The Neutrophil's role during health and disease, new insights, and open questions

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Abstract

Neutrophils are the most abundant leukocytes in human blood, constituting 40 to 80% of the total circulating white blood cells. These cells participate as part of the host's first line of defense against invading pathogens, performing three main functions: (i) phagocytosis, (ii) degranulation, and (iii) generation of Neutrophil Extracellular Traps (NETs). This review focuses on NETs and the NETosis process. The most studied signaling pathways of NETs induction and dynamics of nuclear decondensation during NETosis are described. In addition, we describe how neutrophils contribute to the production and secretion of cytokines which are important for the recruitment of immune cells and the homeostasis of the immune response. We also review how the non-specificity of cytotoxic proteins in NETs and prolonged host exposure to proinflammatory cytokines contribute to chronic inflammatory diseases, cancer, autoimmune diseases, and, more recently, COVID 19. Although fundamental aspects about the induction, process, and termination of the signaling pathways of NETs have been studied in recent years, conventional NETosis assays have been limited to end-point assays, taking measurements only when the experiment is over. NETosis end-point studies measure the response of a cell population to an inductor at different concentrations, resulting in the loss of the response at the individual level and temporal evolution of the NETosis process. Therefore, there is still a growing interest in developing comprehensive studies at the individual cell level, the temporality of NETosis, and dynamic stimulation conditions, which could lead to a better understanding of the pathologies in which neutrophil-derived cytokines and NETs participate.

Key words: Biomarkers,cytokines,inmunecells, neutrophils, neutrophil extracellular traps (NETs).

Resumen

Los neutrófilos son los leucocitos más abundantes en la sangre humana, constituyendo del 40 al 80% del total de glóbulos blancos circulantes. Estas células participan como parte de la primera línea de defesa del huésped contra patógenos invasores, realizando tres funciones principales: (i) fagocitosis, (ii) desgranulación y (iii) generación de Trampas Extracelulares de Neutrófilos (NETs). Esta revisión se centra en las NETs y el proceso de NETosis. Se describen las vías de señalización más estudiadas de inducción de NETs y la dinámica de descondensación nuclear ocurridos durante la NETosis. Además, describimos cómo los neutrófilos contribuyen a la producción y secreción de citocinas, que son importantes para el reclutamiento de células inmunes y la homeostasis de la respuesta inmunitaria. También revisamos cómo la inespecificidad de las proteínas citotóxicas de los NETs y la exposición prolongada del huésped a citocinas proinflamatorias contribuyen a las enfermedades inflamatorias crónicas, el cáncer, las enfermedades autoinmunes y más recientemente COVID 19. Aunque los aspectos fundamentales sobre la inducción, el proceso y la terminación de las vías de señalización de los NETs se han estudiado en los últimos años, los ensayos convencionales de NETosis se han limitado a estudios de punto final. Los estudios de punto

final de NETosis miden la respuesta de una población celular a un inductor a diferentes concentraciones, lo que resulta en la pérdida de la respuesta a nivel individual y la evolución temporal del proceso de NETosis. Por lo tanto, todavía hay un interés creciente en el desarrollo de estudios integrales a nivel celular individual, la temporalidad de la NETosis y las condiciones de estimulación dinámica, que podrían conducir a una mejor comprensión de las patologías en las que participan las citocinas de neutrófilos y los NETs.

Palabras clave: Biomarcadores, células inmunes, citocinasneutrófilos, trampas extracelulares de neutrófilos (NETs).

Introducción

Neutrophils are a type of polymorphonuclear myeloid-derived leukocytes, recognized as key actors of the immune response against pathogens (Niels Borregaard, 2010a). Due to the neutrophil´s requirement to perform active migration from blood vessels to the site of the infection quickly and efficiently, they are highly mobile and sensitive to stimuli. Furthermore, neutrophils are recognized as the first leukocytes recruited in an inflammatory site (Fousert et al., 2020) and are regarded as the principal effectors during acute inflammation.

Over the past 50 years, there has been rapid growth and interest in the field of inflammatory disease (Turner et al., 2014). Indeed, new research approaches aim to elucidate how neutrophil's main signaling molecules (i.e., cytokines and chemokines) contribute to pathological processes (Poeta et al., 2019).

