Review Article

Understanding the role of neutrophils in acute respiratory distress syndrome

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ABSTRACT

Acute respiratory distress syndrome (ARDS) is difficult to treat and is associated with a high mortality rate. The most severe form of coronavirus disease 2019 (COVID-19) also leads to life-threatening ARDS. Neutrophil counts are positively correlated with disease severity in ARDS. Neutrophil activation not only plays a significant role in immune defense against infections, but also causes tissue damage and leads to inflammatory diseases. Activated neutrophils rapidly migrate to inflamed lung tissue, releasing toxic granular contents and generating neutrophil extracellular traps. In the last few decades, it has become apparent that neutrophils occupy a central role in ARDS pathology. In this review, we summarize the neutrophil inflammatory responses and their relationships to ARDS. According to the current literature, understanding the function of neutrophils may be helpful in the treatment of ARDS.

Acute respiratory distress syndrome (ARDS) is a life-threatening respiratory condition with an increasing incidence rate, ranging from 20 to 40%, and a mortality rate of approximately 40% [1,2]. On account of the outbreak of coronavirus disease 2019 (COVID-19), 29–42% COVID-19 patients developed ARDS and 15–52% of COVID-19 ARDS cases resulted in fatality [3,4]. Initially, the American-European Consensus Conference for ARDS defined acute lung injury
Overview of lung inflammatory injury

The pathogenesis of ARDS is complex and classified as direct or indirect. Most commonly, direct injury results from infectious damage to lung tissue. The causes of lung injury by infectious stimuli are not fully known. Pneumococcus and influenza A virus originally infect bronchial epithelium, induce upper airway and alveolar damage, recruit neutrophils and macrophages, and amplify cytokine and chemokine production \cite{13,14}. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects pulmonary tissues, resulting in accumulation of massive amounts of immune cells and leads to an inflammatory cytokine storm. In COVID-19 ARDS patients, cytokine storm is a common characteristic. Patients with COVID-19 ARDS have elevated plasma levels of inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8, granulocyte colony-stimulating factor, interferon gamma, and tumor necrosis factor alpha (TNF-α). An excess of inflammatory cytokines further causes immune cell infiltration into inflamed lungs to induce alveolar damage and diminish lung function \cite{3,15,16}. Another cause of direct lung injury is mechanical tissue damage, such as pulmonary contusion and inhalation of injurious materials. Pulmonary contusions primarily contribute to ARDS in traumatic patients by priming the innate immune response and enhancing the activity of toll-like receptor 4 resulting in excess production of pro-inflammatory mediators \cite{17,18}. Additionally, indirect pulmonary injuries can be explained by the “2-hit hypothesis” which states that an inflammatory response related to extra-pulmonary area stimulus (first hit) is followed by systemic inflammatory response (second hit) that can induce lung injury. Common triggers of indirect lung injury induced by systemic inflammatory response include sepsis, shock, acute pancreatitis, bone fracture, and massive blood transfusion \cite{19,20}. It has been shown that severe systemic inflammatory response resulting from sepsis produces numerous circulating inflammatory mediators. These mediators reach the bronchial and alveolar tissues and activate resident macrophages that attract inflammatory cells. Excess recruitment of immune cells not only occludes lung microcirculation but also releases cytotoxic products to damage surrounding tissues \cite{21,22}. Moreover, the pathogenesis of transfusion-related acute lung injury (TRALI) occurs through antibody-dependent mechanisms. The first hit contributes to the underlying inflammatory condition of the patient. The second hit is related to antibodies in the transfused blood product \cite{23}. Neutrophils are thought to mediate the development of TRALI. The antibodies from transfused blood components bind to the pulmonary endothelium followed by accumulation and activation of neutrophils. Activated neutrophils undergo respiratory burst and release ROS, release proteolytic enzymes by degranulation, and form NETs, which further contribute to lung inflammation \cite{24}.

