Novel concepts of acute lung injury and alveolar-capillary barrier dysfunction

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Perspectives

Herold S, Gabrielli NM, Vadász I. Novel concepts of acute lung injury and alveolar-capillary barrier dysfunction. Am J Physiol Lung Cell Mol Physiol 305: L665–L681, 2013. First published September 13, 2013; doi:10.1152/ajplung.00232.2013.—In this review we summarize recent major advances in our understanding on the molecular mechanisms, mediators, and biomarkers of acute lung injury (ALI) and alveolar-capillary barrier dysfunction, highlighting the role of immune cells, inflammatory and noninflammatory signaling events, mechanical noxae, and the affected cellular and molecular entities and functions. Furthermore, we address novel aspects of resolution and repair of ALI, as well as putative candidates for treatment of ALI, including pharmacological and cellular therapeutic means.

Alveolar-capillary barrier dysfunction; molecular mechanism; pharmacological and cell-based therapy; pulmonary edema; zaaacute lung injury/acute respiratory distress syndrome

Immune Cells and Inflammatory Signaling Pathways in ALI

One of the central concepts in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) is that an unbalanced quantity or quality of the inflammatory response aggravates epithelial or endothelial injury. This includes a dysregulated recruitment of leucocytes and/or an exaggerated activation of these cells; inappropriate expression of cytokines, lipid mediators, or reactive oxygen species; enhanced activation of death receptor signaling (97, 98, 140, 207, 240); or an uncontrolled activity of platelets or the coagulation cascade (154). In general, these responses are initiated and maintained by recognition of danger- or pathogen-associated molecular patterns (DAMPs/PAMPs) by highly conserved pattern recognition receptors such as TLRs (Toll-like receptors), NLRs (NOD-like receptors), RLRs (RIG-I-like receptors), C-type lectin receptors, and AIM2 (absence in melanoma 2-like) receptors (178, 259, 260).

Macrophages. A well-balanced innate and adaptive immune response is necessary to clear infection, to prevent systemic spread of microbes, and to finally resolve inflammatory infiltrates without causing injury to the alveolar-capillary barrier. Alveolar macrophages are composed of different subsets that reside in the lung or are recruited from the circulation upon an inflammatory stimulus. They are key orchestrators of the immune response and form the first line of defense in pathogen elimination, and, later on, they promote clearance of apoptotic debris to allow inflammation resolution and tissue repair (48, 91, 139). Recruited, “exudate” macrophages were shown to attenuate alveolar epithelial cell injury by release of the anti-inflammatory IL-1 receptor antagonist (IL-1ra) in ALI associated with gram-negative pneumonia (92). In other models of lung injury, e.g., in viral pneumonia, they may instead contribute to aggravation of inflammatory injury by expression of the death ligand TRAIL or by p38/MK2-dependent TNF-α expression (14, 97, 170). For example, it has been recently demonstrated that infiltrating macrophage precursors regulate ongoing neutrophil recruitment and loss of barrier function in ALI associated with gram-negative bacterial infection; therefore, monocyte depletion has been proposed as a potential therapeutic approach. However, monocyte/macrophage depletion might result in loss of important beneficial anti-inflammatory effects of these cells (52, 222), and a recent trial failed to prove significant improvement of LPS-induced systemic and pulmonary inflammation in humans after depletion of circulating monocytes (11).

In later stages of ALI, macrophages phagocytose apoptotic neutrophils and excess mononuclear cells by mechanisms that have recently been identified in detail (110, 215). A deficiency of resident alveolar macrophages worsens influenza-related pneumonia and lung injury in mice, leading to an increase in neutrophils and their harmful mediators (163). This dichotomy reflects the broad functional plasticity of macrophages, which has been associated with their polarization phenotype in lung diseases in a time-, organ compartment-, or pathogen-dependent context. Macrophage polarization toward a proinflammatory, infectious M1 or an anti-inflammatory, healing or profibrotic M2 phenotype is thereby likely induced during the recruitment process into the air spaces or within the inflamed alveolar compartment and is associated with the transcriptional programs induced by DAMP/PAMP sensing, local mediator profile, and cell-cell interactions (69, 91). The macrophage polarization state may therefore represent a central determinant of whether macrophages are protective or detrimental in different entities of ALI (167, 169), and elucidating the molecular signals driving defined functional phenotypes of macrophages may offer future therapeutic targets for ALI treatment.

