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Pathogenesis of indirect (secondary) acute lung injury

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Abstract

At present, therapeutic interventions to treat acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) remain largely limited to lung-protective strategies, as no real molecular–pathophysiologic-driven therapeutic intervention has yet become available. This is in part the result of the heterogeneous nature of the etiological processes that contribute to the state of ALI/ARDS. This article sets out to understand the development of ALI resulting from indirect pulmonary insults, such as extrapulmonary sepsis and trauma, shock, burn injury or mass transfusion, as opposed to direct pulmonary challenges, such as pneumonia, aspiration or lung contusion. Here, we consider not only the experimental and clinical data concerning the roles of various immune (neutrophil, macrophage, lymphocyte and dendritic) as well as nonimmune (epithelial and endothelial) cells in orchestrating the development of ALI resulting from indirect pulmonary stimuli, but also how these cell populations might be targeted therapeutically.

Keywords

apoptosis; ARDS; Fas; silencing; small interfering RNA

The incidence of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) in the USA has been reported to be 79 and 59 per 100,000 people per year, respectively [1–5]. Based on the exponential growth of the population, the incidence will likely double in the next 25 years [4]. The highest incidence of ALI is seen during sepsis, with approximately 25% of all ARDS cases stemming from severe sepsis [6] and 7% of intensive care patients eventually developing ALI/ARDS [6,7]. ALI/ARDS are associated with a lethality of approximately 40%, accounting for around 75,000 deaths per year in the USA [2,4]. Premorbid conditions [8] and underlying pathophysiology [9] markedly influence the

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mortality rate seen with sepsis, and represent the highest risks factors for mortality from ARDS (up to 50%) [6,10,11].

Hypoxemia, diffuse bilateral infiltrates of the lung often accompanied by pulmonary edema, reduction of lung compliance and a decrease in the functional residual capacity of the lungs are key features that characterize ALI and ARDS [1,2]. The degree of hypoxemia differentiates between ALI (associated with mild hypoxemia [$\text{PaO}_2/\text{FiO}_2 < 300$]) and ARDS (associated with severe hypoxemia [$\text{PaO}_2/\text{FiO}_2 < 200$]). However, there is still a lack of consensus in these definitions, in particular as they relate to severe ALI. In this regard, the use of a particular lung injury score defined from a combination of the ratio between $\text{PaO}_2/\text{FiO}_2$, alveolar consolidation based on chest radiography, the level of positive end-expiratory pressure (PEEP) and compliance has provided a more comprehensive approach to defining severe ALI [3].

At present, therapeutic interventions to treat ALI or ARDS remain limited. Lung-protective ventilation, including low tidal volume and low inspiratory pressure ventilation, has been associated with increased survival rates [12,13]. Recruitment maneuvers with high PEEP so far have had no impact on overall mortality but may improve hypoxemia-associated mortality [14]. Prone positioning, high-frequency oscillatory ventilation, inhaled nitric oxide and glucocorticoids are also used, but have so far failed to alter mortality rates (reviewed in [15]).

Thus far, no real pathophysiologic-driven therapeutic intervention has become available. This fact necessitates continued basic scientific research in this field to elucidate the pathomechanisms associated with ALI and ARDS. In 2002, the National Heart, Lung and Blood Institute (NHLBI) convened a working group to establish future research directions in ALI [16]. They concluded that more basic research to detail the cellular, molecular and physiologic mechanisms that protect the lung from biomechanical stress and injury and potentially modulate systemic inflammation was needed [16]. In addition, they found that the heterogeneity and the imprecisely defined phenotypes of patients with ALI/ARDS should be addressed in studies [16]. As ALI is a condition which can occur as a result of multiple insults and diseases, it is likely that the underlying pathophysiology leading to failure of the lung may be quite different, despite the fact that a common end state may be present. In this regard, differentiating ALI based on the underlying condition has been widely neglected. This heterogeneity may be one important reason why results from basic ALI research differ and why clinical trials often fail to show improved overall survival while, quite frequently, improvement in various physiological parameters or even subgroup-specific survival can be observed.

