The pulmonary endothelium in the Acute Respiratory Distress Syndrome – insights and therapeutic opportunities

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ABSTRACT

The pulmonary endothelium is a dynamic, metabolically active layer of squamous endothelial cells ideally placed to mediate key processes involved in lung homeostasis. Many of these are disrupted in the Acute Respiratory Distress Syndrome (ARDS), a syndrome with appreciable mortality and no effective pharmacotherapy. In this review, we consider the role of the pulmonary endothelium as a key modulator and orchestrator of ARDS, highlighting advances in our understanding of endothelial pathobiology and their implications for the development of endothelial targeted therapeutics including cell-based therapies. We also discuss mechanisms to facilitate the translation of preclinical data into effective therapies including the application of biomarkers to phenotype ARDS patients with a predominance of endothelial injury and emerging biotechnologies that could enhance delivery, discovery and testing of lung-endothelial specific therapeutics.

INTRODUCTION

The pulmonary vasculature is a homogenous layer of squamous endothelial cells lining the entire pulmonary circulation. Having initially been thought of as an inert, static structure, the lung endothelium is increasingly recognised as a dynamic, metabolically active organ that modulates several key regulatory functions including: leukocyte diapedesis, intravascular coagulation, vasomotor tone and solute and fluid trafficking via regulation of barrier permeability.

The pulmonary endothelium is distinct from the systemic vascular bed, in that it is exposed to the highest oxygen tension, whilst maintaining low-pressure blood flow. Coupled with the lung possessing the highest abundance of endothelial cells relative to total cell population and its vast surface area, it is ideally placed to interact with blood-borne cells and vasoactive mediators to sense mechanical, chemical and cellular stimuli. Whilst this allows the endothelium to regulate local (and possibly systemic) inflammation, disruption of lung endothelial homeostatic mechanisms transforms it from a primarily anti-inflammatory phenotype to an activated pro-inflammatory phenotype that propagates lung parenchymal inflammation [1].

Disruption of lung endothelial homeostasis manifests clinically as Acute Respiratory Distress Syndrome (ARDS). ARDS is characterised by acute inflammation of the gas exchange surface of the lung. Dysregulated inflammation promotes the pulmonary accumulation of leukocytes and platelets, whilst activation of pro-coagulant pathways and disruption of alveolar capillary membrane barrier function leads to hypoxia, hypercapnoea and pulmonary oedema. Thus, ARDS presents clinically with acute onset of breathlessness and hypoxaemia in the presence of diffuse pulmonary oedema on the chest radiograph, with the majority of patients requiring mechanical ventilation. Risk factors for ARDS can be divided into two groups, depending on whether injury to the lung is direct such as pneumonia, with predominantly epithelial injury, or indirect blood-borne insults, such as severe sepsis, with a predominance of endothelial injury (table 1). Although mortality in ARDS has temporally declined, it remains between 25 and 35% [2] and there is currently no licensed effective pharmacotherapy, highlighting the need for novel therapeutic strategies. Contemporary management focuses on treatment of the underlying cause and organ support whilst avoiding iatrogenic injury, most notably with low tidal volume and pressure mechanical ventilation and a conservative fluid management strategy [3].

Table 1 Indirect and direct ARDS - distinguishing features

	Indirect ARDS	Direct ARDS
Causes	Severe Sepsis Trauma Blood product transfusion (TRALI) Pancreatitis Cardio-pulmonary bypass Burns	Pneumonia Aspiration Smoke inhalation Pulmonary contusion Reperfusion injury
Clinico-pathological Hallmarks	Neutrophilic alveolitis Hyaline membranes Microthrombi Probable predominance of endothelial injury Imaging and plasma evidence of (non-pulmonary) pathology e.g. pancreatitis	Neutrophilic alveolitis Hyaline membranes Microthrombi Probable predominance of alveolar epithelial injury Chest imaging evidence (CT) of initiating process e.g. lung contusion
Proposed Biomarkers	Angiopoetin-2 von-Willebrand factor Soluble thrombomodulin Interleukin-8 Soluble ICAM-1	Surfactant protein-D Receptor for advanced glycation end-products Krebs von den Lungen-6 Club cell 16

CT: Computed Tomography; ICAM-1: intracellular adhesion molecule 1; TRALI; transfusion associated lung injury.

In this article we focus on our current understanding of the role of the pulmonary endothelium in orchestrating and propagating ARDS, and further explore the endothelium as an emerging pharmacological target in ARDS. It is important to note that the pulmonary endothelium is structurally, morphologically and functionally distinct from the systemic vasculature (reviewed in [4 5]). Accordingly, this review will address data generated in relevant models of pulmonary endothelial injury, with reference to recent specific expert reviews where appropriate.

STRUCTURE AND FUNCTION OF THE PULMONARY ENDOTHELIUM

The pulmonary endothelium forms a single layer of mesenchyme-derived, non-fenestrated endothelial cells. This serves as a semi-permeable barrier separating the pulmonary circulation from the lung interstitium, regulating macromolecule, nutrient, leukocyte and fluid transfer.

The integrity of this barrier is determined by homophilic interactions between neighbouring endothelial cells via intercellular junctions (tight junctions and adherens junctions; reviewed in [6]). These junctions link endothelial cells and are served by cytoskeletal microtubules and actin microfilaments to facilitate both maintenance of barrier function and modulation of signal transduction in response to the tethering and contractile forces exerted on the endothelium during mechanical ventilation [7].

Tight junctions are formed by the fusion of the outer layers of the plasma membranes and are comprised of occludins, claudins and junctional adhesion molecules coupled to cytoplasmic proteins and linked to the endothelial cell actin cytoskeleton by the zona occludens family (ZO). Adherens junctions (AJ) are composed of cadherins, primarily vascular endothelial cadherin (VE-cadherin), that bind intracellular catenin proteins (including p120-catenin, a VE-cadherin stabilising protein) that in turn bind to other protein partners in the actin cytoskeleton. AJ are mediated by calcium dependent association of cadherin proteins and regulate the paracellular transport (the predominant pathway) of cells and solutes between the blood and the

interstitium. Hence AJ, and specifically VE-cadherin, are key regulators of paracellular permeability, which determines leukocyte transmigration and oedema formation [8] whilst cell membrane scaffolding proteins called caveolins regulate trans-endothelial trafficking (transcytosis) of macromolecues including albumin [9 10]. Data suggest that transcellular permeability increases may precede and subsequently trigger paracellular permeability via Src-mediated phosphorylation of caveolin-1 [10].Endothelial cells are tethered to the extra-cellular matrix (ECM) via interaction between cell surface integrins and their ECM ligands, which are organised in focal adhesion plaques [11].

