Neutrophil Extracellular Traps (NETs) "A Two way Sword" in Periodontitis

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Abstract: *Neutrophils are the most abundant immune cells in humans and are the first line of defense against invading pathogens. They employ a wide array of anti-microbial strategies, most notably phagocytosis, Reactive Oxygen Species production, Intracellular and Extracellular degranulation to attack and eliminate pathogens. However, in 2004 Brinkman discovered a novel neutrophil-mediated defense mechanism, which showed that, upon encountering bacteria, neutrophils release a mesh-like structure capable of capturing and killing microbes. These web-like traps contain a backbone of DNA/histones and are peppered with anti-microbial peptides that are present within the neutrophil granules. These are called Neutrophil Extracellular traps (NETs) and the process of NET formation is known as "NETosis". Periodontitis is an infectious-inflammatory disease of humans, affecting the gingiva, bone and the supporting tissues of the teeth characterized by PMN infiltration in the gingival crevice and formation of NETs. The main function of NETs appears to be evacuation of dental plaque Pathogen-Associated Molecular Patterns (PAMP). However, when there is excess (Exagerrated NETosis), or dysfunctional NET production (Impaired NETosis), bacterial evasion of NETs, and decreased removal of NETs, it is associated with periodontal disease progression.*

I. Introduction

Periodontal disease is a pathological inflammatory condition of the gingiva, bone and supporting structures of the teeth. The two most common periodontal diseases are **Gingivitis,** which is the inflammation of the gums at the necks of the teeth and **Periodontitis,** inflammation and destruction of the bone and the supporting tissues of the teeth. Gingivitis is initiated by local accumulation of bacteria (dental plaque) adjacent to the tooth and in the subgingival areas. These bacterial antigens and their metabolic products (Endotoxins) stimulate the epithelial and connective tissue cells to produce inflammatory mediators that result in an inflammatory response recruiting Neutrophils to the site[1] as depicted in Figure.1
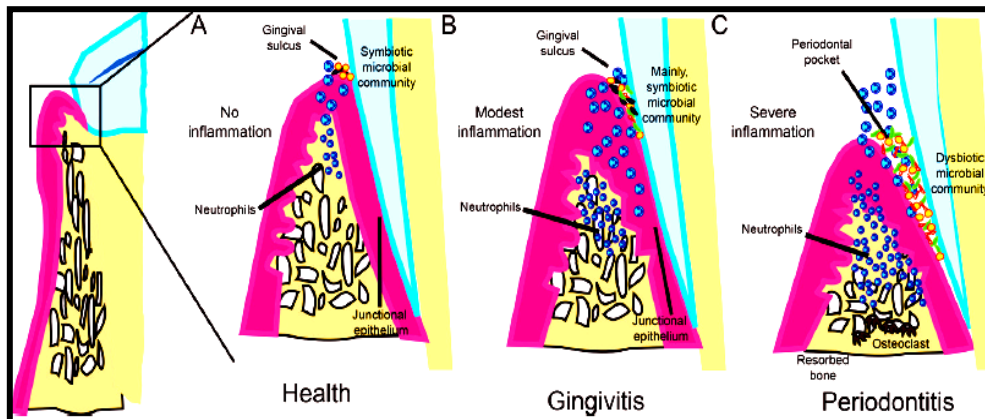
Figure 1. Shows neutrophil infiltration in the subgingival region (A) In health, few neutrophils are recruited to the gingival sulcus to maintain symbiotic microbial community and the gingiva is not inflamed. (B) During gingivitis more neutrophils are recruited to the gingival sulcus and the gingiva is moderately inflamed. The junctional epithelium is starting to detach from the tooth. (C) During periodontitis a majority of neutrophils are recruited to the periodontal pocket and the gingiva is severely inflamed (reference.2 ;Carlos Rosales,2017)

Neutrophils are highly specialized cells equipped with machinery for the destruction of microorganisms. Neutrophil responses to infection can be divided to ''3 R's'' Recruitment, Response, and Resolution[3]. Thus an immune response is generated, and the pro-inflammatory cytokines (IL-1ß, TNF-α, and MMPs) are produced by inflammatory cells. In susceptible individuals, this inflammatory process eventually extends to involve and destruct the deeper connective tissues causing bone resorption and eventually loss of teeth[1].

