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REVIEW

Neutrophil Extracellular Traps: An Emerging Therapeutic Target to Improve Infectious Disease Outcomes

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Neutrophils possess a diverse repertoire of pathogen clearance mechanisms, one of which is the formation of neutrophil extracellular traps (NETs). NETs are complexes of histone proteins and DNA coated with proteolytic enzymes that are released extracellularly to entrap pathogens and aid in their clearance, in a process known as NETosis. Intravascular NETosis may drive a massive inflammatory response that has been shown to contribute to morbidity and mortality in many infectious diseases, including malaria, dengue fever, influenza, bacterial sepsis, and severe acute respiratory syndrome coronavirus 2 infection. In this review we seek to (1) summarize the current understanding of NETs, (2) discuss infectious diseases in which NET formation contributes to morbidity and mortality, and (3) explore potential adjunctive therapeutics that may be considered for future study in treating severe infections driven by NET pathophysiology. This includes drugs specifically targeting NET inhibition and US Food and Drug Administration–approved drugs that may be repurposed as NET inhibitors.

Keywords. neutrophil extracellular traps; neutrophil activation; NETosis; innate immunity; inflammation.

In the recent pandemic, early coronavirus disease 2019 (COVID-19) phenotypes characterized by persistent and excessive inflammatory responses posed significant challenges to clinical management, as evidenced by high fatality rates. Autopsies revealed the presence of neutrophilic infiltrates and fibrin deposition in lung airspaces [1] and blood vessels [2]. Additionally, emerging research has uncovered crucial, yet underrecognized mechanisms of immune dysregulation, specifically the increased release of neutrophil extracellular traps (NETs) by circulating and infiltrating neutrophils in COVID-19 patients [3, 4]. Collectively, these findings suggest that NETs and their associated collateral damage, such as lung epithelial injury [5] and the induction of immunothrombosis, could be key drivers in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) immunopathology and host damage. While present in SARS-CoV-2, this vicious cycle of NET-induced dysregulated inflammation and coagulation is also present in other infectious disease states. This underscores the pressing need for more comprehensive data and therapeutic approaches targeting these innate immune components in treating systemic infections.

NEUTROPHIL MECHANISMS FOR PATHOGEN KILLING

When neutrophils encounter pathogens, they can phagocytose (engulf) them and eliminate them by fusion with their cytoplasmic granules containing proteases, defensins and other antimicrobial peptides, or reactive oxygen species (ROS) [6]. To tackle larger pathogens, neutrophils can also create NETs [6], web-like structures composed of DNA, histones, and antimicrobial proteins that entrap and neutralize invading microorganisms.

During the acute phase of infection, neutrophils leave the bloodstream to target tissues, guided by chemokine gradients and chemical signals [7, 8]. These signaling molecules activate neutrophils, as observed with CXCL8, which triggers ROS production and induces L-selectin shedding [9]. Other inflammatory mediators like complement component C5a also contribute to neutrophil recruitment to infection sites [7] and activation of NET formation when primed by interferons [8]. Activated platelets may also guide neutrophil migration into inflamed tissue and induce NET formation [10]. Immunothrombosis is a bidirectional process between the innate immune system and coagulation that is initiated by tissue factor (TF), a high-affinity receptor that is expressed on perivascular and immune cells (including neutrophils). In response to blood vessel damage or inflammatory mediators (eg, endothelial cells or leukocytes), TF acts as a cofactor to activate the extrinsic pathway of the coagulation cascade that leads to

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the activation of factor X (the common pathway), which ultimately produces thrombin. In the course of combating infection, innate immune cells detect pathogen-associated molecular patterns (eg, bacterial lipopolysaccharides) via pattern recognition receptors (eg, TLR4), resulting in TF release that activates the coagulation cascade [11]. The end product is thrombin, which not only converts fibrinogen to fibrin but also activates platelets. The creation of a fibrin mesh that entraps pathogens is further enhanced by the presence of activated platelets that amplify inflammatory processes through the release of cytokines [11]. The latter attracts immune cells, including neutrophils that release NETs, thereby further provoking "immunothrombosis" in blood vessels. However, intravascular NET formation can lead to the occlusion of microvessels independently of fibrin, resulting in severe systemic inflammation and massive cell death in affected areas.

