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Kinase signaling as a drug target modality for regulation of vascular hyperpermeability: a case for ARDS therapy development

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Teaser:

The endothelial vascular permeability barrier has an important role throughout the body's extensive vasculature, and its disruption leads to vascular hyperpermeability (leakage), which is associated with numerous medical conditions. In the lung, vascular hyperpermeability can lead to pulmonary edema and acute respiratory distress syndrome (ARDS), the most severe and deadly complication of viral and bacterial infections, trauma and radiation exposure. There is currently no pharmacological treatment for ARDS with the only approved options being focused on supportive care. The development of effective treatments for ARDS has a potential to turn infectious diseases such as bacterial and viral pneumonia (including COVID-19) into manageable conditions, saving lives and providing a new tool to combat future epidemics. Strategies that aim to protect and augment the vascular endothelial barrier are important avenues to consider as potential treatments for ARDS and other conditions underlined by vascular hyperpermeability. We propose the activation of the MAPKAPK2 (MK2) kinase pathway as a new approach to augment the endothelial barrier and prevent or reverse ARDS and other conditions characterized by vascular barrier dysfunction.

Introduction

Endothelial cells line all blood vessels in the body, forming a tightly regulated barrier that maintains organ integrity and ensures delivery of oxygen and other nutrients to all essential organs. The endothelial permeability barrier plays a key part in maintaining the integrity of the air–liquid interface in the lung and regulates the passage of components between intravascular and interstitial spaces in response to injury and inflammation. Vascular hyperpermeability underlies >60 medical conditions, including inflammatory bowel disease, brain edema, acute kidney injury and diabetic retinopathy.

In the lung, vascular endothelial barrier dysfunction can lead to acute respiratory distress syndrome (ARDS), a serious condition associated with significant morbidity and mortality. Although acute lung injury has been described as early as World War I in response to trauma or influenza, ARDS was first described as a syndrome in 1967[1] and later characterized in 1981 by Brewer et al.[2] as "the wet lung of trauma". The underlying mechanisms of ARDS include diffuse injury to epithelial cells that line lung air spaces and the microvasculature surrounding them, surfactant dysfunction, activation of the immune system and dysfunction of the body's regulation of blood clotting. In effect, ARDS and the accompanying fluid accumulation in air sacs impair the ability of the lungs to exchange oxygen and carbon dioxide. ARDS diagnosis is usually based on the observed ratio of partial pressure of arterial oxygen to fraction of inspired oxygen (PaO_2:FIO_2 ratio) being <300 mm Hg. A group of American and European researchers introduced a definition of ARDS in 1994 which was updated in 2012 to what is currently known as the Berlin Definition of ARDS,[3] endorsed by major European and American respiratory associations. The Berlin Definition classifies ARDS based on the degree of hypoxemia measured as the PaO_2:FIO_2 ratio in the presence of other features of the disease including radiographic, lung compliance, respiratory pressure and volume criteria. ARDS is described as mild (200 mm Hg < PaO_2:FIO_2 ≤ 300 mm Hg), moderate (100 mm Hg < PaO_2:FIO_2 ≤ 200 mm Hg) or severe (PaO_2:FIO_2 ≤ 100 mm Hg).[3] More recently, some lung researchers proposed a modification of the ARDS definition that can be employed in under-resourced settings that does not rely on chest radiography, mechanical ventilation and/or blood gas measurements; but focuses on the peripheral capillary oxygen saturation (SpO_2) to FiO_2 ratio, which is easier to measure than arterial PaO_2.[4] The latter proposal reflects a belief that ARDS is underdiagnosed when the strict Berlin criteria are required for diagnosis. This point was also highlighted during the COVID-19

crisis, including a proposal to widen the definition to include patients receiving high-flow nasal oxygen.[5]

ARDS remains a significant medical, public health and economic problem even without expanding the criteria for diagnosis. A commonly cited study has estimated ARDS annual incidence in the USA at 190 000 with 38% mortality.[6] A cross-sectional study in 50 countries reported ARDS prevalence to be 10% in intensive care patients and 23% in ventilated patients.[7] Using the Berlin definition, the mortality was estimated to be 34.9% in mild ARDS, 40% in moderate ARDS and 46.1% in severe ARDS in the latter study.[7] Although ARDS burden has always been appreciated by the medical community, it has received more attention during the current COVID-19 pandemic. Although the majority of COVID-19 patients recover from the viral infection, ~14% require hospitalization with oxygen support and 5% require intensive care unit admission including mechanical ventilation[8] owing to ARDS. Of those who become critically ill, the dreaded complications are dyspnea and hypoxemia that rapidly progress to ARDS within 1–2 weeks after disease onset.[9] As with SARS and MERS,[10] ARDS is a leading cause of death in COVID-19 patients.[11]

