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VIEWPOINT

Neutrophils and COVID-19: Nots, NETs, and knots

Carl Nathan

The pace of the COVID-19 pandemic makes it timely to take stock of evidence for the involvement of neutrophils and NETs, to weigh the implications, and to increase efficiency in clinical trials.

Compounding the mysteries of COVID-19, the initial months of the pandemic were marked by a dearth of published autopsy reports amid the plethora of deaths. Word-of-mouth had it that postmortem COVID-19 lungs displayed diffuse alveolar damage and lymphocytic infiltrates. In April 2020, Barnes et al. (2020) published what may have been the first report of the histology of lungs from open necropsies of COVID-19 patients. What they saw in three of three subjects was different from diffuse alveolar damage and remarkable for a prolonged viral infection: intense neutrophilic infiltration of pulmonary interstitial (extravascular, interalveolar) spaces and alveoli. Barnes et al. (2020) proposed that neutrophil extracellular traps (NETs) could play a prominent role in COVID-19 and recommended interventions accordingly.

Though only a few months have passed, the urgency of achieving a fuller understanding of COVID-19 and the rapid pace of research make it appropriate to take stock of this hypothesis. Are neutrophils prevalent in the pulmonary interstitium and alveoli in COVID-19 patients or not? Are NETS present or not? These are the "nots" of the title. "Knots" refers to the complex interrelationships among neutrophils, thrombosis, coagulation, and complement in COVID-19.

Neutrophils or not?

Aside from the three cases noted by Barnes et al. (2020), I found English-language reports of open autopsies of 277 people who succumbed to COVID-19 in Italy (Carsana, 2020), Germany (Edler et al., 2020), Switzerland, and the United States (Magro et al., 2020; five additional references will be provided on request). The following tabulation excludes cases where the terms "sepsis," "purulence," or "abscess" were mentioned.

Interstitial neutrophils were only reported in the three cases of Barnes et al. (2020) and two cases described by Magro et al. (2020). One or two of the Barnes et al. (2020) cases for which no clinical information was provided may have corresponded to those of Magro et al. (2020). In contrast, intra-alveolar neutrophils were noted in 15 subjects (5.4%) who were described as having "bronchopneumonia" and in another 42 subjects (15.2%) who were not described as having bronchopneumonia. "Neutrophilic pneumonia" was reported in a preprint by Veras et al. in 6 of 10 (60%) ultrasound-guided postmortem lung samplings of COVID-19 patients (Veras et al., 2020 *Preprint*). Should the accumulation of neutrophils in the alveoli in some of these cases be ascribed to a superimposed bacterial or fungal infection? The authors of these studies made no mention of seeing bacteria or fungi inside or alongside the neutrophils in the sections they examined, nor did they report the results of bacterial or fungal cultures.

Examining bronchoalveolar lavage fluid (BALF) from living subjects, Zhou et al. (2020) compared the cells recovered from eight individuals with COVID-19 to those from 46 subjects with community-acquired pneumonia, which is generally bacterial in origin, and from 20 healthy subjects. Neutrophils were the one cell type most selectively elevated in the BALF of subjects with COVID-19 (Zhou et al., 2020). This lends support to the idea that the intra-alveolar neutrophils found at autopsy in 20.6% of the 277 cases surveyed above are often a feature of COVID-19 itself, rather than the result of superinfection. The elevated CXCL8, CXCL1, and CXCL2 also detected in the BALF (Zhou et al., 2020) may account for the accumulation of neutrophils in the pulmonary alveoli. As neutrophils migrate up a concentration gradient of chemokines, they can encounter concentrations that activate them to secrete cytotoxic products, as discussed below.

Strikingly, however, some autopsy series reported no cases with intra-alveolar neutrophils (e.g., 0/80; Edler et al., 2020), even as others reported intra-alveolar neutrophils in 81.5% of their cases (31/38, excluding five cases with "abscesses"; Carsana, 2020). The latter finding is comparable to what Veras et al. saw (60%) in their series (Veras et al., 2020 *Preprint*). COVID-19 is notoriously heterogeneous in its severity. However, one might expect to find a substantial commonality of pathological features in people whose disease was severe enough to kill them. Indeed, convergence of pathology toward a common end stage is borne out by almost all the other pathological pulmonary features noted in these reports, such as diffuse alveolar damage, hyaline membranes, interstitial lymphocytic infiltrates, and microvascular thrombosis and hemorrhage.

