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Neutrophil Extracellular Traps – The catapult of periodontal disease

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ABSTRACT

Neutrophils are highly versatile and sophisticated cells which act as the body's first line of defense. They act as effectors in both innate and adaptive immunoregulatory networks. Neutrophil extracellular traps are a new and unexpected antimicrobial activity of neutrophils wherein upon encountering bacteria, neutrophils release a meshlike structure capable of ensnaring and eliminating microbes. Studies have shown that periodontitis is characterized by neutrophil infiltration and the formation of neutrophil extracellular traps (NETs). This review is intended to highlight the structure and formation of NET, NETosis, NET in health & disease and the role of NET production in periodontal pathophysiology.

Keywords— *NETs, Neutrophil Periodontitis Immunity*

1. INTRODUCTION

Neutrophils, the major antimicrobial phagocyte of the innate immune system, are terminally differentiated white blood cells, equipped with a plethora of microbicidal and pro-inflammatory mechanisms and form the first line of defense against pathogenic insults [1]. They arrive at the site of infection and play a critical role in pathogen clearance, recruitment and activation of other immune cells and tissue repair. Neutrophils combat microbes by employing three major strategies: Phagocytosis, degranulation and the release of neutrophil extracellular traps.

In 2004, Brinkmann et al described a hitherto unrecognised and unusual mechanism by which neutrophils immobilise pathogens extracellularly. NETs are webbed like structures composed of decondensed chromatin heavily impregnated with different antimicrobial compounds, including histones and antimicrobial peptides from azurophilic, specific and gelatinase granules [2]. NETs can only be generated by mature neutrophils as immature neutrophils lack the molecular machinery to transduce signals to trigger their production. NETs are released by a process called Netosis, which is an active form of cell death resulting in rupture of plasma membrane and release of decondensed chromatin and granular contents into the extracellular space [3].

Periodontal diseases are chronic inflammatory diseases of the supporting tissues of the teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament, alveolar bone with pocket formation, recession or both [4]. Periodontitis arises because of the aberrant host response to a pathogenic biofilm and the neutrophils are the immune cell type involved inn periodontal inflammatory response. It is seen that neutrophils from patients with periodontitis could either exhibit constitutive hyperactivity and a raised baseline level of NET production or they could be hyper-reactive, resulting in excessive NET production in response to bacteria [5]

This review is intended to highlight the structure and formation of NET, NETosis, NET in health & disease and the role of NET production in periodontal pathophysiology.

2. NEUTROPHILS AND THEIR ROLE IN PATHOGENESIS OF PERIODONTAL DISEASE

2.1 Structure of neutrophil:

Neutrophils, first described by Paul Ehrlich form the first line of defense of the human innate immune system. These myeloid-derived, professional antimicrobial phagocytes can kill pathogens extracellularly, link the innate and adaptive arms of the immune response, and help promote inflammatory resolution and tissue healing [6]. Structurally neutrophils are round cells approximately 12-14 pm in diameter containing a multilobed nucleus, several types of granules and subcellular organelles.

Table 1: Granules present in Neutrophil

Granules of neutrophils	Contents	Function
Primary or azurophilic granules	• Myeloperoxidase • Human neutrophil defensins • Lysozyme • Azurocidin • Serine proteinases • Elastase • Cathepsin G • Proteinase 3 • Esterase N	They fuse with phagocytic vesicles resulting in the delivery of their contents to the ingested organism[6]
Specific or secondary granules	• Apolactoferrin • Plasminogen activator • Collagenase • Lysozyme • Gelatinase	Release of granule contents may modify the inflammatory process[7]
Tertiary granules	• Matrix metalloproteinases • Lysozyme	Facilitate extravasation via matrix metalloproteinase mediated degradation of basement membrane[7]

2.2 Functions of Neutrophils

- Important effector cells in the innate arm of the immune system
- Maintains homeostasis by phagocytosis, production of reactive oxygen metabolites, and degranulation of cytotoxic proteins
- Antimicrobial functions by phagocytosis, degranulation, and the release of neutrophil extracellular traps
- A diverse repertoire of functional responses
- Transcriptionally active complex cells involved in the production of cytokines, resolution of inflammation and macrophage regulation for long-term immune responses[8]