Orgin of Neutrophils

They are the most abundant white blood cells in humans (40%-60%)[4] from the PMNs family together with basophils and eosinophils[5]. They originate from the bone marrow and differentiate from granulocyte/macrophage progenitor cells; measuring 12-14μm. approximately $1\text{-}2 \times 10^{11}$ cells are generated per day with a circulating life span of 5.4 days[6]

following which they undergo apoptosis and express phosphatidyl-serine phospholipids on their surface ("death signals"), facilitating their removal by macrophages[7].

Naming of "Neutrophils"

They possess a unique polymorphic nucleus segregated into 3-5 lobules (Figure.2) and named as Neutrophils because of their **"neutral"** staining with wright stain[8]
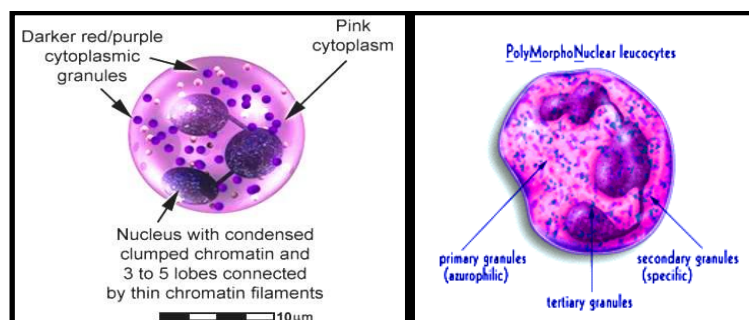

Figure.2 morphology of neutrophils and cytoplasmic granules (reference.9 Hager,2010)

Neutrophils contain numerous granules in the cytoplasm, that are specialised lysosomes and grouped as primary, secondary, and tertiary and so referred as **"granulocyte."**(Table.1)

Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles
Azurocidin	CD11b/CD18	Acetyltransferase	Alkaline phosphatase
Bacterial/permeability-increasing protein	Cathelicidin	CD11b/CD18	CD11b/CD18 (CR3)
	Collagenase	Cytochrome b558	CD14
Cathepsins	Cytochrome b558	Gelatinase	CD16
Defensins	fMLP-R	Leukolysin	CR1
Lysozyme	Lactoferrin	Lysozyme	Cytochrome b558
Myeloperoxidase	Leukolysin	Natural-resistance-associated macrophage protein 1 (NRAMP1)	FRP
Natural serine proteases	Lysozyme		fMLF-R
Cathepsin G	Neutrophil gelatinase-associated lipocalin (NGAL)		
Neutrophil elastase			
Proteinase 3			

Table.1 Cytoplasmic granules of neutrophils

Functions: The secondary granules promote phagocytic capacity, while the primary and secondary granules each contribute majorly to the anti-microbial arsenal[14].

Neutrophils in Periodontal Inflammation

Neutrophils play a pivotal role in controlling the periodontal microbiota. Matured neutrophils from the circulating blood are attracted and recruited to the infected areas of the periodontium eg. subgingival region, by inflammatory mediators that induce the expression of E or P selectin on the surface of endothelial cells, which bind to mucins on the neutrophil cell surface.