A negatively charged extracellular layer of proteoglycans, glycoproteins and glycosaminoglycans (GAGs) that line intimal surfaces, the endothelial glycocalyx, may act as an additional barrier to large molecules and circulating cells. Data from a murine ARDS model suggested that the glycocalyx modulated neutrophil diapedesis via heparinase mediated glycocalyx shedding and consequent exposure of neutrophil adhesion molecules [12], whilst *in vitro* human data proposed that the sialic acid component of the glycocalyx maintained barrier function via regulation of cell-matrix and cell-cell interactions [13]. Despite these and other observations (reviewed in [14]), it remains unclear whether and how the glycocalyx contributes to the pathogenesis of human ARDS.

The endothelium performs additional regulatory roles in gas exchange, vascular tone and coagulation (reviewed in [15]). As an integral component of the alveolar-capillary unit, it is structurally and functionally optimised to facilitate perfusion-ventilation matching. Hence, lung endothelial cells regulate the synthesis and metabolism of vasoactive compounds such as nitric oxide and endothelin-1, potent regulators of pulmonary vascular tone. Furthermore, the endothelium also produces both pro- and anti-thrombotic substances which act both locally and remotely to regulate coagulation. It separates blood borne cellular (e.g. platelets) and humoral (e.g. coagulation factors) components of the coagulation cascade from pro-thrombotic substances in the lung interstitium and alveolar space.

PULMONARY ENDOTHELIAL ACTIVATION IN ARDS

PATHOBIOLOGY

In health, the lung endothelium adopts a predominantly inhibitory effect on inflammation and coagulation. However, upon 'activation' by a range of stimuli including hypoxia, cytokines (e.g. Tumour Necrosis Factor Alpha (TNF) and Interleukin (IL)-1β), chemokines (e.g. Interleukin-8 (IL-8)), thrombin, and bacterial endotoxins, including lipopolysaccharide (LPS) and interactions with activated inflammatory cells, a shift towards a pro-inflammatory phenotype occurs [1]. Indeed, dysregulated endothelial activation and the resultant loss of homeostatic mechanisms are aspects of ARDS pathobiology that may distinguish it from self-limiting, localised insults, for example bacterial pneumonia [1].

Accordingly, lung endothelial cells are increasingly recognised as orchestrators of the inflammatory response. In experimental influenza models, the pulmonary endothelium was a key regulator of innate cellular and cytokine responses, if not the actual source of cytokine release [16]. In addition, endothelial cells express various leukocyte adhesion molecules including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin [17]. These pro-inflammatory responses may exhibit calcium dependency; TNF and IL-8 release from lung microvascular endothelial cells stimulated with LPS correlated with an increase in intracellular calcium [18], whilst cytosolic calcium oscillations induced pro-

inflammatory gene transcription and endothelial E-selectin expression [19]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) production by activated cells saturate local antioxidants and contribute to tissue injury directly via; down-regulation of VE cadherin [20]; up-regulation of neutrophil adhesion molecule expression and release of neutrophil chemotactic factors [21].

Activated endothelial cells also assume a pro-coagulant phenotype to limit damage to lung microvasculature and localise infection. This is characterised by increased expression of platelet adhesion molecules, intra-alveolar and intravascular fibrin deposition and release of activators of the extrinsic coagulation cascade [22], in particular nitric oxide [23]. Moreover, up-regulation and activation of tissue factor and loss of the ability to activate protein C and S results in capillary thrombosis and extra-vascular fibrin deposition, thereby contributing to the increased dead-space fraction that correlate with clinical outcomes [24].

CLINICAL AND THERAPEUTIC SIGNIFICANCE

Whilst potentially propagating injury, the expression and release of pro-inflammatory molecules has driven research firstly into utilising these molecules as biomarkers of ARDS and secondly as putative pharmacological targets. Moreover, there is increasing evidence that the alveolar and vascular compartments are biologically distinct despite the alveolar capillary membrane disruption seen in ARDS supporting the notion that 'phenotypic signatures' identifying patients with site-predominant injury (endothelial vs epithelial) could be generated.

Angiopoetin-2 (Ang-2) is an endothelial growth factor produced by endothelial cells that regulates vascular permeability, promoting cell death and vascular regression. An incremental rise in plasma Ang-2, suggestive of progressive endothelial injury [25] predicted mortality in patients with sepsis related ARDS [26]. Similarly, higher circulating GAG levels (reflecting the integrity of the glycocalyx) were found in those patients with non-pulmonary insults (ie. indirect ARDS patients) although these did not predict outcome [27]. In other studies, plasma levels of soluble thrombomodulin [28], the circulating form of a transmembrane endothelial glycoprotein with anti-thrombotic and anti-inflammatory capabilities, and von Willebrand Factor (vWF) [29], a glycoprotein produced by endothelial cells, predicted mortality in ARDS. Finally, endothelial derived microparticles (EMP), sub-micron vesicles formed during membrane blebbing that shuttle proteins, organelles, lipids, and RNA are an emerging biomarker of lung endothelial activation (reviewed in [30]), particularly in the context of mechanical stretch. Hence, EMP levels were elevated in human macrovascular endothelial cells and animals exposed to pathologic mechanical stress as well as endotoxin [31 32].

Using unbiased latent class analysis, a recent study identified two distinct cohorts of ARDS patients who differed predominantly in their inflammatory profile, and more significantly divergent responses to the application of different ventilator strategies [33]. Whilst this study did not differentiate endothelial injury from epithelial injury, it suggests that patient endotyping may hold value in predicting response to therapy. Coupled with evidence from biomarker studies outlined above, it is becoming increasingly plausible that researchers may soon be able to enroll sub-phenotypes of ARDS patient with a predominance of endothelial injury to enrich enrollment in trials, thus optimizing trial design and potentially outcomes.