Vascular endothelial hyperpermeability as a major component of ARDS

The significant mortality rate seen in ARDS is in part caused by the fact that the pathophysiological processes that lead to ARDS are not fully understood. Despite several clinical trials targeting pathways implicated in ARDS, to date no pharmacological treatment exists, and the current standard of care is supportive: mechanical ventilation and fluid management. ARDS pathophysiology has been reviewed elsewhere,[12] whereas this review is focused on the role of endothelial permeability regulation in ARDS. Epithelial damage and inflammatory processes have been the target of many studies on the pathogenesis of ARDS, in particular in direct lung injury such as ventilator-induced lung injury (VILI), acid aspiration or pneumonia. Injury to epithelial cells is believed to impair clearance of fluid accumulated in lung alveoli. Inflammatory components and cells such as neutrophils have also been implicated in pathogenesis of ARDS. Along with bacterial toxins such as lipopolysaccharide (LPS), a variety of immune modulators such as interleukin (IL)-6 and tumor necrosis factor (TNF)α have also been implicated in ARDS pathogenesis.[13] Whereas targeting epithelial injury or immune components are plausible

approaches to treat ARDS, this review highlights the potential of regulating the intracellular endothelial cell signaling that leads to permeability barrier augmentation to limit interstitial and alveolar flooding.

The fact that ARDS can arise in response to indirect lung injury, such as sepsis or distal trauma, points to factors that lead to endothelial permeability barrier dysregulation as major contributors to ARDS pathogenesis. Immune components such as platelets and neutrophils have been reported to activate endothelial cells. Factors that increase endothelial permeability such as thrombin, IL-1 and TNFα, or that decrease endothelial permeability such as S1P, Slit2N, Ang1 and atrial natriuretic peptide, APC, have been proposed as targets for ARDS therapeutic development (reviewed in Matthay et al.[14]). One challenge to targeting the mediators that lead to alveolar-capillary permeability is that they have the physiologic benefit of enabling the immune system components to reach the site of injury to fight injurious agents. When the inflammatory process keeps this permeability tightly controlled by the vascular endothelial barrier, it has the benefit of enabling egress of inflammatory mediators and blood components to the site of injury while limiting tissue edema or lung flooding. In conditions such as ARDS, however, vascular hyperpermeability develops, which leads to interstitial and alveolar edema. Hence, it is plausible to postulate that the problem originates not in the immune response or inflammatory cells and mediators but in dysregulation of the endothelial permeability barrier.

A case for an endothelial barrier therapeutic in COVID-19 ARDS

Vaccinations and antiviral drugs remain crucial preventive and therapeutic approaches against infectious diseases like COVID-19. Nevertheless, even with effective vaccines, the emergence of new virus variants and novel pathogens that might lead to pandemics in the future remains a significant threat. With respect to antivirals, experience with the influenza virus demonstrates that antiviral drugs are only effective when administered early in the infection. In addition, studies on the development of ARDS in other coronavirus infections suggest that the correlation between viral load and ARDS development is not well established. For all of these reasons, there is a clear need to develop therapeutics focused on the host response to pathogens. Such therapeutics that could prevent or reverse ARDS would turn a fatal condition into a manageable,

or even preventable, disease, which would have a significant impact on individual patient health, public health and the overall economic burden of respiratory infectious diseases. Several unsuccessful host-directed therapeutics evaluated against ARDS[15] have focused on modulating immune mechanisms but have not addressed the endothelial barrier permeability.

Although the pathogenesis leading to ARDS in COVID-19 is not completely understood, dysregulation of the inflammatory response has been suggested to play an important part in severity of disease. By contrast, suppression of the innate immune response, in particular interferon (IFN) signaling, has been implicated in the severity of COVID-19. Meanwhile, hyperactivation of the immune system, or a 'cytokine storm', has been suggested to be a contributor to severity. In this context, consideration of which patients with COVID-19 develop ARDS is informative. Indeed, a pattern of laboratory findings has emerged within the patients presenting with more severe forms of the disease. Neutrophils are the main source of cytokines, and indeed higher numbers of neutrophils and macrophages in pulmonary infiltrates in COVID-19 patients correlate with severity of lung damage.[16] Studies have found that COVID-19 patients who developed ARDS had significantly higher neutrophil counts than those who did not.[16] In addition, lab findings in cases with poor outcomes in hospitalized COVID-19 patients include elevated IL-6 and C-reactive protein (CRP), coinciding with classical ground-glass opacifications or consolidations on chest imaging. Furthermore, significantly elevated D-dimer levels are consistently associated with adverse outcomes in these patients,[17] and elevated IL-6 is strongly associated with a patient's need for mechanical ventilation and has been useful in predicting respiratory failure with high accuracy.[18]

A commonality among these lab indicators is their effect on the endothelial function. IL-6 promotes a sustained loss of endothelial function and related changes to endothelial permeability.[19] CRP, a prototypical marker of inflammation, has likewise been shown to promote endothelial dysfunction.[20] In addition, D-dimer has been shown to be at least independently associated with endothelial dysfunction.[21] Further evidence that the endothelium plays an important part in COVID-19 comes from recent studies,[22] including one that showed that circulating factors from patients with severe COVID-19 disrupt endothelial permeability barrier function.[23] Another study showed that COVID-19 induced anti-spike-protein IgGs to activate

macrophages that target the endothelial barrier.[24]

Recent evidence indicates that patients suffering from respiratory distress from COVID-19 initially retain good lung compliance. Despite the low oxygenation in these patients, it is not until the disease progresses that the low lung compliance typical of ARDS becomes more likely.[17] As such, an approach to prevent this progression to ARDS, and its associated ventilation–perfusion mismatch, would be a crucial tool in decreasing the adverse outcomes in COVID-19 patients. Given the lab indicators associated with adverse outcomes and their relationship to endothelial function and permeability, an approach that targets this permeability itself holds promise as a means of achieving this prevention.