2.3 Neutrophil priming:

Circulating neutrophils exist in a basal state, characterized by non-adherence, a round morphology, minimal transcriptional activity, and a limited capacity to respond to activating stimuli. That limited response protects against unwarranted inflammatory responses and tissue injury [9]. To effectively eliminate invading organisms, neutrophils must be capable of mounting rapid, vigorous responses to activating stimuli. The transition to a state of enhanced responsiveness has been termed priming. Historically the term "priming" was primarily used to describe the augmented reactive oxygen species generation upon neutrophil stimulation because of the depth of knowledge of molecular mechanisms of NADPH oxidase complex assembly, the ease of measurement of ROS generation, and the importance of ROS to antimicrobial activity. The known priming agents include chemoattractants (fMLF, C5a, LTB4, PAF), cytokines (TNFα, GM-CSF, IFN-γ, IL-1β, IL-8, Adiponectin), microbial products (LPS, LAMS, flagellin), others (Substance P, adhesion)[9].

2.4 Neutrophil heterogeneity

Neutrophils display different phenotypes from the time they leave the bone marrow and enter the circulation (fresh neutrophils) to the time they disappear from the circulation (aged neutrophils). This shift in phenotype is known as aging, since it takes place within a single day, and results in various neutrophils with distinct properties. In addition, the microenvironment in different tissues can induce neutrophils to acquire specialized functions [10].

2.5 Role of neutrophils in periodontal disease

Peripheral blood neutrophils from patients with periodontitis have been shown to be both hyperreactive to a microbial stimulus and also hyperactive in the absence of such a stimulus with respect to the release of extracellular reactive oxygen species (in particular, hypochlorous acid release). Neutrophil hyperactivity with respect to reactive oxygen species release may be induced by interleukin-8, interferon-alpha and granulocyte–macrophage colony- stimulating factor, and interferon-alpha also appears to be capable of generating neutrophil hyper-reactivity [11]. Neutrophil interferon-alpha reactivity (interferon-alpha is probably released from dendritic and other tissue immune cells) appears to play a significant role in the resulting release of reactive oxygen species and oxidative stress, and any process that subverts neutrophil chemotaxis and/or apoptosis is likely to enhance this destructive process in susceptible patients. The role of neutrophils in periodontal disease is illustrated in the following flowchart (Figure 1).