One general approach to further subgrouping ALI and ARDS, based on pathophysiologically oriented views, takes into account where the underlying condition is anatomically located. ALI and ARDS can then be of pulmonary (direct) or extrapulmonary (indirect) origin. Direct ALI (pneumonia, aspiration, contusion, embolus, inhalation injury and reperfusion) accounts for approximately 55% of all ALI cases, meanwhile indirect ALI (extrapulmonary sepsis and trauma, burn injury, mass transfusion, bypass surgery, intoxication and acute pancreatitis) accounts for 20% [6]; a further 21% appear to be due to mixed factors contributing to ALI and the remaining 4% have no distinctive underlying pathophysiology [6]. Sepsis is the most commonly encountered condition underlying the development of ALI, with severe sepsis accounting for 46% of direct and 33% of indirect ALI cases [4]. There are several studies indicating that direct and indirect ALI are truly different. Menezes *et al.*, in comparing the development of ALI in response to intraperitoneal or intratracheally instilled lipopolysaccharide (LPS) with identical pulmonary mechanical dysfunction being evident, found that the insult to the pulmonary epithelium

intratracheally lead to a more pronounced lung inflammation and ultrastructural morphologic changes [17]. Gattinoni *et al.*, in comparing nine patients with indirect and 12 patients with direct ARDS, found that lung elastance was higher in pulmonary ALI, whereas, in those individuals with an indirect hit, chest wall elastance and intra-abdominal pressure were increased [18]. However, the long-term (6 months) functional recovery of these patients appeared to be identical [19]. Rocco *et al.* described the differences between direct and indirect ALI as being neither simple or clear, and with overlapping pathogenetic mechanisms and morphologic interactions [20]. Pelosi *et al.* found that, besides pathophysiological differences, extrapulmonary ALI was more susceptible to therapeutic interventions such as PEEP, inspiratory recruitment and prone positioning [21].

In this article, our aim is to discuss the main pathophysiological aspects that are thought to contribute to ALI. We will focus primarily on indirect ALI as this has been a main focus of our group in the last decade. We also intend to consider the current cellular pathological concepts of neutrophil-, epithelial- and/or endothelial-mediated injury, appreciating that these pathological mechanisms cannot be simply divided into direct or indirect ALI, but may rather have to be put into perspective by a more detailed subgrouping of ALI based on underlying conditions.

Pathophysiology of ALI

The pathology leading to ALI/ARDS is not well understood. It is likely to be as heterogeneous as the underlying conditions that induce them. However, it appears that specific pathophysiological events may contribute to different forms of ALI (direct or indirect) and are, therefore, of special importance to consider when developing therapeutic approaches. Irrespective of the initial insult, the final result is that the alveolo-capillary barrier becomes compromised, leading to edema formation in the interstitium as well as alveoli. Gas exchange is compromised and organ dysfunction, including respiratory failure, is the consequence. Histological evaluation of lungs from ALI patients indicated substantial accumulation of activated polymorphonuclear cells (PMNs), diffuse alveolar damage including loss of epithelial integrity and denuded basal membranes, as well as increased pulmonary edema and fibrin-rich membranes [22–24]. From a vascular perspective, microthrombi are present in pulmonary capillaries and injury to the endothelium is evident [25,26].

In cases of direct ALI, the hypothesis would be that the underlying stimuli is restricted to the lung and often associated with direct mechanical, chemical or infectious stimuli, or other direct interactions capable of inducing damage to lung structures. Alternatively, the stimuli for indirect ALI are considered to be derived from outside the lung, from other compartments of the body. Agents proposed to mediate such an extrapulmonary insult to the lung, translating into tissue damage, are, therefore, of particular importance when we consider the pathophysiology of indirect ALI (Figure 1). In this regard, the data that concerns the role of soluble mediators such as cytokines or chemokines, as well as cellular contributors such as neutrophils, will be reviewed as potential candidates.

Neutrophils as potential cellular mediators of indirect ALI

Neutrophils are proposed to have an important role in mediating ALI (Figure 1B–C). When recruited to a site of infection/inflammation, they exert a variety of beneficial functions (phagocytosis, production of reactive oxygen species and nitric oxide species, and degranulation of lytic enzymes) that, when well regulated, enable clearance of the invading pathogen. However, it is also hypothesized that the recruitment of activated PMNs may be potentially harmful when these same functions are dysregulated and directed at otherwise normal host tissue, culminating in injury and organ damage. The activation of neutrophils is

closely related to pro-inflammatory mediators such as TNF, IL-1 β and IL-6, and chemokines such as IL-8 and monocyte chemoattractant protein (MCP)-1. In this regard, Yang *et al.* showed that in peripheral blood PMNs, high levels of activated transcription factors, nuclear factor- κ B or Akt, which regulate proinflammatory genes, were associated with poorer clinical outcome in patients with ALI [27]. This supports a role for activated PMNs in mediating ALI pathology.