Signaling pathways modulating vascular barrier permeability

The factors that affect endothelial permeability and barrier regulation have been reviewed elsewhere.[25] Endothelial cells face various external contractile and shear forces exerted by surrounding contractile tissue and blood flow. In addition, endothelial cells themselves contract in response to various stimuli. The latter contraction is integral to their ability to form a barrier that must open and close in response to inflammation and injury. This functionality allows blood factors to enter the site of injury and mount an inflammatory response. Stimuli such as thrombin, histamine, transforming growth factor (TGF)β or hypoxia transiently activate actomyosin contraction in endothelial cells.[26,27] This contraction can cause gap formation leading to increased endothelial barrier permeability. The role of contractile forces in increasing endothelial permeability has been reviewed elsewhere.[28,29] Weakening or strengthening of the endothelial barrier is believed to be a reflection of the balance between contractile forces and adhesive forces.

Because actomyosin motors constitute the major intracellular contractile machinery, there has been considerable research on the targeting of cell signaling that leads to their activation and inactivation by permeability modulators. At the same time, several studies have highlighted the importance of different types of adhesive junctions in barrier function.[29] Whereas this review focuses on the role of the endothelial cytoskeleton stabilization in barrier augmentation, there has been considerable research on the role of different intercellular junctions in strengthening the endothelial permeability barrier (for review, see[30]). Adherens junctions, tight junctions and gap junctions contribute to formation of a tightly regulated endothelial barrier. These junctions are

regulated by signaling pathways that alter interaction of component proteins such as VE-cadherin, claudins, occludins or connexins, as well as proteins that anchor these junctions to the cytoskeleton. Research that highlights the promise of these molecules as targets for drugs against disorders that affect endothelial permeability such as ARDS has been discussed elsewhere (reviewed in[30]). Other studies have focused on the role of adhesion of endothelial cells to the extracellular matrix in endothelial permeability regulation.[31,32]

In between the contractile and adhesive forces lies the cytoskeleton, which anchors intracellular components as well as adhesive junctions. Actin microfilaments, which constitute one of the three major cytoskeletal components, are also part of the actomyosin contractile apparatus. Hence, researchers have studied actin microfilaments and, to a lesser extent, microtubules and intermediate filaments, for their involvement in barrier regulation. Redistribution of cytoskeletal components has been linked to changes in endothelial barrier permeability. Of the many signaling pathways that regulate cell contractility, adhesion and cytoskeletal structure, we focus on the p38 mitogen activated protein (MAP) kinase signaling pathway and its role in barrier augmentation. In this short review, we make the case that signaling downstream of p38 MAP kinase, which stabilizes the endothelial cytoskeleton, presents a viable target for developing therapeutics leading to endothelial permeability barrier augmentation.

p38 pathway and barrier regulation

The p38 MAP kinase is a descendent of the yeast Hog 1 – a kinase that primarily functions in mediating the stress response that enables yeast to adapt to hyperosmolar conditions.[33] Hyperosmolarity causes water loss and cell shrinkage that can be very detrimental or fatal to yeasts, which respond by increasing synthesis of the osmolyte glycerol. This high-osmolarity-glycerol (HOG) response is under the control of the p38 analog Hog1. The Hog1–p38-mediated adaptive responses, which affect transcription and cell cycle regulation, are preceded by functional modulation by direct phosphorylation of proteins,[34] enabling the yeast to resist the osmotic pressure and maintain cell integrity (reviewed in[35]). The responsiveness of p38 to hyperosmolarity and other stresses is conserved in mammalian cells and, hence, p38 is referred to as stress-activated protein kinase (SAP kinase). We propose that an analogous response is conserved in eukaryotic cells that participate in barrier function such that in this instance the

fluid balance is maintained not at the intracellular level but at the tissue level. In that model, yeast cell shrinkage under osmotic stress is mirrored in eukaryotes by endothelial and epithelial cell contraction, whether due to external mechanical forces or activation of internal actomyosin motors. Activation of p38 signaling in these cells augments the permeability barrier by stabilizing their cytoskeleton through heat shock protein (HSP)27 phosphorylation (Figure 1).