Role of neutrophils in ARDS

Our research, along with other studies, has provided insights into the pathogenic role of neutrophils in various inflammatory states, including sepsis and ARDS \cite{25,26}. Neutrophils are the first immune cells recruited to the site of inflammation following stimulation by chemotactic factors released from damaged pulmonary tissues. Both exogenous and endogenous inflammatory stimuli can be recognized by specific receptors of human neutrophils. This further promotes the recruitment and activation of circulating neutrophils. These activated neutrophils produce several cytotoxic products, including ROS, granular enzymes, NETs, and various pro-inflammatory cytokines \cite{27}. Moreover, these immune products trigger several chemotactic signals to induce positive feedback, further enhancing inflammation. The overwhelming activation of neutrophils contributes to surrounding tissue damage and even lung dysfunction \cite{Fig. 1} \cite{28}. In COVID-19 ARDS patients, higher counts of neutrophil are observed and represent a predictor of poor outcome \cite{29}. Therefore, strategies to diminish neutrophil recruitment and impairing neutrophil immune functions, have been predicted to attenuate lung injury.

Neutrophil recruitment in ARDS

Neutrophil migration from the circulatory system into inflamed lung tissue can be initiated by both infectious and sterile inflammatory stimuli. Neutrophils attach to the vascular endothelium, and then migrate following a
chemokine gradient [30]. These chemoattractive signals are recognized by local immune cells, which produce inflammatory mediators that further boost neutrophil recruitment. In ARDS, endothelial cells activate and capture circulating neutrophils [31,32]. Neutrophils are sequestered through selectin-mediated binding, triggering “inside-out” activation of integrins, such as CD11a/CD18, which binds to intercellular adhesion molecules (ICAMs) from the endothelium [33].

Neutrophil rolling is a form of migration along the endothelial wall and is facilitated by specialized structure like membrane extensions and uropods. The rolling neutrophils become flattened and polarized to generate the forward-moving force, and then slowly crawl along the endothelial cells [34]. This slow rolling, or crawling, delays the neutrophils’ passage through the inflammatory tissue. This phenomenon augments neutrophil recruitment through chemokine signaling. Crawling neutrophils can either pass between the endothelial cells or through them to exit the vessel lumen. Finally, the migrating neutrophils detach from the vessel wall and move into the inflamed respiratory tissues [35–37].

Once activated, pulmonary neutrophils exhibit various immune responses, including increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and release of proteolytic enzymes [38,39]. Overwhelming NADPH oxidase activity and proteolytic enzyme release are associated with surrounding respiratory tissue damage. Therefore, strategies to reduce neutrophil migration may be useful in the alleviation of pulmonary tissue damage [40,41]. Consistently, several chemical compounds were shown to diminish neutrophil migration in animal models. Decreasing neutrophil recruitment decreases production of cytotoxic mediators [42,43], whereas increased neutrophils in pulmonary tissue demonstrably contributes to the pathogenesis of ARDS. Notably, studies of human and animals with neutropenia and ARDS showed poor recovery [11,12]. Evidences that neutrophils promoted both pulmonary inflammation and repair explain these findings. Neutrophils participate in the remodeling of damaged lung tissue through release of MMP-9 and the Wnt/β-catenin pathway [44,45]. The role of neutrophil recruitment in ARDS is complex and still needs to be explored.

**ARDS and neutrophil oxidative stress**

In response to infectious and sterile stimuli, neutrophils contribute to the progression of ARDS through the assembly and activation of the ROS-producing NADPH oxidase complex (NOX2) [46,47]. NOX2, located on the membrane, reduces oxygen into superoxide anion and releases it outside the cell. Superoxide anion is highly reactive and spontaneously dismutates into a more stable hydrogen peroxide (H₂O₂). H₂O₂ can pass through cell membrane and distribute to either extracellular or intracellular areas. The enzyme myeloperoxidase uses H₂O₂ to produce a series of more reactive products, including hydroxyl radicals and hypochlorous acid [48,49].

