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Neutrophils in periodontal inflammation

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Neutrophils, the most abundant of the leukocytes, are myeloid-derived, professional antimicrobial phagocytes that can also kill pathogens extracellularly, link the innate and adaptive arms of the immune response, and help promote inflammatory resolution and tissue healing. Found extensively within the gingival crevice and epithelium, neutrophils are considered the key protective cell type in the periodontal tissues (1–4). Intriguingly, histological evidence (see Figure 1) suggesting that neutrophils form a “wall” between the junctional epithelium and the pathogen-rich dental plaque (2–3). Providing more than a mere barrier function, this antimicrobial wall is thought to function in two ways: as a robust secretory structure (reactive oxygen species [ROS] and bacteriocidal proteins) and as a unified phagocytic apparatus. In other words, the protective power lies in the synergic structure. However, this protection is not without cost as considerable observational, genetic and experimental data have established a clear association between neutrophil infiltration into the periodontal tissues and the severity and progression of inflammatory periodontal diseases (3, 5–7).

Gene activity in neutrophils

While in the bone marrow, neutrophils are transcriptionally and translationally active. During this “*differentiation program*” neutrophils are furnished with a large armoury of pre-formed signaling and anti-microbial molecules, stored in granules (8). In the region of $5\text{--}10 \times 10^{10}$ new neutrophils are produced daily (9). Differentiation occurs over approximately 14 days, after which neutrophils enter the circulation as mature, essentially, terminally differentiated cells that have been traditionally considered to be transcriptionally quiescent. However, recent evidence shows that upon leaving the vasculature for infected or inflamed tissues, such as the periodontium, neutrophils undergo a second burst of transcriptional activity, in a process referred to as the “*immune response program*” (8). During the immune response program cytokines and chemokines, molecules that direct the resolution of inflammation and promote healing, are the prominent translational products (8). While the relevance of neutrophil gene activity in the periodontal tissues has yet to be established, it is important to note that the immune response program renders periodontal neutrophils capable of *de novo* synthesis of multiple factors that may influence disease progression. In other words, periodontal neutrophils are not functionally reliant solely on their granule contents, as previously thought.

Search strategy: This manuscript was informed by a PubMed search for “Neutrophil OR PMN” AND “periodont* OR gingivitis” for articles published between 2004 and 2010 that on November 5, 2010 revealed 386 hits. These data were supplemented by a selection of key articles providing contemporary hypotheses on general neutrophil physiology that were identified by “Neutrophil OR PMN”, limit “review”.

Neutrophil granule diversity

A TEM highlighting a typical, highly granular human neutrophil is presented in Figure 2. The variant membrane-bound, intracellular granular structures of neutrophils, known as primary (azurophilic), secondary (specific) and tertiary (gelatinase) granules as well as the secretory vesicles, are traditionally distinguished by granule-specific biomarker proteins (see Figure 3). However, a huge degree of heterogeneity in neutrophil granule content is now appreciated (8). Granule formation occurs as a continuum with the considerable heterogeneity explained by the “*targeting-by-timing hypothesis*”. This targeting-by-timing hypothesis states that different granule proteins are expressed at different stages of the differentiation program and that these temporally controlled granule proteins are diverted from the constitutive secretory pathway and packaged into granules as and when they are made (8, 10). Thus, the contents of granules packaged late in the differentiation program will be very different than those packaged early in the differentiation program. The ability of the differing granule sub-types to fuse with the neutrophil cell membrane and, thus, degranulate into the extracellular environment or populate the neutrophil cell surface (as opposed to fusing with the phagosomal membrane) is similarly temporally controlled. v-SNAREs (vesicle-soluble NSF [N-ethylmaleimide sensitive factor/fusion protein] attachment protein receptors) are produced in larger amounts as the differentiation program progresses, with vesicle-associated membrane protein-2 (VAMP-2) the best characterized (8, 11). v-SNAREs, such as VAMP-2, enable granule membrane fusion with the cell membrane. Thus, the targeting-by-timing hypothesis also provides an explanation for the alternate functions of the various neutrophil granule compartments. The last granules to form in the bone marrow - the secretory vesicles – contain the greatest concentration of v-SNAREs and, thus, they control neutrophil environmental responsiveness and firm adhesion to activated vascular endothelia in the periodontium and elsewhere. The tertiary granules facilitate extravasation via matrix metalloproteinase (MMP)-mediated degradation of basement membrane (without release of the degradative neutrophil serine proteases [cathepsin G, neutrophil elastase and proteinase 3]. The secondary granules promote phagocytotic capacity, while primary and secondary granules each contribute the major anti-microbial arsenal (8, 12–13). While not an absolute, then, the predominant proteinaceous contents of the various intracellular membrane-bound compartments in human neutrophils are presented in Figure 3. The combination of proteases shown in Figure 3, in conjunction with reactive oxygen species (ROS), are capable of destroying most components of the periodontal soft tissues, not limited to collagen and collagen-derived peptides including the organic component of alveolar bone, fibrinogen, fibronectin, laminin and elastin.