Neutrophils. Despite their important role in pathogen containment, excessive alveolar neutrophil recruitment has been associated with injury to the alveolar-capillary barrier in ALI (136). Neutrophils are margined in the alveolar capillaries in infectious or noninfectious ALI, and this process involves engagement of chemokine-receptor interactions (267) and of different families of endothelial and epithelial adhesion molecules, such as JAMs (junctional adhesion molecules), ICAM-1 (intercellular adhesion molecule-1), PECAM-1 (platelet endothelial cell adhesion molecule-1), and VCAM-1 (vascular adhesion molecule-1), which are upregulated upon release of inflammatory mediators like TNF-α (16, 93, 131). In addition, leukocyte-expressed adenosine receptor A(2b) and eicosanoid expression are attributed a role in pulmonary neutrophil recruitment in LPS-induced ALI or after Pseudomonas aeruginosa infection in mice (102, 124). Once recruited into the
alveolar compartment, neutrophils release proteases or neutrophil extracellular traps composed of chromatin and antimicrobial agents, which may cause endothelial injury (163, 201). The neutrophil CXCL10-CXCR3 autocrine signaling axis was recently found to severely aggravate influenza virus-induced ARDS via generation of reactive oxygen species (ROS) (103). Transgenic mouse models reveal that components of the coagulation cascade impact on neutrophil recruitment or their survival. Plasminogen activator inhibitor-1 (PAI-1) enhances neutrophil recruitment after *P. aeruginosa* infection and exerts antiapoptotic effects on neutrophils via phosphatidylinositol 3-kinase activation in a model of LPS-induced acute lung injury (80, 277), increasing neutrophil load in the air spaces. Tissue plasminogen activator (tPA)-deficient mice are protected from lung ischemia-reperfusion injury through inhibition of neutrophil recruitment, which is mediated by the tPA/LDL receptor-related protein/NF-kB signaling pathway and reduction in PECAM-1 expression (275). Although the role of neutrophils as main drivers of ALI is evident, it has to be emphasized that patients might still develop ARDS under neutropenic conditions. Lung injury in such cases might be mediated by bacterial products like endotoxin, or by exaggerated responses from lung-resident immune cells. In addition to the concept that neutrophils are central drivers of lung tissue injury, recent data suggest that these cells may also induce regeneration signals to immediately repair the injury they have caused to the epithelium during transmigration. These processes involve elastase-dependent cleavage of E-cadherin and nuclear translocation of β-catenin, driving proliferation of epithelial cells (269). These data highlight that the molecular mechanisms of neutrophil-epithelial interactions during injury and repair of ALI are still not fully understood and need further evaluation.

**Platelets and alterations in the coagulation system.** Platelets are key factors in clot formation and homeostasis, but they have recently been attributed an essential role in antibacterial host defense and in the pathophysiology of sepsis and ALI. Platelets express TLRs, allowing them to sense PAMPs and directly kill pathogens by releasing microbicidal molecules and by phagocytosing pathogens (111, 207). In addition, platelets modulate the function of endothelial cells and leukocytes during infection. Platelet-neutrophil interactions have been found to be crucial determinants of alveolar neutrophil recruitment in different ALI models, which are in part mediated by the platelet-derived chemokines CCL5 and CXCL4 and P-selectin (82, 207). Moreover, platelets secrete metabolites such as IL-1ß and tromboxane A2 (TXA2) to increase the bacterial activities of neutrophils, and platelet TLR4 facilitates the formation of microbe-containing neutrophil extracellular traps (NETs) (32). The interactions between platelets, leukocytes, and endothelial cells highlight how cellular components of inflammation and coagulation cross talk to fight infection of the lung. However, when dysbalanced, these interactions might be detrimental to the vulnerable alveolar-capillary barrier (207).

It is well recognized that local impairment of the coagulation and the fibrinolytic pathways contribute to the pathogenesis of ALI (104, 123). Interestingly, direct injury to the alveolar epithelium has been shown to activate platelets and lead to a procoagulant endothelial surface expressing von Willebrand factor, tissue factor, and P-selectin molecules (63). Furthermore, parenteral ascorbic acid infusion was found to be protective against detrimental consequences of sepsis by attenuating the proinflammatory response, preventing sepsis-associated coagulation abnormalities, improving epithelial barrier function, and increasing alveolar fluid clearance (68). The dysregulated coagulation system, among many others, also plays an important role in the pathogenesis of pulmonary hypertension associated with ALI (188).

**Inflammatory mediators and signaling events in ALI.** A variety of endogenous inflammatory mediators, signaling events, or transcriptional programs were recently shown to contribute to disease progression in different models of lung injury (Fig. 1). In particular, an endogenous inhibitor of the mitogen-activated protein kinase (MAPK) signaling pathway, MKP-2, was identified as one of these triggers in pneumonia-associated ALI, since mkp-2 null mice demonstrated attenuated proinflammatory cytokine production as well as decreased neutrophil infiltration without delayed bacterial clearance (44). Similarly, induction of MKP-1, a MAPK activator, was protective against ventilator-induced lung injury in an NF-kB-dependent manner (182). Lyso phosphatic acid (LPA) is a bioactive phospholipid, which plays an important role in ALI by inducing the release of chemokines and lipid mediators. Knockdown or genetic deletion of the LPA receptor 1, interacting with the TLR4 coreceptor CD14, showed significant protection against LPS-induced inflammation but had no effect on barrier function (272). Kim and colleagues (120) recently demonstrated that pulmonary inflammation and injury can be modified by both the iron transporter divalent metal transporter 1 (DMT1) and the iron status, since DMT1-deficient rats show increased injury after LPS instillation. Importantly, activation of the inflammasome by both endogenous danger signals or pathogens amplifies inflammation, and its influence on host defense or organ injury depends on the extent and duration of this response (56). Endogenous inhibition of its main effector, IL-1ß, by IL-1ra attenuates ALI in *Klebsiella pneumoniae*-induced ALI, and high systemic levels of IL-1ra are associated with reduced susceptibility to ARDS in humans (92, 158). Deletion of caspase-1 and caspase inhibitors were found protective in mouse models using LPS challenge (207), and recent studies demonstrate that IL-18 is increased in mice with ALI with inhibition of caspase 1 or depletion of IL-18 decreasing lung injury, highlighting an important role of the inflammasome. In patients with sepsis-induced ALI, elevated levels of IL-18 or markers of caspase-1 activation correlate with morbidity and mortality (55). Finally, the TLR4 pathway in conjunction with oxidative stress and oxidized phospholipids has been highlighted as crucial driver of injury in different ALI models in which TLR4 mainly acts as a DAMP sensor (105, 211, 255).