Once the neutrophils are bound, they start to roll along the endothelial surface. Here, chemokines (interleukin-8), and complement factors (C5a, and N- formyl-peptides from bacteria) activates the neutrophils, which start to express integrins (lymphocyte function-associated antigen- 1). These integrins bind to cell adhesion molecules (ICAMs) on the surface of endothelial cells and establish a tight adhesion that arrests neutrophil migration[15] (Figure.3)
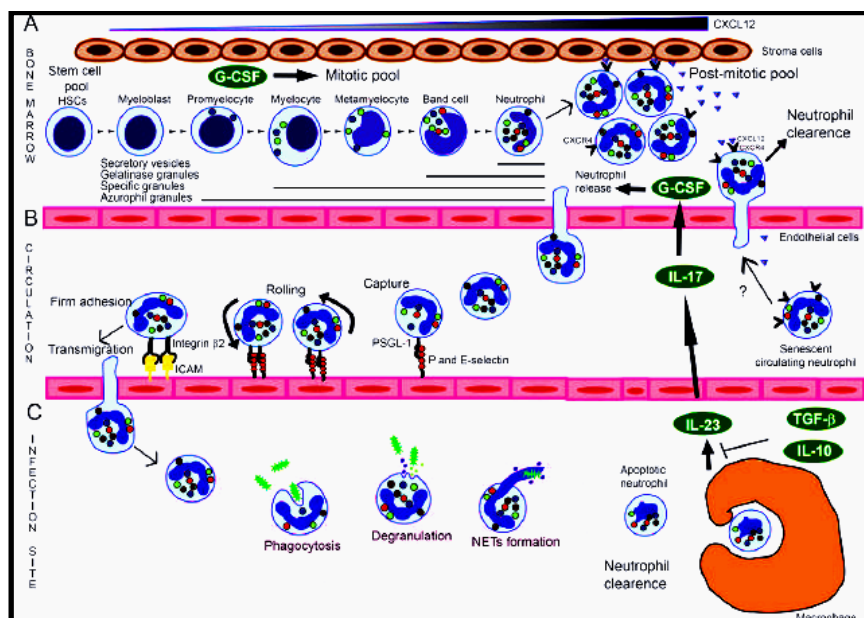

Figure.3 Neutrophil homeostasis involves production, trafficking, and clearance of these cells. (A) Production of neutrophils takes place at the bone marrow. Neutrophils mature in the bone marrow accumulating different granules and also produce secretory vesicles. (B) Neutrophils mobilization to infection site through a leukocyte adhesion cascade that includes capture, rolling, firm adhesion, and transmigration of neutrophils.(C) Neutrophils kill bacteria by phagocytosis, degranulation, and NETs formation. (reference.2 ;Carlos Rosales,2017)

Following this, neutrophils traverse through endothelial gaps and migrate towards infected region and engulf the microorganisms into the membrane-bound compartment known as phagosome[15]. Subsequently, the intracellular granules fuse with phagosome (Figure.4) to form a phagolysosome, and discharge their contents. In these phagolysosomes, neutrophils can periodontal pathogens by both oxidative (respiratory burst) and non-oxidative (lytic or proteolytic enzymes) mechanisms[4].
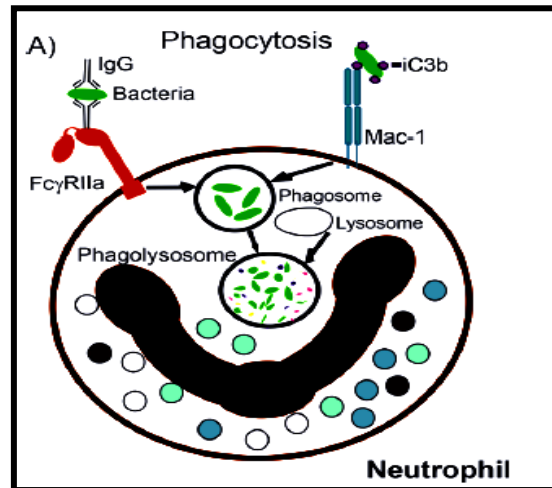

Figure 4. Phagocytosis.(A) Neutrophils recognize opsonized pathogens through Fc Receptors (FcγRIIa) or complement receptors (Mac-1) on their membrane. The pathogen is internalized into a nascent phagosome, which matures by fusing with lysosomes forming a phagolysosome. (reference.2 ;Carlos Rosales,2017)

Intracellular killing of Neutrophils

In the **Oxygen-independent mechanism**, the secondary and tertiary intracellular granules are released first which contain anti-microbial proteins which facilitate neutrophil migration and action. Next the primary azurophilic granules are discharged. These granules harbor bacterial permeability increasing protein and MPO[16].The antimicrobial action of Cathelicidin, alpha defensins, and serine proteases disrupts the integrity of the bacterial cell membrane. Other granule-derived antimicrobial mechanisms include destruction of peptidoglycan (lysozyme), iron sequestration (lactoferrin, neutrophil gelatinase-associated lipocalin (NGAL) and degradation of proteolytic bacterial virulence factors (elastase)[14].

