Review Article

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Mechanisms of SARS-CoV-2-induced lung vascular disease: potential role of complement

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Abstract

The outbreak of COVID-19 disease, caused by SARS-CoV-2 beta-coronovirus, urges a focused search for the underlying mechanisms and treatment options. The lung is the major target organ of COVID-19, wherein the primary cause of mortality is hypoxic respiratory failure, resulting from acute respiratory distress syndrome, with severe hypoxemia, often requiring assisted ventilation. While similar in some ways to acute respiratory distress syndrome secondary to other causes, lungs of some patients dying with COVID-19 exhibit distinct features of vascular involvement, including severe endothelial injury and cell death via apoptosis and/or pyroptosis, widespread capillary inflammation, and thrombosis. Furthermore, the pulmonary pathology of COVID-19 is characterized by focal inflammatory cell infiltration, impeding alveolar gas exchange resulting in areas of local tissue hypoxia, consistent with potential amplification of COVID-19 pathogenicity by hypoxia. Vascular endothelial cells play essential roles in both innate and adaptive immune responses, and are considered to be "conditional innate immune cells" centrally participating in various inflammatory, immune pathologies. Activated endothelial cells produce cytokines/chemokines, dynamically recruit and activate inflammatory cells and platelets, and centrally participate in pro-thrombotic processes (thrombotic microangiopathies). Initial reports presented pathological findings of localized direct infection of vascular endothelial cells with SARS-CoV-2, yet emerging evidence does not support direct infection of endothelial or other vascular wall cell and thus widespread endothelial cell dysfunction and inflammation may be better explained as secondary responses to epithelial cell infection and inflammation. Endothelial cells are also actively engaged in a cross-talk with the complement system, the essential arm of innate immunity. Recent reports present evidence for complement deposition in SARS-CoV-2-damaged lung microcirculation, further strengthening the idea that, in severe cases of COVID-19, complement activation is an essential player, generating destructive hemorrhagic, capillaritis-like tissue damage, clotting, and hyperinflammation. Thus, complement-targeted therapies are actively in development. This review is intended to explore in detail these ideas.

Keywords

COVID 19, hypoxia, endothelial cell, inflammation, complement

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Introduction

The worldwide pandemic of coronavirus disease 2019 (COVID-19) caused by infection with severe acute respiratory syndrome (SARS) coronavirus (CoV)-2, a member the *Betacoronavirus* genus in the subfamily *Orthocoronavirinae*, which has led to more than 2.5 million deaths (https://coronavirus.jhu.edu), urges a focused search for the underlying pathogenic mechanisms and treatment options.[1–3] The lung is the primary organ affected in patients with COVID-19, wherein the primary cause of mortality is hypoxic respiratory failure, resulting from acute respiratory distress syndrome (ARDS), with severe hypoxemia, often requiring

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assisted ventilation.[4] While similar, in some ways, to ARDS secondary to other causes, lungs of patients with COVID-19 exhibit distinct vascular involvement features, including severe endothelial injury and cell death via apoptosis and/or pyroptosis, widespread capillary inflammation, and thrombosis.[5,6] Furthermore, the pulmonary pathology of COVID-19 is characterized by focal inflammatory cell infiltration, impeding alveolar gas exchange and resulting in areas of local tissue hypoxia, consistent with the idea that a feedback loop exists between viral infection and hypoxia in disease progression. Vascular endothelial cells play an essential role in both innate and adaptive immune responses, and are considered to be "conditional innate immune cells" centrally participating in various inflammatory and immune pathologies.[7,8] Activated endothelial cells produce cytokines/chemokines, dynamically recruit and activate inflammatory cells and platelets, and centrally participate in pro-thrombotic processes (microangiopathy).[9–11] Endothelial cells are also actively engaged in a cross-talk with the complement system, an essential arm of innate immunity. Recent reports present pathological findings of localized direct infection of vascular endothelial cells with SARS-CoV-2, yet widespread endothelial cell dysfunction and inflammation exists, raising the possibility of indirect activation, through infection, of other susceptible cell types, which cause hyperinflammation and aberrant anti-viral responses.[6,12–15] Furthermore, recent reports present evidence for complement deposition in SARS-CoV-2-damaged endothelium, further strengthening the idea that, in severe cases of COVID-19, complement activation is an essential component, generating destructive hemorrhagic, capillaritis-like tissue damage, clotting, and hyperinflammation.[12,16,17] Thus, complement-targeted therapies are actively in development.[18] Based on our work on the role of complement in pulmonary hypertension (PH) and respiratory virus infections, we herein review evidence and examine the possibility that localized SARS-CoV-2 infection and hypoxia act synergistically on microvascular endothelial cells to drive widespread and excessive complement-dependent pro-inflammatory and microthrombotic responses in COVID-19-induced lung injury.[19–23] These ideas are summarized in Fig. 1. Herein, we review evidence for a potential role of complement in COVID-19-induced lung injury.

Evidence that COVID-19-induced severe ARDS is a distinct state of endotheliitis

The leading cause of mortality in patients with COVID-19 is hypoxemic respiratory failure from ARDS.[31] Pathological studies, primarily based on an autopsy of patients with the disease, provided evidence for a set of distinct features of COVID-19-associated lung injury, which cannot be accounted for by the cellular processes underlying classical ARDS.[12,14,32–34] One recent study revealed that the lungs from COVID-19 patients have three pathological features distinct from those of H1N1/influenza lung injury:[14] (1) evidence of severe endothelial injury associated with intracellular SARS-CoV-2 virus and disrupted endothelial membranes; (2) widespread vascular thrombosis with microangiopathy and occlusion of alveolar capillaries; (3) a unique form of intussusceptive angiogenesis, not traditionally observed in ARDS. This study further suggested direct SARS-CoV-2 infection of lung vascular endothelial cells (previously seen in kidney endothelial cells of the glomerular capillary loops),[13] with accompanying infiltration of inflammatory cells, evidence of endothelial and inflammatory cell death, disruption of intracellular endothelial junctions, cell swelling, and loss of contacts with the basement membrane. Other studies also support significant local inflammation, alveolar hemorrhage, and thrombosis[4,35–37] and indirectly implicate endothelial infection.[5,8,11,13,37] It should be noted, however, that, following these reports, many investigators have raised serious questions regarding whether pulmonary vascular endothelial cells are directly infected with the virus.[28,38]

The SARS-CoV-2 spike protein binds to host cells via angiotensin-converting enzyme 2 (ACE2) and viral entry is

