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EC PULMONOLOGY AND RESPIRATORY MEDICINE

Review Article

The Pathogenic Roles of Neutrophil Extracellular Traps (NETs) in Acute Lung Injury

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Received: January 23, 2018; **Published:** February 15, 2018

Abstract

Acute lung injury (ALI) is a syndrome of alveolar-capillary damage, resulting in hyper-permeability and non-hydrostatic pulmonary oedema. It is most commonly caused by sepsis, gastric aspiration, shock and trauma, and carries an extremely high mortality. The pathophysiology of ALI is poorly understood and therefore pharmacological treatments have not been successful. In the last decade, neutrophil extracellular traps (NETs) have been discovered and their pathological roles have been well characterized. One major role of NETs is its involvement in the development of ALI. NETs function as a part of the innate immune response but have been demonstrated to be associated with transfusion-related ALI and infection-related ALI. Extracellular histones, DNA, platelet-neutrophil interaction and coagulation activation could be the crucial factors in the development of NET-associated ALI. These factors as well as NETs therefore become potential therapeutic targets in ALI.

Keywords: *Neutrophil Extracellular Traps (NETs); Acute Lung Injury (ALI)*

Acute lung injury (ALI) is a syndrome of inflammation and increased vascular permeability characterized by acute and persistent widespread alveolar and capillary endothelial cell damage resulting in non-hydrostatic pulmonary oedema [1]. The hallmarks of ALI include inflammation, neutrophil activation and migration, and subsequent dysfunction of the alveolar-capillary membrane. It eventually causes severe hypoxemia and low lung compliance, with consequent acute respiratory distress syndrome (ARDS) and atelectasis [2]. ALI and ARDS are differentiated, according to the American-European Consensus Conference (AECC) criteria, on the basis of P_aO_2/FiO_2, where ALI is defined as a $P_aO_2/FiO_2 \leq 300$ mmHg, whereas ARDS is a $P_aO_2/FiO_2 \leq 200$ mmHg [3]. The 2012 Berlin criteria has removed the term ALI, and has instead separated ARDS into grades of severity based on P_aO_2/FiO_2 (Table 1) [4].

ARDS Severity	PaO_2/FiO_2 (mmHg)	Mortality (95% CI)
Mild	200 - 300	27% (24% - 30%)
Moderate	100 - 200	32% (29% - 34%)
Severe	< 100	45% (42% - 48%)

Table 1: *Categorization of Acute Respiratory Distress Syndrome according to the Berlin Criteria.*

ALI is highly prevalent, making up 7% of intensive care unit (ICU) admissions per annum. An American prospective study found an annual incidence of 78.9 patients per 100,000 [5]. Currently, the incidence of hospital-acquired ALI/ARDS appears to be decreasing. This may be due to improved ICU approaches [6]. However mortality rates of ALI/ARDS range between 27 - 45%, depending on its severity. The majority of deaths are related to the development of multi-organ failure (MOF) rather than primary respiratory failure [7].

Aetiology

There are both direct and indirect causes of ALI/ARDS with the commonest being sepsis, severe trauma, shock, pneumonia and gastric aspiration (Table 2). Less commonly, it can be caused by acute pancreatitis, transfusions, drug reactions, and fungal and parasitic infections. Sepsis is the biggest risk factor for ALI/ARDS whereas pneumonia (bacterial or viral) is the most common cause [8].

	Direct Pulmonary Insults		Indirect Pulmonary Insults
Airway	Pneumonia	**Circulation**	Sepsis
	Aspiration		Shock
			Trauma
	Inhalation injury		Major transfusions
			Pancreatitis
Circulation	Embolism (fat, amniotic fluid)	**Neurogenic**	Head trauma
			Intracranial haemorrhage
	Reperfusion injury		Drug overdose (narcotics, sedatives, TCAs)

Table 2: *Etiology of Acute Respiratory Distress Syndrome.*

Pathology

ALI begins with an acute inflammation of the alveolar-capillary membrane, damaging pulmonary endothelial and epithelial cells. This increases the permeability of the membrane, causing an influx of fluid rich in inflammatory cells and mediators. This results in pulmonary oedema, increasing the risk of sepsis and impairing surfactant synthesis. Eventually, there is alveolar collapse [8,9]. Patients may gradually recover from this acute phase if the epithelium retains the ability to reabsorb the oedema [11]. In severe cases, progression to the fibroproliferative phase occurs. The alveoli become filled with mesenchymal cells and fibroblasts. These cells deposit collagen and induce neovascularization. Ultimately, the patient will develop clinical signs of fibrosis [12].

