NEUTROPHIL EXTRACELLULAR TRAPS (NETS): ROLE IN DISEASE

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ABSTRACT

Neutrophil extracellular traps (NETs) are web-like structures composed by a chromatin backbone, histones and antimicrobial proteins. NETs constitute yet another mechanism deployed by neutrophils to immobilise and kill microorganisms, thus contributing to the host innate immunity. Neutrophils cast NETs upon stimulation by a variety of stimuli, including bacteria, protozoa, fungi, viruses, their products and also host factors like chemokines, complement and activated platelets.

NETs production or NETosis occurs as a result of activation of neutrophil PKC, Raf-MEK-ERK and NADPH oxydase signalling pathways. Driven mostly by peptidylarginine deiminase 4 (PAD4) citrullination of histones, the hallmark of NETosis is chromatin decondensation, rupture of nuclear membrane and release of nuclear and granular contents into the cytoplasm, prior their release into the extracellular space. NETs control propagation of pathogens by entrapping them within the lose chromatin web and kills them with the antimicrobial molecules –granule proteins and histones- present in high concentrations within the chromatin network.

Despite contributing to host defence, aberrant NET formation may damage tissues and activate inflammatory cells, contributing to several pathologies, including sepsis, systemic inflammatory response syndrome, autoimmune diseases and thrombosis.

This review presents an overview of our current knowledge of NETs physiology and their role in fighting and propagating disease.

Keywords: Neutrophil extracellular traps; histones; sepsis.

ABSTRACT

Las trampas extracelulares de neutrófilos (NETs, de su sigla en inglés) son estructuras tipo red, compuestas por un esqueleto de cromatina, histonas y proteínas microbicidas. Las NETs constituyen un mecanismo más utilizado por los neutrófilos para inmovilizar y eliminar microorganismos, contribuyendo de esta manera a la inmunidad innata del huésped. Los neutrófilos producen NETs en respuesta a diversos estímulos, que incluyen bacterias, protozoos, hongos, virus y productos derivados; además de factores del huésped como quimiocinas, complemento y plaquetas activadas.

La producción de NETs o NETosis ocurre como resultado de la activación en el neutrófilo de vías de señalización intracelular dependientes de PKC, Raf-MEK-ERK y NADPH oxidasa. El sello distintivo de la NETosis es la decondensación de cromatina, producida principalmente por la citrulinación de histonas catalizada por la enzima peptidyl arginine deiminasa 4 (PAD4). Este evento es seguido de ruptura de la membrana nuclear y liberación del contenido granular del neutrófilo en el citoplasma, seguido de su liberación al medio extracelular. Las NETs inmovilizan patógenos atrapándolos en sus redes de cromatina laxa, controlando así su diseminación, y los eliminan mediante la actividad microbicida de proteínas granulares e histonas, que están presentes en concentraciones elevadas en las redes de cromatina. A pesar

de contribuir a la defensa del huésped, la formación aberrante de NETs puede ocasionar daño tisular y activar células inflamatorias, contribuyendo así a diversas patologías como sepsis, síndrome de respuesta inflamatoria sistémica, enfermedades autoinmunes y trombosis.
Esta revisión presenta un repaso del conocimiento actual sobre la fisiología de las NETs, y su papel combatiendo y desencadenando enfermedades.

Palabras clave
Trampas extracelulares de neutrófilos; histonas; sepsis.

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Introduction

Neutrophils are the most abundant white blood cells, accounting to 60% in normal conditions and represent the first line of defence against infection. Neutrophils present two morphological hallmarks: their distinctive polymorphic nucleus comprising 3-5 lobules, making the nucleus more flexible and allowing neutrophils to extravasate through endothelial gaps to reach sites of infection; and the granules, containing acid hydrolases and antimicrobial peptides. Until recently, the known strategies as key players of the host innate immune response were two: 1) phagocytosis, which involves ensnaring the microorganism in a phagosome and fusion with specialised lysosomes with antimicrobial enzymes that kill the microorganism intracellularly; and 2) degranulation, process that involves antimicrobial molecules release at sites of infection. In 2004 a third strategy was described by *Brinkmann et al*: the release of neutrophil extracellular traps (NETs) [1].
NETs comprise a backbone of extracellular DNA associated with histones and granular antimicrobial components such as the proteases neutrophil elastase (NE), cathelicidins, proteinase 3 and gelatinase; myeloperoxidase (MPO), cathepsin G and lactoferrin [1-3].