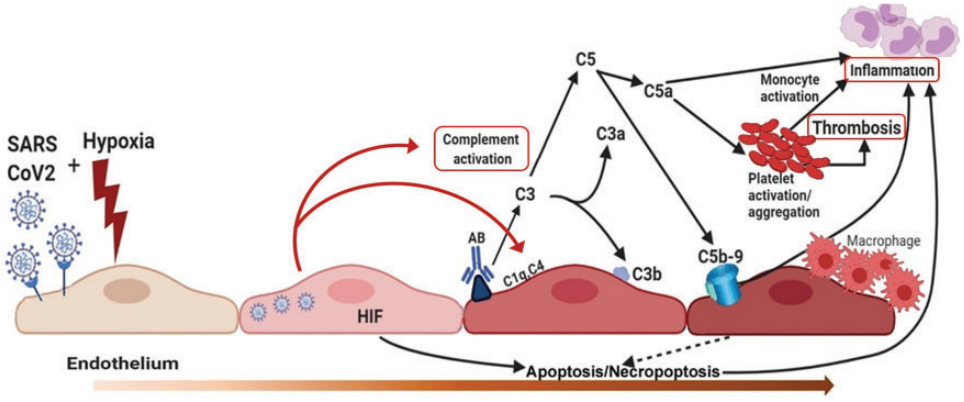

Fig. 1. SARS-CoV-2 does not productively infect pulmonary vascular cells in vitro. Hypothetical scheme demonstrating potential interactions of SARS-CoV-2 and hypoxia in driving endothelial injury/dysfunction resulting in endothelial death, inflammation, and thrombosis.[24–30]

facilitated by cell surface proteases, including TMPRSS2, the liposomal cysteine proteases cathepsins B and, L (CTSB, CTSL), the furin protease present in the secretory pathway and endocytic compartments, and other factors, such as neuropilin-1.[39–43] In the absence of ACE2 receptor and these proteases, it has been suggested that, in some cells, an alternative route of entry exists, whereby SARS-CoV-2 binds to cells via basigin (BSG, also known as CD147).[44] A recent study evaluated these signaling pathways regarding the possibility of supporting infection in three different types of endothelial cells: blood outgrowth, lung microvascular, and aortic. Compared to nasal epithelial cells or Vero-E6 cells (African green monkey kidney fibroblast cell line), endothelial cells expressed low to undetectable levels of ACE2 and TMPRSS2 but comparable levels of BSG.[45] Endothelial cells showed no susceptibility to live SARS-CoV-2 or SARS-CoV-2 pseudo-virus entry or infection.[45,46] However, these cells did display susceptibility to both Ebola and vesicular stomatitis virus infection. Even in the presence of inflammation, where an endothelial cell was pretreated with IL-1β, the cells remained refractory to SARS-CoV-2 infection. A separate study demonstrated that primary human endothelial cells lack ACE2 receptors at the RNA and protein levels, and that SARS-CoV-2 is incapable of directly infecting endothelial cells from pulmonary, cardiac, kidney, or brain tissues, even after stimulation with a variety of factors (e.g., IL-1β, TNF-α, IL-6).[27] In contrast, pulmonary endothelial cells transfected with ACE2 receptors can be infected, indicating that endothelial cells are permissive for SARS-CoV-2 replication.[27]

The failure of these monoculture systems to identify a potential for endothelial cell infection by SARS-CoV-2 could be due to the nature and deficiencies of the endothelial monolayer culture systems. Therefore, more complex ex vivo systems have also been used to investigate the cellular tropism of human CoVs, including SARS-CoV-2. Hui et al. compared virus tropism and replication competence of SARS-CoV-2 with SARS-CoV, Middle East respiratory syndrome (MERS)-CoV and H1N1 influenza A virus in ex vivo cultures of the human bronchus and lung.[25] Immunohistochemical staining showed that SARS-CoV-2 extensively infected ciliated cells, non-ciliated mucus-secreting (goblet) cells, and club cells, but not basal cells, of the bronchial epithelium. In cultured lung parenchymal cells, there was positive staining for SARS-CoV-2 antigen in the spindled, morphologically epithelial type 1 pneumocytes. In this study, double staining showed no co-localization of viral antigen in macrophages. Importantly, there was no evidence of infection of vascular endothelium in blood vessels of the lung, in contrast with that previously reported for MERS-CoV.[25,47] Similarly, Hou et al. used high-sensitivity RNA in situ mapping to show: (1) the highest ACE2 expression in epithelial cells of the nose with decreasing expression throughout the lower respiratory tract, which was (2) paralleled by a striking gradient of SARS-CoV-2 infection in proximal (high) versus distal (low) pulmonary epithelial cultures. COVID-19 autopsied lung studies identified focal disease and, congruent with culture data, SARS-CoV-2-infected ciliated and type 2 pneumocyte cells in airway and alveolar regions, respectively.[24]

Described animal model, the human-lung only mice, which are immune-deficient mice implanted with authentic human lung tissue, allow for the study of SARS-CoV-2 in a single platform with direct comparisons of experimental outcomes.[48,26] In this model system, the human lung tissue displayed robust virus replication, and sustained activation of the innate host immune response. SARS-CoV-2 was noted predominately in the epithelium. No viral antigen was detected in the human CD34 expressing endothelial cells. Viral antigen was clearly identified in cells, which express pro-SP-C (alveolar type II pneumocytes), acetylated alpha-tubulin IV (ciliated cells), and a few vimentin-expressing (mesenchymal) cells, but was not detected in alveolar type I cells or club cells. Studies in animal models of SARS-CoV-2 infection have also failed to identify any obvious signs of endothelial infection despite clear evidence of endothelial dysfunction and thrombosis.[49,50]

Another potential target cell of SARS-CoV-2, that could participate in the endothelial dysfunction observed during infection, is the pericyte, specialized cells embedded in the basement membrane of the vessel wall. Recent studies have suggested that ACE2 is highly expressed in pericytes of certain tissues, especially the brain and heart, making them targets for infection.[51,52] At present, there is no histologic confirmation of pericyte infection in patients with fatal SARS-CoV-2 infection. Reports have suggested pericyte loss or detachment in lung tissue, consistent with the idea that infection of the cells leads to endothelial instability and endothelium-mediated thrombosis.[28] Fig. 2 summarizes current data on SARS-CoV-2 lung cellular tropism.

