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Neutrophil extracellular traps-periodontal implications of netosis: A literature review

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Abstract

Neutrophils are the primary line of innate immune defense against infectious diseases. Numerous researches in the field of neutrophil cell biology explain their role in skewing the immune response in various infections or inflammatory disorders revealed their importance within the regulation of immune response. It has been demonstrated that neutrophils release neutrophil extracellular traps (NETs) for the containment of infection and inflammation. NETosis, being an alternate style of death is that the creation of web-like chromatin decondensates by suitably primed neutrophils as a response to stimulus geared toward containing and eliminating the identical. NETs are implicated in periodontitis and COVID-19 as both these disease form a state of inflammation and be a source of constant harm to the tissues involved. This review thereby highlights the emerging role of neutrophil extracellular trap production in the regulation of immune response and its implication in periodontal disease predisposing towards adverse COVID-19 related outcomes.

Keywords: Neutrophils, NETs, Periodontitis, COVID Implications

Introduction

Periodontitis is a chronic inflammatory disease of the periodontium with a dysbiosis of pathogenic flora within the plaque biofilm being its etiological stepping stone. (Bascones-Martínez *et al.* 2009) [1].

Neutrophilic polymorphonuclear leukocytes (neutrophils) are the predominant cells of the innate immune system which along with the eosinophils, basophils and mast cells, comprise the granulocyte lineage. They are fundamental in the periodontal inflammatory-immune response, for bacterial clearance. In susceptible patients, dysfunctional neutrophils fail to eliminate pathogenic bacteria, resulting in discordance between the host and the biofilm. (Scott and Krauss 2012) [2] Smit *et al.* 2012 associated periodontitis with an elevated systemic inflammatory burden, associated with an increased risk of age-related conditions, such as rheumatoid arthritis (RA) [3]. Neutrophil Extracellular Trap (NET) as reported by Brinkman in 2004 has been described as an alternative form of cell death owing to the accumulation of antibacterial substances within a web of chromatin and neutrophil granule proteins. [4] NETosis is a catastrophe occurring at a cellular level prompting in cell death and may often cause bystander damage directly or through autoimmune mechanisms.

Neutrophil Biology and Homeostasis

Neutrophils are the major antimicrobial phagocytes which commence development within the bone marrow, as myeloblasts- the precursor cells, with a generation of approximately $1-2 \times 10^{11}$ cells daily. (Savil *et al.* 1989) [5]. These precursors differentiate into promyelocytes and finally into specific neutrophil myelocytes. Upon infection, there is a transient release of neutrophils which migrate to specific sites by chemotaxis and release various inflammatory mediators all in course due to a heightened demand placed on the immune system. (Metcalfe 1991) [6]. After an average circulating life span of ~5.4 days, they undergo apoptosis resulting in a surface expression of phosphatidyl-serine phospholipids on their surface ("death signals"), thereby facilitating phagocytosis via macrophages. (Pillay *et al.* 2010) [7].

The light microscopic structure, reveals an approximate diameter of 12 -15 μm with a lobulated nucleus [6]. The cytoplasm is replete with antimicrobial peptides [7] and enzymes required to synthesize arachidonic acid derivatives with either inflammatory properties like thromboxanes and leucotrienes [8], or negative regulators of inflammation like prostaglandins, lipoxins, or protectins. These substances are produced within the neutrophils [2] or in conjunction with other cells using transcellular pathways to supply lipoxins [9], they also produce chemokines and cytokines (from the tumor necrosis factor (TNF) superfamily), as well as angiogenic and fibrogenic factors, and pattern recognition molecules like, collectins, pentraxins and ficolins. Additionally, they require an enzymatic complex - nicotinamide adenine dinucleotide phosphate (NADPH) oxidase type 2 (NOX2) liable for the assembly of reactive oxygen species during the respiratory burst.

