Neutrophils—the Sentinels of Periodontal Innate Immunity

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Abstract

The neutrophils play a pivotal role in the innate immune response to periodontal microorganisms. They are rapidly recruited to the dentogingival area where there is accumulation of dental plaque. The antimicrobial armamentarium that they possess not only eliminates the microbes but also partly contributes to the tissue destruction seen in periodontitis. Hence, their action is akin to that of a double-edged sword. Defects in neutrophil numbers or genetic abnormalities in neutrophil migration, chemotaxis and phagocytosis manifest as severe forms of aggressive periodontitis, emphasizing the importance of these cells in innate immunity. Over the past few decades, extensive research in the field of neutrophil cell biology has revealed fascinating facts about this cell. The discovery of neutrophil extracellular traps (NETs) is a novel concept and is considered as the central aspect of innate immunity. In addition to being a key defense cell, the neutrophil not only regulates the immune response but also provides a link between innate and adaptive immune system. The apoptotic neutrophil exerts anti-inflammatory effect. This review focuses on neutrophil functions, microbial killing mechanisms with special focus on neutrophil extracellular traps and the role of neutrophils in the pathogenesis of periodontal disease.

Keywords: Neutrophil; Polymorphonuclear leukocyte; Innate immunity; NETs; Periodontal disease

Introduction

Infectious diseases are caused by microorganisms, which have the advantage of reproducing and evolving much more rapidly than their human hosts. In response, the human body heavily invests in cells dedicated to defense, which collectively form the immune system [1]. The immune system is a complex, highly regulated set of processes that require the host to detect changes in host cells or undesirable exogenous cells [2]. The RAISON D'ETERE of immune system is to distinguish self from nonself and thereby enable survival in a hostile environment [3].

The oral immune system is part of an extensive and specialized compartmentalized mucosa-associated lymphoid tissue (MALT). The primary function of the immune system of the mouth is to protect the teeth, jaws, gingivae and the rest of the oral mucosa against infection [4]. Two types of immunity, innate and adaptive have evolved and work together to provide defense to diverse challenges. Innate or nonspecific immune mechanisms are present from birth in all individuals, and are the first line of defense against all exogenous challenging organisms. The mechanisms are nonspecific, that is, there is no discrimination between different challenges [2]. The mechanisms of innate immunity include physical barriers such as epithelium, fatty acids, mucus and cilia; soluble factors such as proteins of complement cascade, chemokines (that induce leukocyte migration) and cytokines (that modulate leukocyte function), and leukocytes other than T cells and B cells [3].

Periodontal disease is considered to be a mixed infection wherein pathogens act directly or indirectly in destruction of tooth supporting structures. The main etiological factor is the accumulation of microbial plaque in the dentogingival area. The host reacts to this bacterial challenge by activating its defense mechanism in an attempt to localize and eventually eliminate the pathogens [5]. Defenses against infection comprise a wide range of mechanical, chemical, and microbiologic barriers that prevent pathogens invading the cells and tissues of the body. Saliva, GCF, and the epithelial keratinocytes of the oral mucosa all protect the underlying tissues of the oral cavity and in particular the periodontium. If these primary defenses are breached, then the cellular and molecular elements of the innate immune response are activated. Recognition of pathogenic microorganisms and recruitment of effector cells (e.g., neutrophils) and molecules (e.g., the complement system) are central to effective innate immunity. The specific recognition of periodontal pathogens and the events that lead to activation of neutrophils in the periodontium are orchestrated by a diverse range of cytokines, chemokines, and cell surface receptors [6].

The host reaction to gingival microorganisms is characterized in part by an influx of polymorphonuclear neutrophils, [7] considered to be the first line of innate immunity.