Non- granule specific enzyme LL-37 is present in the gingival epithelium. LL-37 is effective against Aggregatibacter actinomycetemcomitans (Aa) present in localized aggressive periodontitis. Increased concentrations of alpha-defensins are found in diseased periodontal tissues[17]. In hypoxic periodontal pockets, the non-oxidative mechanisms are critical although neutrophils may be capable of generating ROS in periodontal pockets with oxygen content as low as 1-3%[18].

The **oxygen-dependent mechanisms** involve a non-mitochondrial generation of reactive oxygen species (ROS)[19]. Upon activation of neutrophils the transmembrane and cytosolic subunits of the large NADPH-oxidase complex assemble at the phagosomal membrane and transfer electrons to molecular oxygen producing superoxide (O_2^-) (Table.2). Superoxide either spontaneously or catalytically (driven by superoxide dismutase (SOD)) dissociates into hydrogen peroxide (H_2O_2). H_2O_2 in turn is converted to hypochlorite (HOCL) by myeloperoxidase. HOCL is the most bactericidal antioxidant in neutrophils[16]. The chlorination of bacterial targets inactivates the membrane proteins and disturbs the replication site for DNA synthesis[20].
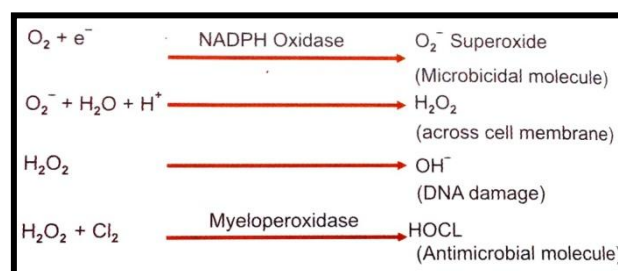

Table.2 Oxidative mechanism

These mechanisms further leads to activation of complement system and activation of other immune cells and tissue repair[21].

Extracellular killing by Neutrophils

Activated neutrophils entrap microbes in the extracellular space through release of a web of fibres containing DNA, histones, and granule-derived bactericidal molecules. **These Neutrophil Extracellular Traps (NETs) are another weapon in their armory**[8].

This article reviews the formation, importance and pathways of NETs. Along with Beneficial role of NETs in periodontal health parallel with a pinch of dark side of NETs in the periodontal disease pathogenesis and the lesson learnt from the Neutrophils.

What are Neutrophil Extracellular Traps?

This novel paradigm in innate immunity was discovered by **Brinkmann et al. in 2004**[22]. NETs are new antimicrobial Extracellular killing mechanism of neutrophils and an important strategy to immobilize and kill the invading microorganisms, thus preventing microbial dissemination. NETs have a DNA backbone, along with decorated bactericidal substances such as histones, human neutrophil elastase (NE), lysozyme, bactericidal permeablility increasing protein, human peptidoglycan- recognition protein S, and other PMN proteins[23,24].

Structure of NETs

NETs have complex structures composed of smooth 'threads' approximately 15 -17nm in diameter, which likely represent a chain of nucleosomes from unfolded chromatin and hence it can be degraded by DNases and not by proteases. This process of NET formation is known as **"NETosis"**

High resolution scanning electron microscopy (SEM) shows that NET threads are studded to variable extents with globuli of 30-50nm in diameter. Several threads can be arranged into "cables" that can be upto 100nm in diameter. These cables then form complex three dimensional structures that resemble webs covering areas of several μm^2.

In time-lapse fluorescence microscopy of neutrophils, NETs appear to be flexible and surround the cell resembling like clouds and floating[15].

Stimuli for NETs

The stimuli that induce NETosis can be broadly classified into microbe associated, inflammatory, and endogenous (sterile) triggers[25]. Microbial stimuli are from bacteria, fungi, protozoa, and viruses. Bacterial stimuli include whole bacteria as well as cell surface components of gram-positive and gram-negative bacterial lipoteichoic acid and LPS, as well as breakdown products of prokaryotic proteins, such as fMLP[26]. Bacteria such as staphylococcus aureus[27,28], streptococcus sp[29,30], haemophilus influenzae[30], klebsiella pneumoniae[32], Listeria monocytogens[33], Mycobacterium tuberculosis[34] and Shigella flexneri[22], yersinia[35]and members of the oral microbiome, including porphyromonas gingivalis[36].