Collectively, these observations support the idea that the vascular dysfunction and thrombosis, observed in severe COVID-19, could be the result of factors released either by adjacent infected cells, i.e. epithelial cells, or by circulating systemic inflammatory mediators.[45,53] What seems clear is that SARS-CoV-2 pathogenicity involves amplification of cellular damage via the activation of immunological and cellular injury systems not exclusively accounted for by SARS-CoV-2 direct cytopathic effects. Thus, mechanistic insights into the origin(s) of the high degree of endothelial dysfunction and pro-inflammatory activation (endotheliitis) in COVID-19 patients could lead to a better understanding of the progression of pulmonary involvement, with a potential impact on the morbidity and mortality of the disease.

Activation of the complement cascade is involved in COVID-19 lung vascular disease

The complement system is a critical part of the host immune response to bacterial and viral infections.[54] In the late 19th

SARS-CoV-2 human airway and lung cellular tropism

Primary Human Cell Type	Productive Infection	Reference
ciliated epithelial cells (upper and/or lower airway)	susceptible	24-26,29,30
alveolar type II pneumocytes	susceptible	24,26
alveolar type I pneumocytes	mixed results (subset infected)	24,26
goblet cells	mixed results (subset infected)	24,25,29,30
club cells	mixed results (subset infected)	24-26,30
basal cells	resistant	25,29
lung macrophages	resistant	25
primary lung microvascular endothelial cells	resistant	24,27 and unpublished observation
pulmonary artery endothelial cells	resistant	28 and unpublished observation
blood outgrowth endothelial cells	resistant	28
lung vascular endothelial cells (type not specified)	resistant	25,26
primary lung pericytes	resistant	unpublished observation
primary lung fibroblasts	resistant	24 and unpublished observation

Fig. 2. SARS-CoV-2 human airway and lung cellular tropism: the following references correspond to the references shown in Fig. 1.

century, it was identified as a heat-sensitive, non-specific "complement" to the more specific humoral immunity pathways, i.e., its name—complement system.[55] The complement system has been described as one way the innate immune system can detect and respond to foreign antigens. It is now recognized that the complement system comprises nearly 60 proteins, including several receptors and regulatory proteins.[56] Complement activation has direct cytotoxic effects, and it can amplify danger signals and augment inflammatory responses. These protein components of the cascade are inactive in their native state, but activation of the pathway causes proteolytic cleavage of several pathway proteins. These cleaved proteins, in turn, form enzymes ("convertases") that lead to further protein cleavage. Activation of the system occurs through three major pathways.[57] The classical and mannose binding lectin (MBL) pathways are canonically activated by IgM/G and carbohydrate moieties on pathogens, respectively. They converge at C2aC4b, which acts as a C3 protease or convertase that mediates the cleavage of C3 into C3a and C3b fragments.[58] There is also the Alternative pathway, which can act as a C3 amplification loop, that can contribute significantly to complement activation from the classical and MBL pathways, and can itself be triggered by altered/injured surfaces, such as damaged or foreign tissue.[59] The resulting activation fragment C3b then joins C2aC4b from the classical/MBL pathways, or C3bBb from the Alternative pathway to form a C5 convertase, which cleaves C5 into C5a and C5b fragments, the latter of which, wherein C5b can associate with C6, C7, C8, and C9 to form C5b-9, the so-called membrane attack complex (MAC).[59] It is also clear that a pathway, now termed the Extrinsic pathway, exists, and comprises a collection of proteases, including thrombin, kallikrein, elastases, that possess certain convertase activity, some of which are part of the coagulation cascade.[60] Once the complement cascade is triggered, many effectors are generated, including (1) the opsonins (C3b, C4b), which can mark cells or foreign invaders for phagocytosis; (2) the anaphylatoxins (i.e., C5a, C3a, C4a), which are broad-spectrum immune activators known to promote a variety of immune processes including immune cell chemotaxis, NETosis, production of cytokines, inflammasomes, reactive oxygen species (ROS), and eicosanoids, all of which are known to participate in a variety of vascular injuries; and (3) the MAC (C5b-9), which causes cell lysis and other forms of collateral damage, that are observed when complement is activated, including the release of damage-associated molecular patterns (DAMPs; e.g., hyaluronan and ATP) that can further activate complement in a self-perpetuating cycle.[61,62] Because of this vast array of proteins with inflammatory and destructive capabilities, the complement system is very tightly regulated through a number of inhibitory proteins that are constitutively present in the serum, local tissue microenvironments, and on cell surfaces.[63] Endothelial cells are continually exposed to high concentrations of complement proteins in plasma. They express several different complement regulatory proteins on their outer membranes, however, and are ordinarily very efficient at regulating complement activity

on the surface (Fig. 3).[64] The soluble complement regulator factor H also adheres to ligands on endothelial cells, thereby providing an additional mechanism of protection. Nevertheless, congential or injury-associated impairments in complement regulation can leave endothelial cells susceptible to complement-mediated injury. This association is well established in atypical hemolytic uremic syndrome, a cause of thrombotic microangiopathy.[65]

Complement pathway signaling is critical for protective host immune responses to bacterial infections, as well as some viral infections, such as influenza virus and flavivirus.[22,66,67] Interestingly, several viruses, including herpesviruses, poxviruses, flaviviruses, and retroviruses, encode genes that help to evade detection by the complement system, providing further evidence that complement is important in host anti-viral responses.[23] It is known that, upon activation of the complement pathway, the anaphylatoxins C3a, C5a, and, to a much lesser extent, C4a have important immunostimulatory roles with regard to both vascular permeability and inflammatory cell recruitment.[68] C3a and C5a, in particular, are noted for their often distinct roles in causing mast cell degranulation and initiating a cytokine storm, thus contributing to endothelial injury and/or acute lung injury.[69,70] It should also be noted that complement activation, in conjunction with the presence of neutrophils, has been shown to increase vascular permeability, such as observed during infection with SARS-CoV and/or MERS-CoV.[71] Perhaps consistent with these observations regarding complement is the fact that it has been reported that serum C5a levels are predictive of ARDS development, and that in mice with genetic absence of C5, animals are protected from bacterially induced ARDS.[72,73] Recent studies have suggested significant dose-response effects of inflammation and coagulation, wherein plasma C5a concentrations exceeding 10 nM correlated with hyperactivation of immune and endothelial cells.[74] Also interesting, in the context of the SARS-CoV-2 outbreak, are reports that baseline complement activation increases with age, and that age clearly is associated with COVID-19 outcomes.[75]