The expression of cell surface receptors and adhesion molecules also provides a putative platform to apply advances in pulmonary endothelial immunotargeting. Coupled with Advanced Drug Delivery systems (ADDs, such as liposomes, nanocarriers and host carriers) this methodology may facilitate targeting of specific

aspects of lung endothelial injury in ARDS. For example, antioxidants conjugated with antibodies to the endothelial determinant PECAM-1 inhibited endothelial activation and reduced VCAM-1 expression in murine lung injury [34]. Similarly, dexamethasone loaded nanogels targeted to ICAM-1 accumulated in murine LPS-injured lungs and blocked expression of ICAM-1 and VCAM-1 at 24 hours [35]. In an effort to both target the lung endothelium and enhance biologic effect, researchers have fused single-chain fragments of PECAM-1 antibodies to recombinant thrombomodulin (TM) and endothelial protein C receptor (EPCR). Using this dual-targeting approach, a 5-fold increase in receptor activation compared to isolated TM or EPCR targeting was observed as well as amelioration of lung injury parameters [36]. Translation of these methods to clinical use will be challenging, and costs may be prohibitive, nonetheless, these novel methods of targeted drug delivery hold promise.

MECHANISMS OF ENDOTHELIAL BARRIER DISRUPTION AND INJURY IN ARDS

Loss of barrier integrity, characterised by the formation of reversible intercellular gaps between endothelial cells, is accepted as the ultra-structural basis for the increased permeability pulmonary oedema observed in ARDS [37]. A range of circulating (TNF, IL-6, LPS), released (reactive oxygen species (ROS), histamine) and physical (mechanical stretch) effectors disrupt the endothelial barrier, principally by causing aactivation of the actin-myosin contractile apparatus which cause dispersion of cortical actin filaments and increased prominence of stress fibres which extend throughout the cytoplasm. Actinomyosin contraction of these stress fibers increases tension and is proposed to cause cell contraction, pulling cells apart and compromising barrier integrity [7]. Contractile machinery is regulated by the phosphorylation status of the critical actin binding protein, myosin light chain (MLC) on Ser-19 or Ser-19/Thr-18. This is controlled through an interplay of the calcium/calmodulin dependent MLC kinase (MLCK, phosphorylation) and Rho regulated MLC phosphatase (MLCP, dephosphorylation) [38]. Hence, MLCK in particular, plays an essential role in both barrier disruption and restoration in an agonist specific manner [39-41] (figure 1).

Although data specific to the lung endothelium is comparatively limited, regulatory small GTPases including RhoA, Rac1, cell division control protein 42 (Cdc42) and Rap1 are central intracellular regulators of the actin cytoskeleton and thus barrier function. Broadly, Rho negatively regulates barrier function [42] and Cdc42 and Rap1 signaling enhance barrier function [43 44]. RhoA through its regulated signalling circuitry, including the serine-threonine Rho kinase (ROCK), induces phosphorylation of MLC (via MLCK) as well as inhibition of MLCP, inducing cytoskeletal remodelling and barrier permeability [45-47]. Cdc42 directly regulates cortical actin organization as well as a host of proteins including cofilin, MLCK and neural-Wiscott Aldrich syndrome protein (N-WASP) that affect actin organization and cell adhesion to the ECM [44]. Rac1 can either positively or negatively regulate barrier function in a stimulus dependent manner [48-50] whilst Rap1, enhances barrier function via inhibition of Rho and activation of Cdc42 [51 52] as well as a co-operative association with VE-cadherin [53].

Other intracellular mediators include cyclic AMP (cAMP), nuclear factor kappa B (NFκ-B) and focal adhesion kinase. An increase in cAMP levels in response to a range of mediators reduces vascular leakage through activation of protein kinase A (PKA) and the guanine exchange factor, exchange protein activated by cAMP (Epac) [54 55]. PKA inhibits RhoA activation and EC contraction [56] and Epac (via Rap1) enhances VE-cadherin junctional integrity and actin reorganization [53]. FAK, a non-

receptor tyrosine kinase regulates turnover of focal adhesion formation by binding to focal adhesion proteins as well as enhancing AJ formation [6]; in experimental ARDS models (including conditional FAK deletion), decreased FAK expression was associated with lung oedema as well as albumin and neutrophil influx [57].

MLC-phosphorylation independent mechanisms of barrier disruption also exist. Endothelial cell apoptosis via mediators including TNF [58] and influenza virus [59] may contribute. Tyrosine phosphorylation of cytoskeletal proteins and adhesion molecules including VE-cadherin, β-catenin and p120 via tyrosine kinases including Src [60] may induce disassembly of the catenin-cadherin complex [61 62] whilst microtubule disassembly independent of MLCK and Rho has been reported [63 64].

The angiopoietin-Tie2 signalling axis (the endothelial tyrosine kinase Tie2 and its circulating ligands angiopoetins 1-4) merits specific mention as a mediator of barrier disruption as it represents one of the most extensively studied barrier-regulating mechanisms. Ang-1 is constitutively expressed in a range of cell types and mediates barrier integrity and endothelial quiescence via steady activation of the Tie2 receptor, which is abundantly expressed in endothelium. Ang-2, released from endothelial cells in response to a diverse range of mediators [65 66], acts as a functional antagonist of Ang-1 at the Tie2 receptor, mediating cytoskeletal rearrangement [25] and junctional disruption [67]. Thus, mice heterozygous for Ang-2 were protected from lung injury compared to wild type mice in sepsis models [67]. Ang-2 may play additional roles in leukocyte endothelial interactions [66]. In the clinic, circulating levels of Ang-2 correlated with increased pulmonary oedema and mortality in ARDS patients [26] and predicted the development of ARDS in critical illness [68] further supporting a central role for Ang-2 in the endothelial injury of ARDS.

The role of damage-associated molecular patterns (DAMPs, native molecules released after tissue injury) and in particular mitochondrial DNA (mtDNA) production in barrier disruption is an emerging area of investigation. Hence, circulating levels of mtDNA are elevated in critical illness. [69] In this context, they are potent inducers of the inflammasome via Toll-like receptor-9 (TLR-9) [70], activating leukocyte mediated lung injury when injected *in vivo* [71] and endothelial barrier disruption *in vitro* [72]. A bacterial challenge in isolated mouse lungs induced mtDNA release which was associated with endothelial hyper-permeability; this effect was replicated with exogenous mtDNA and attenuated by blockade of TLR-9 [73]. Further elucidation of mechanisms of mtDNA release and their interplay with ROS as well as intracellular signalling pathways are required but this represents an intriguing line of investigation, if not a potential therapeutic target. Whilst previously thought to contribute primarily to lung epithelial injury and repair [74], pathogen-associated molecular patterns (PAMP) signalling via pattern recognition receptors (PRRs) may also contribute to lung endothelial barrier dysfunction. Accordingly, influenza virus infection up-regulated PRR expression (specifically TNF receptor 1) in a range of relevant models including human lung autopsy specimens, with resultant endothelial leak and apoptosis following exposure to *Staphylococcus aureus* derived PAMPs *in vitro [75].*