Maintaining a balance between effectively eliminating pathogens and limiting overactive responses that can harm vascular health is a complex challenge for neutrophils. Factors such as tissue-specific microenvironments, the microbiome, and pathological conditions, along with intrinsic factors like the expression of aging-regulating receptors (eg, CXCR2 and CXCR4), regulate neutrophils that favor a proinflammatory phenotype [12].

NETosis: A KEY INNATE IMMUNE SYSTEM PROCESS WITH COLLATERAL HOST DAMAGE

NET formation is a tightly regulated and multifaceted process for trapping and killing microbes and tumor cells, enhancing immune responses, and promoting coagulation [13]. NET formation is initiated by innate immune receptors and influenced by the local environment, cytokines, and pathogen size and virulence factors. Two main pathways have been described:

1. Slow, lytic pathway: This pathway involves the assembly of NETs intracellularly, followed by slow cell membrane rupture.
2. Fast, nonlytic pathway: In this pathway, nuclear chromatin is expelled rapidly along with degranulation and the release of prestored granule proteins. These components are then assembled extracellularly, leaving behind an anucleated cell capable of phagocytosis, aptly referred to as a "zombie neutrophil" [13].

NETosis BIOMARKERS

NETs consist of an extracellular network of DNA, oxidants, and proteolytic enzymes of both cytosolic and granular origin. These include neutrophil elastase, myeloperoxidase (MPO), PAD4, cathepsin G, gelatinase, lysozyme C, leukocyte proteinase 3, lactoferrin, defensins, calprotectin, cathelicidins, HMGB1, actin, and histones. Circulating surrogate markers of NETs have been identified in plasma, such as complexes of DNA and MPO, citrullinated histone H3, cell-free DNA, and neutrophil elastase [14]. Markers of endothelial injury induced by NETs include von Willebrand factor and its protease, ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin motifs 13). These markers have been used to risk-stratify the prognosis in COVID-19, where significant morbidity and mortality have been associated with NET-driven pathophysiology [15].

THE ROLE OF NETs IN THE PATHOPHYSIOLOGY OF INFECTIOUS DISEASES

Elevated levels of circulating NETs have been associated with poor clinical outcomes in patients with sepsis [16]. NETs have also been demonstrated in cases of severe bacterial pneumonia [17]. The bacteriostatic rather than bactericidal effects of NETs and induction of inflammation have led to dynamic discussions regarding the risk-benefit of NETosis to the host [18]. DNA, a key component of NETs, exerts direct antibacterial activity through membrane disruption and chelation of essential cations [18]. DNA also facilitates complement-mediated killing of bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [19]. While nearly all bacteria can elicit NET formation, some organisms have evolved strategies to evade NETs, including NET degradation, resistance to the antimicrobial components of NETs, or by inhibiting NET formation altogether. For a comprehensive understanding of the interaction between different bacterial species and host NETs, we refer readers to the excellent review by Schultz et al, which summarizes current knowledge on several bacterial species [18].

NET release also plays a significant role in the pathogenicity of severe respiratory viral infections [20, 21]. Studies have shown elevated plasma NET release in patients with influenza, which can increase the permeability of alveolar epithelial cells. The extracellular histones of NETs have been detected in nasal aspirates of influenza-infected patients. While histones have been shown to inhibit influenza in vitro, they may exacerbate acute lung injury. In a mouse influenza infection model, treatment with antihistone antibodies resulted in a significant reduction in lung injury, suggesting that targeting NETs, especially their histone component, could be a potential therapeutic approach for severe influenza [18]. High levels of NETs correlate with poor prognosis of severe influenza A infections [22]. NET release has also been observed in respiratory syncytial virus (RSV) infections, and in the lungs of children with severe RSV infection, resulting in airway obstruction [21].

Dental caries and periodontal disease are the primary source of most viridans streptococcal endocarditis [23]. Periodontal disease is a complex and multifactorial condition characterized by dysbiosis between the gingival microbiome and the host immune response, in which NETs play an integral role. In patients with periodontal disease, there is a significant increase in total and apoptotic neutrophils in the gingival tissue compared to those without the disease. The increased presence of NETs is

believed to result from multiple factors, including impaired NET degradation and bacterial escape from NET-mediated antibacterial effects. Consequently, neutrophils become trapped in the local tissues, leading to augmented tissue destruction [24].