As is clear from Figure 3, multiple receptors are stored in the v-SNARE-rich secretory vesicles and can be readily transported to the cell surface. The interaction of selectin adhesion molecules present on the neutrophil surface with activated vascular endothelium, as is present in abundance during periodontal inflammation, is sufficient to induce the intracellular calcium fluxes required to induce secretory vesicle fusion with the cell membrane (8, 16). Human neutrophils express all known TLRs (TLR1 – 10) with the exception of TLR3 with v-SNAREs enabling the transfer of TLRs that signal from the surface (TLR1, 2, 4 and 5) (17–18). The importance of TLRs in recognizing periodontal, and other, pathogens by innate cells is addressed in detail in **Chapter 5: Innate cellular responses to the periodontal biofilm**. Other signaling molecules transposed to the neutrophil surface include extrinsic apoptosis receptors (e.g., FAS, TNFRI, TRAIL-R2); apoptosis inhibitors (IL-1R, TLR4); and anti-apoptosis receptors (e.g. for IFN γ and GM-CSF). While this array of environmental sensing receptors are present intracellularly prior to exiting the bone marrow, it is not until inflammatory stimuli are encountered that these signaling molecules are transported to the cell surface, rendering them functional.

Bacterial killing mechanisms of neutrophils

Until the recent growth in our understanding of the important roles played by epithelial cells in innate immunity (see **Chapter 5: Innate cellular responses to the periodontal biofilm**), neutrophils have been considered the first line of defense against infectious agents. Neutrophils can destroy periodontal pathogens by both oxygen-dependent (the “*respiratory or oxidative burst*”) and oxygen-independent (lytic and proteolytic enzymes) mechanisms. Functional neutrophil NADPH oxidase, required for ROS generation, is a composite of seven or more proteins. Cytosolic NADPH oxidase components are translocated to the phagosome membrane upon “*neutrophil priming*” (engagement by individual, or a consult of, pro-inflammatory messengers), where they assemble into NADPH oxidase complexes in association with phagosomal cytochrome *b558* (17, 19). NADPH oxidase catalyzes the production of superoxide anions. Granule proteins also contribute to the production of ROS within phagosomes, most notably myeloperoxidase (MPO), which produces $\text{OH}^\cdot$ and singlet oxygen as well as HOCl from H_2O_2 and chloride (17, 19). The strong oxidant combination generated by the respiratory burst and myeloperoxidase is sufficient to kill most prokaryotes through compromise of bacterial phospholipid bilayers; fragmentation/inactivation of proteins; and induction of DNA damage.

Several granule proteins are involved in oxygen-independent bacterial killing. In hypoxic periodontal pockets, this may be critical, although neutrophils may be capable of generating ROS in periodontal pockets with oxygen concentrations as low as 1–3% (20). Indeed, the generation of neutrophil serine protease-deficient mice has evidenced the importance of cathepsin G and elastase in the destruction of both Gram positive and Gram negative microbes (21–22). Azurocidin (hCAP 37kDa), cathelicidin (LL-37), human neutrophil peptides (HNPs; α -defensins), lactoferrin, elastase and lysozyme are all neutrophil-derived molecules with direct or indirect antimicrobial activities (23–24). The most common mechanism of neutrophil-derived antimicrobial action is disruption of the integrity of the bacterial cell membrane (cathelicidin; α -defensins; serine protease; azurocidin). Other granule-derived antimicrobial mechanisms include destruction of peptidoglycan (lysozyme); iron sequestration (lactoferrin; neutrophil gelatinase-associated lipocalin [NGAL]); opsonization (azurocidin) and the degradation of proteolytic bacterial virulence factors (elastase) (8, 22, 24). For a more complete review of neutrophil granule-dependent killing mechanisms, please see (24).

While β -defensins are predominantly produced by the oral epithelia, neutrophils produce mainly α -defensins (HNP1-4) (25). Increased concentrations of α -defensins have been shown in neutrophils in diseased periodontal tissues (25). It seems that periodontal pathogens (e.g. *P. gingivalis*, *Treponema denticola*) in general may be more resistant to neutrophil phagocytosis and to epithelial (human beta defensin-3)- and neutrophil (cathelicidin)-derived antimicrobials than non-pathogens (e.g. *Actinomyces naeslundii* and *Streptococcus sanguis*) (26). Cathelicidin, e.g., can neutralize the anti-neutrophilic leukotoxin produced by *Aggregatibacter actinomycetemcomitans* (27). However, it seems that neutrophil phagocytosis of *A. actinomycetemcomitans* may be inefficient regardless of strain or ability to produce leukotoxin (28).

This plethora of oxygen-dependent and oxygen-independent antimicrobial factors - in addition to extracellular bacterial traps (see below) - renders neutrophils very efficient at controlling periodontal pathogens and, generally, results in containment of pathogens within the oral cavity. The importance of functional neutrophils in the maintenance of periodontal health will be discussed later in the context of genetic neutrophil defects that predispose to often severe inflammatory periodontal diseases. Conversely, the contribution of overly

robust or prolonged neutrophilic anti-microbial activities to collateral periodontal tissue damage will also be discussed.