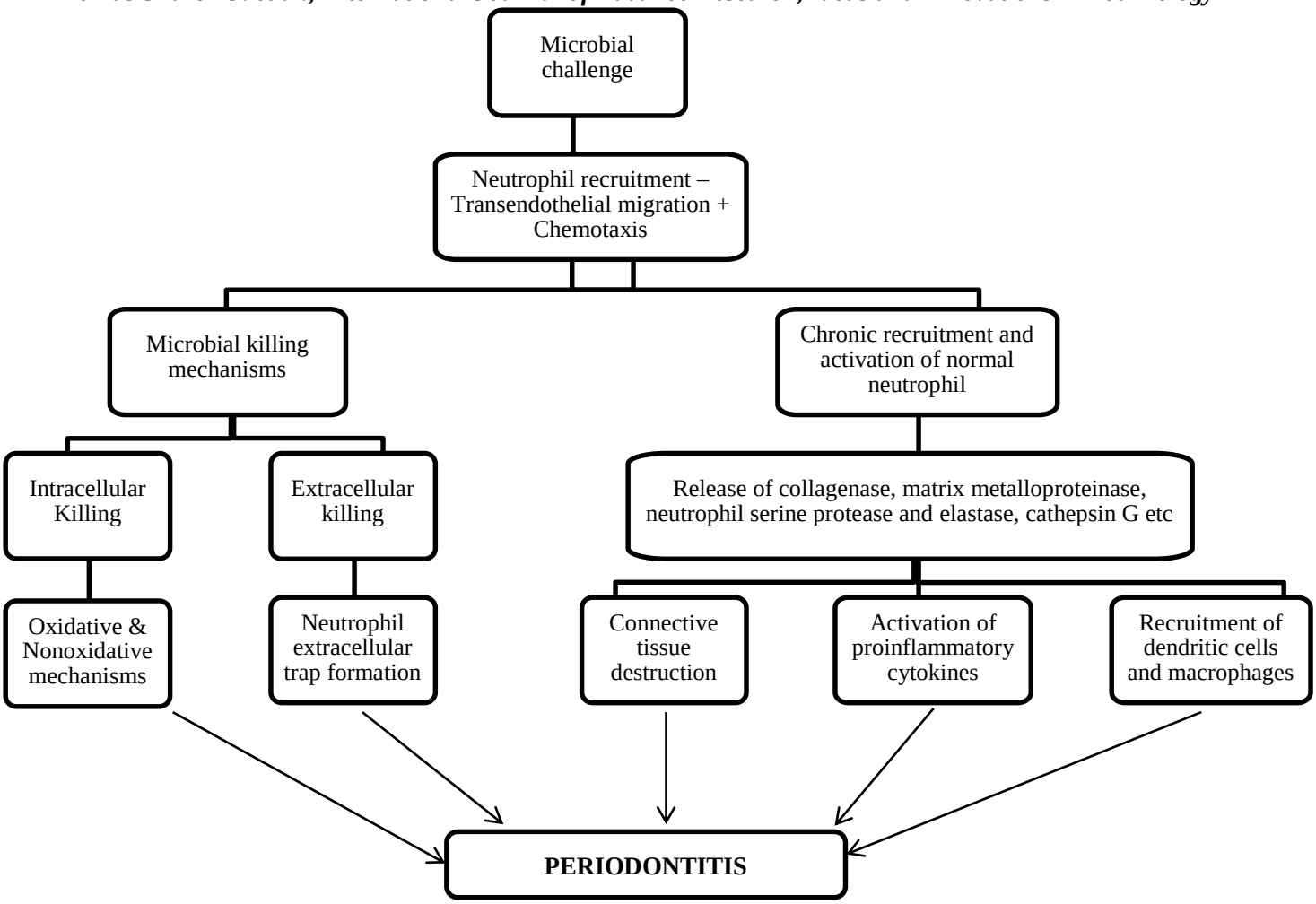

Fig. 1: Flowchart showing the role of neutrophils in periodontitis

3. NEUTROPHIL EXTRACELLULAR TRAP

Brinkmann et al.in 2004 through a series of static and video microscopic studies documented, a neoteric method of neutrophil-mediated microbial killing – the release of neutrophil extracellular traps. The NET formation is a unique immunological phenomenon, analysis of which might provide insight into biological principles such as cell death, regulation of chromatin structure, nuclear envelope disassembly and membrane dynamics [3]. As a result of stimulation by certain inflammatory signals, the neutrophil undergoes a programmed sequence of events that consequently releases its entire nuclear chromatin (DNA and associated histone-rich protein backbone), along with the embedded, granular cathelicidin antimicrobial peptides,and is actively extruded by the neutrophil as a type of biologic 'spiders web' into the extracellular space or tissue. As the immature neutrophils lack the molecular machinery to transduce extracellular signals via membrane receptors and second messengers to trigger the enzyme systems necessary for their production, neutrophil extracellular traps are released only by mature neutrophils [3].

3.1 Structure of NETs

Nuclear chromatin comprises double-stranded DNA wrapped tightly around a histone protein-rich backbone within a double-helix structure forming nucleosomes. As the genes are transcribed, the local region of chromatin unwraps to a looser structure that associates with RNA polymerases, called 'euchromatin', and nontranscribing regions are more tightly packed and are referred to as 'heterochromatin'[2]. Neutrophil extracellular traps released during neutrophil extracellular traposis consist of nuclear DNA and various histones and, most importantly, high-resolution scanning electron microscopy demonstrated that they are studded or 'decorated' with globuli of 30–50 nm in diameter[3] that contain the multiple cathelicidin antimicrobial peptides which originate within the neutrophil granules (lysosomes) and which co-localize into the web-like mesh that forms[3].

