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Consequences of Extracellular Trap Formation in Sepsis

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Abstract

Purpose of Review—This review will focus on *in vivo* findings derived from animal models of sepsis regarding the trapping role of NETs which is difficult to assess *ex vivo*. The NETotic response of neutrophils at sites of sterile injury or autoimmune disease is destructive as no antimicrobial advantage to the host is realized and dampening NETosis is largely beneficial. In early stages of local infection or in sepsis, the trapping function of NETs may help abscess formation and limit microbial dissemination.

Recent Findings—The trapping function of NETs limits bacterial dissemination keeping an abscess from becoming bacteremic or confining tissue infection to local sites. Once containment is lost and disease has progressed, the best therapeutic approach suggested by animal studies to date is to inhibit PAD4 and prevent NETosis rather than attempting to neutralize caustic NET components. Prognostic value may best be realized by taking cell free DNA, citrulllinated histones, neutrophil function and counts of immature granulocytes into consideration rather than rely on any one measure alone.

Summary—The trapping function of NETs may supercede the value of antimicrobial function in the early phases of sepsis such that degradation of the DNA backbone is contraindicated.

Keywords

NETs; Sepsis; Neutrophils

Introduction

Sepsis is currently defined in 2016 as "*life-threatening organ dysfunction caused by a dysregulated host response to infection*" [1,2**]. This updated definition was put forth by a task force of specialists convened by the Society of Critical Care Medicine and the European Society of Intensive Care Medicine. A clinical scoring system for organ dysfunction is newly provided (Sequential Sepsis-related Organ Failure Assessment; SOFA) allowing for more uniformity in assessment of the septic patient. A clinical distinction is now drawn between sepsis and septic shock wherein septic shock denotes a subset of septic patients

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Conflicts of interest

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with underlying hemodynamic alterations together with cellular/metabolic abnormalities that contribute significantly to mortality.

Sepsis is the leading cause of death of hospitalized patients worldwide [3]. A syndrome of physiologic, pathologic, and biochemical abnormalities induced by infection, sepsis becomes life-threatening when the inflammatory response to the infection persists without resolution and tissue damage progresses to multiple organ failure [2**]. Later stages may include immune suppression and dysfunction [4]. However, there is currently no available FDA-approved treatment and the continued disappointment in devising effective treatments for sepsis was described in a commentary by Derek Angus entitled *"The search for effective therapy for sepsis: Back to the drawing board?"* [5]. In sepsis, neutrophils become systemically dysregulated and mediate much of the morbidity and mortality associated with the disease [6]. The clinical Holy Grail would be to gain iatrogenic fine control of neutrophil functions by dampening tissue destruction while maintaining full antimicrobial capacity.

Initiation of NETosis

NETosis is a neutrophil effector mechanism in which cells extrude a mesh of chromatin fibers complexed with granule-derived antimicrobial peptides and enzymes [7**]. NETs both physically contain microbes within the chromatin network and exert extracellular microbiocidal activity by associated granular enzymes.

NETosis is initiated by activation of Protein arginine deiminase 4 (PAD4) which postranslationally deiminates histone arginine to citrulline. This neutralization of histone positive charge allows for relaxation of bound chromatin. Progression to NETosis includes chromatin decondensation, nuclear translocation of elastase, nuclear envelope disintegration, formation of an intracellular vacuole mixing DNA and granular contents, followed by extrusion through the plasma membrane. The kinetics of "classical" NETosis are slow, lagging hours post-stimulation, require reactive oxygen species (ROS), and culminate in a non-viable cell [8**]. Rapid "vital" NETosis occurs within minutes of stimulation, independent of ROS, and likely leaves a viable enucleated cell still capable of migration and phagocytosis [9**]. Whether NETosis proceeds *via* the "classical" or "vital" path may be a consequence of the ligand inducing the response as well as environmental factors including the presence of extracellular matrix [10,11,8**]. Temporal differences are not likely due to the need for *de novo* gene expression [12]. Given that sepsis is most often polymicrobial with opsonized and non-opsonized microbial ligands proffered to a broad repertoire of neutrophil surface receptors, it is likely that all modes of NETosis are operative within the septic patient.