Delivery of neutrophils to the periodontal tissues

Neutrophil recruitment requires adhesion to, and transmigration through, blood-vessel walls at sites where the vascular endothelium is activated by pro-inflammatory mediators. The human junctional epithelium is never sterile meaning that, even with optimal plaque control, neutrophils will still be stimulated to exit the gingival microvasculature, enter the periodontal tissues and, subsequently, migrate firstly toward endogenous, epithelial- (such as IL-8 and IL-1 β) and serum-derived (plaque activated C5a) chemoattractants then preferentially toward exogenous chemotactic signals (such as LPS and fMLP) produced by plaque bacteria in the gingival crevice. Indeed, high levels of bacteria and individual neutrophil chemoattractants, including stromal cell-derived factor-1 (SDF-1 α or CXCL12) (29), are found in the gingival crevice, which can result in the recruitment of vast numbers of neutrophils into the periodontal pocket (30). Indeed, neutrophils can employ glycolysis to meet their energy requirements whereas macrophages must rely on oxidative phosphorylation. Hence, neutrophils are the key phagocytic defenders in the periodontal pocket. Essentially, the stronger the inflammatory stimulus, the greater the epithelial and endothelial activation and the larger the number of neutrophils recruited.

Some oral pathogens produce virulence factors that inhibit neutrophil transmigration into the periodontal tissues; promote leukocyte longevity; or are cytotoxic to recruited neutrophils. The HAD family serine phosphatase, SerB, produced by *P. gingivalis*, for example, may play an important role in preventing granulocyte recruitment to the periodontal tissues as, in a rat model, there is greater neutrophil infiltration upon infection with an isogenic Δ SerB mutant compared to wild type bacteria (31). On the other hand, neutrophils treated with supernatants from monocytes that have been exposed to *P. gingivalis* LPS exhibit suppressed apoptosis relative to supernatants from naïve monocytes, suggesting a longer functional life for neutrophils (32). The best characterized, plaque-derived anti-neutrophil molecule is the leukotoxin, LtxA, produced by *A. actinomycetemcomitans* (33). LtxA is a member of the RTX (repeat in toxin) group of toxins that targets α L/ β 2 (LFA-1; Cd11/CD18b) adhesion molecules on the surface of hematopoietic cells and is highly cytolethal to neutrophils through induction of apoptotic cell death and/or formation of pores in the innate cell membrane (33–34).

Neutrophil extracellular traps (NETs) in periodontal pockets

NETs are extracellular, neutrophil-derived chromatin fibers combined with various granule proteins (including elastase, cathepsin G, myeloperoxidase, bactericidal/permeability-inducing protein [BPI], lactoferrin, peptidoglycan recognition proteins and MMP-9) that bind and kill bacteria, and other pathogens, as well as destroying microbial virulence factors, presumably proteolytically (35–36).

Several oral pathogens have long been known to produce factors that can subvert the immune response. The discovery of NETs provides a mechanism by which innate cells can “fight back” or, in other words, interfere with bacterial virulence factors extracellularly. NETs may also control innate cell life span through an alternate mechanism of eukaryotic cell death, known as NETosis. An abundance of NETs containing trapped bacteria can be visualized in periodontal pockets (pocket surface, GCF and pus) (37–39), as is shown in Figure 4. The study of NETs in altering pathogen-host interactions is in its infancy and the role of NETs in protecting against periodontal diseases has yet to be established. However, NETs are clearly present in the periodontal crevice and, furthermore, it has been hypothesized that bacteria that produce DNases capable of degrading the chromatin NET

backbone may be more virulent than those without DNase (35, 40). Additionally, DNase-producing bacteria may facilitate the maturation of a pathogenic plaque biofilm. It is hoped that the next few years will provide fascinating insights in the role played by NETs in the maintenance of periodontal health and how periodontal pathogens may have evolved strategies to subvert NET-mediated bacterial killing.

Neutrophil survival in the periodontal tissues

On leaving the bone marrow, neutrophils generally remain in circulation for less than 12 hours. Circulating neutrophils that do not extravasate into inflammatory compartments are catabolized in the spleen (1). Neutrophils that do enter tissues undergo apoptosis after 1 to 2 days and are subsequently cleared by macrophages. Conventional extrinsic (e.g. TNF-, FAS ligand-, TRAIL-induced; caspase-dependent and -independent) and intrinsic (spontaneous, constitutive, and, e.g., ROS-, mitochondrial-induced in senescent cells) apoptotic pathways are clearly operational, as recently reviewed (17). However, other less well defined cell death programs are also active in neutrophils, including phagocytosis-induced cell death and NADPH oxidase dependent-NETosis, while neutrophils also appear to have a large autophagic capacity (17, 41–43). It is important to note that the *de novo* production of pro-inflammatory cytokines is suppressed, and most primary effector functions down-regulated, during apoptosis (17, 44–45). During apoptosis multiple, redundant macrophage receptors facilitate effective recognition, engulfment and clearance of neutrophils. Such pro-phagocytotic molecules include complement receptors, the phosphatidylserine receptor and several other scavenger receptors, such as MARCO and CD36 (17, 41). Furthermore, the uptake of apoptotic neutrophils induces the production of anti-inflammatory cytokines, such as TGF β , in the phagocytosing cell (17, 46). Several periodontal pathogens have been shown to induce phagocytosis-induced cell death in innate cells, including *Fusobacterium nucleatum* and *P. gingivalis*, as well as other bacteria known to inhabit the oral cavity, such as *Neisseria gonorrhoeae* (17, 47–48). Induction of apoptosis presumably benefits periodontal pathogens by preventing phagocytosis. In general, however, apoptosis of inflammatory cells, particularly neutrophils, is key to the resolution of apoptosis and prevention of collateral tissue damage by degradative neutrophil enzymes and ROS that would otherwise be released into the periodontium upon neutrophil necrosis and/or degranulation. In other words, apoptosis is a non-phlogistic process.