CANDIDATE THERAPIES TO ENHANCE ENDOTHELIAL BARRIER FUNCTION

The endogenous lipid growth factor sphingosine-1-phosphate (S1P) enhances barrier function through a series of signalling pathways that maintain cortical actin, focal adhesions, and tight junctions (reviewed in [76]). S1P receptors are highly expressed on pulmonary endothelial cells. Accordingly, S1P and its analogues reduced vascular leakage in small and large animal lung injury models [77 78] as

well as dampening the cytokine storm in a murine influenza model [16]. The clinical application of S1P and its analogues is currently limited by systemic toxicity, most notably immunosuppression prompting the use of FTY720 (an S1P analogue) in multiple sclerosis [79]. Moreover, in a murine model, prolonged application of S1P agonists worsened vascular leakage and promoted fibrosis [80] whilst the S1P pathway has been linked to dysregulated fibrogenesis of idiopathic pulmonary fibrosis [81]. Safer analogues with promising, if not superior preclinical data, may however be on the horizon [82].

Based on the preclinical and clinical data outlined above, the Tie2 axis represents an attractive therapeutic target. A stable variant of Ang-1 protected against systemic microvascular dysfunction and restored endothelial barrier function in a murine sepsis model, [83] whilst Ang-1 therapy rescued barrier disruption *in vitro* from ARDS plasma high in Ang-2. Recent work has demonstrated that a specific pharmacological inhibitor of VE–protein tyrosine phosphatase (VE-PTP) catalytic activity, AKB-9778, which activates Tie2 [84], blocked lung neutrophil recruitment in LPS challenged mice and stabilized lung endothelial junctions via Rap1 [85]. A novel Tie-2 agonist rescued mice from severe influenza up to 3 days after infection [59]. Improvements in vascular leak were attributed to Tie-2 mediated attenuation of endothelial cell apoptosis as cellular proliferation was unaffected. Notably, maintenance of barrier function did not impair leukocyte transmigration. This observation supports data from study in the systemic circulation suggesting independent regulation of these 2 processes [86 87].

The renin-angiotensin system (RAS) is a complex network orchestrating blood pressure, electrolyte and fluid homeostasis that has been implicated in the pathogenesis of ARDS. Angiotensin-converting enzyme-2 (ACE2) is a endogenous modulator of RAS highly expressed in the lung endothelium and alveolar epithelium [88] which diverts potentially injurious Angiotensin II (Ang II) signalling via conversion of Ang II to Ang 1–7 and inactivation of angiotensin receptors, thus negatively regulating RAS [89]. Studies in knockout mice have demonstrated that loss of ACE2 worsened sepsis and acid aspiration induced lung injury. [90] ACE2 is reported to be the receptor for the SARS coronavirus induced lung injury [91]. More recently, compelling data suggest that ACE2 has a central role in the development and progression of the potentially lethal avian influenza viruses H5N1 [92] and H797 [93 94]. Gain of function ACE1 polymorphisms are also associated with ARDS susceptibility and worse outcome [95]. None of these studies offer clear mechanistic insight but ACE2 signaling in the lung is likely to be mediated by alveolar epithelial cells [88 96]. A Phase IIa trial of a recombinant ACE2 compound in both direct and indirect ARDS patients Clinical Trials.gov ID: NCT01597635 has recently completed recruitment and interim results are awaited.

HMG-CoA reductase inhibitors (statins) exert pleomorphic, anti-inflammatory, immunomodulatory, and antioxidant effects on endothelial cells to promote cytoskeletal rearrangement, decrease oxidative stress and modulate gene expression [97 98]. Hence, statins attenuated vascular leak in a range of murine ALI models [97 99 100]. Despite promising pre-clinical data, including an *in vivo* human inhaled LPS model [101], these findings were not translated into clinical improvements in two recent clinical trials [102 103]. Given the extensive data supporting a biological effect on the endothelium, it is intriguing to speculate that these trials may have been enriched by the enrolment of specific endotypes of indirect ARDS patients to optimize outcomes.

A recent phase I clinical trial of 26 patients demonstrated mortality benefit in ARDS (n=37) treated with interferon-beta-1a [104]. The proposed mechanisms of benefit were modulation of inflammation (possibly neutrophil endothelial interactions) and

endothelial barrier function via CD73 mediated dephosphorylation of adenosine monophosphate. Whilst non-randomised, the mortality benefit (24% absolute reduction) in this study suggests that targeting the lung endothelium may hold promise as a viable therapeutic strategy in ARDS. A randomized controlled trial is imminent to confirm these findings. This study was also notable by the generation of human (*ex vivo*) data to support animal data prior to early phase trials, a paradigm that should be increasingly adopted.

The pleiotropic effects of the tyrosine kinase inhibitor imatinib, particularly in attenuation of vascular permeability induced by a broad range of mediators (discussed in [105]) have stimulated study into its efficacy as a barrier enhancing agent in ARDS. Despite imatinib's association with peripheral oedema [106], case reports have suggested clinical improvements in idiopathic vascular leak [107] and bleomycin induced lung injury [108]. Substantiating these clinical observations, imatinib attenuated thrombin and histamine induced barrier dysfunction *in vitro* [109] and pulmonary vascular leak in clinically relevant murine models [105 109]. Given its multiple sites of action (Table 2), further mechanistic work is required to progress Imatinib as a potential therapy in ARDS.

Other candidate therapies have shown promise, but again mechanistic understanding in the pulmonary vasculature is not sufficiently advanced. Atrial natriuretic peptide (ANP) protects against lipopolysaccharide (LPS) mediated lung microvascular leakage by blocking nuclear factor kappa B (NF-κB) activity [110], while concurrently enhancing VE-cadherin localisation to AJ [111]. Recent murine data support an additional role for ANP in microtubule stabilisation an emerging mechanism in regulation of endothelial cell permeability [112]. Of note, a previous small clinical trial demonstrated physiological improvements with an infusion of ANP [113]. Adrenomedullin (AM), a ubiquitously expressed peptide hormone that binds calcitonin receptor-like receptor (CRC) on lung endothelial cells promoting intercellular adherence, improved endothelial barrier function in preclinical ALI and VALI models [114-116]. Given the abundance of binding sites on the pulmonary endothelium, AM also holds promise as a lung endothelial imaging tool using 99mTechnetium labelling [117]. Finally, Hepatocyte Growth Factor (HGF) has recently been shown to suppress LPS-induced endothelial activation and barrier disruption possibly via a guanine nucleotide exchange factor [118 119].