PMN recruitment & organ damage

Early PMN accumulation has been observed in the lung tissue [25,26] as well as in bronchoalveolar lavage fluid (BALF) of ARDS patients [22]. Warshawski *et al.* isolated PMNs from ARDS patients, labeled and reinfused them back to the patients and found a substantial accumulation of labeled PMNs in the lung [28]. Rinaldo *et al.* and Azoulay *et al.* found that when ALI developed during neutropenia, it rapidly progressed once PMN counts were restored [29,30]. Furthermore, the degree of neutrophilia in BALF has been correlated with poor prognosis in septic ARDS [31]. However, there is some debate as to how neutrophils are retained in the lung. The emigration of activated PMNs and passage through the endothelium is regulated via adhesion molecules (Figure 1) [32]. Among them, L-selectin (CD62L) on PMNs appears to be involved in the initial rolling process of PMNs on the endothelial surface [33], while CD11b/CD18 on PMNs then mediate a tighter contact between them [34]. CD31 or PECAM-1 is needed in the final step for the vascular diapedesis of leukocytes [35]. In this regard, trauma patients (injury severity score >16) showed an immediate increase in PMN L-selectin expression followed by a gradual decrease. PMN CD11b expression exhibited an immediate increase for the first 3 h after trauma [36]. In contrast, a significant drop in L-selectin expression on PMNs in response to trauma has been described [37] along with upregulated CD11b/CD18 and downregulated L-selectin expression on PMNs after abdominal surgery [38]. Doerschuk *et al.* described this adhesion molecule-independent recruitment mechanism in the lung [39]. In this study, PMN emigration in response to *Escherichia coli*, *E. coli* LPS, *Pseudomonas aeruginosa*, IgG immune complexes and IL-1 has been reported to be mediated through CD11b/CD18, but this does not appear to be the case in response to *Streptococcus pneumoniae*, Group B *Streptococcus*, *Staphylococcus aureus*, hyperoxia or C5a [39]. Even in CD11b/CD18-dependent migration, blocking of CD18 only reduced PMN emigration by 60–80% suggesting that other redundant mechanisms mediated these effects [39]. In this regard, neutrophils do not seem to require adhesion molecules during their initial contact to the endothelium but they appear to be needed to allow neutrophils to stay adhered for a longer period of time [32]. This appears true for both direct and indirect lung injury. Activated PMNs from ARDS patients appeared even more rigid than those from septic patients [40]. PMNs need to deform to pass through the lung capillaries. Inflammatory mediators such as C5a, IL-8 or endotoxin can induce neutrophils to become stiffer, increase in size and prolong transit through lung capillaries facilitating endothelial–neutrophil interactions [32].

As mentioned previously, neutrophils are able to release a variety of harmful substances, such as proteolytic enzymes, reactive oxygen/nitrogen species, cytokines and chemokines, which may be injurious to the adjacent endothelial cell and, after diapedesis, to the alveoli itself. As such, it has to be taken into account that all these products exert primarily beneficial effects when directed against pathogens, but might become harmful under other conditions. For example, the proteolytic activity of PMN elastase, which facilitates transendothelial migration, may also induce epithelial cell death [41]. Donnelly *et al.* have linked elevated elastase plasma levels with the progression of trauma patients to ARDS and a need for mechanical ventilation [42]. Gando *et al.* also showed markedly elevated plasma elastase levels in ARDS patients [43]. Kodama *et al.* found that plasma levels of 220 ng/ml and higher in systemic inflammatory response syndrome patients were associated with

increased risk of developing ALI/ARDS [44]. Inhibition of neutrophil elastase improved respiratory function during experimental septic ALI [45] and has been shown to be an independent predictor of survival in septic patients with ARDS [46]. However, previous studies did not show such an effect in a heterogeneous ALI patient population [47]. In addition, deficiency of elastase and/or cathepsin G was accompanied by increased susceptibility to fungal infections but decreased susceptibility to experimental endotoxin-mediated inflammatory ALI [48]. The inhibition of matrix metalloproteinases and elastase prevented lung dysfunction and reduced sepsis-induced ALI in pigs [49]. Ricou *et al.* found the ratio between gelatinase and its natural antagonist, tissue inhibitor of metalloproteinases-1, to be elevated in late phases of prolonged ARDS [50]. Collagenase can also be found in the BALF of ARDS patients [51].