NETs are "traps" because their physical sequestering function prevents microorganism dissemination. Recently, *in vivo* imaging has provided insight into the containment function of NETs, which may prove to be its essential value in septic host defense. This containment comes at the expense of significant tissue damage, the balance of which must be weighed when considering NETosis as a therapeutic target in the septic patient.

Animal Models of NETs in Sepsis and Systemic Inflammation

Although NETs have been shown to have microbiocidal or microbiostatic activity *in vitro*, DNase release of ensnared microbes was shown to leave these organisms in a viable state [13]. Whereas NET effects on microbial viability and proliferation are amenable to quantification *in vitro*, whether NETosis is a critical component of anti-microbial host defense and important in limiting microbial burden *in vivo* is less straightforward. This section summarizes lessons learned from murine models of infection, sepsis, and endotoxemia regarding the role of NETs [14**–25] (Table 1). No one animal model can recapitulate clinical outcome exhibited by septic patients with variance in predisposing conditions and in disease etiology, severity and progression. The reader is referred to an outstanding review of the essentiality of continued, but wiser, use of murine models, in efforts to understand the biology of critical illness and to identify new therapeutic modalities relevant to human disease [26*].

NETs and Sepsis: When and how to intervene

The following section will describe findings derived from the application of animal models such as cecal ligation and puncture (CLP), bacterial installation or infusion, or the reductionist endotoxemia model has offered insight into the NETotic contribution to the pathobiology of infection and sepsis. As with any novel mechanism underlying a disease with no adequate therapy, NETs present a target for septic treatment. However, animal studies caution that the type and timing of intervention be carefully considered based on the stage of the disease. Therapeutic interventions may be proposed at the level of: (1) inhibiting NETosis; (2) dissociating the DNA meshwork; or (3) neutralization of caustic NET components such as elastase and histones [27*]. Evidence suggests that the beneficial role of NETs as a means to contain microbial dissemination is best realized in localized infections, such as abscess formation or early trapping in the vasculature [9**]. In early infection, NETosis may contribute to microbial sequestration thereby keeping the disease from becoming systemic. At this stage, DNase disruption of NETs would be contraindicated. Once containment is overwhelmed, or the patient responds with a systemic inflammatory response, then intervention may be aimed at neutralizing tissue damaging components of NETs. Moreover, inhibiting NETosis at advanced stages such as with PAD4 inhibitors might prove even more advantageous. Reductionist models of sepsis such as endotoxemia have shown a protective advantage in either the prevention of NETosis or the dissolution of intravascular NETs. Microbiocidal function of NETs does not seem to be a hallmark of host protection during sepsis *in vivo* [7**].

Bacterial Containment

Staphylococcus aureus, among the most common causes of bacteremia worldwide [28], is a potent inducer of NETosis. Staph infections begin locally but have a propensity for dissemination [4]. Yipp *et al.*, discovered that treating a localized abscess with DNase increased bacteremia and diminished skin bacterial content, suggesting that disrupting the NET structure compromises microbial containment [9**].

Neutrophils accumulate within the microcirculation of vascularized tissues during sepsis [29]. In mice challenged with endotoxin or *Escherichia coli*, NETosis is induced in liver sinusoids through a novel mechanism that includes the bridging of activated platelets with neutrophils *via* LFA-1as associated with significant liver damage [14**]. *E. coli*-infected animals had significant bacterial trapping in sinusoid NETs. Similar to the abscess model, infusion of DNase disrupted these NET structures, releasing sequestered microbes into the bloodstream and the lung. *S. aureus* or viral infection of the liver caused similar pathology suggesting that this is a broad spectrum mechanism of host defense [14**].