Being myeloid-derived cells, neutrophils are produced in the bone marrow. Given their high numbers and relevance in acute inflammation, up to ~ 55-60% of the bone marrow is dedicated exclusively to the production of neutrophils. Under normal conditions, up to 2x1011 cells are produced per day (Niels Borregaard, 2010b); however, this can increase to 1012 cells per day during inflammation or infection (Liew & Kubes, 2019). Neutrophils can also be found in organs such as the spleen, liver, or lung and have been proposed as potential reservoirs of mature neutrophils. These reservoirs allow the neutrophils to deploy rapidly to nearby sites of inflammation or infection (Liew & Kubes, 2019).

Neutrophils are considered short-lived cells with a lifespan of 5-9 h (Liew & Kubes, 2019). However, their lifespan increases up to 5.4 days during inflammation (Pillay et al., 2010). The increase of both cell number and lifespan of neutrophils during infection ensures the continuous presence of prepared neutrophils at the site of infection, contributing to an adequate immune response in the presence of pathogens.

Neutrophils are considered the most abundant type of leukocytes in the blood, constituting 40 to 80% of humans´ total circulating white blood cells (Ley et al., 2018). In circulation, mature neutrophils exhibit a segmented nucleus, are approximately 7 to 10 µm in diameter and have a cytoplasm enriched with granules and vesicles, filled with proinflammatory proteins (Niels Borregaard, 2010b). Neutrophil granules are classified into four types:

Primary granules, also known as azurophilic granules: contain myeloperoxidase (MPO), neutral proteases, cathepsin G, neutrophil elastase (NE), and proteinase 3. These granules are responsible for releasing proteins and peptides directed towards the destruction and digestion of pathogens and are the first to occur during the development of neutrophils (N. Borregaard et al., 1990; Sheshachalam et al., 2014).

Secondary granules: specific granules contain proteins such as b558 and lactoferrin associated with iron and copper sequestration (N. Borregaard et al., 1990; Sheshachalam et al., 2014).

Tertiary or gelatinase granules: contain matrix metalloproteinase 9 “MMP9”, essential to degrade the extracellular matrix and activation of IL-1β, among other functions.

Finally, the fourth type of granules, known as secretory granules, contains serum

albumin and cytokines (N. Borregaard et al., 1990; Sheshachalam et al., 2014).

The proteins inside the granules are highly cytotoxic and are released to the surrounding environment as a defense mechanism against pathogens (Kobayashi & DeLeo, 2009). Besides degranulation, neutrophils effector functions include phagocytosis, the production of reactive oxygen species (ROS), chemokines, and cytokines to recruit other immune cells that maximize the host's immune response (Scapini & Cassatella, 2014).

Despite neutrophils effector functions being highly efficient in the adequate elimination of pathogens with minimal adverse effects for the host, these effector mechanisms may be insufficient to control massive bacterial infections or the attack of other large pathogenic cells (Liew & Kubes, 2019). An alternative mechanism performed by neutrophils was first described by Brinkmann et al. in 2004, which involves releasing DNA fibril structures to the extracellular space, which act as pathogen capture networks. These networks are composed of DNA decorated with azurophilic granule proteins that increase the ability of neutrophils to capture and kill pathogens (Brinkmann et al., 2004) and are known as Neutrophil Extracellular Traps (NETs). The production of NETs is a consequence of a regulated form of cell death called NETosis (Remijsen et al., 2011). The term NETosis was first accepted in 2012 as a type of granulocyte death, different from apoptosis and necrosis, demonstrated by the insensitivity of NETosis to the inhibition of caspase and necrostatin, considered essential mediators of apoptosis and necrosis, respectively (Mesa & Vasquez, 2013). The antimicrobial role associated with NETs has been widely studied, demonstrating its effectiveness in the elimination of a broad group of pathogens such as:

(i) Bacteria, including *Shigella flexneri, Pseudomonas aeruginosa, Escherichia coli, Shigella sonnei, Salmonella enteritidis, Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus albus, Staphylococcus aureus, and Propionibacterium* (Brinkmann et al., 2004).

(ii) Virus, inhibits viral replication by blocking the PKC pathway or by histones promoting viral aggregation and neutralization, leading to a significant decrease in viral replication (Hoeksema et al., 2015).