The interaction between *S aureus* and neutrophils is a dynamic process with significant implications for various infections, including endocarditis and pneumonia. *Staphylococcus aureus* has multiple mechanisms to both induce and evade NET-mediated killing, contributing to catastrophic pathophysiology in the bloodstream and respiratory tract in the most severe infections. NET induction by *S aureus* is mediated by several mechanisms: (1) surface lipoproteins via TLR2/1; (2) platelet activation and aggregation via binding to aIIbb3 integrin, FcgRIIa, or GPIBa binding mediated through microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) clumping factor A, fibronectin binding protein A and B, protein A, and iron-regulated surface determinant B; (3) α-toxin-mediated platelet release of β-defensins; and (4) Panton-Valentine leukocidin and other exotoxins [25].

Severe *S aureus* pneumonia can develop following influenza infection, and this association is not specific to any particular virulence factor. It is believed that during influenza infection, a significant number of neutrophils are recruited into the alveolar space. Subsequently, coinfection with *S aureus* "ignites" these neutrophils to undergo NET formation. Interestingly, surfactant, which is mainly (80%) composed of phospholipids and produced by type 2 alveolar cells, can inhibit NET formation. This suggests that surfactant acts as a local regulator, mitigating the inflammatory consequences of NETosis and possibly controlling excessive inflammation. Damage of type 2 alveolar cells in severe *S aureus* pneumonia can undermine this important defense, further exacerbating tissue injury. We refer readers to an excellent summary of the pathophysiology of postinfluenza pneumonia [26].

NETosis also plays an integral role in the pathophysiology of *S aureus* endocarditis, revealing the intricate interplay between bacteria, immunity, and coagulation [27]. Platelets are rapidly recruited to sites of vascular injury and activated by von Willebrand factor and collagen released by endothelial cells. The activated platelets recruit additional platelets and attract neutrophils to the site of endovascular infection. This interaction between platelets and neutrophils triggers NETs and formation of vegetations. *Staphylococcus aureus* possesses several virulence factors that enable it to defend against potential NET-mediated killing within the vegetations. These factors include adherence proteins like Eap, serine protease EpiP, and nucleases nuc1 and nuc2. This complex microenvironment within the vegetation serves as a battleground where *S aureus* attempts to manipulate NET formation while simultaneously safeguarding itself from being entrapped and killed by NETs. We refer readers to an excellent review by Meyers et al further describing the complex relationship of *S aureus* and NETosis [27].

COVID-19

NET formation has emerged as a pivotal factor in contributing to the dysregulated inflammatory responses, immunothrombosis, and organ damage commonly observed in severe COVID-19 cases. Immunothrombosis in COVID-19 has been attributed to complement and TF-enriched NETs [28]. Elevated levels of NETs have been identified in plasma, tracheal aspirates, and lung autopsy tissues from COVID-19 patients [5]. NETs have also been found to infiltrate the vascular, airway, and interstitial compartments in lungs of patients with SARS-CoV-2 infection.

Research on SARS-CoV-2–mediated NETosis suggests its dependence on viral replication, angiotensin-converting enzyme, PAD-4, and serine proteases [5]. Neutrophils collected from patients hospitalized with SARS-CoV-2–related respiratory distress exhibit heightened NET release, increased ROS production, and enhanced phagocytosis compared to healthy controls [29]. The aberrant neutrophil responses characterized by excessive NET release significantly contribute to the pathophysiology of severe COVID-19, making them attractive targets for therapeutic intervention to lessen inflammation and tissue damage. Clinical trials have investigated the role of anakinra, an interleukin 1 receptor antagonist, in mitigating NETosis. In 2022, an emergency use authorization was issued for the use of anakinra in patients hospitalized with severe SARS-CoV-2 based on the SAVE-MORE trial (NCT04680949). Our group has also demonstrated a significant attenuation of NETosis with intravenous immunoglobulin (IVIG) [3]. The efficacy of IVIG has shown mixed results in therapeutic trials, possibly due to its benefits being limited to a subset of younger patients with severe disease treated early in their course [30].