As mentioned earlier, PMNs play a particular role in indirect ALI as, once activated, they can exert harmful effects on the *a priori* unaffected lung tissue. In an experimental setting of indirect ALI stemming from hemorrhagic shock (HEM) followed by polymicrobial sepsis, we found that in response to HEM, circulating PMNs exhibited an *ex vivo* increase in respiratory burst capacity and a decrease in apoptosis [52]. This is consistent with the concept that shock/injury can produce *in vivo* 'priming', the significance of which could be seen when HEM was then followed by sepsis, as recruitment of these PMNs into the lung occurred, along with the development of ALI (Figure 1B–C). Also, when these HEM-primed PMNs were injected intravenously into PMN-depleted animals, which subsequently underwent cecal ligation and puncture to induce sepsis, ALI again resulted [52]. Based on this, using different models of ALI, we [53] and others [54–60] have found that depletion of PMNs may actually serve to decrease injury associated with ALI. Thus, depletion of PMNs prior to HEM and sepsis markedly reduced the extent of lung inflammation and ameliorated lung protein influx and the severity of ALI (Figure 1D–F) [53]. This is in line with findings during transfusion-induced ALI, a form of indirect ALI associated with plasma-containing blood products [57]. Blockade of the PMN chemokine receptor, CXCR2, using antileukinate or adoptive transfer of PMNs isolated from anti-KC or anti-macrophage inflammatory protein (MIP)-2-treated mice following HEM reduced lung PMN influx in response to subsequent sepsis and additionally decreased lung inflammation and lung protein leak [61,62].

PMN apoptosis & clearance

The lifespan of mature neutrophils is between 6–12 h in the peripheral blood. However, this changes once PMNs are activated. Several inflammatory agents, such as LPS, TNF, IL-8, IL-6, IL-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [63–66], have been shown to inhibit PMN apoptosis. The anti-apoptotic effect of ARDS plasma on PMNs appears to be mediated through the GM-CSF receptor [67]. While the mean percentage of neutrophil apoptosis appears to be lower in sepsis-induced ARDS when compared with uncomplicated sepsis [68], in infants receiving extracorporeal membrane oxygenation for severe respiratory failure, survivors had proapoptotic BALF to neutrophils compared with nonsurvivors [69]. These differences, however, may well be a reflection of differences in the pro/anti-apoptotic milieu of the blood versus the alveolus. In the alveoli, PMN clearance usually occurs via phagocytosis by alveolar macrophages. The clearance of apoptotic neutrophils is not associated with the release of harmful proteolytic enzymes and therefore typically occurs without tissue damage. However, PMNs from ARDS patients show decreased apoptosis [70]. Delayed apoptosis provides activated PMNs with a longer lifespan, which in turn allows them to accumulate at local tissue sites of inflammation/infection. Activated PMNs have been shown to exert damaging effects in the lungs [71,72]. However, following granulocyte colony-stimulating factor (G-CSF) treatment in pneumonia

patients, no differences in outcome or time of recovery were noted, while it appeared that complications such as ARDS were decreased [73].

In our model of hemorrhage-induced septic ALI, it is evident that prolonging the lifespan of activated PMNs by using transgenic mice that overexpress the anti-apoptotic protein Bcl-2 in a myeloid-restricted fashion [74], does not exacerbate ALI, even with evidence of a prolonged presence of activated PMNs in the lung [75]. What we did observe, however, was an initial survival benefit when the lifespan of PMNs was further prolonged in these transgenic mice, likely due to an enhanced capacity to clear bacteria in the lung [75]. This is in line with our previous studies using Bcl-2-transgenic mice indicating that during polymicrobial sepsis, the presence of PMNs with a prolonged lifespan is actually beneficial for the outcome [75]. Conversely, in an inflammatory/noninfectious environment, such as systemic inflammatory response syndrome, mimicked by intraperitoneal injection of *E. coli* LPS, prolonging the lifespan of activated PMNs was detrimental to the animals' survival and was associated with an exacerbation of lung injury [75]. Additionally, during noninfectious/inflammatory ALI, failure to clear PMNs from the lungs contributed to increased inflammation and mortality [76]. Together, these data suggest that the tissue environment (infectious vs inflammatory) the neutrophil encounters plays an important role in determining whether PMNs mediate organ damage or not.

These data show that activated neutrophils are likely mediators of indirect ALI. However, it is interesting to note that ARDS has been described as developing in patients with neutropenia [77–79]. In addition, following G-CSF therapy in pneumonia and sepsis patients, the incidence of ARDS was not increased [73,80], and during experimental ALI or pneumonia, PMNs have been shown to migrate to the lungs without exerting deleterious effects [81,82]. This indicates that other mechanisms are likely involved in exertion of lung damage during ALI.

Epithelial cell injury

Type I epithelial cells make up approximately 90% of the pulmonary surface and, while type II epithelial cells account for only 10%, they are critical since they produce surfactant and are important for ion transport [23]. Impairment of the epithelial integrity harms the lung in many ways. First, the epithelial barrier is inherently less permeable when compared with the endothelial barrier, thus, destruction of its integrity prompts a progressive influx of protein-rich fluid into the alveoli [23,81]. Second, the loss of epithelial integrity represents an impairment of the physiologic transepithelial fluid transport and further inhibits the reabsorption of the alveolar edema [83,84].