To further evaluate the role of complement in virus-induced lung injury, Gralinski et al. performed studies in mice using the original SARS-CoV, which emerged in 2002.[21] Intranasal infection of C57BL/6J mice with mouse-adapted SARS-CoV resulted in high-titer viral replication within the lung, induction of inflammatory cytokines and chemokines, and immune cell infiltration in the lung.[76] Using this model, it was found that complement activation occurred as early as one day following SARS-CoV infection. Utilizing mice deficient in complement component C3 ($C3^{-/-}$), the investigators found that $C3^{-/-}$ mice exhibited significantly less weight loss and less respiratory dysfunction despite equivalent viral load. Fewer neutrophils and inflammatory monocytes were present in the lung tissue of SARS-CoV-infected $C3^{-/-}$ mice.[21]

Another group of investigators used an animal model to study a related betacoronavirus, MERS-CoV, which also causes severe acute respiratory failure and high mortality in patients.[77] Using a humanized mouse model expressing the viral cell entry receptor (hDPP4-Tg), in which MERS-CoV infection causes lung injury, Jiang et al. found that

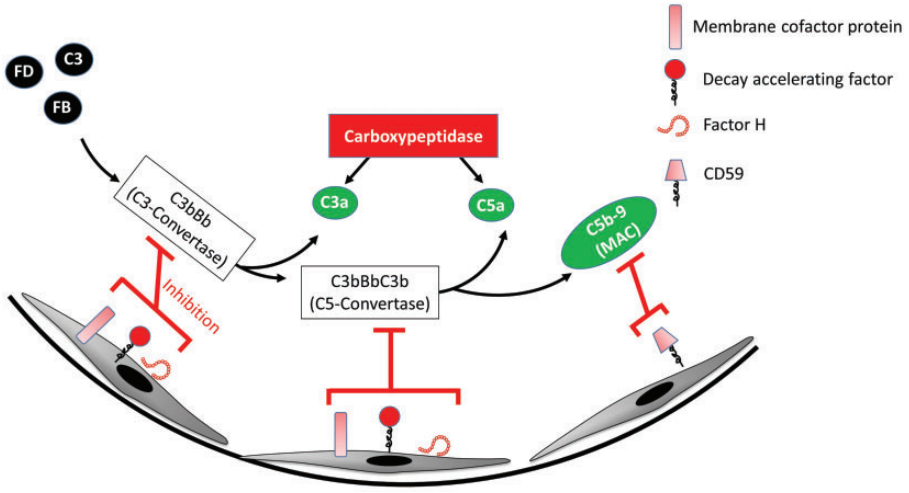

Fig. 3. Complement regulation on endothelial cells: C3, factor B (FB), and factor D (FD) are Alternative pathway proteins that are present in plasma. These proteins can be rapidly activated on susceptible surfaces. Endothelial cells express several different membrane bound complement regulatory proteins, including decay accelerating factor (CD55) and membrane cofactor protein (CD46). In addition, factor H is a soluble Alternative pathway regulator present in plasma that binds to glycosaminoglycans and sialic acid displayed on the surface of endothelial cells. These regulatory proteins inhibit complement activation at the levels of the C3 and C5 convertases. Another regulatory protein, CD59, is expressed on endothelial cells and blocks formation of C5b-9 (membrane attack complex, or MAC). C3a and C5a are soluble peptides generated during complement activation. They are rapidly inactivated by carboxypeptidases in serum. This system of regulatory proteins ordinarily protects endothelial cells from complement-mediated injury. In some diseases, however, impaired complement regulation on endothelial cells is associated with vascular injury.

complement was activated following MERS-CoV infection. This included increased concentrations of C5a and C5b-9 complement activation products in serum and lung tissues. In addition, the authors found that blocking C5a function, by targeting its receptor C5aR, reduced inflammatory responses and alleviated lung damage.[78] These observations further substantiate a critical role for complement in coronavirus-induced lung and lung vascular injuries.

With the emergence of SARS-CoV-2 and the world-wide COVID-19 pandemic, there has been obvious interest in the role of complement in this newest iteration of CoV infection. Magro et al.'s study of five patients with COVID-19 was consistent with other pathologic reports regarding COVID-19, demonstrating clinical and pathologic features distinct from typical ARDS.[12–14] In some cases, COVID-19 respiratory distress can be manifest by relatively well-preserved lung mechanics, despite the severity of hypoxemia, characterized by high respiratory compliance, high shunt fractions, and prolonged requirements for mechanical ventilation.[79] Magro et al. showed that this pathology is accompanied by extensive deposition of components of the alternative and MBL pathways, including MBL-associated serine protease 2 (MASP-2), C4d, C3d, and MAC, which in some areas co-localized with SARS-CoV-2 nucleocapsid (N) protein within the lungs septo-microvasculature (Figs. 4 and 5). The authors speculated that this extensive complement activity/involvement was associated with activation of the C5b-9/MAC, causing microvascular endothelial injury and activation of the clotting pathways. These observations were consistent with high D-dimer levels, found in the five cases assessed in this study. Interestingly, the vascular deposition of C5b-9 is a key feature of many micro-thrombotic syndromes, including the anti-phospholipid antibody syndrome and atypical hemolytic uremic syndrome, which have been shown to respond to anti-complement therapies.[80]

The findings have been extended by experiments, showing that SARS-CoV, MERS-CoV, and SARS-CoV-2 share a common mechanism connecting the viral N-protein to binding and potentiation of a MBL, and calcium (Ca^{2+})-dependent autoactivation of MASP-2, which is a MBL-associated protease that can directly initiate the complement cascade.[16,81] It has been shown that highly pathogenic human viruses, such as HIV, SARS-CoV, and Ebola, all directly interact with components of the MBL pathway and that uncontrolled activation of the complement cascade by MASP-2 leads to enhanced C4 cleavage and complement deposition, and inflammatory lung injury (e.g., ARDS).[23] Similar to the studies of Magro et al.[12] using immunohistochemical staining for MBL, MASP-2, C4a, C3, or C5b-9, Gao et al.[16] demonstrated strong signals in COVID-19 infected patient lung tissue. More recent studies have confirmed these observations.[17] These data suggest that complement components are deposited in type 1 and type 2 alveolar epithelial cells, as well as in inflammatory cells, the exudates (hyaline membranes), and in the alveolar spaces filled with necrotic debris. Other studies have demonstrated that the SARS-CoV-2 spike protein (subunit 1 and 2), but not the N-protein, directly activates the Alternative pathway of complement, providing an additional potential mechanism by which viral gene products activate the complement cascade.[82]