NETs formation

NETs formation can be briefly described by a sequence of events comprising neutrophil stimulation, release of granular contents to the cytoplasm and nucleus; nuclear delobulation and nuclear envelope disintegration, chromatin decondensation and finally, cellular membrane rupture and NETs release (**Figure 1**).
NETs are produced in response to a number of stimuli, such as lipopolysaccharide (LPS), cytokines (IL-8) and bacteria, fungi or activated platelets, upon stimulation of their correspondent neutrophil receptors, namely Toll-like receptors (TLRs), cytokine receptors and Fc receptors [1, 4]. Receptor activation is followed by activation of one or more of signalling cascades involving Raf-MEK-ERK pathway activation [5], Ca^{2+} mobilisation from intracellular and extracellular pools [6], PKC activation and assembly of the NADPH oxidase complex followed by the generation of reactive oxygen species (ROS). ROS, in turn, act as secondary messengers in the process that leads to gradual dissolution of the nuclear membrane and the granules, thus facilitating contact of NETs components within the cytoplasm [7]. Finally, NETs are released and, in most cases, the neutrophil dies. The term NETosis was coined for this type of cell death, to differentiate it from apoptosis and necrosis [8]. Unlike apoptosis, NETosis does not exhibit DNA fragmentation not surface exposure of phosphatidylserine prior cell death; and chromatin undergoes decondensation instead of condensation and nuclear fragmentation typical of apoptosis. NETosis also differs from necrosis, which exhibits preserved nuclear membrane and granules.

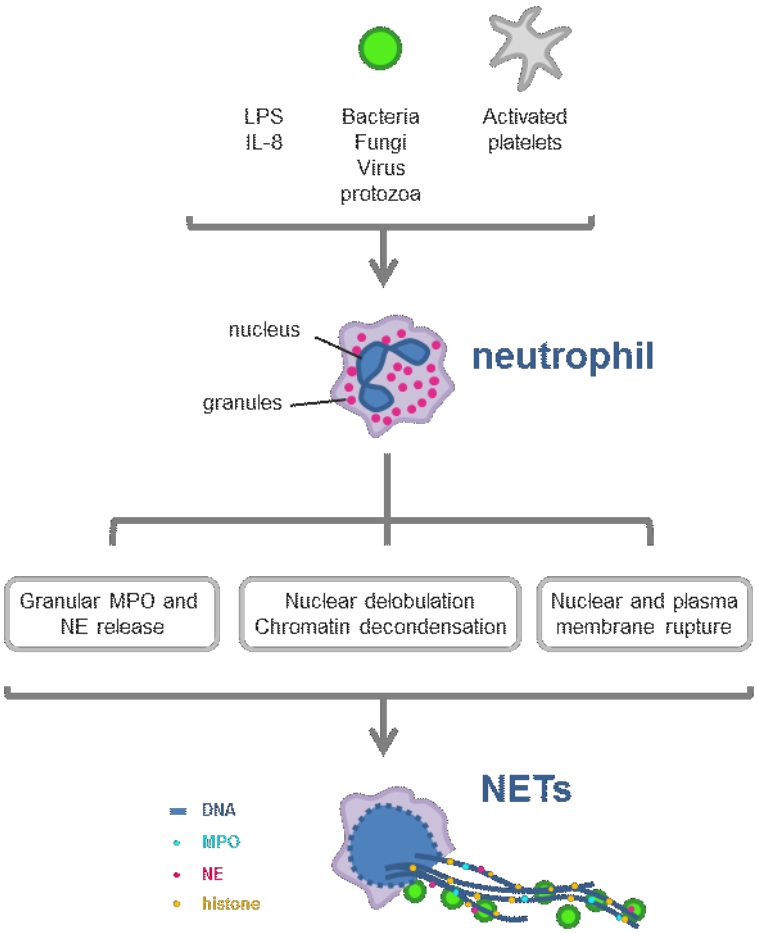

Figure 1 Schematic representation of the key events leading to NETs formation. MPO: myeloperoxidase, NE: neutrophil elastase.