**Noninflammatory Mediators of ALI**

**Gaseous mediators.** Severe hypoxia is a hallmark of ALI. In patients with ARDS requiring mechanical ventilation, regional alveolar hypoxia occurs, contributing to both the pathogenesis and progression of injury (71). Hypoxia is proinflammatory, thereby contributing to damage of alveolar epithelial and endothelial cells (62). Furthermore, it is well documented that hypoxia impairs junctional complexes (31) and downregulates the Na+ transporters Na-K-ATPase and ENaC (49, 257), thus causing both accumulation and retention of pulmonary edema.
in the alveolar space. Patients with ARDS generally require positive-pressure ventilation with high fractions of inspired oxygen causing hyperoxia in the lung. Hyperoxia may also contribute to lung injury primarily by initiating ROS generation (8, 117) and activating the inflammasome (122), leading to altered mechanical properties (198) and apoptosis (23, 144, 148, 263) of alveolar epithelial cells. As a consequence of protective ventilation with low tidal volumes, a number of patients display elevated levels of CO2, termed permissive hypercapnia. The role of hypercapnia in the setting of ARDS is highly controversial (13, 47, 235). Although high PCO2 appears to be anti-inflammatory and reduces oxidative stress (13), it also impairs innate immunity (89), macrophage function (249), alveolar epithelial barrier function (232, 233), and transport capacity (20) and may lead to worse outcomes in mice with lung injury secondary to Pseudomonas pneumonia (74). Since regional hypoxia, hyperoxia, and hypercapnia often cannot be prevented in patients with ARDS, further dissecting and targeting cellular O2 and CO2 sensing, and the specific signaling events initiated by elevated or decreased levels of these gaseous mediators, will be necessary to fight the detrimental effects of altered gas exchange.

Another well-known gaseous signaling molecule, nitric oxide (NO), has both protective and detrimental effects in ALI depending on the site of action and interacting partners. Traditionally, beneficial effects of NO have been associated with soluble guanylyl cyclase (sGC) activation and cGMP production whereas negative effects were attributed to the formation of peroxynitrite (ONOO−) in the presence of superoxide (O2−) or S-nitrosylation of proteins (129). Several mechanisms are responsible for the barrier-protective effects of cGMP (119, 129). cGMP activates cGMP-regulated protein kinases, which in turn attenuate endothelial Ca2+ signaling thereby preventing Ca2+-dependent paracellular gap formation (185). cGMP also regulates cyclic nucleotide-gated membrane ion channels (264). Another, cGMP-independent, protective effect of NO in the endothelium may be related to zinc, which promotes resistance of cells to proapoptotic stimuli (227), since S-nitrosation of metallothionein, a zinc binding protein, causes redistribution of labile intracellular zinc (226).

However, recent studies suggest that cGMP signaling may also have barrier disruptive effects in the alveolar endothelium. For example, it has been demonstrated that high tidal volume (VT)-induced endothelial barrier dysfunction results from a simultaneous increase in NO- and sGC-derived cGMP levels, and the cyclic nucleotide degrading phosphodiesterase, PDE2A, thereby, also downregulating cAMP (206). In line with these findings, in vivo knockdown of PDE2A has now been shown to attenuate alveolar inflammation and barrier dysfunction in mice ventilated with high VT after LPS exposure (195). The complexity of the cGMP and cAMP signaling interplay is further enhanced by the fact that cAMP generated at the

Fig. 1. Schematic representation of inflammatory, noninflammatory, and mechanical stimuli and injuries in the pathogenesis of acute lung injury (ALI). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VILI, ventilator-induced lung injury; NETs, neutrophil extracellular traps; PAF, platelet-activating factor; ATI and ATII, alveolar type I and II cells, respectively.
ubiquitination and proteolysis. Protein homeostasis is regulated by multiple cellular processes, including protein synthesis, folding, trafficking, disaggregation, and degradation, collectively termed proteostasis (7). A critical component of the proteostasis machinery is the ubiquitinproteasome system (UPS). UPS has multiple fundamental functions including degradation of unwanted, misfolded, and damaged proteins and regulation of cellular processes such as proliferation, apoptosis, degradation of unwanted, misfolded, and damaged proteins and (UPS). UPS has multiple fundamental functions including proteostasis machinery is the ubiquitinproteasome system (UPS). UPS has multiple fundamental functions including degradation of unwanted, misfolded, and damaged proteins and regulation of cellular processes such as proliferation, apoptosis, inflammation, innate and adaptive immune response, and cell cycle (94). Ubiquitination and proteolysis play a critical role in several pathogenic mechanisms of ALI (237), including, but not limited to, the regulation of inflammatory responses (6), several pathogenic mechanisms of ALI (237), including, but not limited to, the regulation of inflammatory responses (6), and smoke inhalation injury-induced ALI (132).

Ubiquitination and proteolysis. Protein homeostasis is regulated by multiple cellular processes, including protein synthesis, folding, trafficking, disaggregation, and degradation, collectively termed proteostasis (7). A critical component of the proteostasis machinery is the ubiquitinproteasome system (UPS). UPS has multiple fundamental functions including degradation of unwanted, misfolded, and damaged proteins and regulation of cellular processes such as proliferation, apoptosis, inflammation, innate and adaptive immune response, and cell cycle (94). Ubiquitination and proteolysis play a critical role in several pathogenic mechanisms of ALI (237), including, but not limited to, the regulation of inflammatory responses (6), and smoke inhalation injury-induced ALI (132).