ROS are harmful to pulmonary tissues, indicating that reducing ROS production is beneficial to mitigate lung inflammatory injury [50,51]. SARS-CoV-2 infections cause redox imbalance and induce ROS accumulation. Oxidative stress produced from downregulation of superoxide dismutase 3 expression in lung tissues is observed in elderly patients with...
COVID-19, and the oxidative damage is closely related to poor outcome in elderly COVID-19 patients [52,53].

Several mechanisms of ROS are known. First, ROS directly damages the DNA of target cells. Previous research has shown that ROS produced from activated neutrophils induces oxidative DNA damage in respiratory epithelial cells [54,55]. Moreover, hyperoxia with 95% oxygen induces either neutrophil influx or oxidative DNA damage in neonate lung epithelial cells [56]. Second, ROS triggers peroxidation of lipid membranes, thus contributing to target cell lysis. The products of lipid peroxidation are also used as a measure of the level of tissue injury. In animal lung injury models, the level of lipid peroxidation was related to the severity of pulmonary tissue injury [57,58]. Third, ROS induces intracellular protein phosphorylation and transcription factor activation to promote the release of pro-inflammatory mediators. For instance, mitogen-activated protein (MAP) kinases can be activated by ROS to mediate cell death in response to stress, suggesting that the phosphorylation of MAP kinases has a major role in promotion of lung inflammatory injury [59,60]. Moreover, ROS triggers the activation of NF-κB, an important transcription factor that controls pro-inflammatory mediator release, to augment lung inflammatory injuries [61,62]. Research on the anti-oxidant α-tocopherol showed that ROS production and related expression of NF-κB in activated neutrophils was decreased, and lung inflammatory injury was ameliorated in the presence of α-tocopherol [63]. Similarly, another study showed that paraquat, an oxidant inducer, triggered ROS generation and release of inflammatory mediators, such as TNF-α, and IL-1β. The mechanisms of paraquat to heighten lung inflammation included activation of Jun N-terminal kinase (JNK) MAP kinase and NF-κB [64]. ROS damages the pulmonary endothelium, which is the barrier between the blood and vessels. Intracellular calcium signals are activated by ROS to increase endothelium permeability during pulmonary inflammatory process [65,66]. Cooperatively, neutrophil oxidative stress induces pulmonary endothelial and epithelial barrier dysfunctions. Barrier dysfunction increases neutrophil infiltration into lung tissues, and then these accumulated pulmonary neutrophils secrete several cytotoxic agents followed by worsening of pulmonary tissue damage, further enhancing the progression of ARDS [67].

ARDS and NET formation

NETs are extracellular fibrous net-like structures, which have neutrophil granular proteins, such as myeloperoxidase or neutrophil elastase, coated on the backbone structures of DNA [85,86]. The mechanisms that induce NET formation have been explored, but signaling pathways involved are not completely understood. Raf/MEK/Erk signaling is activated to induce ROS production in the process of NET formation. Upon activation, the granular enzymes, myeloperoxidase and elastase, are released into the nucleus to induce nuclear disintegration. These granular enzymes coupled with PAD4 trigger chromatin decondensation and release. This mixture of granular enzymes and chromatin is expelled, followed by cell membrane rupture [87–89].