Discovery of Neutrophil

The first evidence of an organism’s ability to fend off disease came from the research of Russian zoologist, Elie Metchnikoff in the year 1882. On a beach in Sicily, he collected the tiny transparent larva of a common starfish. Carefully he pierced it with a rose thorn. When he looked at it the next morning, he saw a host of tiny cells covering the surface of the thorn as if trying to engulf it. The cells were attempting to defend the larva by ingesting the invader by phagocytosis. Metchnikoff’s theory of phagocytosis laid the foundation of one of the most important aspects of innate immunity. Paul Ehrlich first described neutrophils as polymorphonuclear leukocytes when new fixation techniques revealed lobulated nuclei and cytoplasmic granules containing host-defense molecules [8].

Neutrophils 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 [9]. They are the most
abundant leukocytes in blood (65% to 75% of all white blood cells) [10]. Neutrophils are so named because of their neutral staining with Wright stain. They are round cells approximately 12-14 pm in diameter. The multilobed nucleus contributes to the extreme elasticity of the cell, which is important for the cell to make rapid transit from the blood through tight gaps in the endothelium.

### Subcellular Structure of Neutrophils

Granules are the hallmarks of granulocytes. Immunoelectronmicroscopy and subcellular fractionation reveal that the mature neutrophils contain several types of granules and subcellular organelles. Four well defined types of granules have been identified in neutrophils. The primary or azurophilic granules contain many antimicrobial compounds like myeloperoxidase (MPO), the human neutrophil defensins (HNP-1 to -3), lysozyme, azurocidin, and serine proteinases elastase, cathepsin G, proteinase 3, elastase N, and others. These granules fuse with phagocytic vesicles resulting in the delivery of their contents to the ingested organism. Specific or secondary granules contain apolactoferrin, plasminogen activator, collagenase, lysozyme and some gelatinase. They are largely for release into the extracellular space. Release of granule contents may modify the inflammatory process. Collagenase may degrade collagen thus augmenting movement through collagen and participate in tissue remodelling. Specific granules contain a membrane-bound molecules that are also expressed on the cell surface- CD11, CD18, f-met-leu-phe (FMLP) receptors, C5a receptor etc. Patients who lack specific granules have defective neutrophil chemotaxis and adhesion [11]. The tertiary granules facilitate extravasation via matrix metalloproteinase (MMP)-mediated degradation of basement membrane. The secondary granules promote phagocytic capacity, while primary and secondary granules each contribute the major anti-microbial arsenal [9].

### The Delivery of Neutrophils to the Periodontal Tissues-the Arrival of the Foot Soldiers

The neutrophils patrol the blood in continuous search of prey. Neutrophil recruitment requires adhesion to and transmigration through blood vessel wall at the sites where the vascular endothelium is activated by pro-inflammatory mediators [9]. Transendothelial migration, the directed movement of leukocytes from blood into local tissues central to inflammation, is a selective interaction between leukocytes and endothelium that results in the leukocyte pushing its way between endothelial cells to exit the blood and enter the tissues. Defects in transendothelial migration are associated with aggressive periodontitis; underscoring the importance of this process in periodontal diseases [12]. Neutrophils will be stimulated to exit the blood and enter the periodontal tissues and subsequently, migrate towards endogenous epithelial (IL-8 and IL-1beta) and serum-derived (plaque activated C5a) chemotacticants and then preferentially toward exogenous chemotactic signals (such as LPS and fMLP) produced by plaque bacteria in the gingival crevice [9].

### Microbial Killing by Neutrophils-the Arsenal of the Legionnaire

Neutrophils are equipped with their antimicrobial arsenal to fight invading bacteria. Upon contact, neutrophils engulf the microbes into a phagocytic vacuole, called phagosome. Subsequently, the intracellular granules fuse with the phagosome and discharge their contents to form phagolysosomes. In these phagolysosomes, neutrophils can kill periodontal pathogens by both oxidative (respiratory burst) and non-oxidative (lytic or proteolytic enzymes) mechanisms [10]. The oxygen-independent effectors are stored in three different neutrophil granule subsets. The granules to be released first are the secondary granules and the tertiary granules. These granules contain antimicrobial proteins, such as lactoferrin, lipocalin, lysozyme, and gelatinase as well as metalloproteinases, important in tissue breakdown, which facilitates neutrophil migration and action. Next the primary granules are discharged. These peroxidase-positive granules contain small antimicrobial peptides, alpha-defensins, and antibiotic proteases namely cathepsin G. Azurophilic granules also harbor bacterial permeability increasing protein and myeloperoxidase [8]. The most common antimicrobial action is disruption of the integrity of the bacterial cell membrane (cathelicidin, alpha-defensins, serine protease). 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) [9]. Increased concentrations of alpha-defensins have been shown in the neutrophils of diseased periodontal tissues [13]. 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% [14].