On the other hand, several inflammatory mediators promote neutrophil longevity at sites of inflammation, such as LPS, lipoteichoic acids, C5a, IL-1 α , GM-CSF, G-CSF, and IFN γ , and render these granulocytes resistant to extrinsic ligand-induced apoptosis (FAS and TNF) (49). The consequence is extended functionality (17). Some of the molecules known to extend neutrophil life span also prime neutrophils in a manner that enhances ROS production, and perhaps other functions, when a second activating signal is encountered (17). Obviously, many of these longevity-inducing factors are present in high concentrations in periodontal tissues and gingival crevicular fluid in inflammatory periodontal diseases, particularly LPS produced by the predominantly Gram-negative flora associated with periodontitis. Some pathogens have developed mechanisms for suppression of apoptosis in neutrophils, such as *Chlamydia pneumoniae* (50). However, the potential anti-apoptotic properties of the periodontal microflora have not received much research attention. Specific bacterial pathogens may also promote inflammation through the promotion of degranulation and/or lysis and/or secondary necrosis in neutrophils - most notably *Streptococcus pyogenes* and leukotoxin-producing *A. actinomycetemcomitans* strains (51–53). While the balance between pro- and anti-apoptotic signals for neutrophils in periodontal tissues has not been clearly established - and is likely to vary depending on disease state and environmental influences - there are clearly large numbers of functional neutrophils present in the gingival

tissues during gingivitis and at all stages of chronic periodontitis capable of both killing bacterial pathogens and contributing to collateral tissue damage.

Neutrophils and the promotion of oedema

Neutrophils are thought to play key roles in promoting edema as a consequence of interactions with the vascular endothelia during diapedesis; through the secretion of arachadonic acid derivatives; chemokines, such as CXCL1, 2, 3, and 8; and heparin binding protein (HBP; CAP37 or neutrophil azurocidin 1, AZU1); with neutrophil elastase and cathepsin G also reported to promote vascular permeability, at least *in vitro* (13, 54–56). Of these mediators, the arachadonic acid metabolites may be of particular importance due to their ability to enhance the permeability inducing capacities of other endogenous (e.g. C5a, histamine) and exogenous (e.g. fMLP) pro-inflammatory mediators. Several factors generated *de novo* by neutrophils during the immune response program help to promote edema, most notably TNF, CXCL2, and CXCL8 (13, 57–58). Finally, in addition to edema induced by the aforementioned permeability-increasing (and pro-angiogenic) factors, neutrophils also contribute to edema by damaging the integrity of the vascular endothelium through the actions of reactive oxygen species and serine proteases (13, 59–60).

Neutrophil-mediated tissue destruction

It is generally accepted that the inflammatory nature of the neutrophil response to the oral biofilm can promote a homeostatic imbalance that key to periodontal disease onset and progression. As reviewed in 2010 (3), there are two prominent mechanisms that have been proposed to explain the role of neutrophils in periodontal disease development. These are:

- The **impaired** neutrophil
- The **hyperactive** neutrophil

A third mechanistic category could also explain the role of neutrophils in periodontal disease development:

- Chronic recruitment and activation of the **normal** neutrophil

In other words, it could be that destructive periodontal disease, the prevalence of which increases with age (61), is the price to be paid for the control of periodontal pathogens through the chronic activation and extended longevity of normal neutrophils over many years.

The weight of evidence seems to suggest that overt neutrophil defects generally lead to a predisposition to aggressive forms of periodontitis (3). Impairment in neutrophil function(s) in such cases is frequently genetically (or intrinsically) determined, as discussed further below. However, the importance of the impaired or defective phenotype in disease progression is often exacerbated by environmental factors, most notably by cigarette smoking (62).

Hyperactivity, with respect to neutrophils, has been described in the periodontal literature to mean “elevated function”, e.g., increased enzymic activity, particularly, an increased respiratory burst (3). The term hyperactive is often used synonymously with “primed”, although it is not entirely clear that this is always appropriate. All neutrophils can be primed by various pro-inflammatory mediators but some authors apply a variant meaning (63–64) - a specific and intrinsic predisposition to elevated function in neutrophils in those predisposed to inflammatory periodontal diseases. Therefore, there is a need to clearly differentiate between neutrophils that have been activated, or primed, as a consequence of extravasation and neutrophils that are hyperactive prior to activation by local periodontal

stimuli. That is, neutrophils that are already primed while in systemic circulation. Again, for an in depth review of priming vs impairment of function of neutrophils and the consequences for aggressive vs chronic periodontitis, please see the recent review by Ryder.

Certainly, normal neutrophils that enter the periodontal environment are primed. This is as a consequence of the adhesive interactions with activated vascular endothelium and endogenous pro-inflammatory mediators during the extravasation process (65) and/or exposure to intact periodontal bacteria, such as *P. gingivalis* (66) and *A. actinomycetemcomitans* (67), or bacteria-derived pro-inflammatory mediators, such as fMLP (3, 66).