A summary of the mediators and mechanisms of barrier disruption and enhancement is outlined in table 2 and figure 2.

Table 2 Mediators and mechanisms of barrier disruption and enhancement

BARRIER DISRUPTION

Molecule	Target	Mechanisms	Refs
Endothelial Glycocalyx	Unknown	Loss of endothelial homeostatic mechanisms Disruption of cell-cell & cell-matrix interactions Increased leukocyte adhesion	[12 13]
LPS	TLR4	Intracellular calcium influx via DAG induced TRPC6 channel activation Reduced FAK expression MLCK activation NF-κB induced inflammatory cytokine release	[41 57 120]
Mechanical stretch	IL-6R	RhoA activation Expression of contractile proteins Cytokine release	[121-123]
mtDNA	TLR9	Intracellular calcium influx Cytokine release	[72]
ROS	NADPH oxidase	Intracellular calcium influx & MLCK activation	[20 21]
Thrombin	PAR1	Intracellular calcium influx & activation of MLCK & RhoA	[124 125]
TNF	TNFR1	Promotion of NF-κB induced inflammatory cytokine release & up-regulation of leukocyte ligands eg. ICAM-1	[17]

BARRIER ENHANCEMENT

Molecule	Target	Mechanisms	Refs
ACE2	n/a	Negative regulation of RAS via inactivation of angiotensin receptors Abrogation of Ang II signalling & activity	[89 90]
Adrenomedullin	CLR complex	Attenuation of MLCK phosphorylation Activation of protein kinase A, Promotes intercellular adherence	[115 126]
Ang1	Tie2	Adherens junction assembly via Rac1 activation Inhibition of NF-κB signalling Competitive inhibition of Ang2 mediated barrier disruption	[65]
ANP	NF-κB	Protects against LPS mediated NF-κB signalling Enhanced VE-cadherin localisation to AJ	[110 111]
HGF	MET	Activates Rac-dependent cytoskeletal rearrangement	[118 119]
IFNβ1a	CD73	Up regulation of CD73 enhancing adenosine activity via adenosine dephosphorylation	[104 127]
Imatinib	c-Abl Arg PDGFR	Augmented Rac1 signalling Cytoskeletal rearrangement Inhibition of LPS induced NF-κB signalling	[105 109 128 129]
S1P	$S1PR_1$	Stabilization of adherens junctions via Rac1 Inhibition of cofilin signalling enhancing tight junction formation	[76 130]
Statins	HMGCoA reductase	Abrogation of VEGF signalling via RhoA inhibition & Rac1 activation	[97 99 100]

ACE2: Angiotensin converting enzyme-2; AJ: Adherens junction; Ang1/2; Angiopoetin 1/2; ANP: Atrial natriuretic peptide; Arg: Abl-related gene; CD73: Cluster of differentiation 73; CLR: calcitonin receptor-like receptor; DAG: Diacyglycerol; FAK: Focal adhesion kinase; HGF: Hepatocyte growth factor; HMGCoA: 3-hydroxy-3-methyl-glutanyl-CoA; ICAM-1: Intracellular adhesion molecule; IFN β 1a: Interferon-beta 1-alpha; IL-6R: Interleukin-6 receptor; LPS: Lipopolysaccharide; MET: Hepatocyte growth factor receptor; MLCK: Myosin light chain kinase; mtDNA: mitochondrial DNA; NADPH:

Nictotinamide adenine dinucleotide phosphate; NF-κB: Nuclear factor kappa-B; PAR1: Protease-activated receptor; PDGFR: platelet-derived growth factor receptor; RhoA: Ras homolog gene family, member A; RAS: Renin-angiotensin system; Tie2: Tyrosine kinase receptor-2; TJ: Tight junction; TLR4: Toll-like receptor 4; TLR9: Toll-like receptor 9; TNF: Tumour necrosis factor; TNFR1: Tumour necrosis factor receptor 1; TRPC6: Transient receptor potential cation channel, member 6; S1P: Sphingosine-1-phosphate; $S1PR_1$: Sphingosine-1-phosphate receptor 1; VEGF: Vascular endothelial growth factor

INTERACTIONS OF THE LUNG MICROVASCULATURE WITH LEUKOCYTES AND PLATELETS

Neutrophils are central to the initiation and propagation of inflammation and injury in ARDS and neutrophilic alveolitis is a histological hallmark [131]. Their importance in ARDS is highlighted by the observation that a decline in respiratory function is seen in neutropenic ARDS patients upon recovery of neutrophil counts [132]. Pathway analysis of differential gene expression in sepsis induced ARDS compared to sepsis alone identified a preponderance of genes regulating neutrophil homeostasis and activation [133], further supporting the notion of the role of neutrophilic inflammation.

Despite extensive data from models of the systemic circulation (e.g. murine cremaster vessels and human umbilical vein endothelial cells), our mechanistic understanding of the role of the pulmonary endothelium in neutrophil sequestration is limited. As a consequence of the unique pulmonary capillary microanatomy, the site and mechanisms of neutrophil sequestration are different from the systemic microcirculation. For example, owing to space constraints in the alveolar capillary bed, neutrophils must change their shape to pass through. Moreover, neutrophil rolling on the endothelial surface does not occur [134]. Similarly, neutrophils exhibit selectin and CD11b/CD18 independent sequestration in pulmonary capillaries [135 136]. A role for the endothelial glycocalyx in modulation of neutrophil adhesion molecule expression (ICAM-1) has been proposed [12], however, precise identification of the cognate receptors for neutrophils on lung endothelium remains elusive and further study in refined animal models and novel human models is an urgent unmet need.

Clarification of the role of neutrophil migration in barrier disruption is also required. Activated neutrophils exert negative effects on barrier permeability via secretion of various products such as TNF, complement component 5a (C5a) and arachidonic acid but it remains unclear whether neutrophil migration through the endothelium is damaging *per se*. As outlined above, recent data support the concept that neutrophil migration and endothelial barrier disruption may be independently regulated [59 86 87].

Recent work has focused on the role of the transient receptor potential channels (TRP), specifically TRP vanilloid 4 (TRPV4) receptor, particularly as it is expressed on both neutrophils and the pulmonary endothelium, and specific inhibitors are available [137]. TRPV4 signaling has been implicated in endothelial dysfunction secondary to mechanical [138] and hydrostatic [139] stress possibly via calcium influx [137 140]. Deletion of TRPV4 attenuated both endothelial barrier leak and neutrophil activation in a murine acid injury model [137]. Further, chimeric mouse models demonstrated that attenuation of lung injury was contingent on endothelial TRPV4 as opposed to leukocyte TRPV4 although deletion of TRPV on neutrophils did abrogate injury in isolated perfused mouse lungs [137].