(iii) Fungi, including *Aspergillus nidulans, Candida albicans, Aspergillus fumigatus*, and *Cryptococcus spp*. The ability of NETs to trap and kill large pathogens *in vivo* is critical to fungal defense (McCormick et al., 2010), and parasitic diseases, including *Plasmodium falciparum* and *Toxoplasma gondii*, where NETs prevent the spread of the parasites (Guimarães-Costa et al., 2012).

The broad spectrum of microbes that induce NETosis highlights the important role that neutrophils play as part of the first line of defense within the innate immune response. However, since much of the effective elimination of pathogens by NETs is primarily due to the cytotoxicity of components that include: histones, cathepsin G, NE, MPO, lactoferrin, antimicrobial peptide LL37, pentraxin 3, gelatinase, proteinase 3, produced in cytoplasmic granules of neutrophils (N. Borregaard et al., 1990; Sheshachalam et al., 2014), and because these molecules are highly cytotoxic, deregulated NETosis processes would evoke in the undesirable release of these molecules into the surrounding tissue, causing unwanted damage to the host.

NETosis regulation and dynamic stimulation conditions.

The mechanisms that induce NETosis are highly regulated by large and complex cell signaling pathways (Liew & Kubes, 2019). Likewise, the release of neutrophils from the blood vessels to the site of inflammation is conditioned by an activation process. Neutrophil activation can be a two-step process. Initially, a priming step is necessary before the activation process. Maximum neutrophil degranulation and NADPH oxidase activation have been reported to occur only in cells that have been primed before activation (Guthrie et al., 1984). The priming step includes an initial exposure to mediators such as cytokine, which can be divided into early-phase cytokines (such as TNF-α, histamine,

IL-1β) (Kolaczkowska & Kubes, 2013), and late-phase chemoattractants, pathogen-associated molecular patterns (PAMP), such as endotoxin or growth factors (Summers et al., 2010). Neutrophil activation by chemoattractant includes chemokines such as CXCL8 (also known as IL-8) in humans and its analogs in mice CXCL1 (KC), CXCL2, CXCL5 (LIX), or macrophage inflammatory protein 2 (MIP-2) (Liew & Kubes, 2019). These chemokines send signals through CXCR2 to activate the neutrophils that subsequently migrate through chemotactic processes that promote their adhesion to the endothelium via integrins. Finally, neutrophils extravasate towards the inflamed tissue, where they perform their effector functions (e.g., apoptosis, necrosis, and NETosis) to control pathogen invasion (Pruenster et al., 2009).

However, although the signaling pathways leading to NETosis have been identified, they are considered complex and not yet fully understood (Mutua & Gershwin, 2020). Likewise, the factors that trigger the production of NETs are considered multifactorial since NETosis can be activated by many compounds or stimuli (Zawrotniak & Rapala-Kozik, 2013). Some molecules reported as NET inductors are: proteins exposed on the surface of pathogenic cells, such as Gram-positive and Gram-negative bacteria, or some fungi, even numerous chemical compounds, such as phorbol ester (PMA), hydrogen peroxide, nitric oxide, ionomycin, calcium ions, glucans, mannans and lipopolysaccharides among others (Zawrotniak & Rapala-Kozik, 2013).

Most of the stimuli inducing NETosis are recognized by different surface receptors of neutrophils, known as pattern recognition receptors (PRR), such as Toll-like receptors, including TLR2, TLR4, TLR7, and TLR8, complement system receptors (CR), Fc receptors, C-type lectin receptors (CLR), which activating different molecular mechanisms to forming NETs (Zawrotniak et al., 2017). Therefore, it is known that signaling pathways are not static events, but complex patterns resulting from a broad cross-dialogue between various biochemical and molecular events at different levels, as well as feedback loops, which make them highly dynamic systems. Deciphering how neutrophils signals from the extracellular environment (e.g., integrative or differential) to carry out specific tasks is a complex task, historically limited by the capacity of technical tools (Purvis & Lahav, 2013).