Pro-inflammatory cytokines (TNF-α,IL-8,INFγ)[22,26,37],activated endothelial cells, reactive oxygen species (ROS) such as Hydrogen peroxide, antibodies, antigen-antibody complexes, NO, and TLR-4 activated platelets and by using Phorbol myristate acetate (PMA)(invitro)[25]. Diverse neutrophil receptors can signal to induce NETosis, by binding via TLRs, Fc receptors, and complement receptors.[22,38]

NETosis

NETosis, is a dynamic process that can come in two forms, and mechanisms. One is conventional suicidal NETosis and Non-suicidal vital NETosis. Overall, many of the key components of the process are similar for both types of NETosis, however, there are key differences in stimuli, timing, and ultimate end result[39].

Conventional suicidal NETosis

Fuchs et al in 2007[40] first described in their study that the release of NETs resulted in

Neutrophil's death through a pathway, different from apoptosis and necrosis. The total duration of suicidal NETosis is about 2-4 hours. (Figure.5) (I) several stimuli (e.g., bacteria, viruses, fungi and chemical stimulation from phorbol 12-myristate 13-acetate) initiate NETosis by binding to neutrophil receptors (e.g., Fc receptors, TLRs), which activate the endoplasmic reticulum to release stored calcium ions (II) Elevated cytoplasmic calcium levels increase Protein Kinase C (PKC) activity and phosphorylation of gp91phox[25]. This induces the assembly of NADPH oxidase to assemble into a functional complex at the cytoplasmic or phagosomal membranes (Phagocytic Oxidase-PHOX). (III) Subsequently, PHOX generates ROS[23]. (IV) ROS generation leads to the rupture of granules and the nuclear envelope. Subsequently, the released nuclear,granular and cytoplasmic contents blend. (V) Meanwhile, NE and MPO, stored in the azurophilic granules, migrate to the nucleus. (VI) As a result, histone deimination by peptidyl arginine deiminase 4 (PAD4) and chromatin decondensation contribute to the formation of NETs. i.e NE degrading the linker histone H1, and MPO

enhancing chromatin decondensation[32]. (VII) Finally, the rupture of the plasma membrane causes the release of NETs.(Figure.6)

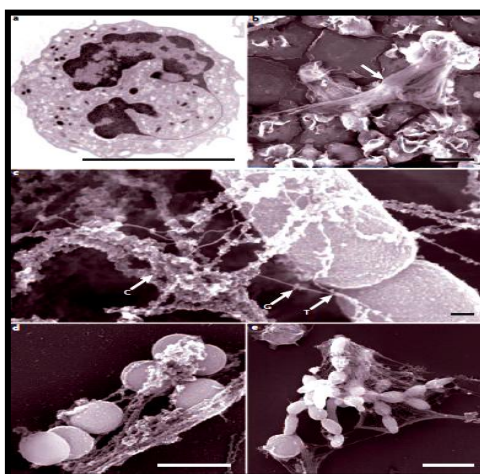In suicidal NETosis the Neutrophil dies and there is loss of viable cell functions like migration and phagocytosis[32].

Figure. 5 Neutrophils and Neutrophil Extracellular traps. NETs can trap Gram-negative bacteria, Gram-positive bacteria and fungi. a) Transmission electron micrograph showing an unstimulated human neutrophil. b) Scanning electron micrograph (SEM) showing stimulated neutrophils forming NETs c) SEM showing a detailed view of NETs trapping Shigella flexneri. d) SEM showing NETs trapping Staphylococcus aureus. e) SEM showing NETs trapping Candida albicans. (reference.15; Volker Brinkmann,2007)
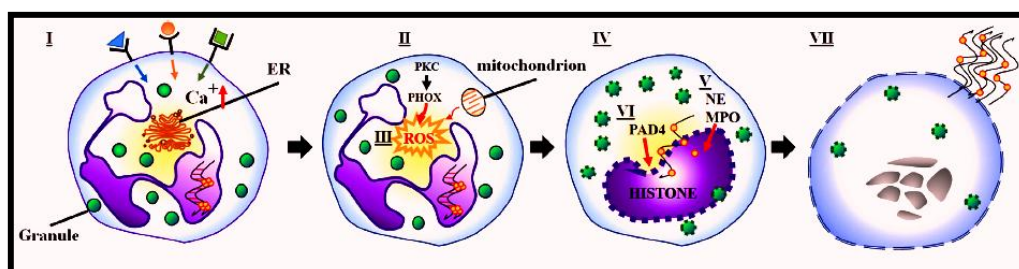