The concept that the lung endothelium may play a role in host defense by facilitating the de-priming of neutrophils, and hence providing protection from neutrophil-mediated remote organ injury, has been proposed. Data from an *in vivo* human model demonstrated that the healthy lung microvasculature retained primed cells,

and subsequently facilitated their de-priming and release (into the systemic circulation) in an un-primed quiescent state [141]. It is conceivable that failure of this mechanism, for example as a consequence of endothelial injury in ARDS, may result in high circulating levels of primed neutrophils, which correlates with the severity of lung injury [142] and which may mediate multi-organ dysfunction. Manipulation of this de-priming mechanism may offer a novel therapeutic approach.

Whilst platelets play a role in a range of pathobiological processes in ARDS (reviewed in [143]), our understanding of platelet endothelial interactions is under-developed and extrapolated from work on the systemic vasculature. In murine ARDS, platelets induced ICAM-1 expression on endothelial cells, propagating neutrophil extravasation [144]. Experimental lung injury models have also demonstrated that platelets induce endothelial activation as evidenced by increased expression of vWF, P-selectin and tissue factor, such that platelet depletion and blockade of platelet binding ameliorated injury [145 146]. Platelets also modulate endothelial barrier permeability through expression of a range of factors, including S1P (reviewed in [147]). Of note, however, in murine pneumonia, thrombocytopenia enhanced lung inflammation and endothelial cell activation suggesting that platelet depletion strategies may be detrimental [148]. The interplay between neutrophil extracellular traps (NETs) and platelets is an emerging narrative in lung injury secondary to blood transfusion [149] and following lung transplantation [150]. Platelet-endothelial interaction may also play a role in regulating alveologenesis (see below, [151]) and hence lung repair after injury.

MODULATION OF THE COAGULATION CASCADE

Activated coagulation and depressed fibrinolysis coupled with low circulating levels of endogenous anticoagulants contribute to the pathological and physiological features of ARDS. Moreover, it is becoming increasingly apparent that inflammation and coagulation in ARDS are intimately linked [152]. Modulation of these effects has thus been an attractive therapeutic target.

The protein C pathway, in particular, has been the focus of extensive research, at least in the systemic circulation (reviewed in [153]). Activated Protein C (APC) is generated from EPCR-bound protein C by thrombomodulin-bound thrombin. EPCR are expressed on both pulmonary artery and lung microvascular endothelial cells [154]. In addition to its anticoagulant activity, APC manifests a myriad of cytoprotective effects, including anti-apoptotic and anti-inflammatory effects through dissociation of APC from EPCR and activation of protease-activated receptor (PAR)-1 (and to a lesser extent PAR-3) biased signalling [153 155]. In the lung macrovascular endothelium, APC enhanced barrier function in a context specific fashion via S1P and RAC1-dependent mechanisms [130].

Despite extensive preclinical data, recombinant APC demonstrated no clinical efficacy in ARDS patients [156] and no reduction in barrier leak was demonstrated with recombinant APC therapy, [157] despite attenuation of the coagulopathy [158]. A multicentre trial of modified (catalytic site irreversibly blocked) recombinant Factor VIIa [159], similarly, showed no benefit. Thus, we have yet to fully harness the abilities of anticoagulants to modulate cell signalling, inflammation and barrier function, in particular via PAR signalling [130 153]. These negative trial data may temper enthusiasm for on-going research in this area. Nonetheless, PAR-1 antagonism has recently shown beneficial effects on neutrophil migration, cytokine release and barrier disruption in a murine pneumonia model [160] suggesting that alternative targets in the pathway may hold therapeutic promise.

THE LUNG ENDOTHELIUM AND REPAIR IN ARDS

The observation that over half of ARDS patients survive [161], suggests that the lung microvascular endothelium and epithelium have a significant capacity for repair and regeneration. The process of repair following ARDS involves both alveolar and endothelial cell reconstitution with restoration of barrier function facilitating removal of alveolar oedema and inflammatory debris. A comprehensive discussion of endothelial repair is beyond the scope of this article and readers are referred to recent expert reviews [162 163]

In addition to circulating progenitor cells, local populations of endothelial progenitor cells (EPC) have been identified in the pulmonary microvascular endothelium [164] and levels of EPCs are elevated in ARDS patients [165 166]. Retrospective analysis of lung tissue from male-to-female haematopoietic stem cell transplant patients provides direct evidence of integration of male endothelial progenitor cells into female recipient pulmonary endothelium confirming the role of EPC [167]. However, the field of circulating EPC remains controversial; their origin and function as well as their ability to effect repair under the hostile environment of clinical ARDS remains uncertain. Moreover, the cell most commonly studied, (identified by Asahara in 1997 [168]) is now recognised as a monocyte with angiogenic features and not a true endothelial progenitor [169]. Furthermore, it has been demonstrated that the majority of endothelial repair, at least after endotoxin-induced lung injury, was affected by tissue-resident progenitor cells, not circulating EPCs [170]. Any therapeutic role of cell-based therapies may be anti-inflammatory, via secretion of paracrine factors, as opposed to mediating endothelial repair *per se.*

Alternatively, endothelial cells may modulate neo-alveolarisation via cross talk with their local niche. Hence, in murine pneumonectomy models platelet-endothelial interaction via stromal-cell derived factor-1 [151] and lung endothelial VEGF signalling (via the production of matrix metalloproteinase 14) [171] induced alveologenesis. Similarly, lung endothelial cells were required to support alveolar stem cell differentiation *in vitro* and lung regeneration *in vivo*; this mechanism was thrombospondin-1 (an endogenous inhibitor of angiogenesis) dependent [172].

These data provide compelling evidence that the pulmonary endothelium participates in the resolution of ARDS. Whether manipulation of these endothelial niches or the administration and mobilisation of EPC populations is a feasible goal in ARDS requires further study. Further definition of the molecular pathways that regulate the cross talk between endothelial cells and the reparative niche, particularly in the context of relevant ARDS models will be invaluable. Whilst primarily directed towards resolution of alveolar epithelial inflammation and injury, data from a clinical trial of mesenchymal stem cells [173] (ClinicalTrials.gov: NCT02097641) will hopefully provide additional insights, particularly regarding the effects of anti-inflammatory strategies on endothelial barrier function and a potential interplay with endothelial cells.