The oxygen-dependent mechanisms involve a non-mitochondrial generation of reactive oxygen species (ROS). A diverse set of ROS is formed during this process [15]. 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 (O2--). Superoxide spontaneously or through catalysis by superoxide dismutase (SOD) reacts to H2O2, which in turn is the substrate for MPO to form the bactericidal acidic acid (HOCL). HOCL is the most bactericidal antioxidant in neutrophils [8]. The chlorination of bacterial targets can inactivate membrane proteins and the origin of replication site for DNA synthesis [16]. In addition, ROS has been implicated in the activation of granule proteases [17,18].

### Neutrophil Extracellular Traps-Nature's Snares to Trap Bacteria

In addition to the intracellular killing mechanisms, activated neutrophils possess another powerful weapon in their armory-the neutrophil extracellular traps (NETs). This novel paradigm in innate immunity was discovered in 2004 by Brinkmann et al. ETs are DNA-based net like fibers that mediate an antimicrobial function outside the cell. They are complexes of nuclear or mitochondrial DNA [19,20] together with proteins such as histones, cell-specific enzymes (myeloperoxidase or elastase) and antimicrobial peptides (e.g., cathelicidins). The DNA itself is not antimicrobial, it is the host proteins bound to the DNA scaffold that give ETs their antimicrobial activity [21]. In the presence of various bacteria, neutrophils have been shown to release their DNA in a net-like fashion to create traps [19]. The molecular mechanism of NET formation is unclear and it is believed that ROS plays a central role. Production of ROS, such as superoxide or H2O2, are essential signaling molecules leading to the induction of a unique cell death program and the elaboration of ETs [22]. NET formation has been described as a novel cell death program [22]. Steinberg and Grinstein termed this novel cell death which is morphologically distinct from the classical cell death program (apoptosis and necrosis) as "NETosis" [23]. In NETosis, global chromatin decondensation and disintegration of the nuclear membrane occur concomitantly with cytoplasmic granule dissolution, allowing the ET components to mix in the cytoplasm prior
to their extracellular release. ETs bind microorganisms, preventing their spread and ensuring a high local concentration of antimicrobial agents capable of inhibiting or killing the invading pathogens extracellularly [21].

**Neutrophil Extracellular Traps in Periodontal Pockets**

Periodontal pathogens have the capability to evade the host defense mechanisms. The extracellular traps of neutrophils are an additional mechanism by which these cells of innate immunity can fight back. A study conducted by Vithov et al., revealed an abundance of neutrophil extracellular traps and some phagocytic neutrophils found on the gingival pocket surface and in the purulent crevicular exudate. Trapping of crevicular bacteria prevents their adhesion to and invasion of the gingival [24]. In their study, Buchanan et al., have hypothesized that DNase expression by pathogen, group A streptococcus is capable of degrading the chromatin NET backbone and thus may escape killing that DNase expression by pathogen, group A streptococcus is capable of degrading the chromatin NET backbone and thus may escape killing. DNase-producing bacteria may facilitate the maturation of pathogenic plaque biofilm [9].

Thus, NET formation by neutrophils helps in the containment of the infection along with decreasing the inflammation by releasing anti-inflammatory lipoxins and lowering pathogen load [26]. Also, NET formation may increase both the spatial and the temporal antimicrobial activity of neutrophils to act on neighboring but non-entrapped microorganisms thus continuing their antimicrobial effects even after their death [27].