During ALI and ARDS, loss of epithelial cells can be noted [25,85] and the extent of this epithelial injury (increased serum protein and proteolytic enzyme influx into the alveolar space) correlates with the clinical outcome of these patients [86,87]. The question now arises, what processes contribute to the demise of lung epithelial cells during ALI? Here, apoptotic cell death is thought to represent an important mechanism contributing to epithelial cell injury during ALI. Bachofen *et al.* reported that patients who died from ARDS exhibited excessive apoptotic alterations in the chromatin of their alveolar type II cells [25]. Subsequent studies confirmed these DNA fragmentations [88] as well as an increased expression of the pro-apoptotic protein Bax (Bcl-2-associated X protein) [89]. Apoptotic cell death can be triggered via several routes. For lung epithelial cells, in association with ALI, activation of the death receptor Fas, with subsequent activation of caspases, appears to be a potential mechanism mediating epithelial cell death. Human lung epithelial cells express Fas and have been shown to be sensitive to Fas-mediated apoptosis [90]. During ALI and ARDS, an increased concentration of Fas and FasL in patients' BALF and lung tissue has

been detected [91,92], and BALF from ARDS patients has also been shown to induce apoptosis in healthy lung epithelial cells *in vitro* [93]. Furthermore, ARDS nonsurvivors exhibited markedly higher FasL concentrations in BALF when compared with survivors [93]. In particular, during septic ALI, an infection severity-dependent activation of the Fas–FasL system in the lung can be observed [94]. FasL concentrations have been reported to be much higher locally than systemically, suggesting its origin is pulmonary tissue [91,92]. In this respect, infiltrating monocytes as well as PMNs appear to be a potential source of FasL [95,96]. FasL may also be cleaved from cell membranes by the activation of matrix metalloproteinases 3 and 7 [97,98]. Considering this fact, one has to ask whether apoptosis of epithelial cells also plays a role in indirect ALI (Figure 1F). We have investigated this in a model of hemorrhage-induced septic ALI and found that lung epithelial cell apoptosis along with neutrophil recruitment to the lung is an early marker of ALI [99]. Fas and FasL mutant animals exhibited less pulmonary epithelial cell apoptosis in response to insult when compared with wild-type animals. The extent of ALI, as assessed histologically and by protein influx, was significantly diminished and this was associated with a survival benefit for Fas-mutant mice when compared with wild-type animals [100]. In addition, a single intratracheal instillation of caspase-3-silencing RNA to mice subjected to HEM and polymicrobial sepsis not only attenuated lung apoptosis and inflammation, but also ameliorated the development of ALI in treated animals. Most interestingly, this experimental therapeutic approach markedly improved 10-day survival of hemorrhaged septic mice [99]. Similar effects were seen after downregulation of the Fas receptor on lung epithelial cells during indirect ALI following HEM and sepsis [101]. However, so far it remains unclear what comes first, apoptosis of lung epithelial cells or the recruitment of neutrophils, and whether neutrophils might even be initiators of lung epithelial cell death or, alternatively, whether the dying epithelial cells attract the activated neutrophils into the lung. Here, results from our laboratory indicate that activation of Fas serves not only to induce apoptosis, but also the secretion of cytokines and chemokines by lung epithelial cells. In murine lungs, activation of Fas initiated a profound inflammatory response, with an early generation of chemokines and subsequent recruitment of neutrophils [100,102,103]; this could be reduced by antagonizing FasL [102]. Triggering of Fas also led to increased expression of TNF- α , MIP-1 α , MIP-2, MCP-1 and IL-6, and compromised the alveolo–capillary barrier [104]. In our experimental setting of hemorrhage-induced septic indirect ALI, we found that the early pulmonary inflammation, which is commonly seen after these insults, was markedly abrogated in Fas- and FasL-deficient animals. In addition, neutrophil recruitment was diminished and the degree of ALI was markedly reduced [100]. Similar results were obtained after silencing the Fas receptor on lung epithelial cells during this insult [101]. Furthermore, *in vitro* experiments revealed that lung epithelial cells were capable of secreting MIP-2, KC and MCP-1 *in vitro* in response to Fas activation through mechanisms involving ERK and potentially FLIP [100]. Instillation of a Fas-activating antibody into transgenic murine lungs, in which lung macrophage numbers are markedly reduced, displayed a similar inflammatory response as seen in wild-type animals, further supporting a role for Fas-mediated, epithelial cell-induced pulmonary inflammation *in vivo* (Figure 1B–D) [100].