Dengue Fever

Dengue fever is associated with dysfunctional innate immune responses leading to excessive inflammation, which can result in severe illness and death. The presence of NET components has been detected in serum samples of patients with more severe forms of dengue, including hemorrhagic fever [31]. In the context of acute infection, NET formation has been shown to be triggered by dengue virus envelope protein domain III (EIII) both in vivo and in vitro. These processes may involve the NLRP3 inflammasome, as evidenced by the suppression of dengue virus EIII-induced NETosis with the use of NLRP3 inflammasome inhibitors. Additionally, NLRP3 knockout mutant mice challenged with EIII exhibit reduced NETosis [32].

Malaria

Malaria remains a significant public health concern in developing countries, causing considerable morbidity and mortality. Neutrophils play a key role in the host response to malaria, but they can also drive inflammation, which may exacerbate the disease [33]. Levels of circulating NETs correlate with

malaria disease severity [34]. In this context of malaria, heme has been identified as a trigger for NET formation in tumor necrosis factor α–primed neutrophils through the activation of protein kinase C, similar to observations in sickle cell disease. Subsequent degradation of NETs by DNase I releases NET components that induce inflammation. For instance, in macrophages, NET components promote the induction of granulocyte colony-stimulating factor, while upregulating ICAM-1, an endothelial cell cytoadhesion protein that binds infected red blood cells. This process leads to the sequestration of cells in the microvasculature, contributing to end-organ damage, particularly in the brain and lungs [35].

POTENTIAL ADJUNCT TREATMENT OF INFECTIOUS DISEASES BY ADDRESSING NETOSIS

Antibiotics

Macrolide antibiotics are particularly well known for their effects in attenuating neutrophil responses, especially in neutrophilic lung diseases like chronic cystic fibrosis pneumonitis. Macrolides have been shown to decrease levels of interleukin 8, a potent neutrophil chemoattractant and stimulant, which contributes to the modulation of NETosis [36]. Studies have demonstrated that pretreatment of neutrophils with azithromycin reduces the release of NETs induced by phorbol 12-myristate 13-acetate [37]. These effects of macrolides in inhibiting NETosis are associated with survival benefits in diseases where NETosis plays a significant role in the pathophysiology, such as cystic fibrosis, sepsis, and pneumonia [38–40].

In a murine model of chronic obstructive pulmonary disease (COPD), erythromycin reduced NETs in the bronchoalveolar fluid of mice chronically exposed to cigarette smoke, a known trigger of NETosis [41]. Erythromycin suppressed ex vivo human neutrophil production of NETs induced by cigarette smoke in COPD patients [41]. In an observational, multicohort study, NETs were identified as a key marker of disease severity and treatment response in bronchiectasis [42]. This study also revealed that low-dose azithromycin was associated with a significant reduction in NETs in sputum from patients with bronchiectasis and asthma over a 12-month period [42]. Although further studies are needed, azithromycin's therapeutic role in neutrophilic airway diseases [42–44] and evidence supporting its direct inhibition of NET production in vitro and in vivo [37, 41] offer promise to the drug's potential therapeutic impact on patients suffering from NET-related diseases.

In the quest for antibiotics to treat multidrug-resistant, gram-negative infections, carbapenem-resistant *Acinetobacter baumannii* (CRAB) has proven particularly challenging. However, macrolides seem to offer significant activity that is not fully captured in standard antimicrobial susceptibility assays [45]. In this context, clarithromycin stands out as a potential contributor to treatment of CRAB infections, as it has been shown to effectively reduce NETs (and consequently, inflammation) in *A baumannii* infection [46].

NONANTIBIOTIC DRUGS THAT INFLUENCE NETOSIS

HMG-CoA Reductase Inhibitors

HMG-CoA (3-hydroxy-3-methylglutaryl–coenzyme A) reductase inhibitors (statins) are widely prescribed lipid-lowering medications used in the primary or secondary prevention of coronary heart disease. Statins possess anti-inflammatory properties that involve the modulation of NETosis through mechanisms still being studied [47]. There is growing evidence supporting their potential as adjunctive therapy for infections. A pilot randomized trial demonstrated that simvastatin improved clinical outcomes in patients with pneumonia [48], and a meta-analysis of several studies points to a possible benefit of statins in COVID-19 [49], both conditions where NETosis plays an important pathophysiological role. Statins were shown to augment the antistaphylococcal activity of neutrophils by promoting NET formation, and patients whose statin treatment was continued during hospitalization for *S aureus* bloodstream infection showed a 54% reduction in 30-day mortality compared to those whose statin was discontinued [50].