In the event of an injury the neutrophil transmigration occurs via leukocyte adhesion cascade, a process initiated by the activation of endothelial cells causing an upregulation of adhesion receptor expression (E- and P-selectins) enables neutrophil rolling. This depends on a transient interaction of selectins with glycoprotein ligands on neutrophils causing an adhesion to occur allowing them to transmigrate into infection or inflammation sites. This is powered by $\beta 2$ integrin interaction adhesion ligands such as intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 on endothelial cells [2, 9, 10, 11].

Cytokines control the expression of endothelial adhesion molecules and chemokines induce integrins to vary conformation into a high affinity state [12]. Neutrophils follow a chemoattractant gradient composed up of complements, like the anaphylatoxin C5a, or alternatively bacterial components, such as formyl-methionyl-leucyl-phenylalanine (fMLF). Eskin *et al* 2012 discovered that the leukocyte adhesion cascade is negatively regulated by endogenous inhibitors like Del-1 (developmental endothelial locus-1), pentraxin 3, and growth differentiation factor [13].

A steady-state of neutrophil level is maintained after being cleared from the tissues, by a process described as a “neurostat” (neutrophil rheostat). The phagocytosis of apoptotic neutrophils reprograms the macrophages which initiates an anti-inflammatory response associated with an increase in synthesis of TGF- β and IL-10 and a consequent decrease in IL-23 synthesis, thereby also reducing IL-17 levels causing less G-CSF production and in consequence, less neutrophil production. This constant loop is termed as “neurostat” [14]. Under physiological conditions activated neutrophils switch to different types of cell death such as autophagy or NETosis [16]. NETosis was accepted for the first time as a specific cell death routine as opposed to apoptosis and necrosis. [15] According to the 2012 classification NETosis has been demonstrated as it is insensitive to caspase inhibition and necrostatin.