The production of the PAD4 knockout (ko) mouse by the laboratories of Wang and Mowen offered an important tool for *in vivo* experimental design directed at the enzyme with the best association with NETosis currently known. Neutrophils from PAD4ko mice are impaired in NETosis in response to proinflammatory agents including LPS, PMA, and H_2O_2 [30,31]. *Shigella flexneri* was used to additionally characterize these PAD4ko mice as unable to elaborate NETs to an intact microbe and with significantly impaired extracellular killing [30]. Phagocytosis was similar to wild-type animals demonstrating no impairment by PAD4ko. Therefore these mice are the best tool currently available to interrogate the role of NETosis *in vivo* [23,19,32**].

In a liver sinusoid NETosis model, mice injected with a sublethal dose of methicillin-resistant Staphylococcus aureus (MRSA) produced pronounced liver infection and tissue damage [32**]. DNase treatment removed extracellular DNA in the infected liver but did not reduce tissue damage because histones and proteolytically-active elastase persisted even though clearance of bacteria was maintained. In contrast, NETosis and associated liver damage was greatly reduced in PAD4ko mice. This supports an organ dysfunction etiology that is not a consequence of the organism *per se*, but a result of collateral tissue injury. It remains to be determined if redundant antimicrobial effector mechanisms allow control of sublethal, low level infection, while the trapping and antimicrobial function of NETs gain heightened significance when the infectious challenge is of greater magnitude.

Neutrophil evasion is a microbial virulence mechanism employed by Group A Streptococcus (GAS) M1 in secreting streptodornase D (Sda1), an extracellular DNase that can degrade NETs. Indeed, Sda1 expression degraded NETs from wild-type PMNs, reducing microbial killing to that of PAD4ko neutrophils [33]. Depletion of Sda1 removed this virulence mechanism and restored Streptococcus killing in wild-type neutrophils. In a subcutaneous GASΔSda1 injection model of necrotizing facitiis, PAD4ko mice were significantly more susceptible to infection with larger lesions containing more viable bacteria, likely due impaired NETosis.

Therefore, using both skin abscess and liver sinusoid models of infection, the Kubes lab showed bacterial trapping and containment by NETs and dissemination when NETs were disrupted. These studies support bacterial entrapment rather than bacterial destruction as the primary NET function in infection. That PAD4ko mice were also shown to be highly susceptible to skin infections such as necrotizing fasciitis demonstrates that NETosis is also fundamental to the surveillance function of the immune system limiting colonization by normal flora [33]. Neutrophils possess numerous bacteriocidal mechanisms and NETs may

contribute to killing efficiency to some extent but may be redundant -cidal means as compared to the unique mode of trapping offered by NETs [34,33].

Cecal Ligation and Puncture

To determine the role of PAD4 in protection from polymicrobial sepsis, wild-type and PAD4ko mice were subject to CLP [19]. The absence of PAD4 had no effect on either survival or bacteremia in a low-grade infection model with 20% mortality, albeit with a narrow observation window. More compellingly, PAD4ko mice showed no survival difference in in a high-grade infection, inducing 80% mortality. However, a slight survival benefit was reported in wild-type compared to PAD4ko when exposed to high-grade CLP with antibiotic treatment; bacteremia was not affected. Cl-aminidine, a pharmacological inhibitor of PAD activity, was shown to improve survival in a CLP model with 50% mortality [35].

Repeated delivery of DNase into the peritoneal cavity in a CLP model with 80% mortality showed a transient increase in mortality at 24h but no overall survival difference [24]. This supports the trapping utility of NETs early in the course of disease by limiting dissemination. Another report using a severe model of CLP that resulted in 100% mortality by 48hr, DNase did not improve long-term survival [15]. A minor survival benefit was seen with antibiotic treatment, however, a significant (50%) survival benefit and improved organ function was seen in animals receiving both antibiotic and DNase, in combination. Administration of DNase to animals receiving LPS as a model of endotoxemia had a notable survival advantage with diminished organ pathology. These data using endotoxima or antibiotic support to minimize the impact of NET sequestration highlight the deleterious effects of NETs in disease progression.