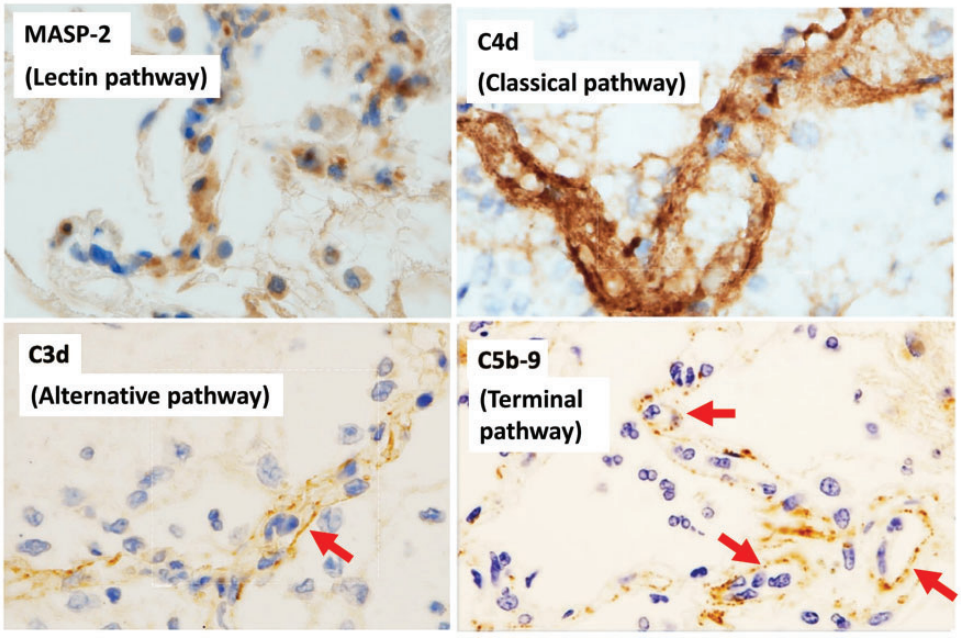

Fig. 4. Local/tissue complement activation in the lungs of COVID-19 patients: Immunohistochemistry analysis of pulmonary autopsy samples. Top left shows MASP-2 deposits localized to the interalveolar septa. Top right shows extensive deposition of C4d in the alveolar septa. Bottom left shows septal capillary distribution of C3d and bottom right shows similar capillary distribution for C5b-9. (Adapted from Magro et al.[12])

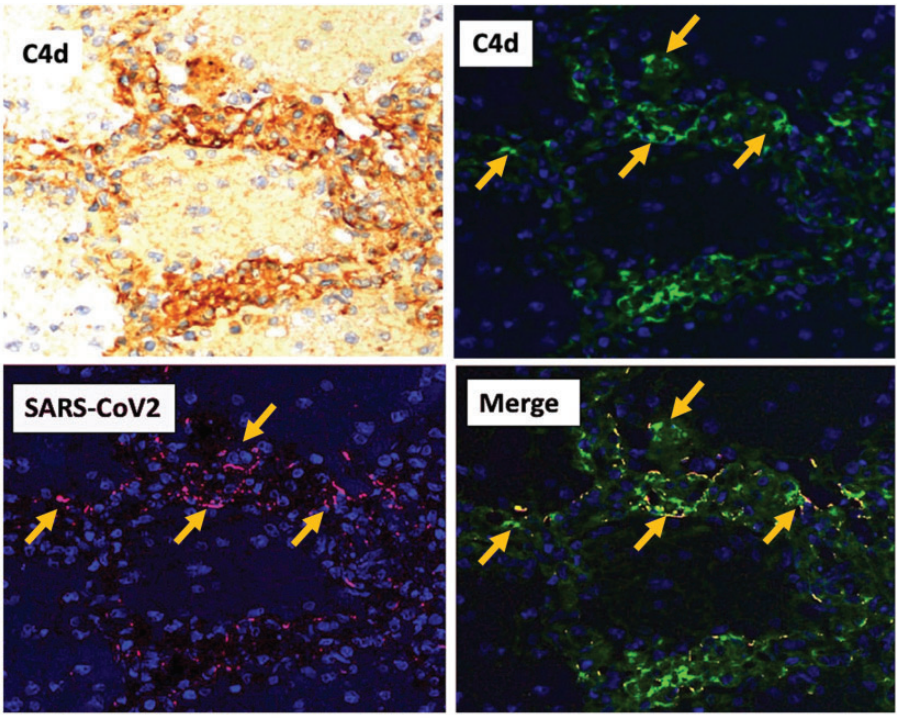

Fig. 5: Co-localization of SARS-CoV-2 and complement activation: Immunohistochemistry analysis of autopsy samples. Top left shows deposition of C4d within the inter-alveolar septa. Top right using NUANCE Software the C4 image appears green and in bottom left the SARS-CoV-2 spike protein shows red. Bottom right is a merged image showing C4d and SARS-CoV-2 co-localization, yellow. (Adapted from Gao et al.[16])

Additional studies of COVID-19 patients have confirmed significant increases in plasma C5a and soluble sC5b-9 levels, and a concomitant activation of endothelial cells.[83,84] The increase was proportional to the severity of the disease and the levels decreased after clinical improvement. At admission, circulating levels of sC5b-9 and C4d were significantly higher in patients with than without respiratory failure. Carvelli et al. observed obliterating endotheliitis associated with an accumulation of C5aR1-positive macrophages around the arteries and in the thrombus.[85] These data support the hypothesis that complement activation and C5a production lead to the chemoattraction and activation of myeloid cells in the lungs and contributes to excessive release of inflammatory cytokines. C5a can also promote the secretion of CCL2,[86] leading indirectly to the recruitment of inflammatory cells. As noted, high levels of C5a may not only be involved in chemotaxis, but other processes, including NETosis and production of inflammasomes, ROS, and eicosanoids, which have been shown to be involved in COVID-19.[70,87] Importantly, C5b-9/MAC-mediated cell death may cause the release of DAMPs (e.g. hyaluronan, ATP, etc.) that can contribute to complement activation in a self-perpetuating cycle.[75,88] There is also evidence that hemolysis (heme release) and myosin release can cause Alternative pathway activation.[89,90] Collectively, these results demonstrate that complement pathways are aggressively activated in the lungs of COVID-19 patients.