Endothelial cell injury

The alveolo–capillary barrier is, from its vascular side, lined with a continuous monolayer of endothelial cells. This monolayer is fused with its basement membrane to epithelial cells, representing the alveolar lining of this construct [105]. Macromolecules are actively and transcellularly transported through endothelial cells [106], while water may also be routed through endothelial water channels [107]. It was recognized early on by Bachofen *et al.* that the vascular endothelium of the lung undergoes substantial ultrastructural alterations in response to ALI/ARDS [25,26]. As mentioned previously in this article, endothelial cells

respond to inflammation with the expression of adhesion molecules, which, during ALI, enable rolling and tethering of activated neutrophils. In contrast to most other organs, where PMN sequestration occurs at postcapillary venules, pulmonary PMN retention takes place within the pulmonary capillaries, representing a complex interconnecting network of short capillary segments where the course from arteriole to venule crosses numerous alveolar walls and includes often more than 50 capillary segments. The blood in this complex network contains 50 times more PMN compared with most other vascular beds (reviewed in [105]). The interaction of the endothelial cell with blood-borne cells and particularly neutrophils makes it an important pathophysiological contributor in the development of ALI. In addition, the vascular endothelium responds to inflammation with the expression of tissue factor and von Willebrandt factor, activating the clotting system [108,109]. Thus, lung endothelial cells actively regulate hemostasis (Figure 1D–F) (reviewed in [110]). In addition, through the release of vasoactive compounds, pulmonary vascular resistance is increased and pulmonary hypertension results [111]. As stated previously, sepsis is an independent risk factor and when the septic focus is extrapulmonary it constitutes 33% of indirect ALI cases [4]. In this regard, it is worth looking at the reaction of endothelial cells to endotoxin as it is a membrane compound of Gram-negative bacteria and, via TLR-4, triggers a variety of immune responses (reviewed in [110]). Endothelial cells can be directly stimulated by endotoxin via TLR-4. A systemic injection of LPS leads to PMN accumulation in the lung [110], whereas TLR-4 blockade effectively attenuates neutrophil accumulation and activation in the lungs, increases in lung permeability and production of inflammatory mediators [112]. Evidence suggests that these effects may not be entirely mediated by the recruitment of activated PMNs to the lungs, but might also be attributed to direct effects of endotoxin on lung epithelial cells. In this regard, endotoxin directly mediates the release of vasoactive mediators and molecules altering lung permeability, such as TNF- α , thromboxane A2 and endothelin-1 [110].

Ischemia reperfusion (I/R)-induced ALI is a form of indirect ALI where the importance of endothelial cell dysfunction is apparent (reviewed in [110]). Pathophysiologically, I/R-induced ALI is associated with the generation of reactive oxygen species and nitric oxide species, as well as the interaction of PMNs with an already activated endothelium. Fullerton *et al.* examined the effects of mesenteric I/R in rats on vasorelaxation in isolated pulmonary artery rings. This indicated that after I/R the vasorelaxation response to receptor-dependent, endothelial-dependent relaxation (response to acetylcholine) and receptor-independent, endothelial-dependent relaxation (response to the calcium ionophore, A23187) was markedly impaired while the endothelial-independent relaxation (response to sodium nitroprusside) remained unchanged [113]. Rehm and colleagues investigated syndecan-1 and heparan sulfate as two components of the endothelial glycocalyx in arterial blood of 18 patients undergoing surgery of the ascending aorta with cardiopulmonary bypass and of 14 patients undergoing surgery for infrarenal aortic aneurysm [114]. They found a substantial increase in both products after both procedures, with higher concentrations after cardiopulmonary bypass [114]. Atochina *et al.* indicate that normoxic lung I/R induces injury to the pulmonary vascular endothelium. I/R led to a time-dependent increase in angiotensin-converting enzyme (ACE) activity in the perfusate of rat lungs [115]. To specifically assess ACE localized on the luminal surface of the pulmonary endothelium, they perfused rat lungs with a radiolabeled monoclonal antibody (mAb) to ACE (anti-ACE mAb 9B9) and found that pulmonary uptake of mAb 9B9 with I/R was markedly reduced [115]. In addition, Nowak *et al.* performed vascular immunotargeting using a conjugate of ACE mAb 9B9 with catalase (9B9-CAT) in an ischemia model of the right lung in rats and found reduced lung injury after I/R by targeting vascular catalase [116].

These examples indicate that in indirect ALI, where the site of injury is of extrapulmonary origin, endothelial cell pathology appears to be suggestive/predictive for the development of

ALI. Although endothelial cell pathology is closely linked to PMN alterations, the aforementioned studies make a strong case for PMN-independent lung endothelial-based mechanisms during indirect ALI.