The co-localization of these granular proteins/enzymes, or indeed of histones (e.g. H1, H2A, H2B, H3, H4 or a complex of H2A–H2B), with the DNA, is critical in discriminating DNA released during cell necrosis from that specific to neutrophil extracellular trap formation. This was demonstrated by Brinkmann et al in their original description, where importantly they verified that neutrophil extracellular trap fibers were DNA structures rather than proteins, because by using DNases they dismantled and dispersed the fiber-like structures, whereas proteases had no such effect. The fiber-like structures were examined using high-resolution scanning electron microscopy and demonstrated strands of varying diameter and length, some of which formed 'cable-like' structures. When produced in multiwall plates in vitro, neutrophil extracellular traps float within the fluid medium, rather like a spider's web does in moving air. They are 'sticky' as a result of their electrostatic charged nature and that they extend over areas of several microns, they are very effective at trapping microorganisms moving in the vicinity[3].

3.2 Netosis – a Novel form of programmed cell death:

NETosis is a novel cell death of neutrophils besides apoptosis and necrosis. Other granulocytes [mast cells and eosinophils] can form extracellular traps upon stimulation, a process renamed as Etosis. In the case of eosinophils, NET like structures containing

mitochondrial DNA and granular proteins have been proposed to contribute to host defense and to allergic responses[12]. NETosis is distinct from apoptosis. NETosis is associated with the disintegration of the nuclear envelope and mixing of nuclear and cytoplasmic material, loss of internal membranes, and disappearance of cytoplasmic organelles. On the other hand, hallmarks of apoptosis such as DNA fragmentation, phosphatidylserine exposure, and caspase activation, are not associated with NETosis [13]. NETosis resembles necrosis in that both membranes are not intact so that they allow intracellular proteins to leak outside the cells. Some intracellular proteins such as myeloperoxidase, S100A8 and S100A9 remain associated with DNA after NETosis, whereas others such as HMGB-1 and heat shock proteins do not . Therefore there is a possibility that, in both NETosis and necrosis, damage associated molecular patterns (DAMPs) such as HMGB-1 and heat shock proteins are released to induce inflammatory responses[14].

Table 2: Difference between suicidal and vital netosis:

Mechanism	Suicidal netosis	Vital netosis
Nature of the exciting stimuli and the timing of the NET release	Slow- takes hours	Rapid
Functional capacity	Absent	Present
Mechanism employed	PMA stimulation and subsequent activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase	Vesicular trafficking of DNA from the nucleus to the extracellular space

3.3 Events in a NET release

The point at which NETs are released from neutrophils is critical because by their very nature, being 'traps', they will impede other functions of the neutrophil e.g. migration to the pathogen, recruitment of other white blood cells and to a certain extent phagocytosis [15]. The complex series of orchestrated events that follow cell stimulation and which define classical neutrophil extracellular traposis are as follows [16]:

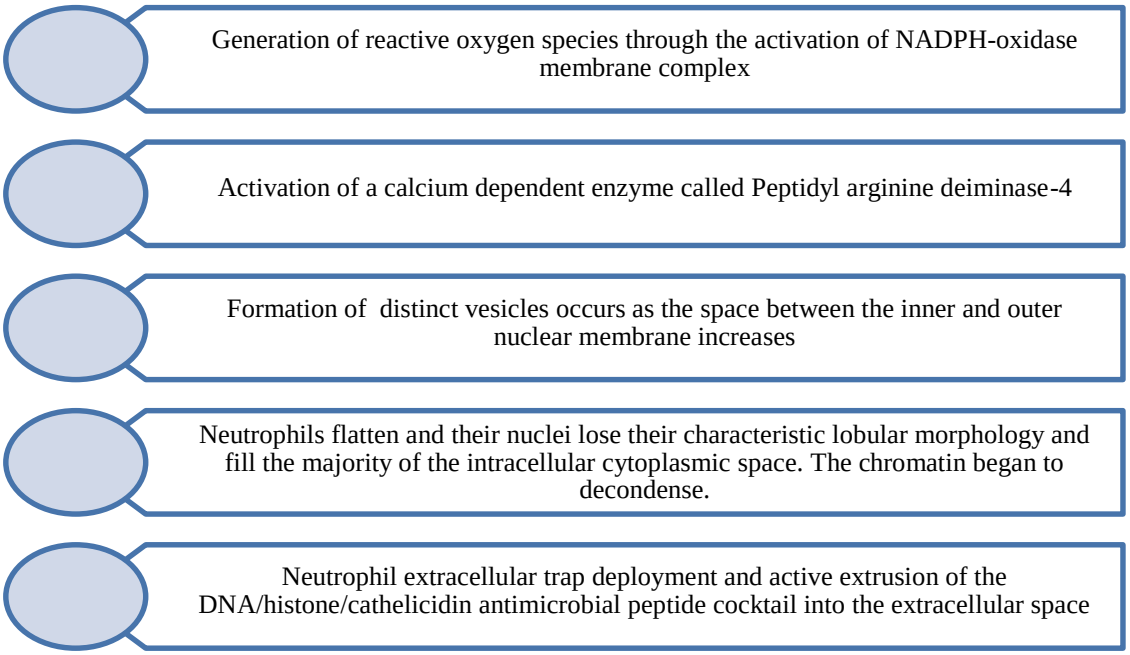

Fig. 2: Schematic diagram showing events in a NET release

Stimuli for neutrophil extracellular trap release include:

- Nitric oxide [17]
- Cytokines [2]
- Microbes and their products, including bacteria, bacterial endotoxins (lipopolysaccharide), other bacterial toxins (e.g. autolysin from S. aureus and alpha-enolase from Streptococcus pneumoniae, yeasts and protozoa/ parasites) [15]
- Antimicrobial peptides such as human beta-defensins (platelet-derived) [18]
- Antibodies such as anti-LL37 and anti-human neutrophil peptide; anti-HNA-3a antibodies (neutrophil alloantigen-3a); and anti-neutrophil cytoplasm antibodies implicated in the pathogenesis of small-vessel vasculitis [19]
- Platelets activated by lipopolysaccharide or collagen and platelet toll-like receptor-4.

3.4 Function of NETS

- The antimicrobial actions of neutrophil extracellular traps comprise two main phases, which include:
- Trapping and immobilizing of pathogens to prevent tissue and systemic spread.
- Pathogen killing by neutrophil extracellular trapembedded cathelicidin antimicrobial peptides.

It has been proposed that histones, which are a core component of the neutrophil extracellular traps (along with the DNA strands), accounting for 70% of its total protein content, can exert antimicrobial effects alongside the cathelicidin antimicrobial peptides that also localize within neutrophil extracellular trap structures. The role of histones within neutrophil extracellular traps is

pathogen specific. The cytotoxic effect of neutrophil extracellular traps is not limited to foreign pathogens as the host's own endothelial and epithelial cells have been shown to be susceptible to neutrophil extracellular traps and their DNase-generated neutrophil extracellular trap fragments [19].

3.5 Non neutrophil derived traps

Neutrophils are not the only immune cell capable of extracellular trap formation. Mast cells have also been shown to be capable of this phenomenon to ensnare S. pyogenes. A primary function of mast cells is to mediate allergic reactions and they are found in tissues exposed to the external environment such as the mucosa of the respiratory tract. Whilst they are capable of phagocytosis they do not perform this at a significant level physiologically. The extracellular traps formed by mast cells (termed MCETs) were shown to be similar to NETs in their composition, dependence on ROS production and required stimuli. A similar NET release process has also been reported in eosinophils whose primary function is to combat parasites. DNA extruded into the extracellular space also occurred in a process independent from cell death and dependant on ROS generation similar to that of NETs. Unlike NETs, eosinophil derived extracellular traps were found to consist of mitochondrial DNA rather than nuclear chromatin and required priming by IL-5 or IFN-γ prior to stimulation with LPS [16].

3.6 Quantification of NETS

NETs can be quantified using various methods including electron microscopy (scanning and transmission), fluorescence microscopy or measuring DNA release. The spectrofluorometric quantification of NET content based on DNA release using DNA intercalating dyes has been frequently used. NETQUANT is an easy to use tool that can quickly and automatically accurately detect cells that are positive for NETs. The NET formation is examined in stimulated neutrophils on glass coverslips using an automated approach. The NETQUANT is written as a standalone app in MATLAB and can be easily installed [20,21].