However, certain gene polymorphisms may lead to the hematopoiesis of neutrophils that exhibit intrinsically elevated function. For example, peripheral, presumably naïve, neutrophils from subjects with a specific (131 H/H) polymorphism in the neutrophil Fc γ receptor IIa (Fc γ RIIa) express higher levels of the degranulation markers, CD63 and CD66b, and release more elastase on exposure to immune serum-opsonized *A. actinomycetemcomitans* than do neutrophils from the more common Fc γ RIIa genotype (131 R/R) (68). Thus, 131 H/H Fc γ RIIa neutrophils are hyperactive without the need for priming. Individuals with 131 H/H Fc γ RIIa are also predisposed to more severe periodontitis than those with the 131 R/R Fc γ RIIa genotype (68). Further, Matthews et al reported that peripheral neutrophils from subjects with periodontitis produced higher unstimulated levels of extracellular ROS than granulocytes from control subjects and that this phenomenon was unaffected by priming by *P. gingivalis* and *F. nucleatum*. On the other hand, effective periodontal therapy reduced Fc γ R-stimulated ROS production (69). Multiple studies have confirmed that peripheral neutrophils isolated from chronic periodontitis patients, compared to control neutrophils, produce higher levels of ROS on exposure to neutrophil activating signals, including LPS and whole bacteria (70–71).

Even when the major endogenous mediators of periodontal tissue destruction, the serine proteases and matrix metalloproteinases (72), are released extracellularly via necrosis, exocytosis/degranulation, or via NETs, there are multiple endogenous inhibitors that limit their activity. These include α 1-proteinase inhibitor (neutrophil elastase and proteinase 3); elafin (neutrophil elastase and proteinase 3); secretory leukocyte protease inhibitor (cathepsin G and neutrophil elastase); α 1-chymotrypsin (cathepsin G); and the tissue inhibitors of metalloproteinases, or TIMPs (MMPs) (22, 73). However, it is thought that proteolytic enzymes that are either surface-associated or present in tight granulocyte-substrate junctions may be protected from endogenous inhibitors and that high, local protease gradients – as occurs in diseased periodontal tissues – can overwhelm the endogenous inhibitory capacity (22). Furthermore, unlike most MMP-producing cells, neutrophils do not contain or manufacture *de novo* the major TIMP, TIMP-1 (73). Total proteolytic activity (74) as well as multiple neutrophil-derived proteolytic enzymes have been shown to be elevated in periodontitis, compared to healthy controls, including elastase (GCF, periodontal tissues) (75–78); proteinase 3 (GCF cell pellet) (74); cathelicidin (GCF and periodontal tissues) (25, 79–81); myeloperoxidase (GCF) (82); and matrix metalloproteinases whose major source in the periodontium is the neutrophil (MMP-2, MMP-8, MMP-9 and MMP-25) (75, 83–88). To this end, it is clear that exposure to and/or phagocytosis of several periodontal pathogens, including *F. nucleatum*, *T. denticola*, *P. gingivalis* and *A. actinomycetemcomitans* leads to the extracellular release of tissue-degrading molecules, such as ROS, MMP-8, MMP-9 and elastase, from neutrophils, as well as pro-inflammatory cytokines, e.g. IL-1 β , that are expected to prolong the inflammatory response (89–91). A prolonged neutrophil response to plaque may also contribute indirectly to compromised wound healing. For example, elastase is efficient at cleaving PDGF receptors from

periodontal ligament cells, compromising proliferative signaling pathways in response to this important growth factor, (92).

Interestingly, recent data has suggested a potential oral-systemic inflammatory activation loop for neutrophils. Dias *et al* have shown that plasma from periodontitis subjects is more efficient than plasma from healthy subjects in priming neutrophils to fMLP responsiveness and in directly inducing the oxidative burst. Antibodies against IL-8, GM-CSF and IFN- α abrogated this superoxide-inducing potential of plasma (93). Others have shown that *F. nucleatum* exposure results in the upregulation of multiple ROS-related genes in neutrophils and that pro-inflammatory transcripts, including several ROS response-related genes, are differentially regulated in cells from periodontitis subjects and healthy controls (94).

In addition to breaking down collagen, and other proteins, to provide a carbon source, the gingipains produced by *P. gingivalis* are capable of modulating multiple factors that influence neutrophil recruitment or function. For example, R- and K-gingipain treatment of the 77 amino acid long variant of IL-8 results in N-terminal cleavage of 5 to 11 amino acids from this chemokine, with the truncated IL-8 molecule exhibiting enhanced chemotactic potency and increased priming activity in neutrophils, as measured by ROS production on subsequent exposure to fMLP. The opposite phenomenon (decreased neutrophil chemotaxis and priming) occurs when the more potent, innate cell-derived 72 amino acid IL-8 variant is treated with gingipains (95). *T. denticola* produces a surface-associated C3 to iC3b converting protease, dentilisin, that promotes ROS production in human neutrophils (91). It is likely that multiple host-pathogen interactions influence the release and activity of endogenous mediators of tissue destruction.