Figure.6 Suicidal NETosis (I) Several stimuli initiate NETosis by binding to neutrophil receptors which activate the ER to release stored calcium ions. (II) Cytoplasmic calcium levels increase PKC activity, which induces NADPH oxidase to a functional complex (PHOX). (III) PHOX generates ROS. (IV) ROS causes rupture of granules & nuclear envelope. (V) NE and MPO translocate to the nucleus. (VI) Histone deimination & chromatin decondensation contribute to NETs. (VII) The rupture of the plasma membrane causes neutrophil lysis & allows the release of NETs. (reference 41; Hang Yang,2016)

Mutations in NADPH complex and the consequence:

In neutrophils, ROS are formed during "respiratory burst" with the involvement of NADPH oxidase complex. **Fuchs et al.**[40] demonstrated that patients with chronic granulomatous disease (CGD) have mutations in NADPH oxidase enzyme system, resulting in a non-functional/ lowly functional enzyme complex, which is unable to synthesize ROS. Since generation of ROS is the most important intracellular event in NET formation, these patients suffer from recurrent life threatening bacterial and fungal infections due to lack of NET formation.

How to treat?

The addition of glucose oxidase enzyme can substitute for dysfunctional NADPH oxidase to generate hydrogen peroxide. This shows that, NET production was not only dependent on ROS, but specifically on hydrogen peroxide, which is a direct stimulus to induce NETs.

PAD4 deficiency:

Neutrophils from mice with PAD4 deficiency causes impaired capacities to form NETs and are highly susceptible to skin infections[42,43].

Mutations in MPO gene

Patients with MPO gene cannot form valid NETs[44],because of insufficient amount of hypochlorous acid[45].

Vital NETosis

Vital NETosis has been reported following both direct microbial exposure and lipopolysaccharide (LPS)**. Pilsczek et al. 2010**[27] demonstrated that Live S aureus induce rapid NET release (<30 minutes) in human and mouse neutrophils, by nuclear envelope blebbing and vesicular exportation, in vitro and in vivo. As a result, this pathway preserved the integrity of the neutrophil's plasma membranes.(Figure.7)
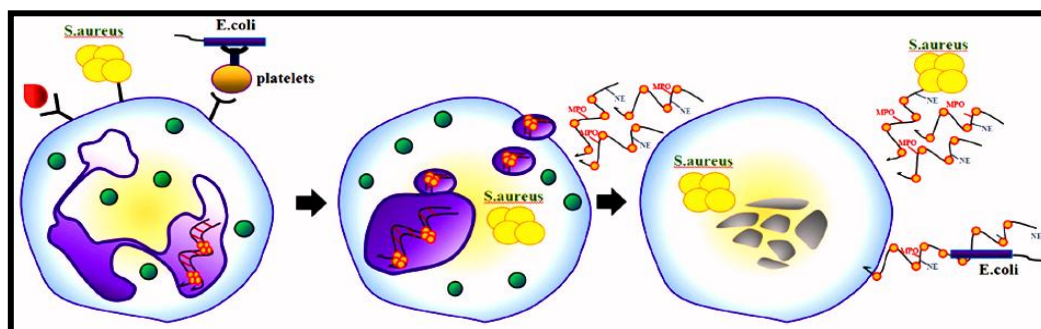

Figure.7 NETosis-inducing stimuli involve TLR4 on platelets. Under these conditions, neutrophils release NETs via blebbing of the nuclear envelope and vesicular exportation. As a result, neutrophils become nuclear cytoplasts, which are still able to migrate and retain several conventional functions of viable neutrophil. (reference 41; Hang Yang,2016)

Clark et al in 2007[46]reported that lipopolysaccharide (LPS) from gram negative bacteria stimulated NETosis within just 30 min involving TLR4 on platelets; whereas both complement receptor 3 and TLR2 are required for vital NETosis following gram-positive infection. In vital NETosis, NETs are released via nuclear budding and vesicular release of NETs .Thus PMN's outer membranes are spared;

Yousefi et al, 2009[47] described another type of vital NETosis dependent on ROS, in which mitochondrial DNA are released instead of nuclear DNA; this process results in NET formation from 80% of neutrophils within 15 min through recognition of C5a or LPS.

