

## Commentary

# Casting a wide NET: an update on uncontrolled NETosis in response to COVID-19 infection

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Dysregulation of neutrophil extracellular trap (NET) formation has been shown to mediate disease pathology in multiple viral infections, including SARS-CoV-2. At the beginning of COVID-19 pandemic, Thierry and Roch wrote a perspective on the mechanisms by which severe SARS-CoV-2 infection may lead to uncontrolled NET formation that leads to acute respiratory distress syndrome (ARDS), systemic vascular permeability, and end organ damage. In this commentary, the progress that has been made in regards to the ideas postulated by the perspective will be discussed, with a focus on the therapeutics that target NET formation.

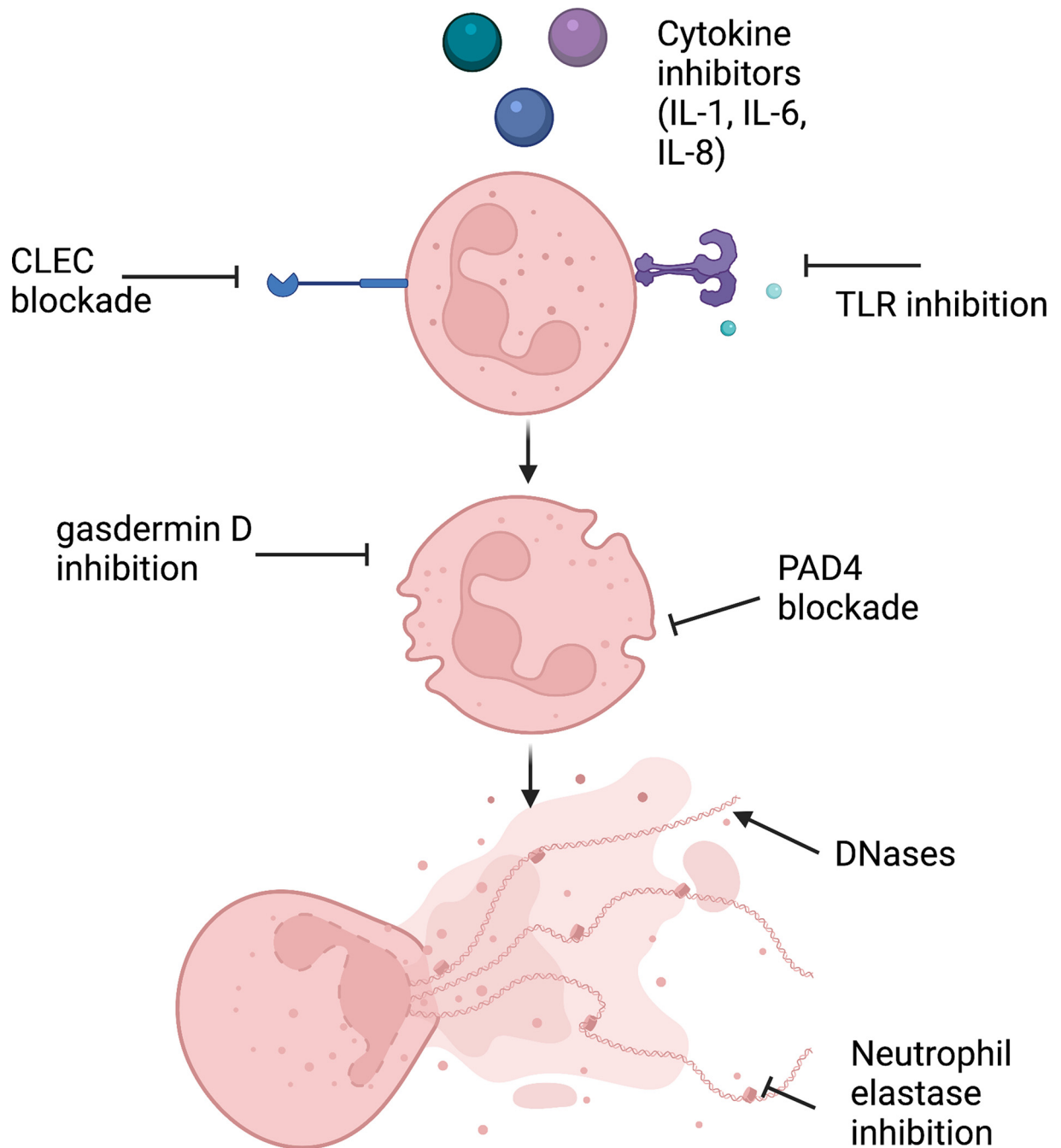
In a seminal 2004 paper, it was reported that neutrophils undergo a form of cell death distinct from apoptosis or necrosis termed neutrophil extracellular trap (NET) formation or NETosis [1]. NETosis is triggered by multiple mechanisms, including pattern recognition receptor (PRR) activation, cytokines (especially IL-8 and IL-1 $\beta$ ), FcR engagement, and activated platelets. This leads to the activation of protein arginine deiminase (PAD4), which converts peptidyl-arginine to peptidyl-citrulline in histones [2]. This results in the destabilization of chromatin followed by the release of chromatin fibers and various granule and cytoplasmic proteins, including elastase, myeloperoxidase, and various cathepsins, among many others. The network of proteins and DNA trap pathogens, limit their dissemination into tissues, and facilitate their killing. Despite their important role in antimicrobial activities, dysregulated NET formation and degradation is increasingly recognized to mediate disease pathology in both infections and inflammatory conditions [3]. In support of this concept, NETs have been shown to control infection in some viral infections such as Chikungunya and poxvirus [4,5] but can exacerbate other viral infections such as dengue virus, rhinovirus, and influenza A [6–9]. In addition, increased levels of NET markers such as myeloperoxidase-DNA (MPO-DNA) are associated with worsened outcomes and multiple organ dysfunction in influenza infection [10].