Neutrophils tune the immune response

Recent evidence shows that neutrophil-derived proteases also modulate chemokine activity (22). For example, limited cleavage of IL-8 (CXCL8) by proteinase 3 increases the chemoattractive potency of this key pro-inflammatory mediator (96), while cathepsin G-mediated cleavage lowers the chemotactic potency of CCL5 (RANTES) (97). Similarly, neutrophil-derived proteases can activate some cytokines, most notably TNF and IL-1 β (98) and inactivate others, such as IL-6 (99). Azurocidin and proteinase-3 upregulate adhesion molecules on the vascular endothelium, while cathelicidin is a potent recruiter of monocytes to sites of bacterial infection (24). Thus, these granule proteins combine to promote the intensity and longevity of the inflammatory response. Neutrophil proteases may also amplify and link the innate and adaptive arms of the immune system. 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 to engage lymphocytes. Both cathepsin G and neutrophil elastase can convert prochimerin into the active form, chimerin (100). Indeed, it seems that neutrophil serine proteases and MMPs can, between them, modulate multiple molecules that play important roles in inflammation and tissue remodeling, including anti-bacterial peptides (e.g., hCAP18 conversion to cathelicidin; azurocidin is chemotactic for, and an activator of, monocytes/macrophages (101)) (27); growth factors (e.g., VEGF and TGF- β release); adhesion molecules (e.g., ICAM-1 and VCAM-1 processing); and a host of cell surface signal transducers (e.g., TLR4 activation; TNFR inactivation) (22, 102–103). While full discussion is beyond the scope of this article, the activities and consequences of neutrophil-derived protease interaction with multiple biological targets has been recently reviewed elsewhere (22, 102–103).

Neutrophil defects and polymorphisms associated with aggressive periodontitis

The importance of fully functional neutrophils to the maintenance of good periodontal health is dramatically highlighted by the large number of genetic neutrophil defects associated with, often, severe inflammatory periodontal disease. The periodontal characteristics of some well-defined neutrophil defects are listed below*.

- **Neutropenias** (< 1500 neutrophils per ml of blood) including **Kostmann Syndrome** (neutropenia with reduced cathelicidin and HNP1-3): susceptibility to recurrent bacterial infection and aggressive periodontitis (3, 25, 104–106)
- **Chediak-Higashi syndrome** (LYST [lysosomal trafficking regulator] mutation resulting in impaired phagolysosome formation): aggressive periodontitis (107–110)
- **Papillon-Lefevre syndrome/Dipeptidyl peptidase I deficiency** (Cathepsin C mutation): (107, 111–113): aggressive periodontitis. Periodontal manifestations of Papillon-Lefevre syndrome in a single patient are shown in Figure 4.
- **A1AT-deficiency**: periodontitis (114–116).
- **Leukocyte adhesion deficiency** (CD18 integrin or sialyl-LewisX mutation): recurrent bacterial infections and aggressive periodontitis (107, 117–120).
- **Granulomatous disease** (defective NADPH oxidase system): susceptibility to bacterial infection and periodontitis (107, 121–122).
- **NA2 polymorphism in Fc γ RIIIb**, a neutrophil-specific antibody receptor: aggressive periodontitis (123–124).
- **Multiple single nucleotide fMLP-R polymorphisms****: aggressive periodontitis (3, 125–129)

The above list is not intended to be exhaustive and the number of potential identified genetic defects that affect neutrophil function and pre-dispose to periodontitis continues to increase. For example, lysosomal-associated membrane protein-2 (LAMP-2) seems critical in directing appropriate phagosomal maturation. LAMP-2 knockout mice (a model of Danon Disease) exhibit reduced bacteriocidal activity and develop severe periodontitis at an early age (135–136). Individuals with neutrophil-specific granule deficiency exhibit recurrent bacterial infections (137), but studies of the oral health of such subjects were not identified by the current search strategy. Interestingly, a polymorphism in p22phox NADPH oxidase (C242T) – a key gene in the generation of the respiratory burst in neutrophils – may also be associated with aggressive periodontitis (138).

The neutrophil in aging subjects

Immunosenescence occurs as we age and is accompanied by an increased susceptibility to multiple infectious diseases, including periodontal diseases (61). Even the acute inflammation associated with experimentally-induced gingivitis may be more severe in older subjects (140–141). There are limited studies on the effects of age on neutrophil function. Of

* Aggressive periodontitis includes earlier terminologies including juvenile periodontitis, early onset periodontitis, and rapidly progressive periodontitis. Aggressive periodontitis occurs in 0.1% to 15% of the population, although valid prevalence estimates are problematic (130).

** Reviews of associations between other single nucleotide polymorphisms, including polymorphisms in IL-1 β , IL-4, IL-10, TNF, TLR, fMLP receptor and human leukocyte antigen (HLA) genes, and susceptibility to periodontal diseases are available (1, 131–134).

those functions so far examined (expression of key receptors (GMCSF-R, TLR4, TLR2); apoptosis; hematopoiesis; chemotaxis; phagocytosis; oxidative burst; and specific activating pathways), it seems that chemotaxis, phagocytosis and capacity to signal via calcium and MAPK-dependent pathways may be impaired, as reviewed in (61, 142–143). Thus age-associated depreciations in neutrophil function may help explain increased susceptibility to periodontitis in aging populations.

Smoking and neutrophil function

Genetic and age-induced defects in neutrophil function increase risk for periodontitis. Similarly, tobacco-induced innate dysfunction appears to contribute to a predisposition to inflammatory periodontal diseases. Indeed, crevicular neutrophil viability has been shown to be consistently reduced in smokers (approximately 75% viable) compared to non-smokers (approximately 85% viable), with phagocytotic capacity also diminished in a potentially dose-dependent manner (40% in heavy smokers; 79% in non-smokers) (144). Furthermore, overall transcriptional activity associated with the immune response program is lower in neutrophils isolated from smokers compared to non-smokers (145).