NETs are thought to be part of an innate immune mechanism for pathogen clearance; however, there are increasing concerns about the potential of NETs to initiate and propagate inflammatory damages in host tissues. Interestingly, both sterile and infective inflammations promote NET formation. During sterile inflammation, the components of NETs were found in lung and plasma of TRALI patients and mice. Treatment with DNase 1 decreased NET formation and lung injury [90,91]. Moreover, NET formation was observed in ventilator-induced lung injury. Previous human and animal studies showed that NET formation in the alveolar space was higher in bronchoalveolar lavage fluid from subjects with ventilator-induced ARDS. However, decreasing NET formation was not related to the shortening of mechanical ventilation [92–94]. During infective inflammation, NETs captured respiratory syncytial virus or influenza virus particles in patients and mice with respiratory infection. Nevertheless, cumulative

ARDS and neutrophil degranulation

Neutrophil granules contain both pro- and anti-inflammatory substances that can be released to kill pathogens or for remodeling tissues. This process is called degranulation [68,69]. Neutrophils release membrane-bound vesicles, which contain various types of cytoplasmic granules associated with the progression of ARDS. The primary granules (also known as azurophilic granules) contain myeloperoxidase and many proteolytic proteins, including neutrophil elastase and cathepsin G. These granular proteins play major roles in microbicidal responses [70]. The secondary granules contain proteins that degrade the extracellular matrix. The tertiary granules contain a few antimicrobials but store several metalloproteases [71,72]. During the inflammatory process, signals from receptors on the plasma membrane are transduced to the cytoplasm, which trigger the delivery of granule-associated proteins to the cell surface and secretion of their contents out of the cells. The regulation mechanisms are numerous, including calcium signaling, src family kinases, MAP kinases, and GTPase-related signaling [73,74].

Neutrophilic infiltration into respiratory inflammatory tissues is a means of defending the host against pathogens, however, overwhelming extracellular release of neutrophil granular enzymes have been implicated in triggering collateral pulmonary tissue damage in ARDS [75,76]. Neutrophil elastase is a primary granular proteolytic enzyme and impacts lung inflammatory injury. Elastase can degrade pulmonary extracellular matrix proteins and cleave epithelial-cadherin to break respiratory cell-cell adhesion. Moreover, elastase has been shown to damage pulmonary vascular endothelial glycocalyx during the pulmonary inflammatory process [77–79]. Myeloperoxidase, the other primary granular enzyme, catalyzes H2O2 to produce toxic ROS, which has been demonstrated to be a local mediator of alveolar damage [80]. The family of matrix metalloproteinases (MMPs) are released from the secondary and tertiary granules. Previous research showed that the expression of MMPs was correlated with the progression of ARDS. The enhanced expression of MMPs was related to inflammation-induced endothelial injury and impaired oxygenation in ARDS [81,82]. Another study revealed that MMP inhibitors suppressed MMP expression in plasma and lung tissue, decreased inflammatory mediator release, and attenuated lung inflammatory injury [83,84].

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NET formation not only obstructed small airways, but also damaged alveolar-capillary to induce lung inflammatory injury [95–97]. The characteristics of lung tissue in ARDS demonstrated intensive neutrophil infiltration of lung tissue and NETs entwined with alveoli were exhibited in infected mice. The progression of inflammation further induced alveolar damage and pulmonary edema. Treatment with DNase I degraded NETs and reduced inflammatory cytokines, indicating that decreasing NET formation ameliorated lung injury and improved survival [98–100].

The severity and mortality of COVID-19 ARDS are correlated to higher neutrophil counts and NET level [29,101]. NETs have the ability to trigger immunothrombosis. The pulmonary capillary from patients with COVID-19 ARDS is filled with microthrombus and NETs. Microthrombi and fibrin deposition in lungs results in reduced oxygenation and further induces ARDS in COVID-19 patients [104–106].

**Conclusion**

Although neutrophil immune responses are involved in ARDS, many questions remain unanswered. Neutrophils produce pro-inflammatory mediators and also augment inflammatory damage, implying that targeting neutrophils is a potential therapeutic for ARDS and COVID-19. However, on the basis of evidence supporting the fact that neutropenia does not attenuate ARDS, perhaps neutrophils play different roles in various phases of acute inflammation. The eventual goal is to determine when and how neutrophil activation is beneficial so as to tilt the balance toward benefit rather than harm.

**Conflicts of interest**

The authors declare no competing financial interests.

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