What could explain such wide-ranging differences in intrapulmonary neutrophils in COVID-19 autopsies? A likely explanation

Department of Microbiology and Immunology, Weill Cornell Medicine, New York, NY.

Carl Nathan: cnathan@med.cornell.edu.

is that while many subjects died of diffuse alveolar damage severe enough to preclude compensation by mechanical ventilation, others died for different reasons and with a shorter course of diffuse alveolar damage. Other causes of death in COVID-19 can include widespread microvascular thrombosis, a large-vessel thrombus, and/or damage to other organs besides the lungs, including the kidneys or the gastrointestinal tract, whose increased permeability can lead to circulation of microbes or microbial products, systemic inflammatory response syndrome, or sepsis. In prolonged inflammatory responses, neutrophils commonly infiltrate affected tissues early; monocytes and lymphocytes typically follow and replace them. Some patients who died of COVID-19 before lung disease was prolonged may still have had neutrophils in their lungs at the time of death. Neutrophils may have disappeared from the lungs of those who died after a longer course of lung disease.

In sum, here is an answer to the first question: neutrophils appear to be prevalent in the alveoli of living COVID-19 patients (Zhou et al., 2020), have been found in the alveoli of about one-fifth of autopsied COVID-19 patients at the time of their death, and were plausibly present in many others earlier. While interstitial neutrophils have been documented very rarely in the lungs of COVID-19 patients at death, they likely passed through the interstitium on their way to the alveoli.

NETs or not?

Within this question lie two others: what are NETs, and how are they detected? Some investigators say NETs are stringy or rod-like structures that form after neutrophils undergo a controlled process of extrusion of nuclear (some say mitochondrial) DNA, lysosomal proteins, and certain cytosolic proteins with or without cell death, and that these structures arise because increased intracellular Ca^{2+} activates peptidylarginine deiminase 4 (PAD4), which then citrullinates histones, linearizing them. Others see NETs as strands of neutrophil-derived DNA decorated with histones, lysosomal proteins, and calprotectin that can be found where neutrophils have lysed. Some wonder whether citrullination might follow rather than trigger the exteriorization of histones, given that cell lysis by any route would expose PAD4 to an activating level of Ca^{2+}. Some declare NETs to be present in tissues if they find extracellular DNA colocalized with citrullinated histones, myeloperoxidase, elastase, and/or calprotectin. Some report "fragments of NETs" when they find some of these molecules in serum, without requiring that they be colocalized.

At the time this was written, a preprint from Veras et al. appears to be the first to document the presence of NETs in fixed lung tissue from people with COVID-19, though the number of subjects was small (Veras et al., 2020 *Preprint*). Veras et al. also found NETs in tracheal aspirates of 12 COVID-19 patients on mechanical ventilation, a circumstance that defines those subjects as having lung disease of a preterminal degree at the time the specimens were collected. Finally, they detected NETs in the plasma of 21 subjects (Veras et al., 2020 *Preprint*). Similarly, Zuo et al. (2020) found higher mean levels of cell-free DNA, DNA-myeloperoxidase complexes, and citrullinated histone H3 in the sera of 50 COVID-19 patients than in the sera of 30 healthy controls (Zuo et al., 2020).

Knots

No matter how variable the detection of neutrophils is in the lungs of COVID-19 patients at death, and no matter what evidence is required for NETs, neutrophils are likely to contribute to the pathogenesis of COVID-19. The microthrombi widely observed in COVID-19 resemble knots of fibrin, platelets, and neutrophils, and can assemble through a skein of host responses to damaged tissue.