The divergent effects of CGMP on the endothelial barrier may contribute to the failure of inhalative NO therapy to improve outcome of ARDS patients and might be governed by subcellular gradients of cGMP and its molecular targets (2, 129). During ALI, NO reacts with superoxide radicals, yielding the reactive nitrogen species ONOO\(^-\), which leads to endothelial barrier hyperpermeability, protein nitration, poly(ADP-ribose) polymerase activation, and vascular endothelial growth factor (VEGF) activation (223), and the use of a metalloporphyrinic peroxynitrite decomposition catalyst has recently been shown to be effective in preventing cytotoxic effects in burn and smoke inhalation injury-induced ALI (132).

Mechanistically, alveolar overinflation results in a response pattern that is linked to pathways of inflammation and cell proliferation, induced by conformational changes of membrane proteins and disruption of cellular walls (biotrauma) or by forces due to cyclic stretch (mechanotransduction) (64). Still, the sensors of these initial stimuli and their downstream pathways are widely unknown. In an effort to identify associations between mechanical and inflammatory responses in the murine lung, Cannizzaro and colleagues (30) defined specific inflammatory pathways that are independently associated with overstretch of the lung parenchyma and loss of lung volume, providing insights into the pathophysiological events that drive local inflammation in VILI. Others defined eNOS-mediated ICAM-1 phosphorylation as crucial for neutrophil recruitment in VILI, a process that was dependent on the Cu\(^{2+}\)-activated cysteine protease calpain (141). As to the molecular mechanisms of ventilation-induced inflammation, the MAPK activator MKP-1 was recently defined as a potential target for modulating regional effects of injurious ventilation (182).

Under normal conditions, lung cells are adapted to cyclic deformations due to normal breathing. Load-bearing elements of cells, mostly the plasma membrane and the cytoskeleton, allow them to adapt to deformation. Plasma membrane unfolding and deformation-induced lipid trafficking along with cytoskeletal remodeling prevent cell wounding. However, in injured, mechanically ventilated lungs, the forces acting on the cells might be excessive or abrupt, increasing the risk of failure of these cell load-bearing components (175, 197). Excessive opening and closing of alveoli, termed cyclic stretch (CS), induces lung damage by directly wounding alveolar epithelial cells and also by affecting epithelial cell-cell interactions (46, 175). It has recently been shown that CS causes calpain-1-induced degradation of the adherens junction-associated protein p120-catenin, leading to interepithelial gap formation (251). Furthermore, CS-induced signal transduction impairs barrier repair by inhibiting cell spreading, cytoskeletal reorganization, and focal adhesion (FA) formation (46). CS-induced alterations of the alveolar epithelial cytoskeleton require Rac1, a member of the Rho family of small GTPases (53, 54). Interestingly, Rho stimulation also exacerbates lung injury in endothelial cells (19). A clinically highly relevant observation is that high fractions of inspired oxygen during mechanical ventilation accelerate VILI by modulating the mechanical properties of alveolar epithelial cells, rendering them stiffer and making them susceptible to cell detachment during CS (101, 198). Moreover, ventilation with high Vt affects the pulmonary expression of microRNAs, of which miR-21 may contribute to the pathogenesis of VILI (242).
Another mechanical stimulus that has gained interest in the last years is interfacial stress, caused by liquid in the alveolar space that may create foams during breathing. A recent work employed microarray technology to demonstrate that interfacial stress leads to rapid and significant changes in the expression of genes involved in stress response and defense pathways (96). However, not only mechanical stimuli contribute to VILI pathogenesis. It is well known that VILI is associated with an increase in circulating factors (244) and it has now been established that these circulating mediators significantly contribute to the VILI phenotype (109).

Pulmonary surfactant is critically required for optimal surface tension in the alveolus and it is well established that high VT ventilation reduces the amount and impairs composition as well as biophysical properties of surfactant (243). It has now been discovered that alveolar epithelial cell deformation induces calcium-mediated ATP release, thereby altering surfactant secretion in the alveoli (193).

Apart from these inflammatory signals, recent approaches defined novel pathways of alveolar epithelial repair after VILI. In response to injury, alveolar type II epithelial cells proliferate, spread, and migrate to cover the denuded basement membrane and partially differentiate into type I cells. The chemokine CXCL12 (stromal cell-derived factor 1α/SDF-1α) was increased in the bronchoalveolar lavage fluid in a rat VILI model and mediated Rac1-dependent migration of alveolar epithelial cells, a response that involved matrix metalloproteinase-2 (MMP-2). Concomitantly, epithelial cell repair and survival in a VILI mouse model was associated with expression levels of MMP-2 (75, 78), highlighting epithelial-regenerative pathways as putative targets for treatment of VILI.

Dysregulation of the Alveolar-capillary Barrier in ALI

Mitochondrial dysfunction, ER stress, cell death, and biomarkers. Cell organelles can be targets of stress stimuli leading to cellular dysfunction (Fig. 2). Interestingly, it has been found that elevated CO2 levels, which often occur in patients with ARDS in part as a consequence of protective ventilation strategies, lead to mitochondrial dysfunction, thereby preventing proliferation of alveolar epithelial cells and thus potentially impairing repair of the alveolar epithelial barrier after injury (246). Metabolites of NO during sepsis also alter mitochondrial function, resulting in ATP depletion and lactate-generating anaerobic respiration, subsequent impairment of Na-K-ATPase function, and therefore lung edema. Of note, the potent antioxidant N-acetylcysteine prevents experimental sepsis-induced pulmonary edema formation and renal dysfunction, possibly by maintaining mitochondrial function and Na-K-ATPase activity (29, 60). Furthermore, oxidant-triggered mitochondrial DNA (mtDNA) damage may initiate endothelial barrier dysfunction in governing lung vascular