The studies mentioned above from Magro and Gao, as well as the new studies by Aid et al. in SARS-CoV-2-infected rhesus macaques, demonstrate a role for complement in COVID-19 associated coagulopathies.[49] The strongest observational evidence so far would suggest a more likely role for complement in severe disease, in which a variety of thromboses, including reticular-form purpura with extensive co-localizing deposition of C3d, C4d, and C5b-9/MAC envirospike proteins, are observed.[12,16] Current work would support the idea that thrombosis is driven by a combination of both systemic and locally activated complement. Some models, though not necessarily reflective of all observations to date, implicate a situation where viral infection of the endothelium leads to the production and release of the viral N proteins that activate complement and drive neutrophil, monocyte, and potentially endothelial production of tissue factor (TF).[91,92] In addition to causing endothelial damage, that can expose subendothelial TF, the complement and coagulation cascades can interact with each other at two key points: (1) MASP-2 can cleave prothrombin and factor Xa and (2) thrombin can cleave C3. This complement-coagulation cross-talk could thus facilitate a positive feedback loop and may explain the striking inflammation that surrounds the reticular-form purpura in severe COVID-19 as evidenced by the work of Magro et al.[12] A schematic of this hypothesis is shown in Fig. 6.

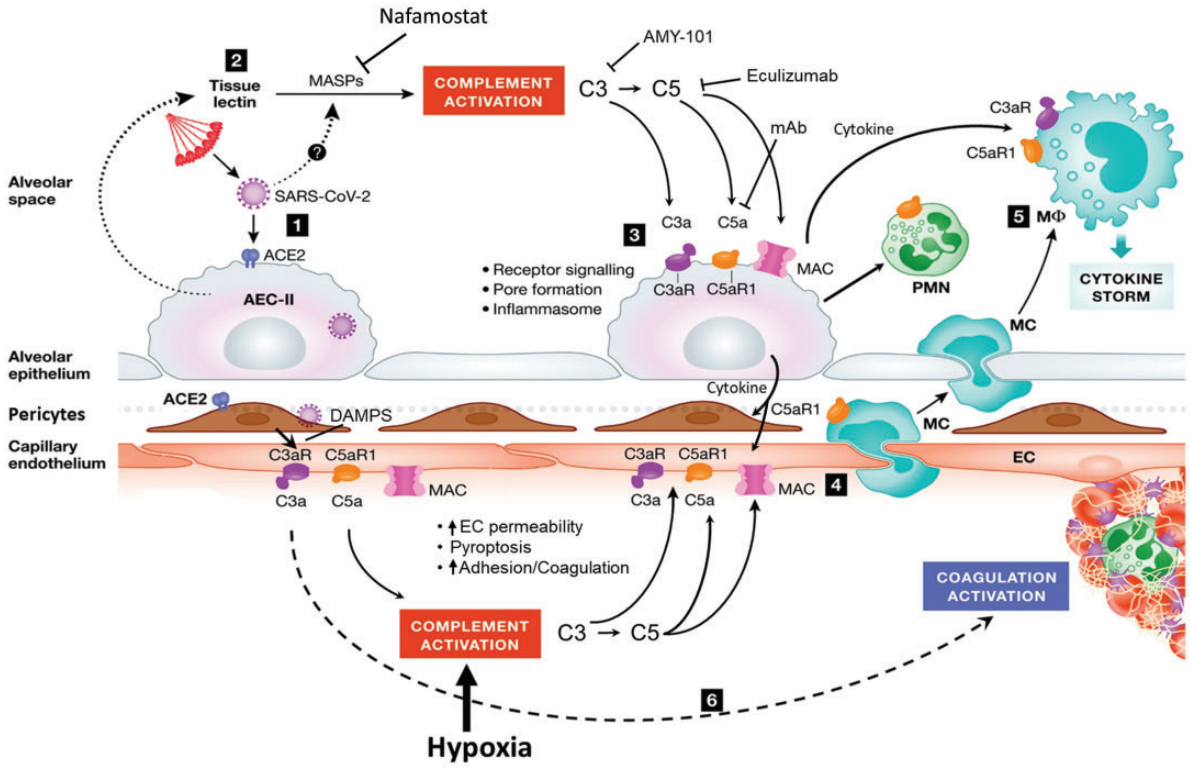

Fig. 6. Hypothetical pathway for complement-mediated inflammation of the pulmonary alveolus in COVID-19: (1) SARS-CoV-2 attaches to type II alveolar epithelial cell (AEC-II) receptor angiotensin-converting enzyme 2 (ACE2). (2) Complement activation is initiated upon recognition of viral glycans by lectins (e.g. collectin-11 and ficolin-1, which are secreted by AEC-II) complexed with MBL-associated serine proteases (MASPs) including MASP-2. Direct binding of MASP-2 to the N protein of SARS-CoV-2 has also been suggested to initiate lectin pathway activation (preprint: Gao et al.[16]). (3) Complement deposition and MAC formation on AECs cause inflammasome activation and cell damage. (4) Release of complement C5a increases vascular permeability and recruitment/activation of polymorphs (PMN) and monocytes (MC) to the alveolus. (5) Monocytes differentiated into inflammatory macrophages (MΦ) overproduce pro-inflammatory cytokines in response to C3a and C5a stimulation. (6) Endothelial cell (EC) activation by C5a and MAC predisposes to thrombus formation, further enhanced through MBL recognition of viral particles in the vascular compartment leading to cleavage of thrombin and fibrinogen by MASPs. (Adapted from Polycarpou et al.[98])

Potential interactions of hypoxia and SARS-CoV-2 on amplifying complement-mediated injury

Obviously, questions remain regarding whether direct infection of vascular cells by SARS-CoV-2 is necessary for complement activation and subsequent cell injury and thrombosis. As noted above, hypoxia, both alveolar hypoxia and systemic hypoxemia, are common findings in a great number of patients with COVID-19. Hypoxia itself is known to have significant deleterious effects on cells of the lung, in particular the endothelial cells lining the pulmonary arteries and capillaries. Mechanisms of hypoxia-induced injury to the lung vasculature continue to be evaluated. Work in ARDS certainly supports the idea that under these hypoxic conditions, complement can be activated.[94] The Stenmark laboratory has been interested, for some time, in the mechanisms involved in hypoxia-induced pulmonary vascular disease.[95,96] Recent studies have uncovered a potential role of complement activation in the vascular diseases both initiated and perpetuated by hypoxia.[19,20] Herein, we provide evidence to support the idea that hypoxia could be interacting with SARS-CoV-2-induced lung injury changes to drive severe lung disease in the microthrombotic angiopathy that characterizes COVID-19.

