Neutrophils set extracellular traps to injure lungs in COVID-19

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As evidence accumulates to establish the clinical and pathological manifestations of acute respiratory distress syndrome (ARDS) in the respiratory failure and fatalities in critically ill patients with COVID-19, intensive research efforts are ongoing to elucidate causative mechanisms underpinning the development of ARDS.[1] Studies on various respiratory viral and bacterial infections have identified neutrophil extracellular traps (NETs) as potent mediators of ARDS, [2-3] NETS are released from neutrophils by a cell death mechanism known as NETosis, and NETs contain a DNA-histone backbone that carry granule proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO). When first discovered, NETs were appreciated for their role in immobilizing and killing various microbial pathogens in the extracellular space, However, persistent and excessive release of NETs can lead to extensive cytotoxic responses, culminating in tissue injury and organ failure.

Early clinical data on COVID-19 patients from Wuhan, China, published in March 2020, raised a red flag, as the investigators observed a progressive rise in neutrophilia in all non-survivors compared to survivors with mild to moderate disease.[4] Subsequently, numerous clinical reports confirmed neutrophilia in severely ill COVID-19 patients, in parallel with increased plasma levels of neutrophil-specific chemokines, NETs and neutrophil-to-lymphocyte ratios (NLR)[5]. These initial studies predicted neutrophilia and elevated NLR levels as early indicators of risk factors for severe COVID-19 illness.[6] However, it was unclear whether neutrophilia and/or plasma NETs reflect their elevated levels in the lungs, until one of the first postmortem lung analyses of COVID-19 patients performed by Barnes and co-workers identified the abundant presence of neutrophils and NETs in the airway and alveolar air spaces.[7]

Given this background on NETs release in COVID-19, the prospective cohort study by Ouwendijk et al. in this issue of *Journal of Infectious Diseases* measured NETs formation in the lower respiratory tracts (LRT) and plasma samples of hospitalized COVID-19 patients with ARDS.[8] These investigators performed a longitudinal and correlative evaluation of NETs formation in 77 patients, who were confirmed as SARS-Cov-2 PCR-positive with variable degrees of ARDS illness: 10% with mild ARDS, 64% with moderate ARDS, and 24% with severe ARDS, as determined within 3 days post-admission to the ICU. Strikingly, mortality rate was 30% among hospitalized patients, and 70% of patients recovered during the 30-day follow-up. The most noteworthy finding from this study is the discovery of active NETosis in the infected lungs with persistent release of NETs, observed even after virus clearance from the lungs. These results provide strong evidence for the pathogenic association of NETs in severe COVID-19.

Although many studies have analyzed temporal changes in plasma NETs, the formation of NETs in viral-infected lungs was identified only in lung autopsies of COVID-19 patients. The current study provides an evidence for continuous NETs release in highly delicate respiratory compartments during progression of the disease. The aberrant release of NETs in the lower respiratory tract of SARS-CoV-2-infected patients could potentially be toxic to the alveolar-capillary barrier. The close proximity of the released NETs components (e.g.,. NE, MPO, and histone proteins) triggers direct cytotoxic effect on alveolar epithelium and endothelium resulting in loss of alveolar integrity. In addition, NETS components also act like damage-associated molecular patterns and induce thrombotic and inflammatory responses in various acute infections. Furthermore, finding persistent release of NETs even when virus replication ceases in patients with severe ARDS, suggests that accumulation of NETs in the infected lungs elicits more collateral damage in COVID-19. Importantly, this study provides data on active NETs release, identified using MPO and citrullinated histone 3

(citH3, a marker for NETs formation) in bronchiolar and alveolar regions from damaged areas of the lungs. This study also presents important prospective cohort data on the dynamics of plasma NETs in disease progression in patients with COVID-19. The temporal changes in plasma NETs also paralleled the increased neutrophil-specific chemokines. The investigators also reported that increase in plasma NETs correlates with lung severity and PaO2/FiO2 values. Thus, detection of high plasma NETs may serve as predictor for severe COVID-19.

The methodology employed in this prospective study represents a valuable tool to unravel the progressive acute pulmonary pathologic changes in COVID-19 patients. The longitudinal analyses of bronchoalveolar lavage (BAL) samples offer opportunities to examine (a) alveolar epithelial damage, (b) vascular endothelial injury and leakage, (c) platelet activation, (d) platelet aggregation, and (e) parenchymal hemorrhage during the course of SARS-CoV-2 infection. Temporal analyses of these parameters together with lung CT imaging will enhance our understanding of the pathophysiology of severe COVID-19. In addition, this study also highlights the differences between measuring histone-DNA versus MPO-DNA to determine NETs in lower respiratory tract and plasma samples. Although histone-DNA was also measured as a marker for NETs, these levels were notably higher compared to MPO-DNA. Given that dead cells, such as from epithelium, can be a source of histone-DNA, measuring MPO-DNA would be a better choice to evaluate NETs in plasma and BAL fluids. Importantly, the current study indicates the feasibility of temporal analyses of BAL fluid samples, which can yield valuable information, particularly on how the progressive thrombotic lesions evolve in the pulmonary vasculature during the persistent generation and accumulation of NETs in the lower respiratory tract. The double immunostaining analysis to visualize spatial orientation of NETs release in bronchioles and alveoli provides valuable data on in situ interactions of NETs with alveolar epithelium,

endothelium and also immune cells in the lung micro-environment. The longitudinal BAL analysis, as tested in patients admitted into ICU, would help to identify and monitor superinfections. Several clinical trials are in progress on various drugs that dismember NETs or inhibit toxic effects of NETs components (e.g.,. NE) and may provide a good basis for experimental evaluation of clinical and pathologic observation to assess therapeutic efficacy of drug treatments in clinical trial settings.

One of the limitations of this study was the analysis of thrombosis using only CT pulmonary angiography, which failed to capture microvascular thrombosis or small platelet aggregates leukocyte-platelet aggregates that are formed in the tiny pulmonary capillary vasculature. Aberrant coagulation effects, including heightened platelet aggregation, pulmonary vascular thrombosis, and endothelial injury have been observed in lung autopsy analyses of COVID-19 patients.[9] Evaluation of platelet activation, changes in the platelet receptor repertoire and signaling events that promote platelet aggregation are critical to identify pathological changes in the pulmonary vasculature during progression to ARDS. Co-infections with bacteria (e.g., *Streptococcus pneumoniae*) or respiratory viruses (e.g., influenza) are also observed in critically ill COVID-19 patients,[10-11] and can potentially aggravate NETosis and acute lung pathology. The current prospective report does not mention the possibilities of secondary bacterial, viral or fungal infections in patients with COVID-19admitted into the ICU during the 30-days follow up studies. In addition, it would be important to understand the role of leukocyte-platelet aggregates (macrophage-platelet or neutrophil-platelet), as recent studies have linked platelet-inflammatory cell interactions in aggravated inflammation and acute lung injury [12]. This study identified high accumulation of extracellular histones in the BAL samples as well as lung histopathology of severely ill COVID-19 patients. These findings are critical, as released extracellular histones

accumulated in the lung alveolar air spaces have implications for extensive alveolitis, vascular damage and thrombosis in severe influenza pneumonia.[13]

The Ouwendijk et al. study highlights active NETosis that occurs during clinical progression in patients with COVID-19, thus supporting the significant contribution of NETs to ARDS pathophysiology. The current prospective cohort study will also aid in identifying novel biomarkers of disease severity, as well as in designing therapeutic interventions to prevent NETs formation and to inhibit toxic constituents of NETs that exacerbate acute lung injury and ARDS in COVID-19.

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