An interesting consideration regarding the use of the CLP model to understand the role of NETs in sepsis was raised by Sørensen and Borregaard [7**] who questioned whether the serosal surface of the peritoneum is sufficient to allow adherence of NETs and, in turn, containment of bacteria. In this regard, the peritoneal cavity has not as yet been shown to support NET immobilization. Therefore, findings to date merit cautious interpretation.

Histones released as a component of extracellular chromatin are directly toxic to endothelial cells and contribute to vascular dysfunction, organ injury, and death in sepsis [25]. Anti-histone antibodies protected mice from mortality following TNF, LPS, or CLP challenge [23,25]. Administration of purified histones to naïve animals mimicked many hyperinflammatory hallmarks of sepsis including neutrophil margination, damaged endothelium, intra-alveolar hemorrhage, and thrombosis. Subsequently, histones were shown to activate platelets and inhibit activation of protein C leading to thrombosis [36].

Another study using the CLP model showed a survival benefit after infusion of anti-histone H4 antibody but, as in the case of DNase therapy, survival was only improved with antibody was given together with antibiotic [25]. Using LPS infusion at a dose to cause 80% mortality, anti-H4 antibody reduced mortality to 20%. It may be of significance in terms of therapeutic design that in order for the antibody to be most effective, a 6 hour delay of

treatment with both antibiotic and anti-histone antibody relative to CLP was incorporated into the treatment protocol, perhaps because of the antimicrobial functions of histones in innate immunity. No such delay was needed in the LPS model to observe a survival benefit [25]. The excitement raised by these studies identified extracellular histones as a therapeutic target in sepsis and other hyperinflammatory diseases. However, removal of NETs by DNase, or neutralization of histones, did not protect the liver vasculature as effectively as prevention of NETosis as in PAD4 or Elastase knockout mice [32**]. This work offers experimental evidence for the therapeutic advantage of preventing NETosis rather than dissociating or blocking tissue-damaging components of NETs after exocytosis.

Intervention and Prognostic Opportunities

Interventional strategies to block NETosis would be counterproductive if they caused overt immune suppression or subverted other neutrophil effector functions during a case of active sepsis, particularly in the case of sepsis where infection is pathognomic of the disease. Therefore, an "Ideal" NET inhibitor should prevent trap release but preserve other antimicrobial effector functions [37]. Therapeutic approaches regarding specific attenuation of NETosis during sepsis includes: 1. Infusion of anti-citrullinated antibodies [25,38], 2. blocking platelet:PMN interactions *via* the peptide MKEY and anti-Mac1 antibodies [39]; 3. An interesting approach towards preventing NETosis while preserving neutrophil function is the finding of Van Avondt *et al.* that cross-linking the signal inhibitory receptor on leukocytes-1 (SIRL-1) obviating NET release from opsonized as well as nonopsonized *S. aureus* [40]. Additionally, indirect targets such as Peroxisome proliferator-activated receptor gamma (PPARγ) and Phospholipase D2 (PLD2) with modulating effects on NETosis have been considered [16,17]. Temporally appropriate inhibition of NET release may be therapeutically more efficient that attempts to neutralize histones, DNA or other NET components after the cat is out of the bag.

A consequence of a NETotic response is the increased levels of circulating cell free DNA and citrullinated histones which have been pursued for their potential as a clinical biomarker of illness severity and progression [41]. In a retrospective observational study of 80 septic patients, one study showed the cfDNA levels were predictive of intensive care mortality [42]. Further refinements in the technology of objectively quantifying blood cells for evidence of NETosis are ongoing [43]. However, a recent longitudinal study of interest has shown that combined measures of cf-DNA, plasma citrullinated histones, phagocytic capacity of blood neutrophils and number of immature granulocytes, when considered together, was predictive of the development of sepsis in patients as early as one day following severe burn injury [44**].