As we have recently reviewed (146), tobacco smoke exposure induces systemic neutrophilia (147) and exerts profound effects on human neutrophils including compromised neutrophil maturation (147–148) and inefficient effector function (148–150). Key to the development of destructive, inflammatory periodontal disease may be the tobacco-induced induction of neutrophil-derived elastase (151–152) and metalloproteinase release (148, 153–154); combined with compromised phagocytotic and bacteriocidal capacities, including diminished ability to kill *P. gingivalis* (148, 155–156) and impaired chemotactic responsiveness (150).

Neutrophils express functional receptors for nicotine and cotinine (148, 157), as well as tobacco-derived aryl hydrocarbons (158). Neutrophils also express receptors for several endogenous immunomodulatory factors that have been shown to be dysregulated in tobacco smokers. These factors include IL-8, ICAM-1 and TNF (150, 159–162). The $\alpha 7$ nicotinic acetylcholine receptor (nAChR)–triggered cholinergic anti-inflammatory pathway is a key endogenous mechanism by which an overly robust pro-inflammatory response to infection is prevented (163–165). Neutrophils express abundant $\alpha 7$ nicotinic acetylcholine receptor (nAChR), as shown in Figure 6, from an early stage of differentiation (148). The role of the cholinergic anti-inflammatory pathway in suppressing pro-inflammatory cytokine production during the neutrophilic immune response program remains to be clarified. However, clearly, $\alpha 7$ nAChR agonists are capable of reducing the expression level of the key adhesion molecule CD11b and suppressing transalveolar neutrophil migration in animal models of pulmonary inflammation and other inflammatory diseases (166–170). Furthermore, nicotine-induced suppression of the oxidative burst and *P. gingivalis* killing in neutrophils is probably $\alpha 7$ nAChR-dependent, and certainly α -bungarotoxin (an $\alpha 7$ nAChR inhibitor) sensitive (148). A model of the mechanistic consequences of the inappropriate, i.e. not infection or trauma induced acetylcholine driven, engagement of $\alpha 7$ nAChRs on neutrophils is presented in Figure 7.

Anti-neutrophil therapeutics

As recently and extensively reviewed, a sub-antimicrobial dose of doxycycline hyclate, an antagonist of many MMP species, is the sole therapeutic designed to modulate the host response that is currently available as an adjunctive treatment for periodontitis (184). While such treatment is not strictly speaking neutrophil-specific, it is nevertheless pertinent to note that neutrophils represent the predominant source of destructive MMPs (MMP-8 and MMP-9) in diseased periodontal tissues (184).

Many identifiable genetic neutrophil disorders are potentially fatal, therefore periodontal therapy is not necessarily the first priority. Because of this, and because such diseases are uncommon, studies examining the outcome of treating the underlying neutrophil defect on oral health are few in number. However, there are some encouraging data available. Neutropenias, for example, can be treated with recombinant granulocyte-colony stimulating factor or by hematopoietic stem cell transplantation (185). A recent case report on G-CSF treatment, combined with regular maintenance therapy, in a subject with cyclic neutropenia with periodontitis proved encouraging (186). In Papillon-Lefevre syndrome, it has been suggested that regular maintenance therapy combined with early and aggressive antibiotic therapy can protect patients from edentulism (112).

As is appreciated by pulmonary scientists, the ideal anti-neutrophil therapeutic would take advantage of unique neutrophil surface receptors and signaling proteins allowing manipulation of neutrophil function in a way that does not compromise antibacterial capacity (9). Although many classes of inhibitors are under investigation, including LTB₄ antagonists, CXCR2 antagonists, long-acting b₂-agonists and HDAC2 activators (187), it remains to be seen if dental research will embrace the development of neutrophil-specific therapeutic approaches to the prevention and treatment of inflammatory periodontal diseases.

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Figure 1. Interface of bacterial plaque and crevicular neutrophils within the periodontal pocket Neutrophils in the periodontal pocket forming a wall against the plaque biofilm. Neutrophils cannot engulf the large biofilm structure *in vivo*. The formation of a wall against the biofilm may be a protective mechanism. Nevertheless, an attempt is made to engulf the surface layer of this biofilm (yellow arrows). During this process of frustrated phagocytosis, enzymes within neutrophil lysosomal granules (red arrows), products of the oxidative burst, and other pro-inflammatory substances may be released directly into the pocket and/or the underlying tissue, where they have a predominantly destructive effect. *Figure and text reproduced with permission, from(3).*

Figure 2. T.E.M. image of a typical human neutrophil

The multi-lobed nucleus and granule-dense cytoplasm are clearly apparent. Scale bar represents 2 μ M. The image is taken from a manuscript currently under consideration for publication by *Cell Death and Disease* (14).

Figure 3. Contents of the intracellular membrane-bound compartments of human neutrophils

^aCD63 and CD68 are degranulation markers; ^bProteinase 3; ^cVAMP2 is key in granule membrane-cell membrane fusion and exocytosis; ^dNADPH oxidase subunits involved in ROS production (Gp91phox; p22phox); ^eTLR1, -2, -4, -6 and -8; ^fCRCX1, -2 and -4 as well as chemokine receptors 1, -2 and -3; ^gcomplement receptor 1 (CD35), c1qR.

Data presented in Figure 3 are combined from (8, 12–13, 15).