The two main pathways that induce NETs formation, and the most studied, are:

a) NADPH oxidase (Nox)-dependent NETosis. This pathway can be activated by molecules such as PMA, lipopolysaccharides (LPS), or bacterial components; this pathway is controlled upstream by different kinases, such as protein kinase C (PKC) or mitogen-activated kinases (MAPK). In the Nox-dependent pathway, the enzymes MPO and NE are translocated from the azurophilic granules to the nucleus, inducing histone degradation and promoting chromatin decondensation (Ravindran et al., 2019).

b) Nox-independent NETosis. It occurs due to an increase in the intracellular calcium concentration [Ca^{2+}]. This pathway is induced through stimuli such as calcium ionophores (a23187, ionomycin), the increase in [Ca^{2+}] allows the formation of calcium and peptidyl arginine deiminase 4 (PAD4) complexes, which are then translocated to the nucleus. PAD4 produces histone citrullination inside the nucleus, leading to chromatin relaxation (Ravindran et al., 2019).

Correlation of NETs and diseases

NETosis is widely regulated through complex signaling pathways and activation mechanisms to prevent the release of cytotoxic compounds in unwanted situations, i.e., if the host is not under infection or inflammation conditions. However, the nonspecific effects of the granular proteins released during NETosis can lead to an uncontrolled inflammatory response that causes tissue injury in the host (Mutua & Gershwin, 2020). Therefore, the process can be both beneficial and counterproductive, acting also against host cells. So, although NETs can protect the host against microbes, excessive NETosis can be harmful. Recent discoveries *in vitro* and animal models demonstrated the crucial role of NETs in the pathogenesis of some diseases such as:

Autoimmunity

a) Psoriasis is a chronic disease characterized by demarcated erythematous plaques on the skin. Neutrophils are recruited to psoriasis lesions, where they clump together, forming spongiform pustules and Munro micro-abscesses and producing proinflammatory cytokines such as IL-6, IL-8, and IL-17. These compounds have been shown to promote NETosis (Pinegin et al., 2015).

b) Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by high levels of IFN-α with the activation of self-reactive B cells. The possible production of autoantibodies against nucleic acids released by neutrophils undergoing NETosis has been reported as a contributor to the pathogenicity of the disease. (Pan et al., 2020).

c) Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by persistent synovial inflammation that leads to cartilage and bone damage in the joints. Circulating neutrophils from RA patients have been reported to be more easily stimulated to NETosis than those from healthy subjects. (Chen et al., 2018).

d) Type 1 diabetes mellitus (DM1) is an autoimmune disease characterized by the destruction of pancreatic β cells leading to hyperglycemia. An increased risk has been reported in the development of neutropenia in patients with DM1, and neutrophils can be found in the infiltrates of the pancreatic islets, inducing the formation of NETs under conditions of elevated TNF-α (Berezin, 2019a).

e) Small blood vessel vasculitis (SVV) is a systemic disease in which patients have inflammation of the blood vessels, leading to organ damage. The etiology of the disease is unknown; however, it has been shown that SVV patients have antineutrophil cytoplasmic antibodies (ANCA), which leads to endothelial damage (Mutua & Gershwin, 2020).

Chronic inflammatory diseases

a) Gout is an auto-inflammatory disease in which inflammatory responses are enhanced by the deposition of monosodium urate crystals (MSU) in the joints, attracting leukocytes and inducing NETs that promote inflammation (Mutua & Gershwin, 2020).

b) Inflammatory bowel diseases (IBD) are characterized by uncontrolled chronic inflammation that affects the gastrointestinal tract. Ulcerative colitis (UC) and Crohn's disease (CD) represent the two main forms of IBD (Mutua & Gershwin, 2020).

Metabolic diseases

a) Type 2 diabetes is a chronic metabolic disease characterized by the accumulation of glucose in the bloodstream (hyperglycemia) and insulin-insensitive cells. Hyperglycemia has been reported to predispose neutrophils to release NETs, and consequently, NE, MPO, and cfDNA are found in high concentrations (Berezin, 2019b).

b) Obesity is a metabolic condition characterized by the accumulation and excess deposit of adipose tissue. An association between obesity and chronic inflammation has been shown with increased neutrophil activity, increased superoxide radicals, and NET formation (Mutua & Gershwin, 2020).