Reversible permeability inducers such as thrombin, histamine, TGFβ, hypoxia and other stressors also activate p38 MAP kinase signaling, which has been implicated in regulation of some inflammatory responses. As a result, this activation has been associated with permeability barrier compromise, among other detrimental effects. This hypothesis appeared plausible in the framework of p38 regulation of expression and circulation levels of certain cytokines, some of which are known to modulate barrier permeability. Hence, it was proposed that inhibiting p38 and downstream kinases, such as MAPKAPK2 (MK2), which are activated in inflammation, should limit associated processes. Because permeability is part of inflammation, it would seem likely that interventions aimed at limiting inflammation should limit endothelial barrier permeability. This thinking also benefits from development by drug companies of more-potent and -specific inhibitors of these kinases that have provided powerful tools in this area. These inhibitors have been proposed as drugs against a variety of diseases that involve the immune system, including ARDS. However, none of these kinase inhibitors has proved successful in clinical trials, and p38 and MK2 inhibitors seem to share mechanism-based toxicity that targets the gastrointestinal epithelial barrier.

Based on our research, we propose that reversible permeability inducers activate p38 signaling in endothelial cells by way of limiting barrier permeability rather than increasing it, and that inhibiting this pathway will dysregulate the response to injury thus resulting in a weaker barrier. The key to this idea is to focus on p38 signaling in endothelial cells rather than its effects on inflammatory cells, cytokine production and other cell responses. We focus on endothelial cells because barrier failure appears to be on the critical path in certain diseases. But, as seen below, lessons from evolution suggest that this pathway might be just as important in other types of barriers such as gastric epithelial barriers.

p38 signaling and immune response

Interest from researchers and pharmaceutical companies in p38 signaling was initially triggered by its potential as a target for inhibition because of its involvement in mediating immune

responses. But studies on bacterial pathogenesis point to another direction: it was shown that p38 signaling is needed to protect the nematode, *Caenorhabditis elegans*, against pathogens such as *Pseudomonas aeruginosa*.[36] It was demonstrated that mutations in MKK or MKKK (the two kinases immediately upstream of p38) that lead to reduced p38 activity correlate with increased susceptibility to pathogens. Not only was this gene protective against the gram-negative *P. aeruginosa* but further evidence has accumulated that p38 activation occurs as part of a cell-protective response in the case of cytolysins. These bacterial toxins can trigger cell lysis but are known to trigger other signaling effects at lower sublytic concentrations. In particular, they have been shown to activate p38 signaling. Because activation of p38 signaling occurs during inflammation, it has been suspected to be a mediator of the damaging effects of bacterial cytolysins. Another study, however, describes how, in the case of cytolysins, p38 activation occurs as part of a cell-protective response.[37] In that study, activation of p38 was ascribed to potassium loss as a result of pore formation. In another study by Huffman et al., the authors used a genetic approach to identify genes that render the *C. elegans* susceptible to the cytolysin Cry5B, a toxin made by *Bacillus thuringiensis*.[38] Using gene arrays, this group demonstrated that p38 is induced by the toxin, and that its knockout makes *C. elegans* particularly vulnerable to the cytolysin. The work was extended to mammalian cells as this group went on to demonstrate that inhibition of p38 in hamster kidney epithelial cells renders them susceptible to the mammalian cytolysin aerolysin which is made by the human pathogen *Aeromonas hydrophila*.[38] In another study it was revealed that p38 signaling is key to resistance of *C. elegans* to pathogens, and that its regulation is integrated at the MAPK kinase level and phosphatase level via MEK1 and MKP7.[39] The role of p38 signaling in innate immunity and host defense in *C. elegans* extends to an opportunistic *Proteus* species.[40] Indeed, a reduction in p38 activity has been linked to immunosenescence in *C. elegans*.[41]

An argument for p38 signaling being a protective rather than deleterious component of the innate immune response is that it is equally activated by pathogenic *P. aerouginosa* and non-pathogenic *Escherichia coli* in *C. elegans*.[42] Indeed, p38 has been shown to contribute to host defense against all intestinal bacterial[43] pathogens tested to date in *C. elegans*.[44] In addition to its established role in protecting the intestinal epithelial barrier against different pathogens in *C. elegans*, p38 has also been shown to play a similar part in epidermal host defense against physical injury and the fungal pathogen *Drechmeria coniospora*.[45] A review on the use of *C.*

elegans as a model for innate immunity has also indicated that p38 disruption is crucial for susceptibility of *C. elegans* to human pathogens *Salmonella typhimuruim* and *Candida albicans.*[43] Yet another study revealed that probiotic bacteria extend the life of *C. elegans* through a p38-dependent mechanism.[46]

Streptococcus pneumoniae bacteria are a common cause of pneumonia and ARDS, and their toxin pneumolysin (PLY) has been proposed to mimic the effects of pneumococcal infection in rats[16] and mice.[47] It has also been reported to increase endothelial barrier permeability.[17,18] PLY belongs to the class of cholesterol-dependent pore-forming toxins that can cause cell lysis (cytolysins). At lower doses these toxins affect signaling pathways including the p38 pathway. Because activation of p38 signaling occurs during inflammation, it has been suspected as a mediator of the damaging effects of PLY. However, and as discussed above, the role of p38 activation by cytolysins has also been described as part of a cell-protective response.[19] Indeed, a member of this family, inerolysin (INY), produced by the microbiotic *Lactobacillus iners* has been suggested to protect against pathological organisms through activating p38 in epithelial cells.[10]