Figure 4. Extracellular neutrophil traps (NETs) in the periodontitis

TEMs of pocket epithelium biopsies are presented. (A) Overview of neutrophil extracellular traps on the pocket epithelium surface. The pronounced blackening is characteristic for neutrophil extracellular traps. The open arrows indicate neutrophil extracellular traps threads. (B) Detail of (A). The threads (open arrows) consist of individual fibers that run in parallel. (C) High magnification reveals that neutrophil extracellular traps consist of uniformly thick fibers (small solid arrows) with a diameter of nearly 15–17 nm. (D) Solitary bacteria (indicated by solid white arrowheads) entrapped by crevicular neutrophil extracellular traps. (E) A bacterium (solid white arrowhead) adherent to the epithelium surface is entrapped by neutrophil extracellular traps. A longitudinally running neutrophil extracellular trap fiber is delineated with white bars. Small solid white arrows: a multitude of cross-sectioned neutrophil extracellular trap fibers. (F) A scheme of a crevice. On the left:

an epithelial cell with an adherent bacterium and bacteria in different stages of internalization. In the middle: periodontal crevice. Neutrophil extracellular traps build a 'firewall', protecting the gingiva from bacteria dispersing out of the subgingival plaque. The neutrophil extracellular traps, together with the entrapped bacteria, are continuously pushed into the oral cavity by the crevicular exudate outflow. Open white arrow: direction of the crevicular exudate outflow. On the right: subgingival plaque. Solid black arrow: a bacterium dispersing from the subgingival plaque. *Figure and text reproduced, with permission, from (39).*

Figure 5. Periodontal manifestations of Papillon-Lefevre syndrome

A and B, Photographs showing marked red inflamed and swollen gingivae. **C and D,** Radiograph showing severe loss of alveolar bone. Images reproduced, with permission, from (139).

Figure 6. Promyelocytic and neutrophilic HL-60 cells express $\alpha 7$ - nicotinic acetylcholine receptors

A. $\alpha 7$ nAChR Western blot: Lane 1. Mouse frontal brain extract (10 μ g; positive control); Lane 2. Lysate (40 μ g) of promyelocytic HL-60 cells; Lanes 3 to 5. Lysate (40 μ g) of five day, DMSO-induced (neutrophilic) HL-60 cells without nicotine, or with 10^{-6} M nicotine, or with 10^{-4} M nicotine treatment, respectively. $\alpha 7$ nAChR-specific antibodies (Santa Cruz SC-5544) bound to protein bands exhibiting a relative molecular mass of 55.0 KDa in all lanes. Densitometric analysis (*data not shown*) revealed that $\alpha 7$ nAChR-specific band intensities were significantly increased in the DMSO-treated samples, relative to promyelocytic cells ($n = 5$, $*p < 0.05$), but that this differentiation-associated increase $\alpha 7$ nAChR protein level was not influenced by nicotine exposure during differentiation in any statistically significant manner.

B. $\alpha 7$ nAChR Immunofluorescence staining (x1000): Fixed five-day, DMSO-induced HL-60 cells were incubated with a polyclonal rabbit anti- $\alpha 7$ antibody (Santa Cruz SC-5544) before staining with a FITC-conjugated anti-rabbit antibody (Santa Cruz SC-2253). $\alpha 7$ nAChR-specific staining was evenly distributed across the cells. A similar cellular distribution of $\alpha 7$ nAChR was observed in promyelocytic cells (*data not shown*).

C. Negative control for $\alpha 7$ nAChR Immunofluorescence staining (x1000).

This figure was originally published by BioMed Central in (148).

Figure 7. Consequences of $\alpha 7$ nicotinic acetylcholine receptor engagement by various agonists in innate cells

The $\alpha 7$ acetylcholine receptor-initiated anti-inflammatory pathway can be stimulated by various stimuli. The endogenous innate cell $\alpha 7$ nAChR ligand is acetylcholine that is produced by the vagus system in response to infection or other tissue insult (171–172). The function of acetylcholine release is to limit an overly exuberant cytokine response to infection. Many therapeutic agents are under development that attempt to take advantage of this suppressive pathway (173). Eventually, such drugs may prove efficacious in the treatment of multiple inflammatory diseases and conditions, including septic shock, pancreatitis and inflammatory bowel diseases, as well as periodontitis (174–176). Cigarette smoke contains large amounts of a potent, exogenous $\alpha 7$ nAChR agonist, nicotine (177–178) (163). The primary stable metabolite of nicotine, cotinine, is also an efficient $\alpha 7$ nAChR agonist (179). The consequences of $\alpha 7$ nAChR engagement by acetylcholine, pharmaceutical agents or nicotine/cotinine is a suppression of the inflammatory response (174, 177, 179–180). When triggered at the appropriate time, $\alpha 7$ nAChR engagement would normally be beneficial. In the case of nicotine, it has been shown that $\alpha 7$ nAChR engagement also results in the suppression of the oxidative burst (148, 181), a critical bacterial killing mechanism in phagocytic innate cells. Furthermore, nicotine- $\alpha 7$ nAChR interactions may also stimulate the rapid release of matrix metalloproteinases and other proteolytic enzymes (148) (152) (182) from innate cells. Therefore, chronic, inappropriate nicotinic stimulation of innate $\alpha 7$ nAChRs in smokers may increase susceptibility to infection with periodontal pathogens by suppressing the inflammatory response and phagocytic killing required to fight infection. Additionally, increased protease release from nicotine-exposed innate cells may promote protease-protease inhibitor imbalances that contribute to the tissue remodelling apparent in periodontal diseases (183).

Figure reproduced and text adapted from (146).