P2Y12 Inhibitors (Ticagrelor/Clopidogrel)

Ticagrelor has been successfully used to clear an endovascular *S aureus* infection that was refractory to even salvage antibiotic therapy, with the added benefit of restoring normal platelet counts [51]. Clopidogrel has been associated with reduced mortality in patients with *S aureus* bacteremia [52]. In addition to its effects on platelets, ticagrelor has been shown to attenuate NETosis [53]. NET inhibition may have advantages in patients after myocardial infarction, placements of intracardiac stents, and other vascular pathologies [54], as well as in protecting the host from adverse outcomes in infection. Moreover, ticagrelor has been associated with reduced infection-related mortality in post hoc analyses of cardiovascular clinical trials [55].

Intravenous Immunoglobulin

IVIG has been shown to attenuate NETosis in a dose-dependent manner [3]. Additionally, IVIG has shown potential benefits in infections where NETosis plays a significant role in the disease pathophysiology, such as sepsis [56, 57]. In the context of COVID-19, the effectiveness of IVIG has been variable, as some smaller studies reported improved clinical outcomes while others did not, suggesting a heterogeneous disease where only certain subsets of patients may benefit from IVIG treatment [3, 30, 58].

Metformin

Metformin, a drug commonly used to treat type 2 diabetes, has been shown to blunt NETosis, translating into promising results

in diabetic patients with COVID-19. Further studies investigating the impact of metformin on clinical outcomes in diabetic patients with infections characterized by NETosis-driven pathophysiology are particularly intriguing [59, 60].

Miscellaneous Drugs

Several hormones and drugs have demonstrated the ability to inhibit NETosis, providing potential therapeutic options for various diseases. Progesterone [61], raloxifene [62], propofol, nutrilipid [63], β-2 adrenoceptor agonists [64], and disulfiram [65] are among the substances that have shown inhibitory effects on NET formation. In addition to these agents, DNA-targeted treatments have also been explored to reduce NETs in the lung [66, 67]. Long-acting nanoparticle DNase had shown promising results in suppressing neutrophil activities triggered by SARS-CoV-2 [66], and nebulized DNase (dornase alpha) has been investigated in a case series of ventilated SARS-CoV-2 patients and was found to decrease the fraction of inspired oxygen [67]. Ongoing clinical trials exploring the use of DNase treatments in patients with SARS-CoV-2 offer the potential for further insights and therapeutic options (NCT04541979).

Colchicine, the well-known tubulin inhibitor, has been shown to inhibit NETosis and ameliorate lung injury in an animal model of acute respiratory distress syndrome [68]. Additionally, it has been found to improve cardiac remodeling following acute myocardial infarction [69]. Recently, a new-generation tubulin inhibitor called sabizabulin has shown great promise in a randomized clinical trial involving COVID-19 patients. The trial reported significant relative reductions in death (55%), intensive care unit days (43%), mechanical ventilation (49%), and overall hospital stay (26%) among the treated patients [70]. These compelling results led the data and safety monitoring board to recommend early termination of the trial due to the drug's favorable findings [70]. However, despite the

Table 1. Summary of Clinical Relevance of NETosis in the Pathophysiology of Disease

Infectious Diseases	Autoimmune Disease	Pharmaceuticals That Modulate NETosis
Influenza pneumonia	ANCA vasculitis	Macrolides
RSV pneumonia	Systemic lupus erythematosus	HMG-CoA reductase inhibitors
SARS-CoV-2		Ticagrelor
Dengue fever		Intravenous immunoglobulin
Malaria		Metformin
Dental caries		Progesterone
Staphylococcus aureus		DNAse
Pneumonia		Propofol
Bacteremia		Nutrilipid
Endocarditis		Colchicine
		Sabizabulin

Abbreviations: ANCA, anti-neutrophil cytoplasmic antibody; HMG-CoA, 3-hydroxy-3-methylglutaryl–coenzyme A; NET, neutrophil extracellular trap; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