Neutrophils

Neutrophils, a type of short-lived polymorphonuclear granulocytes (PMNs), play an important role in innate immunity against bacterial and fungal infections. They are quickly attracted to a site of infection, attracted by cytokines from activated host cells. They further enhance the immune response by recruiting and activating other immune cells. Neutrophils destroy pathogens through three different mechanisms: (a) phagocytosis and the production of reactive oxygen species (ROS); (b) degranulation and the release of antimicrobial proteins; and (c) the formation of neutrophil extracellular traps (NETs) [13]. NETs were discovered by Brinkmann et al in 2004. They are long chromatin filaments, consisting of either mitochondrial or nuclear DNA, that form web-like structures. These structures are coated with microbicidal histones and granular components. NETs are able to trap and kill extracellular pathogens by exposing them to high concentrations of antimicrobial proteins, including Neutrophil elastase, proteinase 3, histones, LL37, myeloperoxidase, lactoferrin, calprotectin (Table 3), which are helpful in preventing bacterial dissemination [14]. However, in certain clinical situations such as sepsis, they may also cause host tissue damages as listed in table 4 [15].

Protein	Role
Neutrophil Elastase	Cleaves virulence factors
Proteinase 3	Cleaves virulence factors
Histones	Damage cell membrane
LL37	Damage cell membrane
Myeloperoxidase	Produces reactive oxygen species; necessary for *Staphylococcus aureus* eradication
Lactoferrin	Binds iron; antifungal
Calprotectin	Most important antifungal; effective against *Candida albicans*

Table 3: *Antimicrobial properties of neutrophil extracellular traps.*

Protein	Role
Histones	Histones have a high affinity for phosphate groups inside DNA and phospholipids, allowing them to integrate into cell membranes and cause a large calcium influx. This results in endothelial damage, platelet aggregation, cytokine elevation, activation of the coagulation cascade (via interaction with factor XII), and NET formation [16,17].
Myeloperoxidase	Produces ROS which have two effects: 1. Epithelial cell injury, resulting in apoptosis or necrosis [18]. 2. Promote further NETosis [19].
Neutrophil Elastase	Damages endothelium by cleaving actin cytoskeleton, E-cadherin, and VE-cadherin; induces apoptosis of alveolar epithelial cells; promotes pro- inflammatory cytokine release [20].
Cathepsin G	Activates pro-inflammatory proteins and degrades anti-inflammatory proteins [18].
Proteinase 3	Activates pro-inflammatory proteins and degrades anti-inflammatory proteins [18].
LL37	Presents cyotoxic and pro-apoptotic factors to endothelial and epithelial cells [21].

Table 4: *Inflammatory properties of neutrophil extracellular traps.*

NETosis

Activated neutrophils are able to undergo a unique process called NETosis to produce NETs. Neutrophil activation can be induced by a number of factors including activated platelets, thrombin, complement factor 5a (C5a) and lipopolysaccharide (LPS) [15]. Neutrophil activation causes the nuclear DNA to decondense and for the heterochromatin and euchromatin to mix. This process requires histone (H3) citrullination, which is catalysed by peptidylarginine deaminase 4 (PAD4) and is further mediated by neutrophil elastase (NE) and myeloperoxidase (MPO) (Table 5) [22,23]. Decondensation of the chromatin is followed by nuclear membrane rupture [24]. At this time, the cytoplasmic granules also rupture. The chromatin then mixes with the granular components [15]. Recognition of foreign bodies through neutrophil receptors causes cell membrane rupture, releasing the NETs [24]. Neutrophils that are missing NAPDH oxidase (PHOX), NE, MPO or PAD4 are unable to release NETs.

Factor	Function
NADPH Oxidase	Produces reactive oxygen species. NB: patients with chronic granulomatous disease are unable to produce NETs unless treated with H_2O_2 [19].
Neutrophil Elastase	Mediates chromatin decondensation by degrading the linker histone H1 from core histones.
Myeloperoxidase	Mediates chromatin decondensation.
Peptidylarginine Deaminase 4	Catalyzes histone H3 citrullination.