FUTURE DIRECTIONS

The identification and validation of robust biomarkers to phenotype ARDS patients to either identify them as having a predominance of endothelial injury or to predict response to treatment would appear an integral component influencing the success of future trials of novel endothelial therapeutics in ARDS. The application of ‘omics’ technologies such as metabolomics (reviewed in [174]) will hopefully advance this

field in the coming years facilitating personalised therapies within the next decade. Biomarker exploration will ideally also result in the identification of novel drug-able targets. Building on the work by Calfee and others [27 28 33 175], enrichment of patient enrolment into focussed clinical trials of novel endothelial-specific therapeutics using established biomarkers would appear to be a reasonable strategy to optimise patient outcomes.

Further, it is imperative that the heterogeneity between systemic and pulmonary vascular endothelium and between animals and humans is increasingly recognised. Developing better techniques to interrogate neutrophil-endothelial interactions in the lung would seem paramount; the continued development of live imaging in transgenic mice using fluorescent probes to label specific cell types [176] and increased application of isolated perfused lung models [177] as well as human *ex vivo* and *in vivo* models represent mechanisms to maximise the translation of preclinical data into effective therapeutics. It remains to be seen whether so called 'lungs on a chip' [178] which combine biomimetic systems containing microfluidic channels lined by living human cells, will develop into tractable model for lung biology *per se* or specifically ARDS pathobiology. An IL-2 induced injury model recapitulated salient features of *in vivo* ARDS, which were attenuated by a novel TRPV4 antagonist [179]. This technology has the potential to complement (if not replace) current ARDS research platforms, facilitating interrogation of hitherto under-developed aspects of lung endothelial biology and pharmacology in a co-culture platform with biologically relevant cell types (alveolar epithelial cells, lung microvascular endothelial cells and leukocytes or platelets) exposed to physiologically relevant mechanical forces via a stretchable porous membrane [180]. The applications of 3D bioprinting for the study of lung biology [181] similarly represents an enticing, if yet unrealized prospect. Development of such innovative, humanized models coupled with the refinement of existing models will be central to the identification and testing of emerging therapeutics.

CONCLUSIONS

The pulmonary endothelium is increasingly seen as pivotal in both the progression and the resolution of ARDS and is therefore primed as a therapeutic target. Our understanding of endothelial biology, notably neutrophil endothelial interactions in the lung vasculature, is a limitation to potential progress. Despite this, enhancement of endothelial barrier function, in particular, shows promise in preclinical models of ARDS and relevant late phase human trials are imminent. Novel imaging techniques and innovative *in vitro* research platforms may facilitate translation of promising animal data into efficacious endothelial specific therapies. Despite enthusiasm for their introduction, the use of cell-based therapies requires further characterisation of their phenotype, efficacy and safety. In the interim, the application of emerging technologies will assist the search for robust biomarkers of endothelial injury, which will inevitably enhance enrolment into future clinical trials of novel lung endothelial targeted agents.

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FIGURE CAPTIONS

Figure 1: Mechanisms of pulmonary endothelial barrier disruption and enhancement
Barrier disruption results from actin-myosin interaction after MLC-phosphorylation, which is regulated by myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). Activation of the actin myosin contractile apparatus disperses cortical actin and increases actin stress fibre formation, resulting in cell contraction and tensional force applied to AJ proteins. RhoA acts via effector protein Rho-associated kinase (ROCK) to activate MLCK and inhibit MLCP. RhoA activity is inhibited by the GTPases Rap1 and Rac1 as well as cyclic AMP induced protein kinase A (PKA) activation. MLCK activation is modulated by Ca^{2+}which enters the cytosol from endoplasmic reticulum (ER) or extracellular space. Phosphorylation of specific tyrosine residues of cytoskeletal proteins and adhesion molecules including VE-cadherin as well as microtubule disassembly are MLCK independent mechanisms of barrier disruption; Src mediated VE-cadherin phosphorylation leads to VE-cadherin internalisation. Nuclear factor kappa B (NFκ-B) activation promotes a pro-inflammatory state resulting degradation of the endothelial glycocalyx, which may expose neutrophil ligands. Cyclic AMP (cAMP) levels increase in response to a range of mediators to induce activation of protein kinase A (which inhibits RhoA) as well as the guanine exchange factor, exchange protein activated by cAMP (Epac). Epac (via Rap1) enhances VE-cadherin junctional integrity and actin reorganization. Rap1 enhances barrier function via inhibition of Rho and activation of Cdc42 as well as a co-operative association with VE-cadherin. Cdc42 directly regulates cortical actin organization and proteins including MLCK and

neural-Wiscott Aldrich syndrome protein (N-WASP) that mediate cortical actin formation via interaction with focal adhesion kinase (FAK) and actin related protein (ARP) thus strengthening AJ and TJ formation as well as cell adhesion to the ECM. FAK also signals via effector molecules to inhibit RhoA and activate Rac1.

Figure 2: Mediators of pulmonary endothelial barrier function
Thrombin acts via protease activated receptor 1 (PAR1) to induce multiple barrier disruptive mechanisms including calcium influx, via transient receptor potential ion channels (TRP), adherens junction (AJ) protein phosphorylation via the tyrosine kinase Src and RhoA activation. Lipopolysaccharide (LPS), via activation of toll-like receptor 4 (TLR4), increases intracellular calcium and activates myosin light chain kinase (MLCK) as well as induction of nuclear factor kappa B (NF-κB) signalling, promoting inflammatory cytokine production and neutrophil ligand expression. Mitochondrial DNA (mtDNA) acts via toll-like receptor 9 (TLR9) to increase intracellular calcium, activate MLCK and promote actin stress fibre formation. Cyclic mechanical stretch (CMS), via interleukin 6 receptor (IL6R) disrupts barrier function via Rho-independent mechanisms (circulating IL-6) and Rho-dependent mechanisms. Tumour necrosis factor alpha (TNF), via tumour necrosis factor alpha receptor 1 (TNFR1) activates NF-κB. An additional mechanism of TNF induced barrier disruption includes tyrosine phosphorylation of VE cadherin. Barrier protective mediators sphingosine-1-phosphate (S1P) activates sphingosine-1-phosphate receptor 1 ($S1P_1$) to promote MLC phosphorylation and adherens junction assembly via Rac1. S1P signalling may have additional immunomodulatory effects in influenza infection. Hepatocyte growth factor (HGF), via HGF receptor tyrosine kinase (MET) activates Rac1 activity via the adaptor protein IQGAP1. Angiopoetin-1 (Ang1) competes with the functional antagonist Angiopoetin-2 (Ang2) at the tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie2) receptor to promote adherens junction assembly and cortical actin formation through Rac1 and inhibition of NF-κB signalling. Atrial natriuretic peptide (ANP) also mitigates pro-inflammatory NF-κB signalling and RhoA activity. HMG-CoA reductase inhibitors (statins) inhibit RhoA activity. Angiotensin converting enzyme 2 (ACE2) acts via angiotensin 2 type 1 receptor (AT_1R) to inhibit the barrier disruptive effects of Angiotensin 2 signalling and renin-angiotensin system activation. Adrenomedullin (AM) acts via calcitonin receptor-like receptor (CLR) to activate cyclic adenosine monophosphate (cAMP) signalling, mediating barrier enhancement via protein kinase A (PKA) induced RhoA inhibition and endothelial cell contraction as well as Rap1 mediated exchange protein activated by cAMP (Epac) activation.