NETs as biomarkers

Over the last year, the particular interest in studying and quantifying the blood products directly involved in the formation of NETs has grown, such as citrullinated histone H3 (citH3); circulating free DNA (cfDNA); myeloperoxidase (MPO), and neutrophil elastase (NE) (Thålin et al., 2019), due to its potential to be used as biomarkers associated with autoimmune, autoinflammatory and metabolic diseases (Fousert et al., 2020). Therefore, authors such as Thålin et al., (2019) have associated a high plasma content of citH3 (involved in the NETosis process) as a significant indicator of short-term mortality in some cancer patients.

Furthermore, recently the cytoplasmic granular proteins directly involved in the formation of NETs have demonstrated outstanding potential as biomarkers of SARS-CoV-2. High serum levels of cell-free DNA complexes of DNA-MPO and citH3 have been detected in COVID-19, which correlate with the severity of the disease (Huang et al., 2020; Liu et al., 2020; Zou et al., 2020). High levels of interalveolar neutrophils have been reported in post-mortem lung samples from patients with COVID-19 (Blanco-Melo et al., 2020), documenting their presence in up to 81.5% of the total cases studied (Carsana et al., 2020). Other authors suggest that SARS-CoV-2 can directly induce the formation of NETs in healthy neutrophils by a currently unknown mechanism (Monastero & Pentyala, 2017); however, the presence of NETs and neutrophils are involved in the exacerbation of respiratory failure and microvascular injury in COVID-19. The above has led authors, such as Barnes et al. (2020), to propose that neutrophils and NETs could play a prominent role as biomarkers in COVID-19.

Dynamics of nuclear decondensation of neutrophils during NETosis.

In addition to the complex signaling pathways activated during the formation of NETs, complex intracellular changes occur concomitantly (some mechanisms are still unknown), mainly the nuclear decondensation of neutrophils. The release of DNA through the Nox dependent pathway takes about 1 to 4 hours and is quite complex (Papayannopoulos et al., 2010). The reactive oxygen species formed are thought to be involved in the stability of the granules and the nuclear envelope. While proteins stored in neutrophil granules (NE and MPO) are translocated to the nucleus, contributing to histone degradation. They also cooperate with the enzyme PAD4, which catalyzes citrullination of histones (H3 and H4) (Papayannopoulos et al., 2010). The modification of histones leads to the relaxation and decondensation of chromatin, modifying the structure of the nucleus and finally causing the disappearance of the nuclear membrane. Shortly thereafter, DNA moves into the cytoplasm and mixes with granular proteins, and then this mixture is expelled out of the cell.

Despite the knowledge generated in the dynamics of nuclear decondensation during NETosis, over the years, it has been reported that only a fraction of neutrophils can produce NETs, indicating the heterogeneity of the neutrophil population (Fousert et al., 2020). Whether this heterogeneity reflects different activation states (against variable stimuli) or cell subpopulations is unclear. For example, a distinct subpopulation of neutrophils is known to be more predisposed to form NETs in systemic lupus erythematosus (SLE) patients (Fousert et al., 2020). In addition, individuals with chronic granulomatous disease (CGD) have been reported to possess a subpopulation of neutrophils that have limited NADPH-oxidase activity (Moussavi-Harami et al., 2016). Thus, neutrophils do not behave like a homogeneous population. Additionally, the homogeneity of observed phenotypes in these subpopulations is not clear (Rosales, 2018), i.e., several neutrophil phenotypes have been described that exhibit specialized functions even though they theoretically belong to a homogeneous cell population (Hellebrekers et al., 2018). So, It is not clear whether these cells belong to separate parallel lineages originating from the bone marrow (Hellebrekers et al., 2018). In this way, several subpopulations of neutrophils have been suggested in various conditions of health and disease (Sollberger et al., 2018). Therefore, it is essential to characterize NETs in a dynamic way (dynamic-temporal stimulation); this will allow us to understand the relationships between the cellular microenvironment and the NETotic subpopulation response and their specific participation during the development of autoimmunity and various diseases.

