular permeability, increased lung edema, neutrophil accumulation, and worsened lung function.</td>
<td>[41, 53]</td>
</tr>
<tr>
<td>Tmprss2−/−</td>
<td>SARS</td>
<td>SARS-CoV infection in Tmprss2−/− mice showed attenuated inflammatory chemokine and/or cytokine responses</td>
<td>[164]</td>
</tr>
<tr>
<td>COVID-19</td>
<td></td>
<td>Transcriptionally downregulation of Tmprss2 inhibits host SARS-CoV-2 entry.</td>
<td>[51]</td>
</tr>
<tr>
<td>Tmprss2−/−</td>
<td>MERS</td>
<td>Tmprss2−/− murine models infected by MERS-CoV showed improved immunopathology.</td>
<td>[164]</td>
</tr>
<tr>
<td>hACE2</td>
<td>COVID-19</td>
<td>Mouse model of COVID-19 showing similar pattern of hum interstitial pneumonia with infiltration of significant macrophages and lymphocytes into the alveolar interstitium, and accumulation of macrophages in alveolar cavities following SARS-CoV-2 infection.</td>
<td>[6, 7, 54]</td>
</tr>
<tr>
<td>hACE2</td>
<td>COVID-19</td>
<td>This model developed productive SARS-CoV-2 infection and inflammatory pulmonary infiltrates as seen in COVID-19 patients. Evidence of inadequate antiviral activity and potential harms of endogenous type I IFN responses were observed.</td>
<td>[5]</td>
</tr>
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</tr>
</tbody>
</table>

Ace2−/−, angiotensinogen converting enzyme2 knockout; SARS, severe acute respiratory syndrome; C3−/−, complement 3 knockout; hACE2, transgenic mice bearing human ACE2; Tmprss2−/−, Transmembrane protease, serine 2 knockout; hDPP4-Tg, Human Dipeptidyl Peptidase 4 transgene.