Figure 1

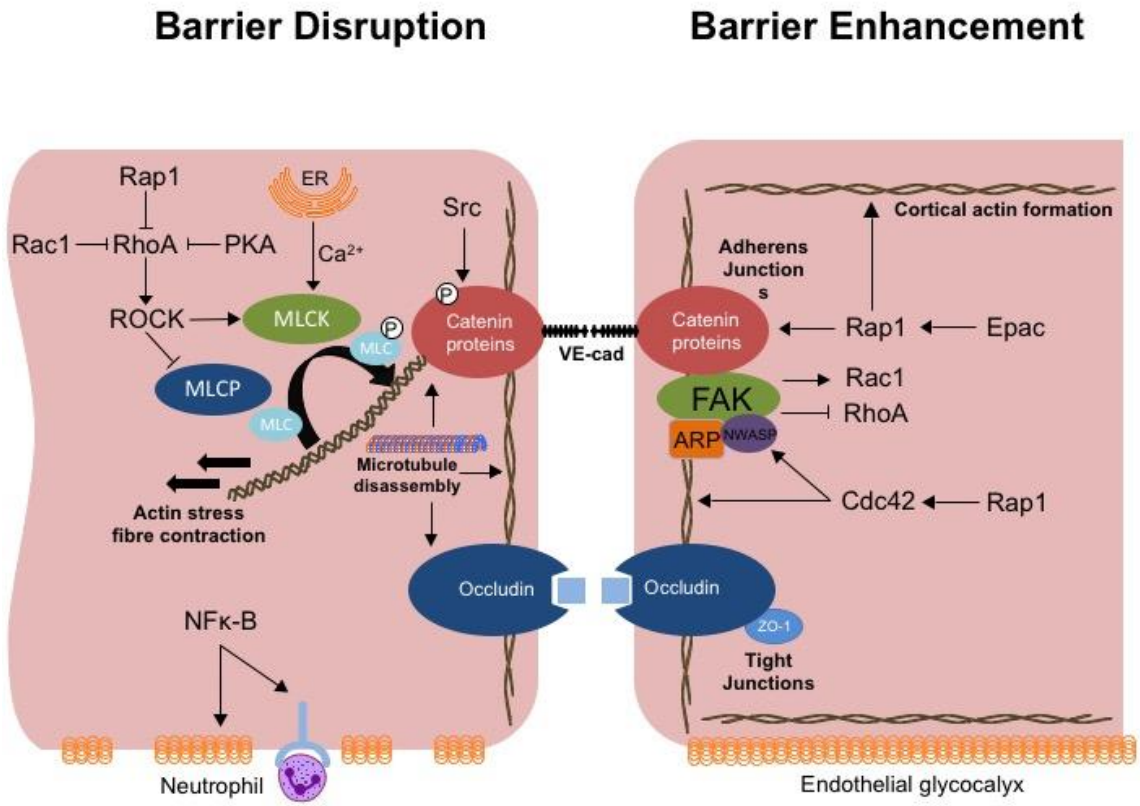
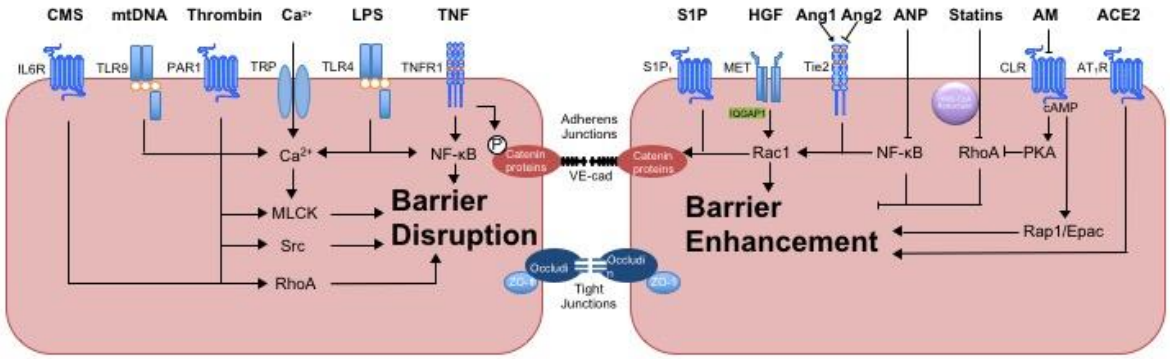

Barrier Disruption
Barrier Enhancement
Rap1
ER
Src
Rac1
RhoA
PKA
Ca2+
ROCK
MLCK
MLC
Catenin proteins
VE-cad
MLCP
MLC
Microtubule disassembly
Actin stress fibre contraction
Occludin
Occludin
NFκ-B
Neutrophil
Cortical actin formation
Adherens Junctions
Catenin proteins
Rap1
Epac
Rac1
FAK
RhoA
ARP
NWASP
Cdc42
Rap1
ZO-1
Tight Junctions
Endothelial glycocalyx

Figure 2


CMS
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Thrombin
Ca²⁺
LPS
TNF
IL6R
TLR9
PAR1
TRP
TLR4
TNFR1
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MLCK
Src
RhoA
Barrier Disruption
Adherens Junctions
Catenin proteins
VE-cad
Tight Junctions
Occludin
ZO-1
S1P
HGF
Ang1 Ang2
ANP
Statins
AM
ACE2
S1P1
MET
Tie2
CLR
AT1R
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Rac1
NF-κB
RhoA
PKA
Rap1/Epac
Barrier Enhancement