Importantly, although some reports linked p38 activation by PLY to cytokine production by epithelial cells,[48] other studies have shown that acute lung injury or ARDS can be linked to the ability of PLY to block p38 activation.[20] These conflicting reports on activation versus inhibition of p38 signaling by PLY could reflect a generalized protective response triggered by all cytolysins (p38 activation) and a pathological effect unique to PLY. Specifically, PLY induces the protein cylindromatosis (CYLD), which de-ubiquitinates TAK1, an activator of MKK3, resulting in inhibition of p38 signaling.[8] PLY has also been shown to activate the phosphatase MKP1 which suppresses p38.[21] The mechanism by which p38 activation protects barrier cells is still not understood. Our results suggest that one possible mechanism by which endothelial cells are protected by p38 activation involves its downstream kinase and substrate MK2, and the substrate of the latter – the small heat shock protein HSP27. These results include our work on another toxin completely unrelated to PLY, the anthrax lethal factor, a component of the anthrax lethal toxin (LeTx). Anthrax LeTx is unique in that it is known to target endothelial cells and induce the vascular hyperpermeability associated with anthrax. As discussed below, LeTx proved to be a useful tool that enables study specifically of the effect on endothelial cells because it has been shown to directly induce endothelial cell injury.

Targeting MK2–HSP27 signaling to treat vascular hyperpermeability in ARDS

Our research on pulmonary vascular endothelial cells suggests that p38 activation can protect barrier cells through downstream signaling leading to cytoskeleton stabilization. In particular, our studies show that p38 activation and phosphorylation and activation of its substrate MK2, which in turn phosphorylates HSP27, leads to stabilization of actin microfilaments and vimentin intermediate filaments in these cells and augmentation of the permeability barrier (Figure 1). We have observed this effect on barrier augmentation with reversible permeability inducers such as hypoxia and TGFβ, where that augmentation can be causally linked to HSP27 phosphorylation and cytoskeleton stabilization.[27]

Previous studies on modulation of endothelial cell signaling and the permeability barrier by hypoxia indicated that regulation occurred through a tight biological control system where hypoxia activates signaling through the Rho-kinase (ROCK)–myosin-phosphatase pathway, which in turn leads to endothelial cell contraction and weakening of the barrier and increased permeability.[49,50] At the same time, hypoxia independently activates p38 kinase signaling leading to phosphorylation of MK2 and its substrate HSP27, which subsequently causes barrier augmentation and decreased permeability.[49,50] These studies indicated that such control could be beneficial when biological systems encounter physiological or pathological stimuli. By initially increasing endothelial permeability, the control system enables blood components to enter the site of injury, and then limits the response via activation of p38-MK2-HSP27 signaling. Disruption of the latter pathway presents a potential mechanism for the persistent endothelial vascular permeability that leads to ARDS. Lung injury in ARDS can be due to direct insult to the endothelium,[51,52] which does not require other immune modulators. For example, although anthrax LeTx has direct action on immune cells, this action is only important for infection and not for the lethality of the toxin.[53,54]

The active component of LeTx, lethal factor, is a protease that has as its only known substrates MKKs such as the p38 activating kinases MKK3/6. We have previously described how LeTx degrades the p38 activator kinase MKK3b in pulmonary endothelial cells and thereby perturbs endothelial barrier permeability by disrupting p38-MK2-HSP27 signaling.[55] Moreover,

overexpressing a phospho-mimicking form of HSP27 restores p38–MK2 signaling and protects the endothelial permeability barrier.[55] In light of these results, we have considered MK2 as the major immediate kinase that directly phosphorylates HSP27 which can be targeted for drug development. Following up on a report that Kaposin B peptides can directly activate MK2, we designed an MK2-activating peptide (MK2-AP) and evaluated it for ability to activate MK2-HSP27 signaling and barrier augmentation.[27,56]. These studies demonstrated the proof-of-concept for developing specific MK2 activator compounds that activated HSP27 phosphorylation in a dose-dependent, rapid and reversible manner. Because these findings point to activation of MK2 as a promising target for prevention and reversal of ARDS, we went on to demonstrate MK2-AP efficacy in an animal model of lung vascular hyperpermeability.[50]

Beyond the proof-of-concept in cell culture and *in vivo* with MK2-AP, we have also searched for likely binding sites at the surface of MK2 that could be involved in the binding of the activation peptide and that could be used for virtual screening experiments. We have assumed that, as with the Kaposin B peptide, activating molecules can bind certain unknown regions of MK2 and block the autoinhibitory domain of MK2.[56,57] Compounds designed or predicted to bind these regions are expected to activate MK2 leading to HSP27 phosphorylation and mitigation of vascular hyperpermeability. Hence, after applying different computational approaches, we propose a novel binding pocket that could bind MK2 activators (Figure 2). Structure-based virtual screening carried out on this binding pocket could lead to the identification of small-molecule drug-like activators of MK2 which could be further developed into therapeutics. We propose an electronegative activating exosite groove on the surface of MK2 that could be crucial for the binding of the described MK2-AP. This zone is also predicted to have the characteristic of pockets binding small drug-like compounds and has not been reported before in the kinase family, at least using co-crystallization approaches.