**Neutrophil-Mediated Periodontal Tissue Destruction**

Ryder in his review has stated that there are two prominent mechanisms that have been proposed to explain the role of neutrophils in the periodontal disease development. These are the impaired neutrophil and the hyperactive neutrophil. A third mechanistic category could also explain the role of neutrophils, which is chronic recruitment and activation of the normal neutrophil [28].

The tissue destruction found in periodontal disease results, for the most part, from the actions of the immune system and related effector mechanisms [29]. Mediators produced as a part of the host response that contribute to tissue destruction include proteinases, cytokines, and prostanolipids. Matrix metalloproteinases (MMPs) are considered to be primary proteinases involved in periodontal tissue destruction by degradation of extracellular matrix molecules. MMPs are a family of proteolytic enzymes that degrade extracellular matrix molecules, such as collagen, gelatin, and elastin. MMP-8 and MMP-1 are both collagenases; MMP-8 is released by infiltrating neutrophils. Several studies have revealed that collagenase is elevated in tissues and GCF associated with periodontitis, as compared with gingivitis or healthy controls. Other proteinases associated with periodontitis include the neutrophil serine proteinases, elastase and cathespin G. Elastase is capable of degrading a wide range of molecules including elastin, collagen, and fibronectin. Cathespin G is a bacterial proteinase that also may function in the activation of MMP-8 [30]. Cathespin G is elevated in the gingival tissues and GCF in adult periodontitis [31]. Elevated levels of elastase in GCF are associated with active periodontal attachment loss, and elastase may provide a convenient clinical marker of disease progression [32].

**Neutrophil Defects and Polymorphisms Associated with Aggressive Periodontitis**

A study conducted by Coeeshott et al. stated that neutrophil-derived proteinases can activate some cytokines, most notably TNF alpha and IL-1β (pro-inflammatory cytokines) [34]. Another study conducted by Bank et al. revealed that the neutrophil proteinases can inactivate the cytokine IL-6 (anti-inflammatory cytokine) [35]. Soehnlein stated that azurocidin and proteinase-3 upregulate adhesion molecules on the vascular endothelium, while cathelicidin is a potent recruiter of monocytes to sites of bacterial infection [36]. Thus these granule proteins combine to promote the intensity and longevity of the inflammatory response. Neutrophil proteinases may also amplify and link the innate and adaptive arms of the immune system [9]. Chimerin, (tazarotene-induced gene 2 protein, TIG-2; retinoic acid receptor responder protein 2) is an important chemoattractant to professional antigen-presenting cells (dendritic cells and macrophages) required

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<td>Agranulocytosis</td>
<td>Almost complete absence of granulocytes or PMNs.</td>
<td>Oral lesions constitute an important phase of clinical aspect. These appear as necrotizing ulcerations of oral mucosa, tonsils and pharynx. Gingiva and palate are involved. Lesion appears as a ragged necrotic ulcer covered by a gray or even black membrane. Patients often manifest excessive salivation.</td>
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<td>Cyclic neutropenia</td>
<td>A periodic or cyclic diminution in circulating PMNs as a result of bone marrow arrest, accompanied by mild clinical manifestations, which spontaneously regress only to recur subsequently in rhythmic pattern.</td>
<td>Patients exhibit gingivitis, sometimes stomatitis with ulceration, which corresponds to the period of neutropenia. In children the repeated insult of an infection often leads to considerable loss of supporting bone around the teeth.</td>
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<td>Leukocyte adhesion deficiency</td>
<td>These patients mostly children have multiple defects in neutrophil and mononuclear phagocyte adhesion dependent functions, including chemotaxis and CR mediated phagocytosis.</td>
<td>Recurrent infections with pyogenic bacteria including severe periodontal disease.</td>
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<td>Hypermagnatobulinemia (Job’s Syndrome), rare complex autosomal recessive disorder.</td>
<td>Defects in chemotaxis of PMNs.</td>
<td>Recurrent infections with opportunistic organisms viz; Staphylococcus aureus and Candida albicans.</td>
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<td>Chediak Higashi syndrome (rare disease) often fatal in early life as a result of lymphoma-like terminal phase, hemorrhage or infection with autosomal recessive mode of inheritance.</td>
<td>Structural defect where in transformation of the azurophilic and specific granules into giant bodies called “megabodies” is characteristic. Functional neutrophil defects include decreased chemotaxis, degranulation and microbicidal activity.</td>
<td>Ulceration of oral mucosa, severe gingivitis, glossitis, periodontal disease, severe bone loss and recurrent pyogenic infection. Mutation in vesicle trafficking regulator gene LYST.</td>
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Table 1: Oral manifestations associated with neutrophil abnormalities.
Specific granule deficiency (SGD) is a rare disease, which is probably autosomal recessive. It represents a failure to pack whole group of proteins (both specific and azurophilic granule protein) into granules. Deficiency of these components results in depressed respiratory burst activity, diminished ability to respond to chemotaxis and poor phagocytosis. Intraphagolysosomal killing is predictably sluggish. Severe periodontitis and ulceration.