So the three major differences between suicidal and vital NETosis are

1. Differences in stimuli and timing[46] 2.Functional capacity of Neutrophils[48] and 3. the mechanisms[27] employed to produce and release NETs.

Microbicidal Activity of NETs

NETs provides a concentrated source of enzymes, Anti-microbial peptides and Histones within the extracellular milieu. The majority of NET associated proteins are Histones,[49] proteins from cytosol and granules account for only 16%, of which Elastase constitutes one third. Elastase is a NET-associated protease, which elicit bactericidal activity and can degrade bacterial virulence factors[22]. Myeloperoxidase from the specific granules are essential for elimination of Staphylococcus aureus[50].Also, Microbes are entrapped due to the electrostatic interactions between the positively charged bacterial surface and negatively charged chromatin fibres[15] Fungi are difficult to be eliminated by phagocytosis[51] and the antifungal activity of NETs has been assigned to calprotectin S100A8/A9[49], which chelates, cation required for fungal growth. NET-bound LL-37 correlates with NET antimicrobial activity, by binding to extracellular DNA to prevent the action of bacterially derived nucleases, thus promoting NET longevity[52]. NETs are also well suited to defense against viral challenge, **Jenne et al.**[53] demonstrated that NETs are produced following the systemic administration of poxvirus in a murine model and are able to protect host cells from the virus.

NETs provide a link between innate and acquired immune responses. NETs can directly prime T-helper cells (CD4+) and T-cytotoxic cells (CD8+), and can elevate T-cell responses by decreasing their activation thresholds. NETs may therefore represent an efficient activator of specific immunity.[54]

NETs in Periodontitis

Chronic periodontitis, is an inflammatory disease of the periodontium, initiated by bacteria and is characterized by an influx of neutrophils into the gingival crevice[55]. The sub-gingival plaque which is situated within the crevice escapes the mechanical removal by tooth brushing and mastication and stays as a tool for persistent infection[56]. Subgingival plaque results in the production of a huge number of solitary bacteria in the crevice and these large quantities of dispersed bacteria in the crevice cannot be controlled by phagocytosis.

These bacteria adhere to the pocket epithelium, triggering colonization of gingiva and invading the deeper tissues[56].

When these dispersed bacteria cross the crevice in order to reach the gingival surface, they are encountered and trapped by the Neutrophil extracellular traps spreading from the gingival surface. These NETs shield the epithelium and reduce the bacterial challenge to a large extent[56].

Vitkov et al., 2009[55] analyzed gingiva biopsies and crevicular exudates from 12 patients with chronic periodontitis and revealed that there was an abundance of NETs and some phagocytic neutrophils in the gingival pocket surface and in the purulent crevicular exudate. NETs found in purulent crevicular exudate effused from the periodontal pocket indicated that, NET products including the entrapped bacteria, were flushed from the pocket by crevicular exudate outflow.

The presence of immense quantity of entrapped bacteria suggests that the phagocytosis might have played a subordinate role in the elimination of huge quantities of dispersed crevicular bacteria, compared with NETosis[57]. Also, the inability of the host to fully eliminate the biofilm through phagocytosis has been partially compensated by NETs.

We know that, NETosis may be activated by the lipopolysaccharides from crevicular bacteria[22], after the transmigrating PMNs reach the crevice. Thus, the combination of NETs and crevicular exudate outflow appears to be a novel defense mechanism for the clearance of crevicular bacteria in chronic periodontitis, thereby evacuating the dental plaque pathogen -associated molecular patterns.

Also, **Josefine Hirschfeld, 2015**[58] showed that, NETs, neutrophil associated proteins, IL-8,1β and TNF were detected within plaque samples and saliva of gingivitis patients; because stimulation of Microbes from biofilm causes release of NETs.

However, NET release is not without a cost, because the concomitant release of cytotoxic molecules can cause host tissue damage. This is evidenced when there is excess (Exagerrated NETosis), or dysfunctional NET production (Impaired NETosis), bacterial evasion of NETs, and decreased NET removal are associated with disease progression.