To date, there has not been a comprehensive proteomic analysis of pulmonary arterial changes in response to hypoxia. In collaboration with Dr Kirk Hansen at the University of Colorado Denver, we used UHPLC-MS approaches to evaluate and quantify changes in both insoluble and soluble matrix-associated proteins in neonatal calves exposed to 14 days of hypoxia and yearling steers who resided at high altitude for at least 15 months. When evaluating only the soluble protein component in both acute and chronically hypoxic exposed animals, we observed a very significant upregulation in complement components, as well as in proteins involved in the coagulation cascade[19] (Fig. 7). This is consistent with our recent observations in plasma of patients with pulmonary arterial hypertension (PAH), showing that there are significant changes in

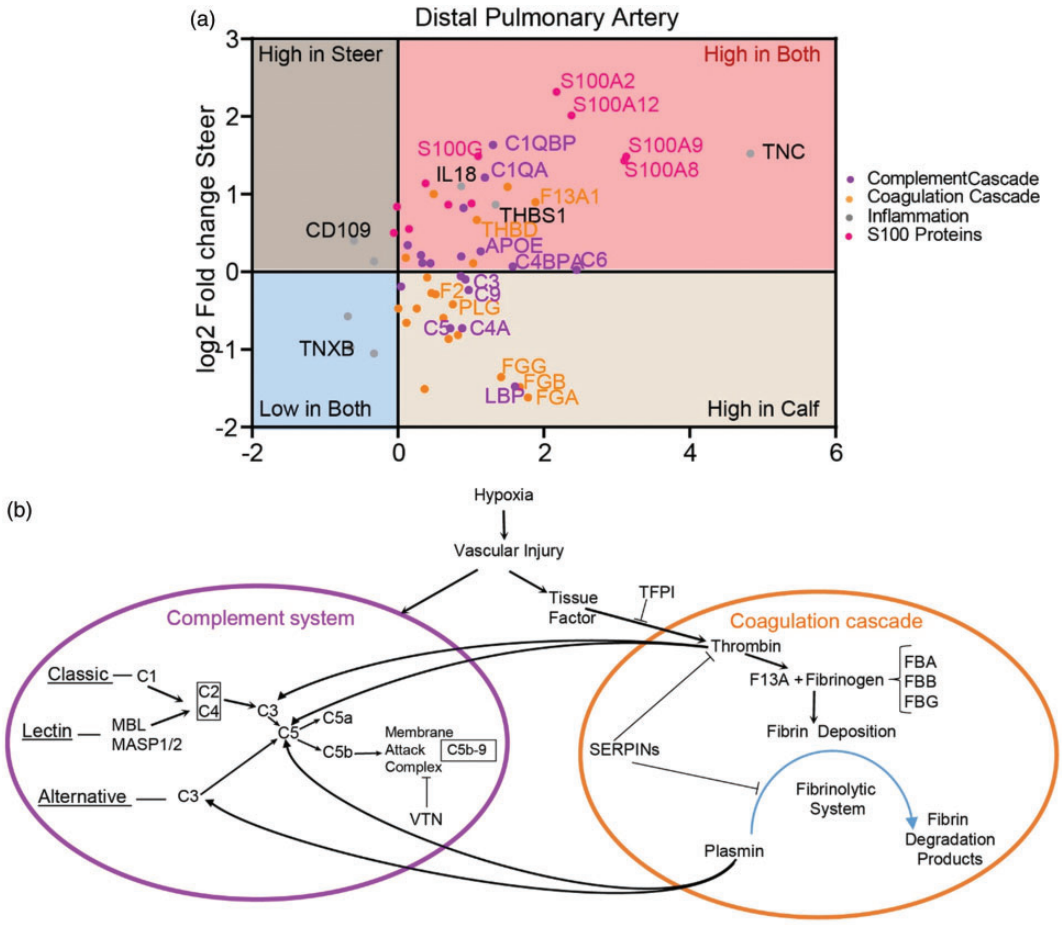

Fig. 7. Innate immune system profiling during hypoxia-driven PH: Innate immune system profiling during hypoxia-driven PH. (a) Comparative fold change analysis of proteins identified in both calf and steer samples and their respective changes in control versus PH conditions in the distal pulmonary artery (DPA). (b) Interactions of proteins identified in the complement and coagulation cascades are shown.

circulating complement components that, when evaluated as a network, correlate with pulmonary hypertensive disease severity and clinical outcome.[19,20]

Moreover, we have recently demonstrated local, pulmonary vascular-specific complement cascade activation in various animal models (mice, rats, calves) of hypoxia-induced PH, in sugen-hypoxia and monocrotaline rat models, as well as in human patients with PAH, as defined by deposition of using the C3d antibody developed by Drs Thurman and Holers.[19,97] (Fig. 8). Furthermore, markedly increased numbers of cells expressing receptors for the anaphylatoxins C5a and C3a (C5aR1 and C3aR1, respectively) were observed in the perivascular areas of rodents with experimental PH.[19,20] Thus even short-term hypoxic exposure can activate complement signaling in the lung.

We also interrogated the role of complement in the early (initiation) stage of hypoxic PH using complement factor B (Cfb)- and, C5-deficient mouse strains. We showed, via RT-PCR analysis, as early as three days of hypoxia, robust augmentation of Cfb, a key component of the Alternative pathway, and increases in complement C3, yet no changes in the expression of complement regulators/inhibitors (complement factor H (Cfh), CD55/Daf). RNAscope in situ hybridization confirmed robust upregulation of Cfb specifically in the pulmonary adventitia of hypoxic mice.[19]

A distinctive feature of PH is early and persistent perivascular inflammation. Using wild-type and complement (Cfb-, C5)-deficient mice, we showed that (1) hypoxia-induced upregulation of pro-inflammatory cytokines/chemokines, Csf2/GM-CSF and Ccl2/MCP1, known to be involved in monocyte/macrophage recruitment and pro-inflammatory activation, and (2) perivascular macrophage accumulation—are mediated through complement activation. Collectively, these data are consistent with a hypothesis whereby hypoxia could act together with viral induced complement activation to drive the vascular and particularly the endothelial responses that characterize SARS-CoV-2 induced microangiopathy.