In June 2020, Thierry and Roch wrote a perspective on the mechanisms by which severe SARS-CoV-2 infection may lead to uncontrolled NET formation that causes severe pathophysiological consequences. They pointed out the similar features between uncontrolled NETosis and COVID-19, including acute respiratory distress syndrome (ARDS), coagulation abnormalities, endothelial and epithelial cytotoxicity, and systemic vascular permeability, all leading to end organ damage and sepsis which complicate many cases of COVID-19 and lead to mortality [11]. Increased levels of NETs were previously linked to ARDS in both transfusion- and infection-associated acute lung injury [12,13]. The authors noted that elevated markers of NET formation had very recently been identified in COVID-19 patients, including myeloperoxidase-DNA and citrullinated histone H3 [14]; thus, they suggested that targeting NETosis might be a viable therapeutic option. Since the publication of the manuscript, a large volume of data have been generated in support of the concepts postulated by the authors. I will briefly discuss some of the data that has been generated in regards to NET formation in COVID-19, potential COVID-19 therapeutics that target NETosis, and highlight some still unanswered questions in regard to uncontrolled NETosis in SARS-CoV-2 infection.

Neutrophilia is a hallmark of acute infection, but excessive neutrophil numbers in the bloodstream and an increased neutrophil-to-lymphocyte ratio were noted early in the pandemic in hospitalized COVID-19

Received: 08 May 2022  
 Revised: 13 June 2022  
 Accepted: 17 June 2022

Version of Record published:  
 06 July 2022



**Figure 1. Summary of NETosis-related pathways that can be targeted in COVID-19 therapeutics**

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patients [15,16]. Neutrophils are also elevated in the nasopharyngeal epithelium and in more distal parts of the lungs of COVID patients [17]. Further, post-mortem examination of COVID-19 patients revealed high levels of neutrophil extravasation in pulmonary capillaries, heart, and liver [18]. Additional studies also reported elevated circulating NET markers, including citrullinated histone H3, neutrophil elastase, MPO-DNA, and cell-free DNA in COVID-19 patients relative to healthy controls [19–21]. It was also reported that increased circulating NETosis markers were higher in hospitalized patients that needed mechanical ventilation versus those breathing room air [14]. Further, NET markers are predictive of disease severity and end organ damage in critically ill COVID-19 patients [22]. Increased NETs are also present in the lungs of deceased COVID patients [19,20]. These data lead to the question:

why do neutrophils from COVID-19 patients have a higher capacity for NET formation? It is likely for multiple reasons. First, SARS-CoV-2 particles can directly trigger NETosis in a process that requires viral entry and replication [19]. The neutrophil compartment is dysregulated in COVID-19, as evidenced by an increased occurrence of neutrophil precursors in the blood and dysfunctional mature neutrophils [23]. In addition, the milieu of inflammatory mediators in the circulation may further increase NET formation. Serum from patients with COVID-19 can induce NET formation [20], and specifically IgG purified from COVID-19 plasma can induce NET formation by neutrophils from healthy patients *in vitro* [24]. In addition to IgG triggering NET formation, various autoantibodies, including anti-phospholipid, anti-nuclear, anti-cytokine, and anti-NET antibodies have been reported in COVID-19 patients [24–27]. It is likely that these autoantibodies can influence the progression of COVID-19 disease as well as the ability to clear NETs. For example, anti-NET antibodies from COVID-19 patients were recently shown to stabilize NETs and impair their clearance by DNase [28]. Finally, COVID-19 patients have increased platelet-neutrophil aggregates that also increase NET production and lead to occlusion of microvessels in the lungs and other tissues [29,30].

Several compounds that target NETs were proposed by Thierry and Roch to treat complications associated with COVID-19 infection. In addition, many other NETosis-related therapeutics have been suggested and tested in patients with COVID-19 in the time since the publication of the perspective. Some drugs are already available that target key proteins in NET formation or molecules released upon NETosis, such as gasdermin D, neutrophil elastase, and DNA. Gasdermin D is thought to form pores in the nuclear and plasma membrane that are important for NET formation [31,32]. Disulfiram is an FDA-approved gasdermin D inhibitor that is used to treat alcohol use disorder. It reduced NETs in a mouse model of acute lung injury as well as in SARS-CoV-2 infected golden hamsters [33]. One registered clinical trial tested disulfiram in mild-to-moderate symptomatic COVID-19, but the results are not yet available ([NCT04485130](#)). The neutrophil elastase inhibitor Sivelestat was shown to improve lung function in several animal models of lung injury and can mitigate acute lung injury associated with acute respiratory distress syndrome (ARDS) and sepsis [34]. Further, it is approved for use in Japan and South Korea to treat ARDS, but it did not improve survival after analyses of clinical trials. It has been frequently suggested as a potential COVID-19 therapeutic [35]; however, there are no ongoing clinical trials testing this therapeutic at the time of writing of this manuscript. Several other neutrophil elastase inhibitors are currently in development, including ionelostat and alvelestat that may be tested in COVID-19 infection or other infections. DNase I is an attractive therapeutic option to degrade DNA and disrupt the chromatin structure of NETs. Recombinant DNase I (dornase alfa) is used in the treatment of cystic fibrosis, and has also been previously used in the management of ARDS [36]. Several studies have shown its effectiveness in the management in COVID-19-associated ARDS as well [37,38]. A very recently published study showed the efficacy of combined administration of DNAase plus the IL-6 receptor antagonist tocilizumab and a JAK-1/JAK-2 inhibitor baricitinib to prevent mortality in a group of patients with COVID-19 severe respiratory failure [39]. Thierry and Roch noted that DNase treatment may also need adjunctive therapies to remove toxic proteins such as naked histones. Indeed, elevated circulating histones are reported to associate with coagulopathy and mortality in COVID-19 [40].