3.7 NETs in pathologies

Neutrophils and NETs play a dual role in host homeostasis. They both protect hosts from infectious diseases; however, they also cause pathologic alterations. Current research has identified direct clinical links between neutrophil extracellular traps and certain pathologies like Cystic fibrosis[22], Preeclampsia[23], Rhematoid arthritis [24], Atherosclerosis [25], Tuberculosis [26], Diabetes[27] and other chronic inflammatory conditions[28]. In general, diseases that associate with neutrophil extracellular traps are a result of either the interaction of neutrophil extracellular traps with bacteria or the relationship between neutrophil extracellular traps and other arms of the host immune response, which potentially lead to an aberrant chronic inflammatory response.

4. NETS IN PERIODONTAL DISEASE

Periodontitis is a chronic inflammatory disease occurring as a result of the aberrant host response to a pathogenic biofilm and manifest in susceptible individuals. Neutrophils are the major immune-cell type involved in the periodontal inflammatory response. Peripheral blood neutrophils in periodontitis show both hyperreactivity to plaque organisms and hyperactivity in terms of reactive oxygen species release[29]. The degradative enzymes and autoantigenic components are concentrated within the abundant neutrophil extracellular traps present within the tissue in a process triggered initially by the response of neutrophils to plaque bacteria. The high and concentrated levels of neutrophil extracellular trap-associated molecules could lead to a localized chronic inflammatory response, potentially with an autoimmune component, leading to significant gingival tissue damage[30]. Factors determining the susceptibility of an individual to develop inflammatory periodontitis may be determined by

(a) The type of bacteria inhabiting the gingival crevice,
(b) Whether these bacteria possess virulence factors for neutrophil extracellular trap evasion
(c) The individual's innate ability for neutrophil extracellular trap production.[30]

There are various concepts linking neutrophil extracellular trap formation and periodontitis.

Concepts linking neutrophil extracellular trap and periodontitis			
	Mechanism	**Author**	
PERIODONTITIS	Inflammation	[31]	**NEUTROPHIL EXTRACELLULAR TRAP**
	Hypercitrullination	[32]	
	Pathogenic bacteria	[33]	
	Interferon	[34]	
	Peptidylarginine deiminase	[35]	
	NADPH oxidase	[36]	
	Reactive oxygen species	[37]	
	Interleukin – 8	[38]	
	Gingival crevicular fluid viscosity	[39]	
	Lipopolysaccharide	[40]	
	Autoimmunity	[41]	
	Cathepsin	[42]	
	Neutrophil elastase	[43]	
	ELANE gene	[44]	
	DNAse production	[45]	
	Serine proteases	[46]	
	Genetics	[47]	
	Smoking	[47]	

Future studies may, therefore, identify novel mechanisms involving neutrophil extracellular traps in periodontitis pathogenesis which subsequently lead to new treatment regimes. Currently, the role of neutrophil extracellular traps in the pathogenesis of periodontitis is at an early stage of research and clearly more studies characterizing their involvement are required. The potentially opposing mechanisms (i.e. hypo- or hyper-neutrophil extracellular trap production) may contribute to periodontitis pathogenesis. The degradation and evasion of neutrophil extracellular traps by virulent periodontal pathogens may cause neutrophils to respond by up-regulating the release of neutrophil extracellular traps. Such a response may not result in trapping and clearance of bacteria but instead may lead to the immobilization and localization of neutrophils responsible for periodontal tissue destruction.

5. CONCLUSION

NET production by neutrophils plays an essential role in the immune response to infection. Considerable attention is needed within the periodontal sciences community to explore the characteristics of NET release in response to periodontal bacteria, their composition and functional relevance. The role of both host DNA and microbial DNA production is probably crucial in NET evasion strategies and also in NET retention within tissues and the potential pathological consequences. Given the hyperactivity and hyperreactivity of neutrophils from periodontitis patients in terms of reactive oxygen species release and the role of reactive oxygen species in NET production, it seems likely that NET biology plays a significant role in periodontal health and disease.

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