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## Human neutrophils and oral microbiota: a constant tug-of-war between a harmonious and a discordant coexistence

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### Summary

Neutrophils are a major component of the innate host response, and the outcome of the interaction between the oral microbiota and neutrophils is a key determinant of oral health status. The composition of the oral microbiome is very complex and different in health and disease. Neutrophils are constantly recruited to the oral cavity, and their protective role is highlighted in cases where their number or functional responses are impeded, resulting in different forms of periodontal disease. Periodontitis, one of the more severe and irreversible forms of periodontal disease, is a microbial-induced chronic inflammatory disease that affects the gingival tissues supporting the tooth. This chronic inflammatory disease is the result of a shift of the oral bacterial symbiotic community to a dysbiotic more complex community. Chronic inflammatory infectious diseases such as periodontitis can occur because the pathogens are able to evade or disable the innate immune system. In this review, we discuss how human neutrophils interact with both the symbiotic and the dysbiotic oral community; an understanding of which is essential to increase our knowledge of the periodontal disease process.

### Keywords

neutrophils; periodontitis; symbiotic; dysbiotic oral community

## 1 | Introduction

As humans, we are constantly exposed to bacterial challenges through the air we inhale, our skin and what we eat. However, we can maintain a healthy life due to our innate immune system which has evolved to protect us from the constant threat of infection provoked by the continuous exposure to foreign microbes at each of the accessible entry zones in our body. Not all the encounters that we have with these foreign ‘animalcules’ (initial designation attributed to bacteria by van Leeuwenhoek) result in a threat to our health. Moreover, the

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symbiotic interaction between the host and commensal microbiota has gained more and more attention lately and proven to be beneficial to our health.<sup>1,2</sup> A delicate balance exists between the host and its associated, highly complex microbiota to maintain homeostasis and health.<sup>3</sup> Even though the oral cavity is an example of an area of our body which is heavily colonized by a diverse microbial community, its interaction with the host immune system remains poorly understood when compared to studies done with the gut microbiome.<sup>4,5</sup>

Neutrophils are the main innate immune cell that is recruited in very high numbers to the oral cavity and are responsible for ensuring a healthy periodontal tissue.<sup>6–8</sup> The junctional epithelium, a specialized tissue that surrounds the tooth, generates a chemotactic interleukin (IL)-8 gradient due to its close contact with the oral biofilm community, which results in constant recruitment of neutrophils to the gingival sulcus.<sup>5,9</sup> Neutrophil presence is associated with protection against periodontal disease since increased periodontitis severity has been observed in congenital diseases like leukocyte adhesion deficiency, Chediak-Higashi syndrome, Papillon-Lefèvre syndrome and chronic/cyclic neutropenia which are all conditions in which neutrophil recruitment is compromised.<sup>10</sup> However, in chronic inflammatory diseases such as periodontitis, pathogens have evolved mechanisms to evade neutrophil clearance, which results in neutrophil accumulation in the periodontal tissue, and potential release of the neutrophil arsenal into the extracellular space causing tissue damage, and in severe cases, bone loss.<sup>11</sup> For example, while the oxidative burst response mounted by neutrophils is an efficient strategy for clearance of infection with anaerobic bacteria, several periodontal pathogens such as *Porphyromonas gingivalis* are resistant to oxidative killing.<sup>11,12</sup> Additionally, hyperactive/primed neutrophils can also predispose individuals to develop periodontitis. These neutrophils' enhanced response is characterized by the release of reactive oxygen intermediates, several cationic peptides, and enzymes such as matrix metalloproteinases (MMPs) that results in increased tissue damage and positions the neutrophil as a perpetrator of periodontitis.<sup>13,14</sup> Thus, a delicate balance between neutrophil function and bacterial challenge has to be maintained to ensure periodontal health. This review discusses the role of human neutrophils in maintaining a healthy oral mucosa and evidence associated to how the cell interaction with periopathogens can shift the homeostatic balance into a dysbiotic environment promoting a chronic inflammatory scenario—leading to periodontitis (Fig. 1).

## 2 | Neutrophils and Oral Health: The Result of a Harmonious Coexistence

In the oral cavity, the sentinel traffic of neutrophils through the junctional epithelium into the gingival space is enhanced by the peaceful partnership established with the indigenous symbiotic oral community.<sup>15</sup> Animal studies using germ-free mice (GF) demonstrate neutrophils patrolling the junctional epithelium; however, the number of cells is significantly enhanced by colonization with commensal bacteria.<sup>15,16</sup> Neutrophil migration to this area is facilitated by the high porosity of the junctional epithelium and the IL-8 chemotactic gradient generated locally that guides the high number of neutrophils from the blood vessels toward the crevicular fluid. Here, neutrophils will form a protective wall between the oral community colonizing the tooth and the junctional epithelium<sup>6,7,9</sup> (Fig. 2A).