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Fig. 2. Schematic representation of impaired structures and functions of the alveolar-capillary barrier in ALI. FAC, focal adhesion complexes.
barrier responses to oxidant stress and DNA glycosylase fusion proteins, which are the rate-limiting component of the mtDNA repair machinery, showed a barrier-protective phenotype upon hydrogen peroxide administration (38), highlighting the possibility of targeting the mtDNA repair pathway in oxidant-induced ALI as an alternative to the use of antioxidants, which has not been proven effective. It has also been recently reported that LPS-induced activation of transient receptor potential vanilloid 1 (TRPV1) calcium-selective ion channel may lead to endoplasmatic reticulum (ER) stress-mediated Gadd153 proapoptotic protein expression and cytotoxicity (228). Hyperoxia-induced, mitochondrial matrix-generated oxidant stress is also associated with epithelial cytotoxicity by activation of the intrinsic apoptotic pathway and significantly contributes to mortality in mice (23).

The identification of subpopulations within ARDS patients is critical for patient treatment, for outcome prediction as well as for clinical trial designs because heterogeneous populations reduce power of trial analyses. Thus discovery of novel biomarkers and characterization of gene, protein, and metabolite profiles by use of genomics, transcriptomics, proteomics, and metabolomics are emerging novel areas of ALI research (4, 17, 130, 209, 220).

Cell junctions. Tight junction proteins play a key role in maintaining the integrity of the alveolar-capillary barrier. The claudin family transmembrane proteins are primary structural constituents of the barrier, with claudin-3, -4, and -18 being most predominantly expressed in the alveolar epithelium (127). An emerging concept is that tight junction composition, as opposed to tight junction strand number, determines properties of the barrier (180). For example, whereas claudin-4 levels are associated with intact alveolar barrier function (199), elevated levels of the highly homologous protein claudin-3 increase paracellular permeability of the alveolar epithelium (161). Tight junction proteins are targets of oxidative stress and, thus, stimuli that lead to redox imbalance with ROS overproduction, since pathogens, inflammatory mediators, hypoxia, hyperoxia, noxious gases, alcohol, or cigarette smoke may alter alveolar-capillary barrier function, thereby contributing to ALI (26, 177, 179). Similarly to the epithelium, endothelial tight and adherens junctions are also targets of pathological stimuli during ALI (210, 225, 241) (Fig. 2). For example, thrombospondin-1, a circulating plasma glycoprotein, expression of which is increased in bronchoalveolar lavage fluids of patients with ARDS, disrupts the endothelial barrier by tyrosine kinase-dependent phosphorylation of zonula adherens proteins, vascular endothelial-cadherin, α-catenin, and p120 catenin (72). In line with these findings, preventing LPS-induced downregulation of vascular endothelial-cadherin with a Tie-2 agonist peptide has been shown to improve vascular leakage and survival in mice (50).

Gap formation. Endothelial hyperpermeability may also result from activation-mediated retraction of endothelial cells resulting in paracellular gap formation. The underlying signaling cascade is well determined and involves Ca2+ -induced calmodulin activation of myosin light chain kinases (MLCK) and phosphorylation of myosin light chains, leading to cytoskeletal reorganization and changes in EC morphology (58, 157, 174). In a recent work, high-mobility group protein B1, a late mediator of sepsis, has been shown to induce the formation of endothelial paracellular gaps in concert with actin cytoskeleton reorganization, perhaps providing a potential therapeutic target in ARDS secondary to sepsis since its late action provides with a longer time window for treatment (258).

Injury propagation. Alveolar endothelial barrier function is also regulated by members of the connexin family (Cx) such as Cx43, Cx40, and Cx37, which form functional gap junctional channels in the endothelium and allow cell-cell flux of small molecules and solutes (143, 247). For example, Cx43 has been shown to mediate interendothelial Ca2+ movement that upregulates the leukocyte adhesion receptor, P-selectin, thereby contributing to propagation of inflammation (184). Moreover, inhibition of Cx43 with the peptide inhibitor Gap27 prevents increases in endothelial permeability after focal micropuncture instillation of acid into alveoli at sites of both acid-instilled and acid-free regions, confirming the important role for this gap junctional channel in alveolar endothelial-endothelial cell interaction and injury expansion (183).

Glycocalyx. Another component of the alveolar-capillary barrier that has been recognized to be a critical regulator of barrier integrity is the endothelial glycocalyx, a dynamic luminal layer of glycoproteins, glycolipids, proteoglycans, and glycosaminoglycans (43, 116). Recently, it became evident that the glycocalyx actively regulates barrier function via mechanotransduction (59) and that depletion of sialic acid, which is typically found at the distal ends of carbohydrate chains and contributes significantly to the overall negative charge of cell surfaces, leads to endothelial barrier dysfunction (39), demonstrating the importance of glycocalyx composition in barrier integrity.

Cell-basal membrane interactions. Endothelial cells are attached to the underlying matrix by FA complexes composed of integrins, which sense physical properties of the extracellular matrix (ECM), regulate cytoskeletal organization, and also play an important role in cell signaling. Disruption of FA leads to endothelial barrier dysfunction (268). Interestingly, activity of FA kinase, a central regulator of FA function, is altered by oxidative stress induced by cigarette smoke (145), which is known to increase endothelial permeability in vivo. These effects were attributable to RhoA GTPase activity. Of note, lack of protein kinase C-δ, which is required for LPS-induced alveolar endothelial dysfunction, displays barrier-protective effects via regulation of RhoA (34), suggesting an important role for the GTPase in the pathogenesis of impaired barrier function during ALI.