Conclusion

Physical entrapment within NETs prior to and during sepsis is the predominant beneficial effector mechanism offered by this pathway whereas the contribution of NETosis to microbial killing may be dispensable or redundant with other effector pathways such as phagocytosis and oxidant production. The preponderance of evidence in this regard has been provided by the laboratory of Paul Kubes and collaborators who have maximized the use of

dual laser multichannel spinning-disk confocal microscopy to provide real time *in vivo* evidence of neutrophil function within viable tissues. As such his laboratory has generated a prodigious body of convincing and paradigm-generating data regarding into the host response to injury and infection and much may be extrapolated to the role of NETosis during sepsis. Until a naturally occurring human mutation in PAD4 is identified, it will be difficult to know with certainty the extent to which NETosis is dispensable or redundant in human health.

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Key Points

- Updated definitions and clinical criteria of sepsis should replace prior definitions.
- Containment function of NETs is important during early phases of infection and may limit progression to sepsis and/or sepsis severity. Disruption of NET architecture at this stage should be avoided.
- Animal models of polymicrobial sepsis are only slightly affected by genetic absence of PAD4.
- When taken together, neutrophil function, cfDNA, citrullinated histones and number of immature granulocytes may have prognostic significance in identifying the likelihood of burn patients becoming septic.

Table 1

Effects of NET formation in mouse models of infection

NET inhibition method	Sepsis model	Microbial burden	Survival	Reference
rh DNase	CLP	Increase - blood	Decrease	Czaikoski *et al.* [15]
rhDNase + antibiotic	CLP	Decrease - blood	Increase	Czaikoski *et al.* [15]
PPARγ agonist[a]	CLP	Decrease - peritoneum	Increase	Araújo *et al.* [16]
PLD2$^{-/-}$[a]	CLP	Decrease - blood, BALF, peritoneum, lung, spleen and liver	Increase	Lee *et al.* [17]
DNase	CLP	Decrease - blood, peritoneum and lung[b]	No difference - early treatment increase - late treatment	Mai *et al.* [18]
PAD4$^{-/-}$	CLP	No change - blood, liver and lung	No difference	Martinod *et al.* [19]
Antithrombin affinity depleted heparin	CLP	ND	Increase	Wildhagen *et al.* [20]
DNase	CLP	Decrease - lung (6 h pCLP) Decrease - blood, lung and spleen (24 h pCLP)	ND	Luo *et al.* [21]
DNase	Intranasal administration *Borkholderia pseudomallei*	No difference - BALF, blood, lung and liver	ND	Jong *et al.* [22]
H3cit inhibitors (Cl- amidine, anti-CitH3)	CLP	ND	Increase - Cl-amidine Increase - anti-CitH3	U *et al.* [23]
rhDNase	CLP	Increase — peritoneum and lung (6 h pCLP) no difference - blood, lung, peritoneum and liver [24–40 h pCLP]	No difference[c]	Meng *et al.* [24]
Platelet-depleting serum Dnase LFA1$^{-/-}$ mice	IP injection *Escherichia coli*	Increase — blood and lung	ND	McDonald *et al.* [14]
Anti-H4+antibiotic	CLP	ND	Increase	Xu *et al.* [25]

[a] Increases NET formation and enhances bacterial killing.

[b] Decreases seen in delayed DNase administration (4 and 6 h post CLP).

[c] Increased survival noticed 24 h post CLP that was abolished by 48 h post CLP.

BALF, bronchoalveolar lavage fluid; CLP, cecal ligation and puncture; LFA1, Lymphocyte function-associated antigen 1; ND, not determined; NET, neutrophil extracellular trap.