Exagerrated NETosis in Periodontitis

J.B Mathews et al in 2006[59], showed that peripheral neutrophils from chronic periodontitis patients showed hyper-reactivity following Fcγ receptor and Fusobacterium nucleatum and in the absence of exogenous stimulation, these hyper- responsive neutrophils showed an increased extracellular reactive oxygen species. It is clear that ROS play a crucial role in the classical suicidal NETosis pathway, which implies that periodontal disease may be associated with an excessive production of NETs. Neutrophils from patients with periodontitis show either hyperactivity with raised baseline level of NET production, or they could be hyper-reactive, resulting in excessive NET production in response to periodontal bacteria and local pro-inflammatory mediators. In both the situations, the hyper-responsive neutrophils causes, the degradative enzymes and auto-antigenic components to be concentrated within the NETs which causes significant tissue damage.[60]

The association between periodontitits and rhematoid arthritis is well known[61], **Nesse et al. in 2012**[62], showed that Periodontitis could lead to the initiation of autoimmune inflammatory response that occurs in rheumatoid arthritis. Anti-citrullinated protein antibodies provide a mechanistic link between periodontitis and RA. Peptidyl arginine deiminase enzymes (PAD4) which is involved in NET production, is also necessary for the formation of anti-citrullinated protein antibodies. P. gingivalis, expresses PAD4 enzyme and provides a source for anti-(citrullinated protein) Igs or even stimulate NET production that could potentially provide the link between RA and periodontitis[63].

The LPS component of the cell wall of Gram- negative bacteria is an important pathogen-associated molecular pattern (PAMP) that triggers an innate immune response mainly through the activation of the toll-like receptor 4. LPS is a potent inducer of NETs[22]. The supernatant of dental plaque also triggers NETosis. Elevated blood plasma LPS levels have been seen in aggressive periodontitis[58] which causes exaggerated NETosis.

Impaired NETosis

Ineffective/Impaired NET activity can also contribute to periodontitis pathogenesis. This may be due to NET production by hypo-active neutrophils or by rendering NETs ineffective by evasion of trapping by expressing capsule or degradation via DNase activity[64].

Wartha et al. in 2007[65] showed that, pathogens that have capsules or which can change their surface charge are not entrapped.eg. Pneumococci are captured, but not killed in NETs. This is because of the polysaccharide capsule and lipoteichoic acid (LTA) modification on pneumococci which interacts with NETs.

Another way to avoid entrapment is by the synthesis of nucleases. Eg. The group A streptococcus (GAS) Streptococcus pyogenes, Pneumococcus species, and Staphylococcus aureus synthesize endonucleases that escape them from NETs and allow their penetration into deeper organs.

The result of ineffective neutrophil extracellular trap function would result in bacterial infiltration into the periodontal tissues, evoking a more widespread inflammatory response and culminating in periodontal tissue destruction[64].

Reduced NET production by peripheral neutrophils in older (greater than 50 years) periodontitis patients compared with younger patients in response to a HOCl stimulus[66]. Periodontitis prevalence increases significantly with age [67], and reduced NET production in older individuals suggests that neutrophil responses in periodontitis alter with aging[68].

As stated earlier, Reduced NET production has been associated with Chronic Granulomatous Disease (CGD), because of mutation in NADPH oxidase, and NETosis failure. Out of 368 CGD patients only nine cases were reported and associated with of gingivitis or periodontitis[69].

Clearance of NETs

The timely removal of NETs, Antimicrobial peptides, autoimmunogenic DNA, and entrapped bacterial debris from tissues are essential, or else, these may concomitantly act as a source of host-cell cytotoxicity. NETs get degradated by deoxyribonucleases (DNases), then NETs are opsonized by complement component C1q, which augments macrophage phagocytosis. NETs get degraded within the lysosomal compartment of the macrophages. The process of NET removal resembles apoptosis, an "immunologically silent" and do not release proinflammatory cytokines[70].

II. Conclusion

The NET formation by neutrophils in periodontal tissues is an important strategic response of the immune system in the containment of the infection by immobilizing microorganisms and preventing their dissemination throughout the surrounding tissues and systemically.

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