Nonetheless, microbiota can also influence other aspects of neutrophil functions. It is now recognized that the presence of the resident microbiota also contributes to the steady state of neutrophil production since studies in GF mice reveal a significant decrease in neutrophil numbers.<sup>17</sup> The encounters that neutrophils have with the metabolites or byproducts that are generated by the local microbial community, such as short-chain fatty acids, are important in maintaining both systemic and tissue immunity.<sup>2,18,19</sup> Moreover, the microbiota contributes to the active phenotype of aged neutrophils in circulation and participate in their removal which provides some novel insight into why these cells have such a short life span in circulation.<sup>20</sup>

In periodontal health, the interaction between neutrophils and the indigenous, or commensal, symbiotic microbial community is tightly controlled to prevent tissue damage. Studies comparing GF mice and specific-pathogen free (SPF) mice reveal that the absence of the oral commensal microbiota has no impact on the structure of the gingival tissue; this is in stark contrast to the significant role that the gut commensal microbiota has on the structural formation of the intestinal tissue.<sup>16</sup> As shown in knockout mice, the chemotactic receptor CXCR2 facilitates neutrophil recruitment to the periodontal tissue. This was found since neutrophils are absent in the junctional epithelium of CXCR2 knockout mice while the cells are present in the blood vessels. Interestingly, both CXCL1 and CXCL2, ligands of the CXCR2 receptor, are expressed in the junctional epithelium of GF mice; however, only a significant increase on CXCL2 expression levels, not of CXCL1, is observed in the epithelium of SPF mice when compared to the GF. The presence of the indigenous oral bacterial community enhanced neutrophil recruitment to the periodontal tissue by selectively stimulating the expression of the potent neutrophil chemokine CXCL2.<sup>16</sup> In a recent study, Greer et al.<sup>21</sup> compare the expression levels of CXCL2 and neutrophil location in different areas of the junctional epithelium across the tooth, among GF, SPF and GF mice which were gavaged with single commensal oral bacteria like *Streptococcus* sp. or *Lactobacillus* sp.<sup>21</sup> Neutrophils show a similar location pattern in the periodontium of the SPF mice and bacteria-gavaged GF mice, but this is distinct from the pattern seen in GF mice. Moreover, a positive correlation exists between the neutrophil location and the expression levels of CXCL2 in the junctional epithelium of bacteria-colonized animals. In the presence of the indigenous oral bacterial community, both the numbers of neutrophils as well as the levels of the CXCL2 chemokine are enhanced in the interdental region of the periodontal tissue. This provides novel and valuable information about how the commensal organisms modulate the innate immune response to maintain homeostasis in the oral cavity.<sup>21</sup>

From both mice and human studies, the protective role that neutrophils play in preservation of the oral health is well established, since low neutrophil counts as well as deficiency in neutrophil functional responses have been associated with the clinical manifestation of periodontal disease.<sup>13,22</sup> Neutrophils are very efficient phagocytic cells and have a vast and diverse arsenal of antimicrobial resources to cope with pathogens which will fight back with their own very sophisticated artillery as well.<sup>23,24</sup> An important and efficient antimicrobial mechanism against bacteria challenge is the respiratory burst response, with high consumption of oxygen, that results in the production of reactive oxygen species (ROS) through the activation of the NADPH oxidase complex.<sup>23</sup> A rare genetic disorder known as chronic granulomatous disease (CGD) characterized by mutations in the NADPH oxidase

which results in neutrophils defective in mounting a respiratory burst response, prones these patents early in life to recurrent infections.<sup>25,26</sup> CGD patents can present with higher bacteria colonization and gingivitis compared to normal healthy controls; however, these patents do not manifest with severe periodontitis.<sup>6,13,27</sup>