Elevated numbers of neutrophils circulate in people with COVID-19; in general, the higher the number, the worse the disease. Microthrombi in pulmonary capillaries contain neutrophils. NETs have thrombogenic effects in vitro and have been observed in thrombi in other diseases (de Bont et al., 2019; Laridan et al., 2019). The DNA in NETs can activate clotting factor XII; activated factor XIIa can activate neutrophils (Renné and Stavrou, 2019). Besides autacoids and cytokines, activated neutrophils secrete proteases and ROS. ROS inactivate α1-antitrypsin, α2-macroglobulin, and secretory leukocyte protease inhibitor, three major plasma antiproteases that protect cells and tissues against neutrophil proteases (Nathan, 2006) and may inhibit the transmembrane proteases that promote SARS-CoV-2 infection. Activated neutrophils can damage endothelium; damaged endothelium can promote thrombosis. Disruption of capillaries might foster access of inflammatory intrapulmonary cytokines to the circulation despite destruction of lymphatic drainage. Platelets populate thrombi; platelets and neutrophils can activate each other to promote thrombus formation (Pircher et al., 2019).

Complement may draw the knot tighter in COVID-19 (Magro et al., 2020; Gao et al., 2020 *Preprint*). Anti–SARS-CoV-2 IgM, IgG, and IgA likely activate complement by the classical pathway. The heavily mannosylated spike protein of SARS-CoV-2 may recruit mannose-binding lectin-associated serine proteases (MASPs) and activate complement by the lectin pathway. MASPs can activate coagulation, including by activating prothrombin (Garred et al., 2016). Both α1-antitrypsin and α2-macroglobulin can block the ability of MASPs to activate prothrombin, and as noted, neutrophil-derived ROS can prevent this inhibition. Plasmin in the coagulation pathway can cleave C5 to release C5a. Finally, NETs can activate complement (de Bont et al., 2019). C5a was strikingly elevated in the serum of patients with severe COVID-19 (Gao et al., 2020 *Preprint*). C5a activates neutrophils to release proteases and ROS.

Inferences and implications

In severe COVID-19, it is likely that neutrophils often emigrate from capillaries and venules through the pulmonary interstitium and into the pulmonary alveoli during the course of the disease, whether or not the individual succumbs and neutrophils are still there at the time of death. Evidence for NETs in COVID-19, while growing, is limited. However, it is not necessary to implicate NETs in order to take neutrophils seriously in COVID-19. From this perspective, the experimental interventions recommended by Barnes et al. (2020) seem worth testing, as do other agents that likewise might help untie the pathogenic knots that can lead to microvascular thrombosis and organ damage. Such agents include the ROS scavenger N-acetylcysteine and a neutralizing mAb against C5 (Lam et al., 2020 *Preprint*), both of which are Food and Drug Administration approved, and neutralizing mAbs against the neutrophil-boosting and -activating cytokine GM-CSF or its receptor (e.g., De Luca et al., 2020).

How can so many agents be meaningfully tested? We need to cast "nets" more widely. Most clinical trials in COVID-19 have been of three types: (1) small, often redundant, nonrandomized studies; (2) small,

often redundant, randomized controlled trials (RCTs) with varied protocols and different standards-of-care in the controls, whose results are difficult to aggregate; and (3) multicenter RCTs. Although these studies have been launched in urgency and executed valiantly under extraordinarily challenging conditions, the overall approach is ill-matched to a pandemic of the novelty, lethality, and rate of spread of COVID-19. Studies of type 1 are uninformative. Those of type 2 are underpowered. Those of type 3 can only be mounted for a few of the more than 500 interventions already under study.

Institutions in a position to conduct interventional COVID-19 clinical trials should join collaborative networks that use an adaptive trial design and share a placebo group. Each mechanistically different experimental intervention can be allocated to one trial site for a small, fast, nonredundant RCT whose results are reported in real time to a centralized data safety monitoring board. Only interventions likely to have a major beneficial effect will approach statistical significance in a small RCT. Those with the highest level of statistical significance, and only those, should advance to studies of type 3, in which they can ideally be compared head to head and then in combinations. The National Institutes of Health's Accelerating COVID-19 Therapeutic Interventions and Vaccines public–private partnership is a start and will hopefully coordinate with similar networks around the world. These are "nets" that COVID needs.

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