Clearance of protein-rich edema. Pulmonary edema is a hallmark of ARDS (252). The loss of alveolar-capillary barrier integrity during ALI not only leads to increased filtration of protein-rich edema into the interstitial and alveolar spaces but also impairs the ability of the alveolar epithelium to resolve the excess liquid from the alveolar space due to impaired alveolar fluid clearance (253). Interestingly, in animal models of sepsis, the most prevalent cause of ARDS, impairment of fluid clearance is also evident (15, 40). The main driving force of alveolar fluid reabsorption is a vectorial Na+ transport across the alveolar epithelium creating a Na+ gradient between the basolateral and apical surfaces of the monolayer, which in turn facilitates movement of water out of the alveolar space (153). Research on the Na-K-ATPase and the epithelial sodium channel ENaC, two key components of the Na+ transport machinery, and their regulation (3, 57, 70, 79, 86, 125, 134, 137, 217, 273) remains highly relevant because proper function of the
vectorial Na\(^+\) transport and edema clearance are associated with better outcomes in patients with sepsis and ARDS (253, 270). In recent years, nontransport functions of the Na-K-ATPase have gained increasing attention. It became evident that the Na-K-ATPase also plays a pivotal role in the regulation of alveolar epithelial cell-cell interactions and, thus, barrier integrity (236). Apparently, presence of Na-K-ATPase β-subunit is required for cell polarization and formation of tight and adherens junctions, and the β-subunit itself also functions as a junctional molecule in epithelial monolayers (191, 192, 238, 239). Thus impairment of the Na-K-ATPase during ALI not only prevents resolution of pulmonary edema but also exacerbates its formation. Recently, a fourth subunit of ENaC, termed δ ENaC, was discovered and proved to have unique biophysical and pharmacological properties compared with α ENaC (76, 112). Importantly, in human primary nasal epithelial cells (humans express δ ENaC) up to 40% of amiloride-sensitive sodium transport is associated with δ ENaC (9), and alterations in nasal potential may be indicative of Na\(^+\) channel dysfunction in ALI (146). Of note, children with genetic deletion of genes encoding for δ ENaC are prone to respiratory infections and nasal congestion during winter (113). Most recently, a novel splice variant of δ ENaC, termed δ2 ENaC, has been characterized, which displays divergent features from the δ1 counterpart in biophysics and pharmacology regulation (274).

Lately, the interplay between ENaC and the cystic fibrosis transmembrane conductance regulator (CFTR) channel function has become focus of several research groups. In patients with cystic fibrosis, lack of function CFTR leads to dehydrated mucus, compromised mucociliary clearance, and, therefore, frequent bacterial infections (10, 186). Though controversial, several studies suggest that the loss of CFTR leads to excessive ENaC-mediated Na\(^+\) and water reabsorption (41). Patch clamp studies in alveolar epithelial type I and II cells demonstrated that ENaC activity negatively correlates with CFTR levels in wild-type, heterozygous and homozygous CFTR knockout, and AF508 mutant mice (133). Furthermore, wild-type CFTR decreases the expression of whole cell, functional, and apical surface ENaC (200). In line with those findings, in a porcine model of cystic fibrosis, in which CFTR is absent, alveolar fluid clearance was assessed, revealing that CFTR is required to increase liquid reabsorption after cAMP-mediated stimulation but it was not the rate-limiting factor (42, 138). Recently, a cross talk among ENaC, CFTR, the Na-K-ATPase and the Na-K-Cl cotransporter in the pathogenesis of cardiogenic pulmonary edema has been established (216).

Not only alveolar liquid but also excess protein needs to be cleared from the alveolar space for repair and recovery; however, the mechanisms of protein clearance are incompletely understood and the pathophysiological significance of active protein removal remains a highly controversial topic (87, 121). It has now been demonstrated that, similarly to that of the kidney proximal tubule, excess intra-alveolar albumin can be taken up by the alveolar epithelial cells by a clathrin-mediated endocytic process after albumin bound to the multiligand receptor megalin (21, 265, 266). The system appears to have the necessary capacity to remove large amounts of alveolar albumin (21), highlighting its potential relevance in alveolar epithelial fluid balance in ALI.

### Fibroproliferative Phase and Repair ALI

The acute phase of ALI is characterized by inflammation, cellular damage, apoptosis or necrosis of cells composing the alveolar-capillary barrier, and pulmonary edema formation. This phase, if the patient survives, is often followed by a fibroproliferative phase, which is associated with proliferation of pneumocytes, fibroblasts, and myofibroblasts and deposition of extracellular matrix and results in worse outcomes (151) (Fig. 3). Thus it is not surprising that intensive research focuses on factors that influence progression of early ALI to the fibroproliferative phase as opposed to repair and reestablishment of the normal pulmonary architecture and function (35, 194, 276). Epithelial-mesenchymal transition (EMT), resident lung fibroblast migration, proliferation, transdifferentiation into myofibroblasts, and fibrocyte recruitment take place in the fibroproliferative phase of ALI (25). Receptor for advanced glycation end products (RAGE), which has a role in alveolar epithelial cell spreading and adherence, when impaired, mediates an increase in cell migration and proliferation (28, 84, 90, 262). It has been now revealed that, whereas under basal conditions RAGE is linked to the cytoskeleton by interacting with the family of ezrin/radixin/moesin (ERM), in the presence of inflammatory cytokines this interaction is disrupted by ERM phosphorylation and subsequent ERM interaction with CD44 and actin stress fibers, causing loss of adhesion and an invasive phenotype contributing to EMT (22). Interestingly, a recent work in a model of bleomycin-induced ALI model has revealed that surfactant protein D expression also plays an important role in the regulation of EMT (202). Fibrocyte recruitment also contributes to the fibroproliferative phase of ALI. A novel work has demonstrated a central role for T regulatory lymphocytes in the resolution of ALI fibroproliferation by reducing CXCL12-CXCR4 axis-mediated fibrocyte recruitment (73). Recently it has been recognized that components of the ECM may be released by protease-mediated proteolysis; these fragments are termed matrikines. Apart from an evident impact on ECM structure, these fragments have biological activities impacting lung diseases (24). For example, the role of hyaluronan degradation fragments in the pathogenesis of ALI has been recently described (61, 114).