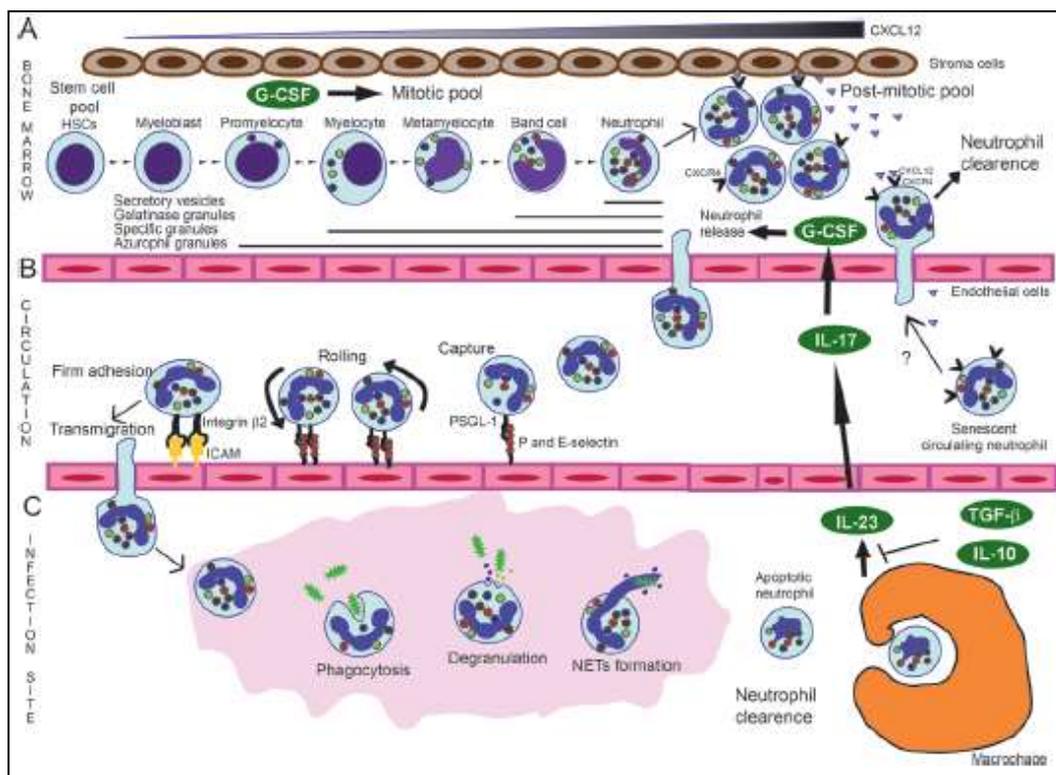


Fig 1 (A): Production of neutrophils takes place at the bone marrow Neutrophils mature in the bone marrow accumulating different granules (arrow heads), azurophil, specific, and gelatinase. Produce secretory vesicles. Neutrophils are released from the bone marrow to the circulation due to CXCR4-CXCL12 interaction (B) Neutrophils mobilization to infection site through leukocyte adhesion cascade that includes capture, rolling, firm adhesion, and transmigration of neutrophils (thin arrows). Senescent circulating neutrophils increase the expression of CXCR4, and respond to CXCL12 (C) Neutrophils kill bacteria by phagocytosis, degranulation, and NET: formation Apoptotic neutrophils are cleared by macrophage phagocytosis. In an infected site macrophages produce IL-23. Which activates IL-17 thus inducing G-CSF that promotes neutrophil differentiation and release from the bone marrow. After macrophages phagocyte apoptotic neutrophils they downregulate the production of IL-23 and produce IL-10 and TGF- β . This events stop the recruitment of neutrophils CXC chemokine receptor IL interleukin G-CSF granulocyte colony-stimulating factor (Adapted from: Carlos Rosales and Eileen Uribe-Querol (June 7th 2017). Neutrophil Role in Periodontal Disease, Role of Neutrophils in Disease Pathogenesis, Maitham Abbas Khajah, Intech Open)

NET

NETs are generated by activated neutrophils extracellularly as web like structures of decondensed nuclear chromatin with a backbone histone proteins and granular peptides. When observed under EM, they measure linearly 15–17 nanometers (nm) in diameter and embedded with 25nm diameter globules and with various substances grouped around this scaffold [16]. They comprise of antimicrobial compounds, (including

histones and antimicrobial peptides (AMPs) derived from azurophilic, specific and gelatinase granules) human neutrophil elastase, lysozyme, bactericidal permeability increasing protein, and human peptidoglycan-recognition protein S (Brinkmann *et al.* 2004) that disarm and kill bacteria extracellularly due to the high serine protease content. [4] Yousefi *et al.* 2009 postulated at NETs being derived from mitochondrial DNA [16]. (Table 1)

Table 1: Components of NET

Net Components		
Nuclear components	Granular components	Cytoplasmic components
DNA	(i) Primary granules: Myeloperoxidase, cathepsin G, Neutrophil, elastase	Calprotectin,
Histones	(ii) Secondary granules: Lactoferrin, pentraxin 3	Catalase
	(iii) Tertiary granules: Gelatinase, peptidoglycan-binding protein	

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Formation of NETS

The literature defines about two models of NETs release:

(a) Suicidal Netosis: is the foremost process of NET formation. It is defined by a slow lytic cell death mechanism. Apoptosis of neutrophils releases NETs within 2–3 hours of exposure to phorbol myristate (PMA) by *S. aureus* or *C. albicans*.

Suicidal NETosis generates two pathways, namely, the Raf-MEK-ERK pathway, NADPH oxidase-dependent pathways, as well as the generation of ROS, and receptor-interacting protein kinase mediated signals (Hakkim *et al.* 2011; Desai *et al.* 2016) [18, 19]. Reportedly, activation of Toll-like receptors (TLRs), Fc receptors, or complement receptors [4], increases the levels of cytoplasmic calcium consequently stimulating the activity of protein kinase C (PKC), the phosphorylating gp91phox, resulting in the assembly of cytosolic and membrane-bound subunits of NADPH oxidase, forming ROS.