Papillon-Lefevre syndrome is a rare autosomal recessive disorder. Reduced PMN motility and reduced PMN bactericidal activity have been reported. There is rapid generalized destruction of alveolar bone and early loss of deciduous and permanent dentition. After the loss of deciduous teeth, tissues heal rapidly and without sequel until the eruption of permanent dentition, when the process begins anew. Recently associated in affected individuals with a mutation in the Cathepsin C gene.

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![Figure 1: Schematic illustration depicting the role of neutrophil.](image-url)
to engage lymphocytes. Wittamer et al. in their study stated that both cathepsin G and neutrophil elastase can convert procherm in the active form, cherm in [37]. Thus neutrophils are emerging as innate immune cells whose activation links both the innate immunity and the acquired immunity. Neutrophils, macrophages, dendritic cells and T cells co-localize at various infectious (i.e. M. tuberculosis) or inflammatory sites, where neutrophils act first as innate immune cells and then provide signals for activation of other innate immune cells (macrophages and dendritic cells) and T cells [38].

**Neutrophil Apoptosis-the Graceful Exit of the Sentinel**

Neutrophil death can occur by apoptosis or necrosis and also by another unique mechanism called as NETosis. NETs are degraded by DNAse, an enzyme found in the serum of healthy individuals [27]. The neutrophils switch from producing proinflammatory mediators (such as prostaglandins and leukotrienes) to producing anti-inflammatory mediators such as lipoxins. The lipoxins promote the phagocytosis of apoptotic neutrophils by macrophages and induce macrophages to synthesize antiinflammatory cytokines such as TGF-beta and lipid mediators (resolvins, protectins, and maresins) that play a key role in inhibiting neutrophil recruitment, their activation and are responsible for clearing neutrophils from the site of inflammation [39]. Thus the dying neutrophil exerts anti-inflammatory effect by modulating the surrounding cell responses (Figure 1).

**Conclusion**

Neutrophils are indispensable for defense against intruding pathogens. They are generated in great numbers in the bone marrow and circulate in blood for a few hours and are recruited to the site of injury or infection where they engulf and kill the invading microorganisms by releasing a cocktail of cytotoxic and proteolytic enzymes. A recent discovery and a novel concept is the existence of NETs, which enable the neutrophil to kill the microorganisms extracellularly. Individuals who have genetic abnormalities with respect to neutrophil migration, chemotaxis or phagocytosis will have severe forms of periodontitis thus underscoring the importance of neutrophil in the innate defense mechanism. Ever since their discovery, these legionnaires have been considered to be unrefined because they cause tissue destruction during the process of eliminating the microbes. The neutrophil is partly responsible for causing periodontal tissue destruction by releasing large-conductance Ca2+-activated K+ channel is essential for innate immunity. Activity of neutrophils is mediated through activation of proteases by K+ flux. Large-conductance Ca2+-activated K+ channel is essential for innate immunity.

**References**