Although several signalling pathways have been described to trigger NETosis, the mechanisms appear to be redundant and despite claims, none of the factors involved proved to act as a crucial step or bottleneck for NETs production. A critical step is, however, the unfolding of chromatin. This reaction consists in histone hypercitrullination and is catalysed by peptidyl-arginine deiminase 4 (PAD4) [9]. This finding led to the publication of several articles highlighting PAD4 activity as a potential target to regulate NET formation. However, the central role of histone hipercitrullination by PAD4 in NETosis, although important, is controversial [10]. For instance, H3cit deficient mice partially retain the ability to form NETs [11] and PAD4 inhibition with Cl-amidine leads in most cases only to partial reduction in NETs production. Interestingly, NE has been shown to modify histones by proteolysis and contribute to NET formation [12].

In addition to the described mechanisms, there is increasing consensus that NET formation might be classified into three different categories:

1. Classical or suicidal NETosis, involves Rac2 and NADPS activation. Rac deficient mice are deficient in NET formation [13] and NADPH deficient animals have impaired NET production [14] and cell lysis [15] via this mechanism. Under this pathway, NET release takes between 1-4 hours.
2. Vital NETosis, nuclear DNA is released by vesicles and is independent from the oxidative burst. This mechanism can occur within 5 min-1 hour [4], faster than suicidal NETosis
3. NETs formed by mitochondrial DNA [16].

Various approaches are used to stimulate neutrophils to study NETosis *in vitro*: phorbol-12-myristate-13-acetate (PMA, a PKC activator), Ca^{2+} ionophores, H_2O_2, IL-8 and LPS are among the most commonly used. By using pharmacological manipulation of neutrophils from human donors it has been possible to establish the contribution of intracellular pathways in NETs production. The exact role of ROS in NETosis is not yet clear, however evidence indicates that at physiological concentrations, the membrane permeable H_2O_2, can initiate NETosis [7] whereas diphenylene iodonium (DPI, a NADPH oxidase inhibitor) [17] and catalase inhibit NET formation [7]. Similarly, ionomycin (a Ca^{2+} ionophore) induces NETosis, whilst chelation of extracellular or intracellular Ca^{2+} with EGTA or BAPTA-AM, respectively, has shown to block NETs production [6]. *In vitro* NETs measurement involves the use of a combination of fluorescent markers for extracellular DNA, such as the cell impermeant DNA marker SYTOX Green; granular proteins, typically MPO or NE; and histones. The simultaneous presence of the three components is preferred in order to fulfil the criteria of NETs definition. The most common methods of detection are multichannel fluorescence microscopy (**Figure 2**) [1] and flow cytometry [1]. Flow cytometry is used to measure NETs formed by cells in suspension and has the advantage of being faster, analyse a large number of cells and being observer independent; whereas fluorescence microscopy can be used on immobilised neutrophils and allows discrimination of necrotic cells from NETs.