[20] Keshari *et al.* confirmed the role of NADPH oxidase-derived ROS in PMA-induced NET release from human neutrophils and demonstrated the involvement of the MAPK/ERK pathway, (mitogen-activated protein kinases) which is an important regulators of autophagy [21].

Kolaczowska 2013 studied its initiation by ROS and NADPH oxidase [22]. Parker *et al.* postulated the involvement of NADPH oxidase-derived ROS in the regulation of NET formation based on its stimulating molecule [23].

The role of ROS in NETosis is ambiguous. ROS may inactivate caspases, prevent apoptosis and autophagy, thereby

leading to the dissolution of cellular membranes. (Remijsen *et al.* (2011) [24].

ROS initiates the release of myeloperoxidase (MPO) from the azurophilic granules of the neutrophils, which translocates to the nucleus. (Papayannopoulos *et al.* 2010; 2011; 2013 and Scherz-Shouval 2011) Furthermore, MPO facilitates histone citrullination (i.e., transformation of positively charged arginine to the neutrally charged amino acid citrulline) weakening the electrostatic attraction, and thereby initiating nuclear disassembly and chromatin decondensation. Leading to ruptured membranes [25, 26, 27, 28]. Metzler *et al.* in a study, found that MPO is essential for NET formation after stimulation with PMA and opsonized *Candida albicans* cells. [29]

Inhibition of NADPH oxidase either experimentally [30, 31] or due to mutation [17, 20] has proven to impede NET formation.

(b) Vital Netosis: does not involve lytic death of the neutrophils. Pilszczek *et al.* 2010 [32] demonstrated a ROS-independent in response to *Staphylococcus aureus* and Byrd *et al.* 2013; Rochael *et al.* 2015 demonstrated the same in *Candida albicans*. [33, 34] It entails the extrusion of chromatin material along with serine proteases from within the nucleus to the extracellular space and in the process preserves the plasma membrane integrity. The active expulsion of NETs necessitates a neutrophil actin cytoskeleton, a microtubular transport system, as well as intracellular calcium (Neeli *et al.* 2009; Parker *et al.* 2012) [23, 35].