Table 5: *Factors required for NETosis.*

Neutrophils Target the Lungs

Although NETs play an important role in defense against pathogens, they are also involved in inflammation and tissue destruction (Table 3 and 4). The main components involved in host tissue damage are histones, MPO, NE and cathepsin G. The lungs are the main target for NETs because neutrophils spend an increased amount of time in the lungs [25]. Studies have shown a neutrophil concentration to be 80 to 100 times greater in pulmonary capillaries compared to the systemic circulation [26,27]. This has been explained by an increased transit time through the lung vasculature as neutrophils must first deform to pass through the smallest pulmonary capillaries [28-31]. During times of inflammation, neutrophils are activated which decreases their deformability [32]. This implies that priming by a systemic inflammatory insult such as sepsis stiffens these cells and traps, which marginates them in the pulmonary vasculature [33,34]. Margination does not immediately lead to ALI; a second insult is required to cause transmigration of neutrophils through capillaries into the pulmonary interstitium [35,36]. If this second insult does not occur, neutrophils will eventually de-prime and return to the systemic circulation [37-39].

Priming has multiple effects on neutrophil function; (a) enhanced respiratory burst activity, resulting in the formation of ROS [40]; (b) decreased deformability due to a change in the shape of neutrophils [41]; (c) shedding of the cell surface adhesion molecule L selectin (CD62L), increasing neutrophil rolling velocity against the endothelial surface; (d) upregulation of cell surface CD11b adhesion molecule, enhancing endothelial adhesion and migration into tissue [42]; and (e) inhibition of apoptosis [43]. These activated neutrophils induce vascular hyperpermeability via three mechanisms: (1) secretion of soluble factors such as tumor necrosis factor-alpha (TNF-alpha), thromboxane A2 (TXA2) and leukotriene A4 (LTA4) causing endothelial contraction [44,45]; (2) contact mediated mechanisms which damage endothelial adherence junctions [46]; and (3) the generation of ROS [35,47].

Furthermore, the production of NETs exposes lung tissue to a variety of cytokines, ROS, tissue-degrading proteinases and cationic polypeptides [48]. Inside the lungs, NETs promote endothelial and epithelial cell injury, resulting in increased endothelial permeability. This is mediated by several molecules, including thrombin, C5a, VEGF, ROS, and platelets [49-52]. C5a, a NETosis induction factor, causes inflammation and vascular hyperpermeability via endothelial cell contraction. NETs can also trap platelets and cause thrombosis resulting in endothelial cell damage [4]. Thrombin itself mediates the release of vascular endothelial growth factor (VEGF) which also contributes to vascular hyperpermeability [51]. ROS released by NETs rapidly decrease endothelial cAMP content and increase vascular permeability [52]. The resolution of ALI/ARDS requires the reabsorption of alveolar oedema across the alveolar epithelium. This process is impaired because NET release of cytokines and oxidants induces apoptosis and necrosis of epithelial cells, causing defects in ion transport mechanisms, preventing fluid reabsorption [11].

Sepsis-Induced ALI

Platelets appear to play an essential role in sepsis-induced ALI. In severe sepsis, LPS activates toll-like receptor 4 (TLR-4) on platelet cell surfaces, inducing their activation. These activated platelets bind to and activate neutrophils, triggering NETosis. Platelet-neutrophil adhesion results in the expression of inflammatory mediators and tissue-factor, resulting in neutrophil and leukocyte recruitment as well as fibrin deposition in the pulmonary vasculature, respectively [53-55]. Activated platelets can also bind NETs, resulting in platelet aggregation. This promotes the formation of microvascular thrombi with subsequent pulmonary ischemia, damaging both endothelial and epithelial cells. Activated platelets also release alpha-granules and transforming growth factor-beta (TGF-B), inducing fibroproliferation [56]. Platelet depletion in a sepsis-model of ALI showed ameliorated gas exchange, reduced permeability, and reduced neutrophil accumulation [16]. Aspirin has been shown to be effective in the prevention of ALI, decreasing the need for mechanical ventilation in septic patients [57-60].

Acid-Induced ALI

Acid-induced ALI occurs due to gastric aspiration. Platelets appear to be a major mediator of acid-induced ALI. Thromboxane A2 (TXA2) has been found to be the main mediator of acid-induced lung injury. Platelet-neutrophil interactions produce TXA2 which

contributes to neutrophil recruitment and platelet-neutrophil aggregation (PNA). TXA2 also causes neutrophil adhesion to the endothelium, causing endothelial cell contraction and increased permeability [56]. Adhesion and increased permeability allow neutrophils to enter the interstitium and undergo NETosis. Gastric aspiration induces P-selectin-dependent platelet-neutrophil interactions in lung capillaries. ALI development is halted when P-selectin is blocked or the amount of circulating platelets is decreased. Anti-P-selectin antibodies have shown a significant improvement in oxygenation, reduced neutrophil transmigration, and reduced protein leakage into bronchoalveolar lavage fluid (BALF) [11,56,61]. Drugs that act by inhibiting P-selectin and TXA2 are currently under development [11]. If successful, they may have major implications in the treatment of acid-induced lung injury.