Monitoring the intracellular processes during NETosis can be of great relevance in identifying and quantifying NETotic subpopulations of neutrophils. Since DNA is the backbone of NETs, this process is commonly studied using DNA fluorescent dyes, such as Hoechst 33342 and Sytox green. Although Sytox green has been widely employed, it eventually leads to unspecified cell death studies. Sytox green is a DNA-binding molecule impervious to cells, allowing the quantification of extracellular DNA. In NETosis, Sytox green binds to neutrophil DNA only when the process is at the breaking point of the cell membrane. Consequently,

fluorescence is recorded (Sytox green), and it is generally inferred that neutrophils developed the NETosis process (Carmona-Rivera & Kaplan, 2016; Khan et al., 2018). The combined use of specific fluorescent markers has demonstrated the ability to assess nuclear decondensation dynamics in a simple and reproducible way (Carmona-Rivera & Kaplan, 2016; Gupta et al., 2018; Khan et al., 2018; Magán-Fernández et al., 2020). Likewise, tools such as high-speed multispectral image flow cytometry have been used to detect and quantify the stages of NETosis that precede cell lysis (Zhao et al., 2015). However, this tool is limited to only taking images of cells actively suffering NETosis, overlooking those cells that have already died or have a delayed response, generating biases in quantifying NETotic populations. On the other hand, live- cell microscopy, such as intravital multiphoton microscopy, spinning-disk confocal intravital, fluorescent labeling, two-photon microscopy, or the IncuCyte ZOOM system, among others (Alasmari, 2020; Gupta et al., 2018), can provide a comprehensive analysis of the different types of cell death (apoptosis, NETosis, and necrosis) that neutrophils can suffer under various stimuli (Gupta et al., 2018). However, live-cell microscopy and in general methods for quantification of NETs are laborious, lengthy, and require labor and specialized equipment to develop.

Neutrophils and cytokines

Finally, neutrophils are also recognized as essential sources of secreted cytokines, either constitutively or by stimulus (Tecchio et al., 2014). Cytokines are produced as an innate immune response to pathogens and contribute to maintaining the inflammatory response (Tecchio et al., 2014). Cytokines expressed by neutrophils include proinflammatory cytokines (IL-1α, IL-1β, IL-6, IL-7, MIF), anti-inflammatory cytokines (IL-1ra, TGFβ1, TGFβ2), chemokines (CCL2, CCL3, CCL4, CXCL1, CXCL2, CXCL3, CXCL8), immunoregulatory cytokines, members of the tumor necrosis factor superfamily (TNF-α, TRAIL, FasL) and angiogenic and fibrogenic factors (Tecchio et al., 2014). Among its primary functions is the regulation of inflammation, so cytokines are essential to play a vital role in regulating the immune response in health. Neutrophil-derived cytokines can be measured in cell-free supernatants or cell lysates using several methods, including enzyme-linked immunosorbent assays (ELISA), radioimmunoassay, immunoprecipitation after metabolic marking, immunohistochemistry, intracellular staining coupled with flow cytometry, commercial multiplex matrices such as FirePlex, confocal microscopy, bead-based assays, among others(Tecchio et al., 2014; Zhou et al., 2010).

Conclusions

Due to the complexity of performing dynamic studies, conventional NETosis assays (e.g., use of specific fluorescent markers and multispectral image flow cytometry) are end- point assays and measure a population's response to an inductor at different concentrations. It quantifies either the released DNA or the number of cells that produce NETs. End-point bioassays result in the loss of valuable information on the response at the individual level and the temporal evolution of the process.

In addition, despite the importance of neutrophil-derived cytokines and chemokines and their involvement in exacerbated cytotoxicity or recruitment of other immune cells, until a year ago, there were no concurrent studies that concomitantly evaluated the NETosis process and the cytokine/chemokine secretion of neutrophils (Tatsiy et al., 2020). On the other hand, although basic aspects about the induction, process, and culmination of the signaling pathways of the NETs are already known, the temporal evolution of the NETosis and the response at the individual cell level under dynamic stimulation must still be clarified in greater detail. For example, it is unknown whether neutrophils actively suffering NETosis concomitantly produce cytokines that exacerbate the pro-inflammatory response or whether the generation of these cytokines is mediated through paracrine or juxtracrine communication. In addition, it is unknown whether differences in the dynamics in the input signals (i.e., stimuli) of a neutrophil affect the output signal (i.e., response) and how neutrophils process the different signals received from the outside environment.

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