Another potential avenue to impact NET formation is blockade of either PRR or cytokine/chemokine signaling pathways that trigger NETosis. This is especially attractive, as pathways involving Toll-like receptors (TLR) and C-type lectins also signal to produce inflammatory cytokines that can lead to a cytokine storm in viral infection. Thierry and Roch suggested that targeting the spleen tyrosine kinase (Syk)-coupled C-type lectins CLEC2 and CLEC5A may be useful as a COVID-19 therapeutic. Both of these lectins were recently shown to trigger NETosis in response to microbes [41]. CLEC5A is abundantly expressed on neutrophils and was previously shown to bind to the dengue virus virion and lead to dengue hemorrhagic fever and dengue shock syndrome [42]. CLEC5A forms a heterodimer with TLR2, with each binding to a distinct portion of the microbial surface. Blockade of a CLEC5A/TLR2 heterodimer leads to attenuation of NET formation in dengue virus infection [41]. CLEC2 is expressed on platelets and to a lesser extent on neutrophils. It has been shown to interact with DC-SIGN to mediate capture of HIV-1 by platelets [43], and was also recently shown to be a PRR for dengue virus [41]. The SARS-CoV-2 spike protein has 22 N-linked glycans [44], but it is unknown whether SARS-CoV-2 particles bind to (Syk)-coupled C-type lectins and trigger NETosis. The spike and nucleocapsid proteins were shown to trigger NET formation, but CLEC interactions were not directly tested [45].

Several chemokine-targeting drugs that affect NET formation have been proposed as treatments for COVID-19 and are in various stages of testing. IL-8 can trigger NET formation by binding to CXCR2 on neutrophils; thus, an anti-IL-8 monoclonal antibody (Humax IL-8) was tested in a clinical trial ([NCT04347226](#)). However, the study was terminated early due to a lack of a significantly different outcome from standard of care for hospitalized COVID patients with severe respiratory disease. An additional phase 2 study of a noncompetitive allosteric inhibitor of CXCR1 and CXCR2 (Reparixin) showed a lower rate of clinical events in COVID patients [46]. A phase 3 trial is currently

being conducted using Reparixin ([NCT04878055](https://doi.org/10.1186/1745-7256-13-10)). The IL-1 receptor antagonist anakinra is currently being tested as a treatment that for COVID-19 with an assessment of NETosis as an endpoint ([NCT04594356](https://doi.org/10.1186/1745-7256-13-10)). A summary of the therapies and pathways targeted in regards to NETosis are shown in Figure 1.

Many unresolved questions remain in regard to NETosis in SARS-CoV-2 infection. Are patients that had severe COVID-19 symptoms and high levels of NETs more likely to develop autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis in the future? Viral infections including herpesviruses, hepatitis B and C viruses, and enteroviruses are associated with the development of autoimmunity, although the virally induced mechanisms that lead to a breakdown of self-tolerance are far from completely understood [47]. It is possible that unchecked NETosis in viral infection could impact the development of autoimmune tendencies. The link between dysregulated NETosis and autoimmune diseases such as SLE, antineutrophil cytoplasmic antibody vasculitis, and rheumatoid arthritis has been reported in both animal models and clinical studies [48]. In one recent systematic review, new-onset autoimmune disease after COVID-19 infection was analyzed. The most common diseases reported were vasculitis and arthritis, although there was a wide spectrum of autoimmune diseases reported [49]. Another important question is whether people who experienced mild-to-moderate symptoms when infected with SARS-CoV-2 also had dysregulated NETosis (albeit to a lesser extent) that also results in the development of autoantibodies. While detailed analyses of the persistence of NETosis markers in COVID patients has not been reported, one study showed that citrullinated histone H3, cell-free DNA and neutrophil elastase were at the same levels as healthy controls four months postinfection [21]. Many patients who have recovered from COVID-19 report persistent symptoms and sequelae long after the primary infection, commonly referred to as ‘long COVID’ [50]. It will be important to study these patients and examine for the presence of residual NETs and autoantibodies that could be responsible for symptoms.

### Data Availability

Data sharing is not applicable to this manuscript.

### Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

### Funding

NIH NHLBI R00HL146888 (to E.B.T.) and NIH NIGMS P20GM104357 to the UMMC Department of Physiology and Biophysics.

### CRedit Author Contribution

**Erin B. Taylor:** Conceptualization, Writing—original draft, Writing—review & editing.

### Abbreviations

ARDS, acute respiratory distress syndrome; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAD4, protein arginine deiminase; PRR, pattern recognition receptor; TLR, Toll-like receptors.

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