Trauma/Shock-Induced ALI

Hypotensive shock can be triggered by trauma or elective surgical procedures despite adequate fluid resuscitation [3,62-65]. Within 24 hours of the initial trauma, the patient is highly susceptible to lung injury on exposure to a secondary insult. Trauma patients often have a dramatically reduced ability to fight infection, making sepsis the most common secondary insult [66]. Extracellular histones are essential to the development of trauma-induced ALI. Large increases in extracellular histones have been noted in trauma patients. In a mouse model, infusion of histones of ultimately resulted in death [67]. It has been proposed that positively charged histones interact with negatively charged phospholipids in plasma membranes. By disrupting the membrane, histones induce a large calcium influx into the cell, damaging endothelial and epithelial cells as well as releasing preformed mediators from leukocytes. Histones also induce NETs which contain more histones, producing a vicious cycle of lung injury. They activate the coagulation cascade and induce platelet aggregation, forming thrombi and furthering endothelial damage [17]. They also cause rapid and profound thrombocytopenia in mice [68]. Anti-histone antibodies have demonstrated decreased histone and NET toxicity both *in vivo* and *in vitro*. Activated protein C (APC) is responsible for cleaving histones however it has been ineffective in the treatment of major sepsis in humans [4,69]. As histones are the major components of NETs, which are essential to the development of ALI, it is reasonable to suspect that they may play an essential role in the development of ALI/ARDS. However, further research is required to determine whether this is true. If so, this will have major implications in the treatment of ALI/ARDS.

Transfusion-Related ALI (TRALI)

ALI most commonly develops within the first hour after the initiation of blood product transfusion and carries a 5 - 10% risk of mortality [70,71]. Blood products containing anti-leukocyte antibodies or bioactive lipids are a second challenge on top of a primary condition, the most common being surgery, trauma or infection, that primes neutrophils. In a mouse model of TRALI, platelets were crucial to neutrophil sequestration and enhanced endothelial permeability [72]. They are activated by TLR4 and secrete TXA2, required for the formation of PNAs which mediate hyperpermeability [61,72,73]. Activated platelets also induce NET formation, which are involved in thrombus generation by platelet aggregation and activation of the coagulation cascade. This furthers endothelial dysfunction [15,73]. Aspirin has shown protective effects against NET production by inhibiting platelet aggregation in TRALI [15,16]. Neutrophil Fcγ receptor interaction with endothelial-bound MHC 1 monoclonal antibody (mAB) is also essential to neutrophil sequestration in the lungs. Mice that lack the Fcγ receptor do not develop MHC I mAB-mediated lung injury *in vivo* [72]. Further research is required to determine whether the Fcγ receptor will prove useful clinically. DNAse 1 inhalation has been shown to improve arterial oxygen saturation in TRALI by preventing antibody accumulation in the alveoli. Depletion of platelets and the neutrophil Fcγ receptor as well DNA degradation may all be implicated in the pharmacological treatment of TRALI. It would also be useful to study the role of histones in TRALI.

Pneumonia (Viral)-Related ALI

In a murine model of influenza pneumonitis, it was found that NETs are essential to the development of ARDS. In macrophage depleted mice, ARDS had 40% mortality. On histology, there was neutrophil recruitment with prominent NET formation. NETs were seen attached to the alveolar epithelium in areas of tissue damage. NET DNA fibers were seen attaching to the capillary endothelium to gain entry into the interstitium. There was a significant increase in T1a and thrombomodulin which are proteins normally present in alveolar type 1 and

endothelial cells, respectively, indicating host tissue damage. Interestingly, neutrophil depleted mice developed no signs of ARDS and had 0% mortality. This indicates that the lack of macrophages is a trigger to neutrophil recruitment which is essential to the development of ARDS secondary to viral pneumonia [74]. DNAse may be implicated as a form of treatment in viral pneumonia-related ALI.

Treatment

Treatment for ALI/ARDS has been extensively studied. To date, the only effective approaches that have been established are prone positioning and positive-pressure mechanical ventilation (PPV) [75,76]. The ARDS Network randomized controlled trial found that using a low tidal volume of 6mL/kg is lung protective, preventing barotrauma and epithelial injury [77,78]. This reduces damage to the alveolar-capillary membrane and thus decreases pulmonary oedema. It also downregulates mechanosensitive pro-inflammatory pathways, reducing neutrophil accumulation in the alveoli [78]. On the contrary, mechanical ventilation at high tidal volumes is a well-known cause of ventilator-induced lung injury [79]. It causes barotrauma, resulting in increased protein and cytokine leakage into the lung, hyaline membrane formation, and increased neutrophil sequestration [80]. Within the past decade, lung protective PPV has reduced the mortality of ALI/ARDS significantly, however an observational study in Spain employing this method still reported a mortality of 47.8%, indicating the need for novel treatment strategies [81].