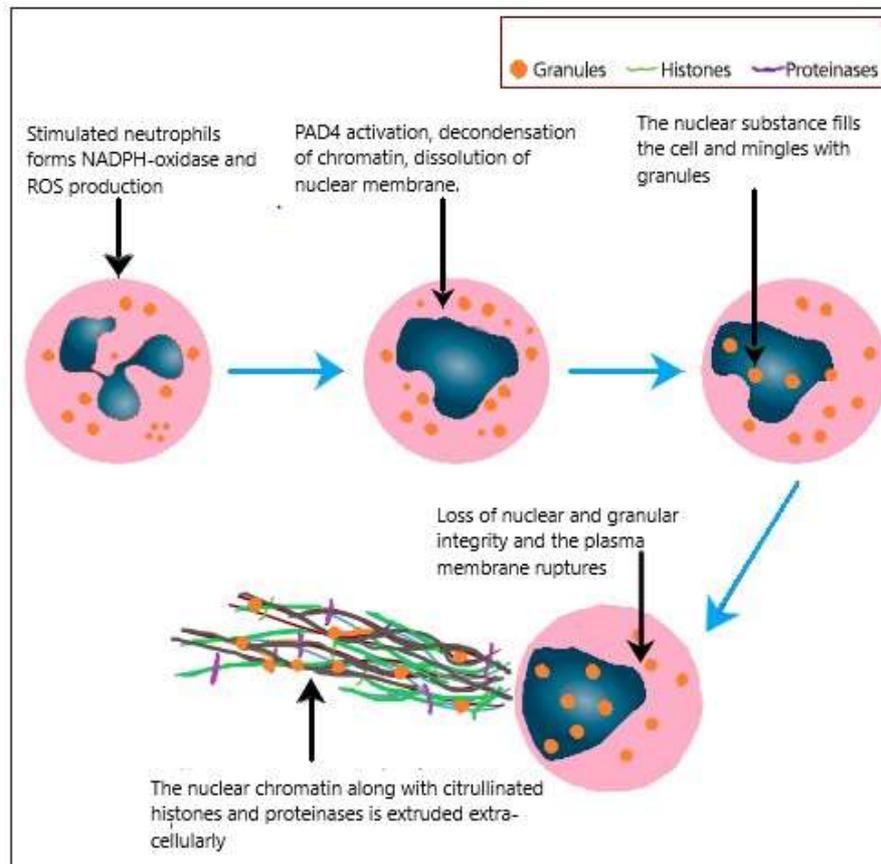


Fig 2: Schematic representation of neutrophil extracellular trap (NET) release. After neutrophil activation and peptidyl arginine deiminase-4 activation, the nuclear chromatin decondenses and intermingles with the granular proteins of the cytoplasm. This chromatin-protein complexes are extruded into the extracellular space as NETs Based on Brinkmann and Zychlinsky (2007)

Inducers of NET Formation

NET formation has been evolutionarily conserved and noted in various mammalian species. Various intrinsic mediators of NET have been described.

Fuchs *et al* 2007 demonstrated hydrogen peroxide as a potent inducer of NET at physiologic concentrations [30]. Khandpur *et al* 2013 observed NETosis induced by inflammatory cytokines- interleukin-17A (IL-17A) and tumor necrosis factor- α (TNF- α) in patients with Rheumatoid arthritis. [36] Rossaint *et al* 2014 reported GPCR-signaling or heteromerization of platelet chemokines to induce NETosis. [37] Warnatsch *et al* 2015 reported that cholesterol crystals formed signaling complexes called inflammasomes to secrete IL-1 β inducing NETosis [38]. Kessenbrock *et al* 2009 suggested that chronic autoinflammatory conditions triggered NETosis [39]. Constantinescu *et al* 2020 reported PMA to be the most common triggering stimulus for NETosis [40].

Johnson *et al* 2017 states, that *Candida glabrata* triggers NETs during planktonic and biofilm growth. [41] The role of *S. aureus*, *A. viscosus*, *A. actinomycetemcomitans* and *F. nucleatum* in the serum for the activation of NET release was examined by Palmer *et al* 2015 [42]. Jenne *et al.* (2013) demonstrated NET production following poxvirus administration in a murine model systemically, enabling host protection from the virus [43]. Other authors have studied that the presence of HIV virus generates high levels of interleukin-10 via c-type lectin CD209 dependent production by dendritic cells enabling NET production. Bryzek *et al* 2017 studied the triggering of NETosis via protease-activated receptor (PAR)-2 signaling, and reported that purified gingipains extracted from *P. gingivalis* strongly induces NETosis [45].

The Antimicrobial Repertoire

NETs have proven effective in combating against the gram-negative and gram-positive bacteria (*E. coli*, *S. flexneri*, *S. aureus* etc.) via MPO enzymatic activity, as well as against parasites such as *T. gondii*. and fungi such as *C. albicans*. The antibodies formed against histones impede the NET-mediated antimicrobial activity [7, 8]. NETs are fungicidal against *C. albicans* owing to calprotectin, which chelates divalent metal ions [46].

The antimicrobial activity of NETs occurs mainly in two stages [20].

1. Trapping and immobilization of microbes to check their dissemination

It has been suggested and supported by high-resolution imaging that NETs display antimicrobial activity by adhering to the invading pathogen and consequently trapping them. This has been affirmed by preliminary studies based on electron microscopy and immunofluorescence on NET stimulation by microbes like *S. aureus*, *S. typhimurium* and *S. flexneri* [4].