Potential therapies for abrogating complement signaling in COVID-19

The observations of local complement activation as evidenced by deposition of C1lq, C4, MBL, C5a, and C5b-9

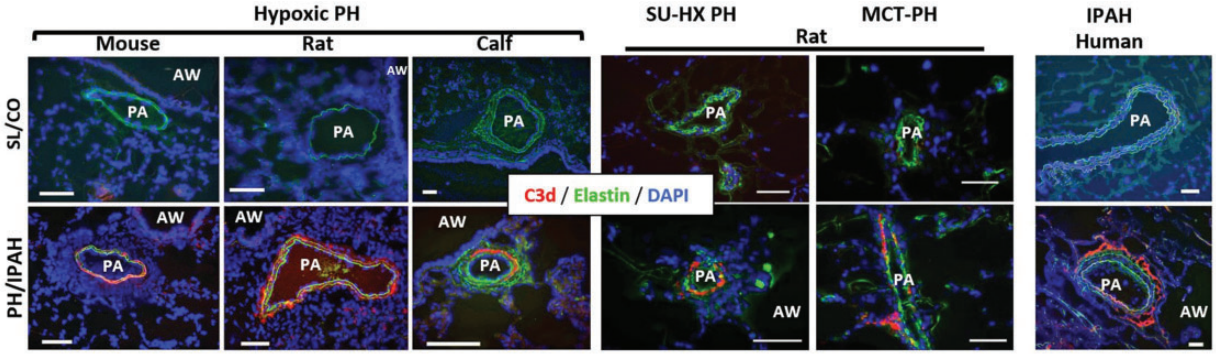

Fig. 8. Activation of the complement cascade, as defined by deposition of C3d (terminal degradation fragment of C3 activation), is observed in a perivascular-specific pattern in the lungs of experimental animal PH models and idiopathic PAH (iPAH) humans. Sections were immunolabeled with C3d-specific mAb (red), which distinguishes tissue-bound C3d from the intact C3 or C3b, allowing assessment of tissue-specific activation of the complement cascade (18). PA: pulmonary artery; AW: airway. Scale, 100 mm.

in the lung interstitial and perivascular spaces in patients dying of COVID-19, as well as reports that C3−/− complement (C3) deficient mice were protected from SARS-CoV lung injury, have raised interest in using anti-complement regimens for the treatment of the disease.[98] C3 activation is upstream of most of the anaphylatoxin signaling (C3a, C5a, and the activation of C5b-9/MAC) and is implicated as an initial effector mechanism that can exacerbate injury.[12,14,16,17,21] Thus, it is not surprising that several groups have initiated small clinical trials using various anti-complement strategies. One of the first studies came from Mastaglio et al., who treated a case of severe ARDS in a COVID-19 pneumonia patient with the complement C3 inhibitor AMY-101.[93] Diurno et al. treated four COVID-19 patients with the C5 blocking monoclonal antibody eculizumab with some success.[99–102] These patients were not as severely ill as might be expected and had only moderately elevated C-reactive proteins that did fall after treatment and all recovered after 14 days. However, Laurence et al. demonstrated the efficacy of eculizumab in three patients with severe COVID-19.[101] Recently, Zelek et al. described preliminary evaluation of the potential benefits of C5 blockade in severe COVID-19.[103] They showed that the C5 blocking monoclonal antibody (LFG316) blocked C5 activity and complement activation for at least four days in treated patients. In four of five patients, there was sustained improvement in the clinical state persisting beyond the duration of C5 blockade. These results suggest that transient blockade of C5 is sufficient to interrupt the hyperinflammatory cycle in COVID-19 and permit recovery. They also suggest that prolonged complement blockade is not only unnecessary for patient benefit, but may be harmful by increasing infection risk.

Interestingly, the Lambris group compared the efficacy of the C5 targeting monoclonal antibody eculizumab with the C3-targeted drug AMY-101.[102] That exploratory study indicated that complement inhibition abrogates COVID-19 hyperinflammation. Perhaps not surprisingly based on the above information, C3 inhibition afforded broader therapeutic control by attenuating both C3a and sC5b-9 generation and preventing Cfb consumption. This was associated with a more robust decline in neutrophil counts and neutrophil extracellular trap (NET) release, faster serum lactate dehydrogenase decline, and more prominent lymphocyte recovery.[102] Another approach has been to block the MBL pathway. Both blocking the interaction between MASP-2 and coronavirus N proteins with anti-MASP-2 monoclonal antibodies (nafamostat), and interfering directly with the catalytic activity of MASP-2, have been found to significantly alleviate SARS-CoV-2-induced lung injury.[16,82,104] A summary of the approaches is seen presented in Fig. 4.

Therefore, data generated thus far support the potential role of complement activation in lung pathology, particularly the endothelial dysfunction that characterizes COVID-19 disease, and suggest that interrupting activated complement signaling is beneficial. However, clearly, we have to recognize that there are very few patients who have been tested, and none have been done in randomized control studies.

Challenges in investigations of the role of complement in COVID-19 lung pathology

A challenge in studying the role of complement in SARS-CoV-2-induced lung injury and with regard to mechanisms in general has been the lack of appropriate animal models or ex vivo systems that show pathology similar to that observed in humans. SARS-CoV-2 has been shown to infect ferrets, cats, rhesus monkeys, and Syrian golden hamsters. However, although both the hamsters and rhesus monkeys develop pneumonia, their cases are much more similar to the mild disease in humans, rather than the severe disease.[49,105] Mouse models have also been developed that use adenoviral or adeno-associated virus transduction of human ACE2 (hACE2), resulting in hACE2 expression in the lungs with and pulmonary pathology observed in response to SARS-CoV-2 infection.[106–108] In addition,

similar to SARS-CoV, mouse-adapted strains of SARS-CoV-2 have been developed that cause more severe disease in mice.[109–111] These animal systems may permit studies of the mechanisms and consequences of virus-induced complement activation in the lung. Currently, we do not have the technologies to determine whether any of the acute or chronic physiologic abnormalities (i.e., increases in alveolar and/or endothelial permeability, sodium transport, altered cell adhesion) are mediated through complement activation. It is also unclear whether complement activation in alveolar epithelial cells alone can ultimately alter the phenotypes of lung vascular cells (i.e., endothelial cells, pericytes, fibroblasts, and immune cells), and if this cell–cell communication is mediated through complement and is a targetable endpoint.

It is also possible that ex vivo cell model systems could also be used to address questions regarding mechanisms of cell injury and, in particular, the role of complement in these responses. It is possible that co-cultures of human epithelial cells with endothelial cells and/or pericytes would be useful. Increasingly, it seems the use of organoids as human blood vessel models may be necessary for experiments such as this. However, even these systems do not take into account the role that alterations in blood flow or local environmental conditions, such as hypoxia and/or acidosis, might play in complement-mediated cell injuries.

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Conflict of interest

The author(s) declare that there is no conflict of interest.

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Authors' contributions

All authors contributed to the concepts presented and to the review of the literature. KRS completed the manuscript with input from each author.

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