There is little evidence base around the pharmacological treatment of ALI/ARDS. Several drugs have been tested but failed to produce any clinical benefit. Beta-2 agonists and glucocorticoids have shown some efficacy in rodents but no effect in humans [82-86]. APC, GM-CSF, surfactant protein C-based agents, nitric oxide and antioxidants such as N-acetylcysteine have been found to be ineffective [47,84,85,87-91]. Eritoran, an LPS-TLR4 binding inhibitor has been shown to reduce pulmonary inflammation in LPS exposed lungs [92,93] and in a phase II clinical trial, it further reduced mortality [94]. However, a multicentre phase III trial found no impact in sepsis-related ALI/ARDS [95].

Conclusions

Accumulated evidence strongly indicates that NETs formation plays a very important role in many types of ALI, including transfusion-related ALI and ARDS caused by infection.

Future Studies

Several therapeutic targets in the treatment of ALI/ARDS have been implicated (Table 6). To note, it may be useful to focus on the deceleration of ARDS rather on reversing severe injury [35]. Before any of these approaches can be used in humans, further research is required on their efficacy. Lastly, a truly successful pharmacological approach would be able to target all causes of ALI. It must therefore target a crucial part of NETs which would be present in all forms of ALI. However, the significance of each NET component in the different types of lung injury, is not completely understood as of yet.

Target	Role	Evidence
Extracellular histones	Major component of NETs causing epithelial and endothelial injury	Anti-H4 and anti-H2A antibodies reduce vascular permeability and lung oedema and reverse coagulation activation [16].
Platelets	Pro-thrombotic.	Aspirin prevents NET formation in transfusion-related lung injury, acid-induced lung injury and sepsis-induced lung injury by inhibiting platelet activation, preventing both aggregation and platelet-neutrophil interaction [96,97].
DNA	Major component of NETs	DNAse1 degrades NET-derived structures, reducing mortality in a murine model [16].
Complement factor 5a	Potent anaphylatoxin, promotes NET formation and extracellular histone release	Infliximab-1 is protective in viral pneumonia [66]. Neutralization of C5a with eculizumab or absence of receptors is protective in sepsis [50].
Transforming growth factor Beta	Released by NET-induced platelets, causing proliferation and chemotaxis of fibroblasts and local transformation into myofibroblasts which then produce extracellular matrix components, resulting in fibrosis in the later stages of lung injury.	No published studies.
Neutrophil elastase	Required for NETosis	Inhibitors have been approved in Japan and South Korea
Spingosine-1-phosphate	Maintain endothelial barrier function.	Intratracheal and intravenous delivery of agonist reduces lung permeability after lipopolysaccharide injection [98].
Tyrosine kinase	Enzyme responsible for phosphorylation of cellular proteins	Imatinib, an inhibitor, is protective against endothelial dysfunction and pulmonary oedema evoked by thrombin, vascular endothelial growth factor, or sepsis [16].
HMG-CoA reductase	Catalyzes conversion of mevalonate to cholesterol	Simvastatin has shown positive anti-inflammatory effects in a randomized controlled trial on patients with established acute lung injury [99].

Table 6: *Potential pharmacological targets in Acute Lung Injury/Acute Respiratory Distress Syndrome.*

Other important targets are anti-histone antibodies, aspirin, and DNAse. Histones have been proven to play a major role in trauma-associated ALI. Since they are the major toxic components of NETs, it is likely that they play an essential role in all forms of ALI, and therefore may have significant implications in the pharmacological treatment of ALI/ARDS. Platelets appear to play an essential role in septic, acid-induced, and transfusion-related lung injury. Aspirin has been proven to be effective in certain clinical scenarios. Interestingly, extracellular histones induce platelet aggregation and rapid thrombocytopenia, however this decrease in systemic platelets does not prevent lung injury. Therefore, the method of aspirin administration in ALI is important. DNA is the central component of NETs and is very toxic to host cells. It can tangle to produce extracellular webs which obstruct blood flow and produce patches of ischemia within the vasculature. DNAse would therefore break apart NETs and prevent major tissue damage.

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