Several recent studies focused on the equilibrium between cell apoptosis and proliferation, repair of injured plasma membrane, and cell migration. During VILI plasma membrane wounding of alveolar epithelial cells occur, which contributes to VILI pathogenesis by initiating or exacerbating inflammatory responses. It is well described that most cells have the ability to repair their membranes; however, the underlying mechanisms remained incompletely understood (175). Recently, we learned that spatiotemporal actin remodeling and endocytic retrieval of plasma membrane are required for cells to repair the injured plasma membrane (77) and that the repair process requires Src-dependent calveolin-1 translocation to the plasma membrane to enhance caveolar endocytosis (115, 250). Furthermore, the molecular mechanism of cell migration after injury, which is necessary to repair and cover sites of wounding, has been described to be mediated by inhibition of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in conjunction with activation of the Akt and ERK pathways decreasing cell stiffness, which results in larger cell deformations required for a more motile phenotype (159).
Similarly, TNF-α also enhances cell migration but reduces cell proliferation after injury (147).

**Putative Candidates for Treatment of ALI**

Although numerous signaling events and mediators have been suggested as putative targets or therapeutics for ALI, the vast majority has failed to prove efficacy in clinical studies. A likely underlying reason is the heterogeneity of the disease, which might be associated with a direct insult by, e.g., infection, aspiration, or injurious ventilation, or an indirect one during situations of severe systemic inflammation, or, frequently, a combination thereof. Efforts to define endogenous pathways or molecules that protect from ALI have recently significantly contributed to our understanding of ALI pathophysiology (5, 36, 51, 81, 95, 99, 132, 150, 176, 189, 226, 245, 254, 256, 261). Imai et al. (106) were the first to suggest that the signals impacting on the balance between angiotensin converting enzymes (ACE) 1 and 2 modulate the degree of inflammatory lung injury after sepsis, and ACE I/D polymorphism was recently associated with mortality risk of ALI/ARDS in patients (152). Angiogenic factors such as sphingosine-1-phosphate (S1P), Robo4 signaling, or angiopoietin-1 have been attributed a central role in preventing loss of or restoring endothelial barrier function (1, 18, 66, 142, 164). S1P is a lipid binding to G protein-coupled receptors expressed on endothelial cells (S1P Rs), which induces actin cytoskeletal reorganization, activation of Rac, relocalization of VE-cadherin and catenin to junctional regions, and reassembly of adherens junctions in vitro (135, 172). S1P enhances endothelial barrier integrity in vivo and in vitro, and small-molecule agonists of endothelial S1P1 suppress hyperinflammation in influenza virus-induced lung injury (224). Interestingly, S1P, in addition to its extracellular effects, also functions as an intracellular second messenger (231).

Further data highlight a protective role of iNOS, which acts in an anti-inflammatory way or mediates infiltrate resolution (48, 118). Finally, the LTB4-BLT1 (leukotriene B4-leukotriene B4 receptor 1) axis was found to promote recruitment of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) regulatory T cells, which are key cellular players in ALI resolution, emphasizing the important role of lipid mediators in resolving ALI (208, 248).

Delivery of pharmaceutical agents to the site of injury is an additional challenge. A novel highly attractive approach is the development of bioresponsive drug delivery systems to optimize control of drug release compared with conventional, diffusion-based polymeric delivery. On the basis of the notion that elastase levels are increased at sites of lung inflammation, Sivadas and colleagues (214) developed inhalable, elastase-sensitive microparticles (MPs). Elastin present in the MP is targeted by elastase at the site of inflammation and the MP is degraded, therefore allowing drug release. The MPs used in the study showed high drug encapsulation properties, enzyme-specific degradability, and lack of toxicity, highlighting a potential role for this delivery system in ALI.
Treatment of ARDS is based on both ventilatory and non-ventilatory strategies. Still, the most significant therapeutic advances are associated with improved ventilator management, prone positioning, or fluid management (83, 165, 242a), but several approaches to treat ARDS based on the modulation of key cellular pathways or transcriptional programs are now in clinical testing. One of these approaches is based on the effects of HMG-CoA reductase inhibitors (statins). Usually applied for the prevention or treatment of cardiovascular disease, statins also exert significant anti-inflammatory, immunomodulatory, and antioxidant effects, primarily on endothelial cells. Statins promote endothelial cell cytoskeletal rearrangement, upregulate endothelial integrin β4, decrease oxidative stress by reducing NADPH oxidase and nitric oxide activity, and affect expression of differential endothelial cell genes relevant to the pathobiology of ALI (33, 213). In different murine models of ALI, statins were found to attenuate the lung inflammatory responses at different levels with concomitant reduction in vascular leak, and in animal models of VILI statins improved barrier function and increased lung compliance (33, 213, 271). With respect to clinical use, a retrospective cohort study of 178 ALI patients revealed that patients treated with statins for other reasons had lower levels of organ dysfunction and sepsis at admission to the intensive care unit, but statin use in this study did not affect the subsequent clinical course when evaluated by multivariate analysis (126). In another study, a cross-sectional analysis of a prospective cohort of 575 critically ill patients, prehospital statin use was associated with a reduced sepsis rate and reduced development of ALI (171). A study evaluating the effects of simvastatin in humans after endotoxin inhalation revealed significant reductions in bronchoalveolar lavage (BAL) fluid neutrophils, myeloperoxidase concentrations, and TNF-α concentration (212). Finally, a phase 2 clinical trial revealed that treatment with simvastatin is safe and may be associated with an improvement in organ dysfunction in ALI, likely mediated by a reduction in pulmonary and systemic inflammation (45).