Complexity of biological pathway

In this short review, we have described background and rationale, as well as our own findings that point to the activation of MK2 as a promising target for prevention, slowing the progression toward and reversal of ARDS. Indeed, MK2-AP has shown efficacy in animal models of lung

vascular hyperpermeability,[58] which has led to our efforts to develop appropriate compounds for human treatment.[50] Although the results we discussed above point to involvement of p38-MK2-HSP27 signaling in response to a variety of agents including bacteria, their toxins or reversible endothelial permeability inducers such as hypoxia and TGFβ, the strongest case for its involvement can be made by the results with anthrax LeTx. Anthrax is typically not thought of as an inducer of ARDS, and anthrax infections are fortunately rare. Yet our understanding of microbial pathogenesis has been greatly enhanced by studying anthrax bacteria and its toxins. The link between anthrax and vascular hyperpermeability is well established, and inhalational anthrax is associated with pleural effusions and pulmonary edema even though no pneumonia is observed. These results highlight how pathogens can target the endothelium without causing the lung epithelial damage often associated with ARDS. Furthermore, because of its known molecular mechanism LeTx can be studied in cell culture and *in vivo*. One challenge in identifying drugs to reverse vascular hyperpermeability, including ARDS, is the lack of reliable and reproducible models.[59] Several models routinely used to study lung inflammation or infection do not produce consistently reproducible vascular hyperpermeability.[60] By contrast, LeTx can produce significant and reproducible pulmonary vascular permeability in Fisher rats, which highlights its value and utility to evaluate drugs that target this process *in vivo*.[58]

There are important corollaries from our LeTx research to the present situation with COVID-19. Although the pathogenesis of coronavirus infections is not well understood, current research suggests that SARS-CoV2 infects cells through interacting with the same receptor to which some other coronaviruses bind: angiotensin-converting enzyme 2 (ACE2). ACE2 receptors are most prevalent on epithelial cells deep in the lung, and, importantly, on vascular endothelial cells, although whether SARS-CoV2 directly infects endothelial cells through ACE2 remains uncertain.[61] Endothelial involvement in COVID-19 pathogenesis has been discussed above, and is also implicated by susceptibility of patients with vascular diseases[22] and the appearance of vascular complications such as strokes and microvascular lesions.[62] It is not clear what other factors contribute to the ability of COVID-19 to infect target cells and research is being conducted to better understand the signaling pathways involved in its pathogenesis. SARS-CoV2 probably affects multiple signaling pathways, including some that are activated by other pathogens. Because signaling pathways activated in response to pathogenic or other types of injury can lead to damage to host tissues, or protection against injury, elucidating that role is very

important in deciding not only which pathway to target but also whether it should be inhibited or activated in treatment strategies. Previous research on the SARS coronavirus has described transient p38 pathway activation in some cells, which appears to mirror activation of several MAP kinases in response to viral infection. However, it is noteworthy that one report noted persistent suppression of p38 in TH8 lymphocytes of patients who developed the disease that lasted for weeks.[63]

MK2 activation as a target modality beyond ARDS

Although the p38-MK2-HSP27 pathway has not been specifically studied in COVID-19, previous reports including our own findings discussed above provide a rationale that supports targeting it in prevention and management of COVID-19 ARDS. It is also worth mentioning that up to 25% of COVID-19 patients suffer long-term symptoms that range from perturbation of sense of smell to respiratory symptoms, fatigue and headaches. The etiology of these persistent symptoms is not well understood although involvement of the immune system has been implicated because they persist beyond viral infection or detectable virus levels in the patient. Another possibility that needs to be considered as contributing to this etiology is barrier damage and persistent consequences. Just as lung injury can be followed by pulmonary fibrosis, there is ample evidence that endothelial injury can lead to vascular remodeling – a form of fibrosis. Considering that we have reported that MK2 signaling affects endothelial physiology as well as fibroblast function and fibrosis[64,65] we propose that MK2 activation is a drug target modality that might ameliorate components of pathogenesis, barrier damage and generate repair. It is possible that long-haul COVID symptoms other than respiratory ones, such as parosmia and perturbed sense of smell, as well as fatigue, headaches and 'brain fog', are related to localized barrier injury followed by incomplete recovery. Headaches and brain fog might reflect a dysfunctional brain endothelial permeability barrier, which is a main component of the blood–brain barrier. In addition, recent findings suggest that perturbed sense of smell in long-haul COVID-19 is related to damage to the nasal-barrier-forming sustentacular cells, which protect olfactory neurons. These cells share with endothelial cells the expression of ACE2 to which the SARS-CoV2 binds.[66] Furthermore, pervious research suggested that activation of HSP27 in these cells enhances their barrier function and protection of olfactory neurons.[67] Hence, activating MK2

might be beneficial in the short term as well as for long term treatment of COVID-19 and related conditions.