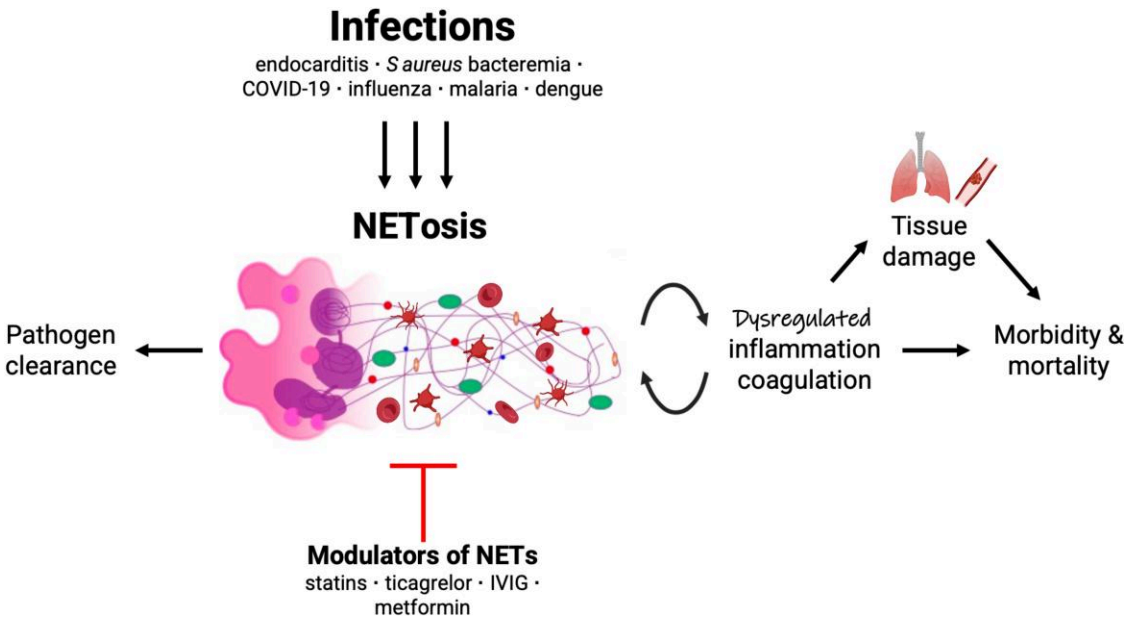

Figure 1. Cartoon schematic depicting the activation and release of neutrophil extracellular traps in a process known as NETosis in response to infections that may facilitate pathogen clearance. However, neutrophil extracellular traps may lead to dysregulation in the host inflammatory and coagulation responses, resulting in tissue injury and death. Several drugs available today have been shown to attenuate NETosis and therefore may be repurposed as adjunctive therapies to reduce morbidity and mortality in diseases where NETosis plays a role in pathogenesis. Abbreviations: COVID-19, coronavirus disease 2019; IVIG, intravenous immunoglobulin; NETs, neutrophil extracellular traps. Figure was created in part with BioRender.com.

promising outcomes, the future development of sabizabulin may face regulatory challenges due to statistical limitations associated with the small size of the study.

CONCLUSIONS

This review discusses NETs in various disease states and the potential pharmaceutical agents that can modulate NETosis (Table 1). NETs are produced by neutrophils by different mechanisms as a host response to clear pathogens, but they can also cause considerable collateral damage to the host in certain situations (Figure 1). The translation of science into clinical practice becomes increasingly challenging as we appreciate the heterogeneity of infectious diseases across different hosts, different pathogens, and even different strains within a bacterial species, each expressing different virulence factors that influence the host–pathogen interaction. However, the literature suggests that tilting the NETosis balance in favor of the host may be a promising approach to reduce morbidity and mortality from serious systemic infection when used as adjunctive therapy alongside antibiotics. Developing reliable biomarkers for NETosis and repurposing existing drugs with NET-inhibiting properties could be crucial first steps in developing effective therapies, opening the pathway for discovery programs seeking new, more selective agents. One of the many lessons learned from the COVID-19 pandemic is that ameliorating the host response may often be more beneficial to patient outcomes than targeting the pathogen itself. Considering NETosis as an integrated systems process across host physiology can improve our understanding of the host response to infection and lead to the development of better drugs to improve patient outcomes.

Notes

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