Granulocyte/macrophage-colony stimulating factor (GM-CSF) is a myeloid growth factor that induces proliferation, differentiation and polarization of macrophages and dendritic cells in different organs including the lung. By its action on alveolar macrophages it significantly contributes to surfactant homeostasis (149, 166). Recently, several publications highlighted GM-CSF to be highly protective in different preclinical models of ALI, including K. pneumoniae infection, influenza virus pneumonia, LPS-induced lung injury, and others (190). A major source of GM-CSF in the lung is the alveolar epithelium (27, 160, 230). Importantly, GM-CSF protects the host both in the early phase of lung infection and during the phase of regeneration of the lung epithelium. Alveolar GM-CSF promotes bacterial or viral clearance by expanding alveolar macrophages or CD103+ migratory lung dendritic cells or by stimulating their host defense capacity (100, 218, 230). With respect to its direct effects on lung barrier, it was demonstrated that GM-CSF exerts antiapoptotic effects and thereby protects alveolar epithelial cells against oxidative stress-induced mitochondrial injury via the Akt pathway (221). In vivo studies reveal that GM-CSF promotes alveolar type II cell proliferation and is indispensable for restoring barrier function following LPS challenge (27). Together, these studies provide strong evidence that GM-CSF improves host defense, attenuates epithelial cell injury, promotes repair of the epithelium, and improves barrier function and gas exchange in ALI. Of note, mice overexpressing GM-CSF in alveolar epithelial cells to high local levels were strongly protected from infection and injury (2, 27, 73), suggesting that high levels of GM-CSF in the alveoli or the alveolar lining fluid needs to be achieved should we consider treatment of patients. With respect to translation into clinical application, GM-CSF levels in BAL fluid were found associated with improved outcome in ARDS (155). Regarding patients with sepsis and sepsis-induced ALI, one study revealed that systemically applied GM-CSF improves respiratory function (187), whereas a more recent trial with a larger patient cohort showed no significant benefit of intravenous GM-CSF delivery (181). Given that high alveolar concentrations of GM-CSF were associated with protection from ALI in animal models and humans, local delivery of GM-CSF needs to be taken into consideration as treatment strategy, particularly for patients with pathogen-associated ALI.

Mesenchymal stem cells (MSC) were found to restore barrier integrity in cultured primary human alveolar epithelial cells, a process that involves release of angiopoietin-1, which inhibits actin stress fiber formation and redistribution of the tight junction protein claudin 18 in epithelial cells (65). MSC secrete multiple effector molecules, including anti-inflammatory cytokines, growth factors such as keratinocyte growth factor, and antimicrobial peptides. These effectors were shown to attenuate features of lung injury, including increased lung endothelial and epithelial permeability, impaired edema fluid clearance, unbalanced inflammation, and infection (65, 85, 128, 154) and were also shown to cross talk with alveolar macrophages via soluble mediators to induce an anti-inflammatory M2 macrophage phenotype (107). In addition, MSC transferred mitochondria to endotoxin-injured alveolar epithelium by connexin-43-based gap junctional channels, a process that restored alveolar ATP production, enhanced surfactant production, and improved epithelial barrier properties (108). Intriguingly, MSC seem to be equipped with a plethora of beneficial factors that they release either directly or within microparticles after reaching the site of injury (156). The concept emerges that they “sense” the quality of injury to release just the right package of factors to clear infection and heal alveolar injury. Although the underlying molecular mechanisms of these processes still need to be defined, this makes them ideal candidates for therapy. Generally, cell-based therapy with allogeneic human MSC is now widely recognized as a promising approach to treat ALI/ARDS (154). An ongoing phase 1 clinical trial will soon reveal first data on the safety of intravenous application of allogeneic bone barrow-derived human MSC in ARDS patients (ClinicalTrials.gov identifier: NCT01775774).

In moving toward a better understanding of ALI/ARDS, it will be important to tease out more of the mechanistic details and the complex interplay between inflammation, signaling events, mechanical stimuli, and the alveolar-capillary barrier. A future task will be to evaluate and integrate the long list of putative therapy candidates and their complex interactions into a rational map. Another approach will be novel therapies based on cell populations with certain differentiation states or functional profiles, which will serve as tool boxes to deliver just the right cocktail of mediators at the right time to improve host defense, attenuate injury, or drive repair. We are now perhaps
closer than ever before to find effective pharmacological and/or cell-based therapies against this devastating syndrome. After over 45 years of intense research, it would be about time.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

S.H., N.M.G., and I.V. drafted manuscript; S.H. and I.V. edited and revised manuscript; S.H., N.M.G., and I.V. approved final version of manuscript; N.M.G. and I.V. prepared figures.

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