Lymphocytes & dendritic cells: active players in ALI resolution phase

Lymphocytes and dendritic cells (DCs) reside in the lung in relatively small numbers. Lymphocytes account for approximately 10% of the cells in BALF obtained from healthy individuals and their distribution is similar to that in peripheral blood. DCs, both myeloid and plasmacytoid DCs, also exist in the lung and reside in airway epithelium, alveolar septae and around pulmonary vessels [117–119].

In this location, these cells are ideally positioned to play a central role in the immune response during infection/inflammation [117,118]. Indeed, during ongoing inflammation, it has been shown that lymphocytes and DCs migrate to the lung where they not only maintain and enhance local immune response, but also regulate this response [117,120,121]. Indeed, a role for DCs and lymphocytes has been shown in a number of lung inflammatory diseases in humans (e.g., asthma, COPD, lung cancer and transplant rejection) [117] and in a number of experimental models of lung infections (e.g., TB, *Pneumocystis carinii*, *Leishmania major* and *Candida albicans*) [117–119].

However, little data is available regarding the role of lymphocytes and DCs in the pathophysiology of ALI. In a murine model of endotoxin-induced lung injury, Morris *et al.* showed that lymphocytes, in addition to neutrophils, infiltrate the lung [122]. Similarly, Nakajima *et al.* showed that following endotoxin administration to the lung, BALF lymphocytes, neutrophils, IL-6, TNF- α and albumin were increased [123]. Analysis of LPS-induced T cells revealed increased Foxp3 and CTLA4 expressions in CD4⁺ T cells (characteristics of a specific subpopulation of T cells with regulatory activities called naturally occurring regulatory T cells [Treg]). In a murine model of indirect ALI, we recently observed a specific and nonredundant role for each lymphocyte subpopulation in indirect ALI pathophysiology [124]. In particular, we showed that CD4⁺ T cells are specifically recruited to the lung and diminish neutrophil recruitment through increased IL-10 production (see Figure 1B) [124]. Most importantly, this appears to be mediated specific by a subpopulation of CD4⁺CD25⁺Foxp3⁺ Tregs [124]. Significantly, our data has recently been confirmed by D'Alessio *et al.* [125] and Aggarwal *et al.* [126] who both have observed a role for Treg in the recovery phase of LPS-induced ALI. Moreover, D'Alessio has shown that the BALF of patients with ALI revealed dynamic changes in CD3⁺CD4⁺CD25^{hi}CD127^{lo}Foxp3⁺ cells [125].

Regarding DCs, we recently showed that plasmacytoid DCs are induced and activated in the lung in a murine model of indirect ALI [127], and play an inhibitory role in the recruitment of pro-inflammatory monocytes through the regulation of lung MCP-1 production (Figure 1B).

Since it is recognized that resolution from lung injury is not simply relief from injurious agents or factors, but rather reflects an actively regulated program involving removal of apoptotic neutrophils, remodeling of matrix, clearance of protein-rich alveolar fluid and engagement of numerous signaling pathways distinct from those involved in acute injury [128], our recent data suggesting that both CD4⁺ lymphocytes as well as plasmacytoid DCs are active players in this process should not be totally surprising, especially as it has been shown that transplantation of DCs can have a positive effect on treating/reducing lung dysfunction seen in murine models of sepsis [129,130]. As such, our results might provide insight into new therapeutic avenues of consideration for the treatment of ALI.

Expert commentary

Heterogeneity and the different phenotypes of ALI/ARDS along with numerous different underlying conditions have been identified as major obstacles in advancing ALI/ARDS research. There is some evidence that dividing ALI into an indirect form (with the injury being located outside the lung) and a direct form (with the injury being located inside the lung) may be a step in the right direction, relative to advancing the classification scheme and potential therapeutic interventions and treatments for ALI. It is likely that the diversity of underlying pathologic mechanisms will prohibit the formulation of a unified pathophysiology of this clinical entity. On the other hand, the mechanisms outlined previously seem to hold true, at least in a variety of indirect and sometimes even direct forms of ALI, seeding the hope that there might be therapeutic options for ALI in the future that might be applied to certain, yet still undefined, subgroups of ALI. In this regard, the activated neutrophil is definitively a paradigm that has been shown to hold true in diverse forms of ALI (Figure 1). Additionally, apoptotic cell death of lung epithelial cells seems to be a mechanism that is evident in a variety of ALI processes. The role of the endothelial cell is probably less clear; however, during indirect ALI it has already been shown to be involved in mediating lung tissue injury. While novel therapeutic approaches can only be developed in clinically relevant animal models, which are usually fairly well defined, their clinical use can only be appraised in large clinical trials. These frequently suffer from the enormous heterogeneity of their patients and may therefore yield confounding results. In this regard, the judicious management of inclusion and exclusion criteria, which takes into account aspects of direct/indirect mechanisms during clinical ALI trials, will be as important as meticulously performed basic science to establish novel therapeutic targets for ALI/ARDS.