Concluding remarks

Vascular endothelial barrier integrity is crucial for maintaining health and survival across species. Therefore, developing therapeutics that restore and reverse the damage to the endothelium has the potential to revolutionize several treatment modalities for conditions as diverse as sepsis and diabetic retinopathy. We have focused in this review on ARDS, which has long been a dreaded complication of several diseases in the clinical setting. Even prior to the COVID-19 pandemic, ARDS support and treatment accounted for US$60 billion in healthcare expenses in the USA alone.[47] The global spread of SARS-CoV2 has created a setting where ARDS cases have acutely risen amid a scarcity of mechanical ventilators, which have long been the only maintenance approach to support ARDS patients. We propose a new drug target modality in the p38-MK2-HSP27 pathway: MK2, as an ideal drug target for treatment of vascular hyperpermeability in ARDS because it is the immediate activator of HSP27 phosphorylation, which augments the endothelial barrier through its direct action on multiple cytoskeletal components. Peptides that act as MK2 activators have been demonstrated to prevent and reverse ARDS-related lung vascular hyperpermeability *in vitro* and in an animal model of lung injury.[55] Hence, such peptides could be developed into therapeutics, as well as serving as valuable tools for development of new compounds using the computational approaches discussed above to treat vascular hyperpermeability. The next steps toward applying these treatment modalities include designing and confirming experimentally novel MK2-activating molecules and advancing them to clinical trials. These novel compounds have great potential as successful treatments for COVID-19-related ARDS, as well as for other conditions resulting in this deadly complication.

Figure legends

Figure 1. The p38 MAP kinase pathway. This pathway is central to regulating endothelial cell barrier permeability. In normal inflammation, permeability inducers activate pathways that weaken or augment the barrier. Balanced signaling enables transient physiologic barrier opening. In Phase I endothelial cell contraction opens the barrier; while in Phase II HSP27-mediated actin and vimentin filament formation strengthens adhesion and closes the barrier. In sepsis, certain pathological agents associated with vascular hyperpermeability (e.g., anthrax LeTx or pneumolysin) weaken the endothelial permeability barrier by blocking HSP27 phosphorylation, tipping the balance toward barrier failure. MK2-activating peptides might overcome these effects by directly activating MK2 and increasing HSP27 phosphorylation.[58]

Figure 2. Structural analysis of MK2 and prediction of druggable pockets. **(a)** The crystal structure of MK2 (PDB ID: 3KA0[68]) is shown in a cartoon representation (orange) with some missing loops predicted by computational means colored in magenta. MK2 is a Ser/Thr protein kinase that comprises 400 amino acids (UniProt entry P49137) divided into a proline-rich N-terminal region (residues 10–40 not visible in the crystal structure), a protein kinase catalytic domain (residues 64–325) and a regulatory domain at the C-terminal position of the protein. The MK2 terminal region contains the so-called autoinhibitory α-helix (only residues 345–364 are visible in this structure, the remaining residues are disordered), painted lime. A small-molecule inhibitor, co-crystallized in the catalytic site, is here shown for orientation. It is known that the C-terminal region (around residues 366–390) represents the p38 MAPK-binding site, also referred to as the docking region. **(b)** Negative and positive electrostatic potentials are mapped onto the molecular surface of MK2. The potentials are on a −5, +5 red-white-blue color map in units of kJ/mol/e. A major electronegative groove that we call here the activating exosite is observed between MK2 residues E290 and D351 and nearby the binding interface of p38. **(c)** Binding pocket predictions identified several possible zones (the molecular surface of MK2 corresponding to these predicted pockets is colored). The calculations were performed with a modified version (re-trained on a larger number of protein–ligand complex) of P2Rank[69], a machine-learning-based tool that proposes putative ligand-binding sites at the surface of a protein. Among the predicted pockets, two best binding pockets (ranked 1 and 3) in the region of the electronegative groove are seen, whereas the pocket ranked number 2 is the catalytic site. The computations required the presence of points lying on the solvent-accessible surface of MK2, these points are shown as small green spheres. Small chemical probes within these pockets are colored according to ligandability scores (ranging from 0 = bad to 1 = good), many of them are painted red (maximum score of 1). It is possible to merge pockets 1 and 3, this defines an interesting zone that could be investigated via structure-based virtual screening computations. We observe that there are no known small molecules that have been co-crystallized in this predicted electronegative pocket thus far and, as such, our proposed cavity is novel and not discussed in the study of Laufkötter et al.[70]). **(d)** Blind peptide docking using as input our positively charged activating peptide (YARAAARQARAHPRNPARRTPGTRRGAPAA)[58] was performed using two different methods (MDockPeP server[71]and the 'piper.py' script of Schrödinger modeling suite (Schrödinger Release 2015-2, Schrödinger, LLC, New York, NY, 2015). The same orientation and pocket-painted molecular surface were kept for orientation. The most likely binding mode for the positively charged activating peptide would seem to be in this

electronegative groove and in contact with the two top predicted binding pockets, near the autoinhibitory helix and binding site for p38, but not directly at the MK2–p38 interface.