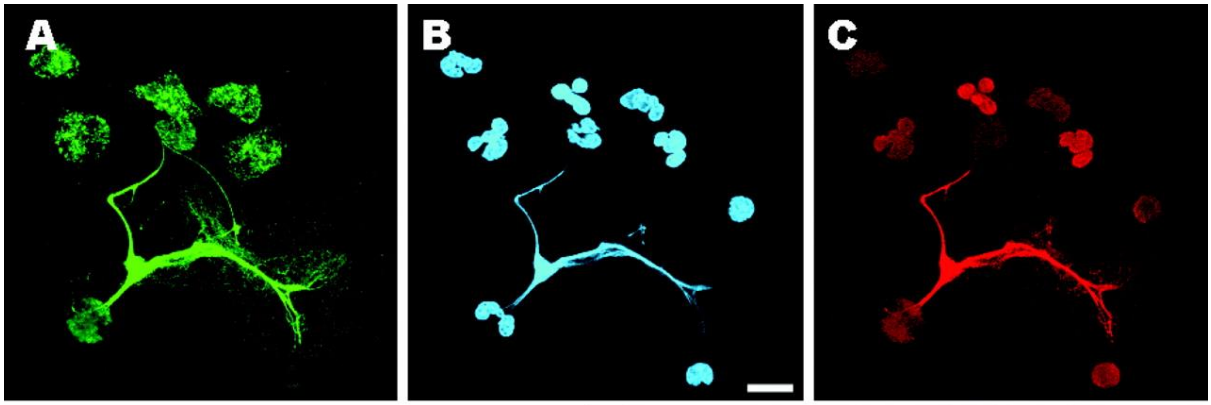

Figure 2 Fluorescence microscopy image of NETs. **A.** Neutrophil elastase, **B.** DNA and **C.** Histones H2A-H2B-DNA complexes. Bar: 10μm. From **Brinkmann V. et al.** [*Science*. 2004 Mar 5; 303(5663):1532-5]. Reprinted with permission from AAAS [1].

Antimicrobial effects of NETs

Since their discovery in 2004, there is increasing and undisputed evidence that NETs act by entrapping and killing microorganisms. One of the main properties of NETs is that they constitute a "sticky" structure to which microorganisms bind to [1, 19-21]. The molecular basis of the binding mechanism is not yet understood, but electrostatic interactions between positively charged NETs components, mainly histones, and negatively charged surface of microorganisms may contribute to the strong physical interaction between NETs and microorganisms. Supporting this notion, the expression of a capsule reduces binding and protects *Streptococcus pneumoniae* against NETs [22].

Once immobilised, microorganisms are susceptible to the action of antimicrobial enzymes and peptides, in an extent and fashion that is unclear and might rely on species susceptibility. Interestingly, not only granular proteins, but also histones, a major component of NETs, and their cleavage products exhibit antimicrobial activity [23, 24].

On one hand, several simple but elegant *in vitro* experiments have provided data confirming bactericidal effect of NETs. NETs associate with *S. aureus* (gram positive bacteria) and

Salmonella typhimurium and *Shigella flexneri* (gram negative bacteria) [1]. This association makes bacterial virulence factors, such as IpaB from *S. flexneri* or α-toxin from *S. aureus*, susceptible to neutrophil proteases like NE; in agreement with the concept that NETs can act by 'disarming' pathogens [1]. Furthermore, activated human neutrophils, in the presence of 10 μmol/L cytochalasin D (to block phagocytosis) significantly decreases *S. aureus* and *S. flexneri* growth by approximately 30%; confirming the bactericidal role of NETs [1, 25]. The underlying mechanism for the ability of neutrophils to kill bacteria comprises the bactericidal permeability increasing protein (BPI) and histones [1]. Histones and derived peptides haven been long shown to kill bacteria [23] with high efficacy, at concentrations as low as 2μg/ml [1].

On the other hand, *in vivo* evidence shows that NETs found in tissues such as lungs and skin contribute to confine bacterial infection in animal models of cellulitis, necrotising fasciitis and pneumonia [11, 19, 20]. Moreover, *in vivo* imaging experiments employing multichannel spinning-disk microscopy on murine models of LPS (endotoxemia) or *Escherichia coli* induced sepsis showed that NETs are formed in the microcirculation of highly vascular organs such as the liver, surmounting the shear forces of the flowing blood [26]. In endotoxemic mice injected with fluorescently labelled *E. coli*, the formation of vascular NETs resulted in a 4-fold increase in the ability to ensnare bacteria from the bloodstream compared with untreated mice [26]. An interesting work from Yipp *et al*. [27] studying Gram-positive skin infections in mice and humans *in vivo* by spinning-disk confocal intravital microscopy, showed that upon *S. aureus* and *S. pyogenes* infection, neutrophils released NETs without cell lysis. In these models, the authors found that neutrophils were able to multitask: carrying out phagocytosis and rapidly NETosis that resulted in limited bacterial dissemination. The presence of anuclear neutrophils containing bacteria, indicating that phagocytosis and NET formation can take place separately in functional compartments of the neutrophil, so that bacteria is retained intracellularly during NETs release. Although loss of nucleus would eventually lead to cell death, enucleation is well described in platelets and red blood cells, which survive in blood for long periods, well beyond the short half-life of ~8h of terminally differentiated neutrophils. This is in agreement with the concept of vital NETosis described by others and suggesting that neutrophil functions do not follow an either-or route, but can act simultaneously or in fast sequence.