Five-year view

Provided that continuous financial support remains available for research in the field of ALI, it is expected that within the next few years we will significantly expand our understanding of the basic mechanism underpinning the development of the different forms of ALI (direct vs indirect). In doing so, we hope those in the field will uncover potential therapeutic targets that are relevant to a specific form of ALI. Clinically relevant double-hit animal models, existing ones as well as those still to be developed, should be a valuable tool to allow a better estimation of the potential benefit of various experimental therapeutic approaches. However, it will still be essential that those therapies uncovered by such experimental work find their way into high-quality, collaborative, multicenter trials, which also respect the complex etiology of the patient populations as well as the heterogeneity of ALI as a condition. Unfortunately, because of the heterogeneity of ALI and the failure of a number of prior therapeutic clinical trials (which makes biotech and pharmaceutical companies cautious), it is unlikely that within the next 5 years we will actually see a significant new molecular/pharmacological cure for ALI come to pass. Nevertheless, the field should still continue to move closer to a true pathophysiological-derived therapeutic approach, as our modeling of this condition continues to improve and hence our understanding becomes more refined.

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Key Issues

- Therapeutic interventions to treat acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) remain limited largely to lung-protective strategies, as no real molecular/pathophysiologic-driven therapeutic intervention has yet become available.
- From a diagnostic/pathological perspective, it would appear that dividing ALI into an indirect form (with the injury being located outside the lung) and a direct form (with the injury being located inside the lung) may be a step of value relative to advancing the classification scheme and potential therapeutic interventions and treatments for ALI.
- Since ALI is a condition that can occur after multiple insults and diseases, it is likely that the underlying pathophysiology leading to failure of the lung may also be quite different, although a common end state may be present.
- Indirect/extrapulmonary/secondary ALI appears to involve priming and/or activation of nonimmune cells such as epithelial/endothelial cells as well as/or immunocytes, such as neutrophils and macrophages.
- Signaling through G-protein receptors, such as CXCL2, appear to be critical to neutrophil priming seen in experimental models of indirect ALI. Their clinical significance is yet to be determined.
- Signaling via death receptors expressed on pulmonary epithelial cells seems to play a role both in lung inflammation and apoptotic responses seen in response to experimental indirect ALI. Their clinical significance is yet to be determined.
- Signaling via reactive oxygen species/nitric oxide species, angiotensin-converting enzyme, Toll-like receptors and TNF receptors plays a role in activating/priming the pulmonary endothelial cell interface for enhanced leukocyte and/or lung parenchymal cell interactions in response to experimental indirect ALI. Their clinical significance is yet to be determined.
- Local pulmonary lymphocytes and/or dendritic cells appear to play roles not only in regulating local lung inflammatory response but also in the apoptotic response to stimuli associated with indirect ALI.

Figure 1. Proposed mechanisms of acute lung injury through hemorrhage ‘priming’ for inflammation (inflamed Epi. [red])/apoptosis (Ao Epi. [grey])/injury and ‘triggered’ by a subsequent infectious insult (see facing page)

The resting lung (A) is primed by divergent inflammatory mediators released during an initial event (e.g., shock and inflammation) that acts on a number of cells in the blood and lung (B). These cells in turn stimulate either separately or concomitantly the proinflammatory response and/or the Ao of a small number of Epi., both through Fas/FasL activation. The release of chemokines primes the AMO, when, at a later time, a subsequent inflammatory/infectious (trigger) event takes place (note this can be antagonized [dashed lines] by the anti-inflammatory actions of pulmonary Treg cells and/or (C) The local EC, pDC). AMO and/or become activated, release chemokines and activating agents that recruit the primed and now activated leukocytes to the lung (D). These activated leukocytes then transmigrate across the endothelium (which actively/passively retracts in response to such cell–leukocyte interactions) (E) into the interstitium and alveoli where they perform their effector roles (in the absence of infection the effector response may be solely injurious). In addition they may propel the inflammatory/apoptotic response into a vicious cycle by further activating Fas through FasL on their cell surface (F).

AMØ: Alveolar macrophage; Ao: Apoptosis; EC: Endothelial cell; Epi.: Epithelial cell; MØ: Monocyte; MCP: Monocyte protein; pDC: Plasmacytoid dendritic cell; PMN: Polymorphonuclear cell; Treg: T-regulatory cell.