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Endothelial Permeability Inducers

e.g., histamine, thrombin, TGF-β

ARDS Toxins
e.g., anthrax LeTx, PLY

Phase I: Barrier Weakening

Actomyosin Activation
Cell Contraction
Disassembled Cytoskeleton

Myosin
Actin

Vimentin

p38

MK2-AP

MK2

HSP27

pHSP27

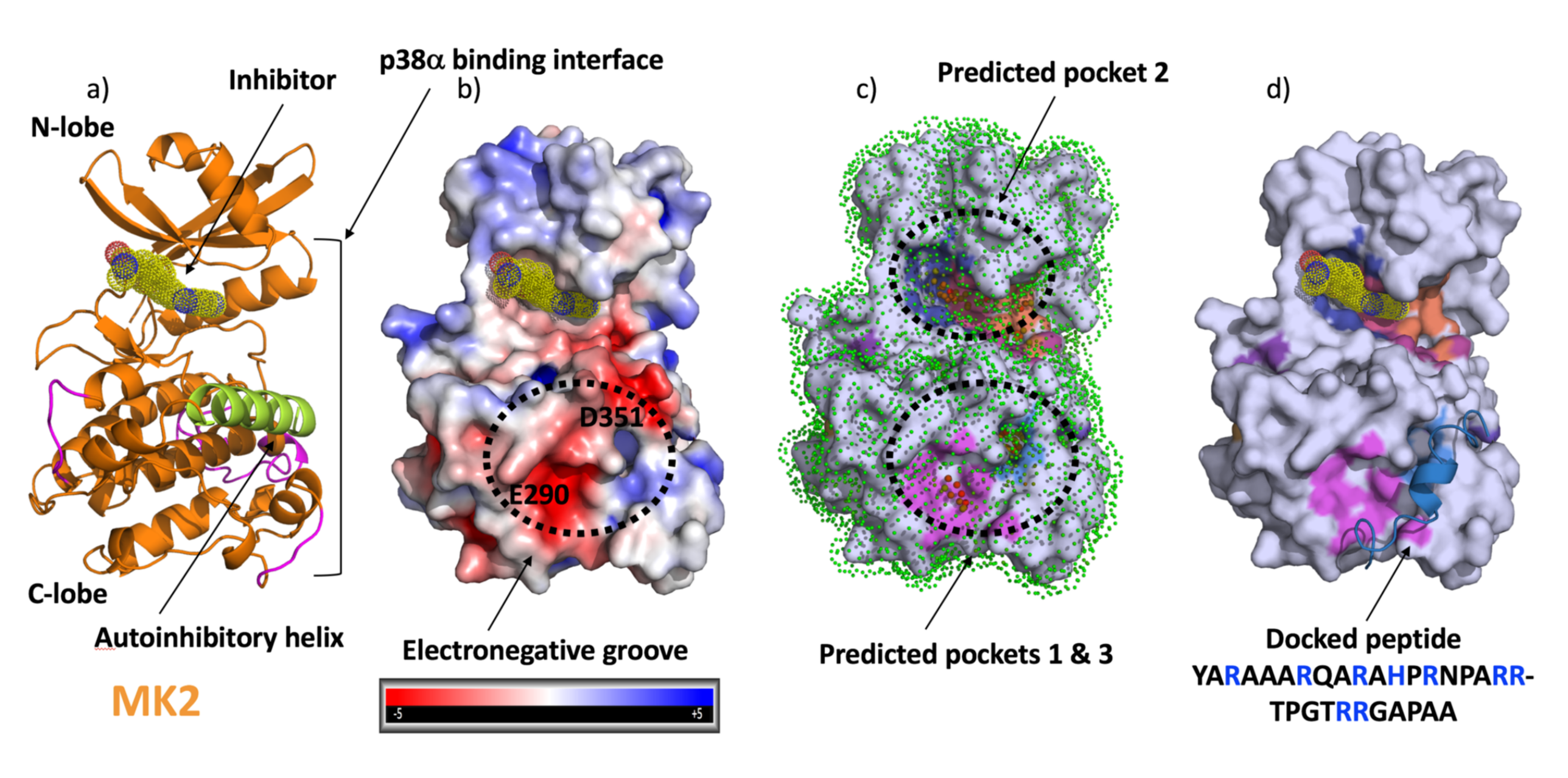
Actin

Vimentin

Polymerized and Stabilized
Cytoskeleton
Stronger Adhesion

Phase II: Barrier Recovery and Augmentation

MK2-AP


a)
Inhibitor
p38α binding interface
b)
c)
Predicted pocket 2
d)
N-lobe
D351
E290
C-lobe
Autoinhibitory helix
Electronegative groove
-5
+5
Predicted pockets 1 & 3
Docked peptide
YARAAARQARAHPRNPARR-
TPGTRRGAPAA
MK2