2. Destruction of pathogen by the antimicrobial enzymes present in NETs

However certain studies challenge the antimicrobial activity of NETs. Menegazzi *et al* 2012 demonstrated that *S. aureus* and *Candida albicans* blastospores, captured by NETs were recovered in cell medium by incubation with DNase [47]. Proteases, antimicrobial enzymes, along with histones and DNA contribute to NET formation and antimicrobial activity. Several authors report of microbial evasion by inactivating NETs by nucleases. These include *Vibrio cholerae*,

Streptococcus pneumoniae, *Staphylococcus aureus*, *Streptococcus agalactiae* [48, 49, 50, 51].

The antibacterial activity of histones has been demonstrated but the physiological conditions related to its presence outside the nucleus, is still unclear. Urban *et al.* states of the use of cytochalasin D to inhibit phagocytic activity of neutrophils, which ensures their antibacterial activity [7, 46].

NETs and Periodontal Implication

The primary function of NETs has been reported to be gingival shielding and bacterial clearance.

Chronic periodontitis patients have hyperreactive systemic neutrophils which release of reactive oxygen species (ROS) in response to both a microbial load as well as even in the absence of an exogenous stimulant (Matthews *et al.* 2007; Wright *et al.* 2007). Periodontal pockets have an adept environment for ROS production, owing to its oxygen tension and a neutral pH which assists in the release of NADPH-oxidase-dependent NETs. High concentrations of NET-associated cytotoxic species is associated cytotoxic AMPs or NET bound components serving as autoantigens (e.g., citrullinated histones or extracellular DNA) causing a host tissue response and periodontal destruction [52, 53].

Palmer *et al.* 2012 suggested that periodontal pathogens belonging to the red and orange complex, to produce deoxyribonucleases which was of prime importance for NETs [54]. However Doke *et al.* suggest that extracellular nucleases and DNAses secreted by the periodontal pathogens enhance pathogenicity by degrading the host NETs [55].

The immune response during periodontitis occurs essentially as an innate immunity with a subsequent increase in antibodies thereby progressing to an acquired immune response. Vitkov *et al.* (2009, 2010) noted the presence of NETs from gingival crevicular samples, which was associated with the periodontal pocket epithelium of chronic periodontitis patients, therefore suggesting that NETs entrap the crevicular microbes, preventing their adhesion and colonization to the gingival tissue. They observed, NETs in abundance with a lack of neutrophils containing phagocytosed bacteria, potentiating NETosis as a defense mechanism within the periodontal pockets. [56, 57] Wright *et al.* 2007, reported that several mediators such as IFN α , are elevated in periodontitis initiate NET release suggesting NETosis to be a feature of periodontitis [53].

Recent studies document, viral evasion of the host defense system as a stimulator of NETosis. There is a report of an unregulated systemic circulation eliciting cytokines, chemokines, immune complexes amounting to inflammation. Mozzini 2020, report of a hyper-inflammatory state or a 'cytokine storm' in the advanced stages of COVID-19. Thus there is a possibility of an increased level of NETs in patients suffering from both COVID-19 and periodontal disease, implying that periodontitis patients are at an aggravated risk of contracting COVID-19. This however requires clinical validation [58].

Conclusion

NETs comprise of the innate immune system. Although most work reports NET-DNA as being derived from nuclear chromatin (NETosis), there's also evidence that NETs released by viable cells is composed of mitochondrial DNA. NETs are produced in response to an array of stimuli, including gram-positive and gram-negative bacteria and their components moreover as host-derived molecules; however, the role of NETs within the pathophysiology of periodontitis

remains to be elucidated. NETs offer a fundamental defense strategy to stop infiltration of bacteria, and this might be important in periodontal tissues. Conversely, it's evident that NETs can also be deleterious if produced in excess or if they're not removed appropriately. Citrullinated and uncitrullinated neutrophil-derived components of NETs may trigger autoimmune responses, which are proposed to underpin the event of diseases, such as RA. On condition that the pathogenesis of periodontitis also involves dysregulated inflammatory responses, NETs could also be a key pathogenic feature. The invention of NETs in 2004 has fueled intense research in many diseases areas, and it's likely that future discoveries in NET biology interprets their association with periodontitis and other chronic inflammatory diseases.

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