A different mechanism is used against fungi, such as Aspergillus nidulans, *Aspergillus fumigatus* and *Candida albicans*. The Zn^{2+} chelator calproctectin, a heterodimer of the neutrophil cytosolic S100A8 and A9 proteins present in NETs, is a critical component of the immune defence against *C. albicans* and *A. nidulans*. Patients with chronic granulomatous disease (CGD), who present deficient NADPH oxidase activity and are therefore impaired in their ability to form NETs, often die of infection with *A. nidulans*. Reconstituting NADPH oxidase by gene therapy rescues NETosis capacity and reduces fungal load in these patients [28].

Likewise, several protozoan parasites and viruses induce NETs. NETosis has been shown upon infection with parasites such as *Leishmania amazonensis* and *Toxoplasma gondii* in which pathogen viability is hampered by NETs. Immobilisation of viruses followed by destruction by MPO and α-defensin is one mechanism employed by NETs during the course of viral infections like influenza and HIV [29]. Hence, NETs facilitate the availability of specialised sets of proteins involved in bacterial, fungal and viral immunity.

Bacterial mechanisms of evasion

While the formation of NETs and their role in host defence were unknown to the scientific community until 2004, several bacterial properties or functions were described before they could be identified as mechanisms of evasion from the action of NETs. Microorganisms express numerous virulence factors to surpass the host's defence mechanisms, produce

bacterial nutrients from host's tissue digestion and simultaneously increase invasive potential. It is widely known that the most virulent bacteria express a repertoire of digestive enzymes, such as proteases, lipases and nucleases. Nucleases production by bacteria such as *Staphylococcus aureus* had been described for a long time [30], yet the specific role of this enzyme was poorly understood until the discovery of NETs. Recently, it has been demonstrated in relevant animal models of infection, that besides increasing necrotising properties, nucleases confer resistance to NET-dependent killing, enhancing the virulence of Gram-negative bacteria like *S. aureus* [25], group A *Streptococcus* [19] and *Streptococcus pneumonia* [20]; indicating that bacteria entrapment by NETs is critical for their subsequent bactericidal action. DNAse production and escape from NETs have also been described in Gram-negative bacteria such as *Neisseria gonorrhoeae*, *Vibrio cholera*, *Yersinia enterocolitica* and *Aeromonas hydrophila* [15],

Besides NETs' backbone digestion, other mechanisms of evasion are used by microorganisms to prevent or escape the action of NETs. The Group A *Streptococcus* (GAS) produces a protease, SpyCEP that cleaves IL-8, decreasing both IL-8-dependent neutrophil endothelial transmigration and bacterial killing by reducing NETs formation [31]. GAS also expresses M1, a fibrillar protein that coats the surface of all clinical isolates and conferes resistance to the antimicrobial action of cathelicidin LL-37, a granular NET component [32]. *Haemophilus influenzae* produces a lipooligosaccharide and DNA based biofilm that confers resistance to the antimicrobial effects of NETs components [33].

NETs and tissue injury

Besides their contribution to innate immunity in host defence, NETs have been also shown to trigger collateral tissue damage and therefore to participate in the pathological mechanisms of several diseases. NETs induced by sterile inflammation induce thrombosis by providing a platform for the coagulation process [34]. It has been shown that histone citrullination by PAD4 is involved in the development of deep vein thrombosis and that both $PAD4^{-/-}$ or PAD4 inhibition with Cl-amidine reduce thrombosis in mice [35]. In acute myocardial ischemia, high levels of circulating cell-free deoxyribonucleic acid (cf-DNA), a NETs marker, have been linked to infarct size [36]. In this case, local myocardial cytokine upregulation induce NETs that promote cardiac micro thrombus and endothelial injury, contributing to myocardial no-reflow [37]. Implementation of a DNase I-based reperfusion treatment strategy attenuates experimental myocardial I/R injury [37]. NETs have been also implicated in autoimmune diseases such as systemic lupus erythematosus (SLE). Anomalous NET formation may be involved in the development of autoimmune responses in predisposed individuals, in agreement with the observation that manifestations of autoimmune responses often follow microbial infections. SLE affects various organs and is characterized by autoantibodies against DNA, chromatin, and DNA-associated proteins, such as NET components. NETs formation promotes autoantibody formation in SLE, and the presence of these antibodies protects degradation of NETs by nucleases and proteases [38], generating an imbalance between NETs formation and clearance that makes SLE patients more susceptible to NETs-induced tissue injury [38]. In sepsis, NETs promote progression towards disseminated intravascular coagulation and septic shock [2, 4]. In addition, liver damage was described 24 hours after the onset of *E. coli* induced sepsis in mice, evidenced by a significant increase of serum levels of alanine aminotransferase compared to non-septic controls [26]. Intravenous infusion of DNAse prevented NETs-induced hepatic injury [26]. Importantly, DNAse treatment improves outcome in sepsis only when it is delayed, so as not to interfere with the physiological role of NETs in targeting the infection [39].

Histone levels in septic patients correlate with episodes of organ failure, and are significantly associated with mortality rates in patients with sepsis [40]. There is also an inverse correlation

between plasma histones and endogenous activated protein C levels (APC), a plasma protease that degrades NETs [40]. Interestingly, administration of APC prevented mortality induced by histone injection in mice [41].
The cytotoxic mechanism of histones involves direct endothelial cell death upon binding to endothelial cell plasma membrane based on positive protein charge [42], although further studies are needed to elucidate their role in disrupting the endothelial barrier. Together, these studies indicate that NETs contribute to disease development and host tissue injury, and highlight the importance of a balanced NETosis-NETs clearance. The disturbances attributed to NETs described above appear to have a common origin in vascular dysfunction, be it disruption of blood flow by accumulation of platelets and red blood cells trapped into the DNA/protein mesh or endothelial injury followed by oedema. In both cases, the final consequence is impaired tissue oxygenation, cell death and organ failure.

Conclusions

NETs play a critical role in innate immunity; however enhanced or prolonged NETs formation or impaired NETs clearance can lead to disease development and progression. More studies are needed to unveil the mechanisms of this recently discovered tool of the immune system in health and disease. The study of NETs pathophysiology constitutes a fascinating field in science that promises potential outcomes on the value of NETs as disease markers and therapeutic targets.

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Dr. Carolina D. Garciarena graduated in Biochemistry and subsequently received a PhD in Cardiovascular Physiology, mentored by Prof Ennis and Prof Cingolani, at the University of La Plata, Argentina. In 2009, she joined Prof Vaughan-Jones group at the University of Oxford as a British Heart Foundation postdoctoral researcher, to explore the role of H^+ and Ca^{2+} in cardiac pathophysiology applying microfluidic techniques and confocal imaging. In 2015 she moved to Ireland to join the Cardiovascular Infection group led by Prof Steve Kerrigan at the Royal College of Surgeons in Ireland. She was appointed as Honorary Lecturer at the School of Pharmacy in 2016. She has published over 25 articles in prestigious peer reviewed journals of the cardiovascular field. Her interests are bloodstream infection, vascular pathophysiology and dynamic bacteria-host interactions; and her research uses live imaging to study dynamic processes within the cardiovascular system in health and disease. Her current work focuses on the study endothelial dysregulation following bacterial infection using in vitro 3D microvessels.

Prof Steven W. Kerrigan is Associate Professor of Pharmacology in the School of Pharmacy in the Royal College of Surgeons in Ireland and Head of the Cardiovascular Infection Research Group which is part of the Irish Centre of Vascular Biology. Prof Steve Kerrigan is an honours graduate in B.Sc Pharmacology from King's College University of London, UK and obtained an M.Sc in Immunopharmacology from the University of Strathclyde, Glasgow, Scotland. He received a PhD in Cardiovascular Infection. Following this he carried out postdoctoral research in the University of California, San Francisco, USA. Prof Kerrigan was appointed lecturer in Pharmacology in the School of Pharmacy in 2008, promoted to Senior Lecturer in 2013 and Associated Professor in 2017. Since his faculty appointment, he has published over 60 articles in leading peer-reviewed international journals, filed 2 patent/disclosures and supervised 9 doctoral candidates to completion as primary supervisor. Accolades include a Health Research Board Training Fellowship (2008), Science Foundation Ireland Career Development Award (2013) and Enterprise Ireland Commercialization Fund (2017).