First, it has been suggested that the inability of CGD neutrophils to mount a robust respiratory burst response might result in less tissue damage which could explain why these patents are less susceptible to periodontitis.<sup>13</sup> In addition, emerging evidence has emphasized the importance of salivary effector functions in the preservation of a homeostatic environment, especially in the context of CGD. Not only does saliva diligently control the number of bacteria that accumulates on the tooth surface via several components with antimicrobial activity, but in response to saliva, neutrophils from healthy and CGD patents can form neutrophil extracellular traps (NETs).<sup>28</sup> Besides the respiratory burst response and granule release, NET formation is another host-defense mechanism that neutrophils can deploy to detain and/or kill pathogens. During NET formation, neutrophils release their nuclear content to form a net of decondensed chromatin that is decorated with antimicrobial granule proteins.<sup>29,30</sup>

It is important to note that not all NETs are created equal; since their initial discovery in 2004 which describes that neutrophil stimulation with phorbol myristate acetate (PMA) will induce NET formation after 2–3 hours, ultimately resulting in a new mechanism of cell death known as NETosis,<sup>29</sup> recent publications have unveiled the finesse and versatility that can be achieved by this neutrophil response which appears to be more tailored than initially believed. Depending on the stimuli that neutrophils encounter, NET formation can be dependent or independent of the activation of the NADPH oxidase and ROS generation.<sup>31</sup> In addition, neutrophils can customize the timing and outcome of NETosis. In response to viable bacteria and fungi, neutrophils undergo vital NETosis where NETs form very quickly (in less than 30 minutes) and the cell remains viable; contrary to PMA-induced NETosis.<sup>31</sup> In the recent study by Mohanty et al.<sup>28</sup>, yet another mechanism for NET formation is described. Saliva-induced NETs, triggered by the interaction between sialyl Lewis<sup>x</sup> and L-selectin, had strong antimicrobial activity against oral bacteria. Notably, saliva-induced NETs form independently of the NADPH oxidase, are resistant to DNAses. Although they did not contain elastase,<sup>28</sup> they did incorporate other neutrophil antimicrobial components like calgranulin and hCAP-18 that have known antimicrobial activities against oral bacteria.<sup>6</sup> In conclusion, CGD neutrophils' saliva-induced NETs with antimicrobial activity toward the oral bacteria could be an important mechanism of how neutrophils maintain homeostasis in the oral mucosa of these patents despite lacking a successful respiratory burst.

Ultimately, neutrophils will release their chromatin DNA to form NETs in an attempt to control the normal symbiotic oral microbiota and maintain homeostasis.<sup>28</sup> However, this beneficial response can be impaired by systemic and local diseases. The saliva from patents with Behçet disease or recurrent aphthous stomatitis, who present with ulcers on their oral mucosa but possess neutrophils with normal functional responses and do not suffer from neutropenia, do not have the ability to induce NETs. Furthermore, the saliva from recurrent aphthous stomatitis individuals collected during periods of no mouth ulcers are able to induce NETs; however, that antimicrobial ability is significantly reduced or absent in saliva

collected during peak periods of aphthous ulcers.<sup>28</sup> One possible explanation for this phenomenon is that the oral microbial community present in recurrent aphthous stomatitis patents is different from the one present in age and gender-matched controls.<sup>32</sup> Nevertheless, at this point, we cannot answer what is the root cause of the change in the oral microbial community in these individuals. Does the mucosal lesion trigger the microbial change? Or, is it the modified microbial community what triggers the ulcers? In these patents, the saliva collected 24 hours before the ulceration event fail to induce NETs due to loss of sialyl Lewis<sup>x</sup><sup>28</sup>; a plausible explanation could be that the modified oral bacterial community present in the ulcer phase is releasing higher amounts of sialidases which manifests as a loss of the protective mucins in the saliva during those ulcerous periods. Yet, it is unknown whether those oral bacteria, the producers of sialidases, are absent or in lower abundance during the periods of healthy oral mucosa. More research is needed to answer some of the above questions, but the answers will be critical to provide fundamental information needed to comprehend the interplay between human neutrophils and the symbiotic oral microbiota.

In addition to NET formation and the respiratory burst response, neutrophils have another potent antimicrobial strategy to combat pathogens.<sup>33</sup> These versatile innate immune cells are members of the granulocyte family of leukocytes, and as such have different granules which are involved in bacterial killing both intracellular and extracellularly, as well as during each step of neutrophils journey from the blood vessels to the infected and/or inflamed tissue.<sup>23,34</sup> Neutrophils have four different types of granules, which can be differentiated based on their protein content and on their functional response. They can either be recruited to the bacteria-containing phagosome or stimulated to undergo exocytosis and release their matrix content to the extracellular environment.<sup>35</sup> Neutrophil granules are mobilized in a hierarchical way in which each granule requires increasingly potent stimulation to induce its exocytosis. Secretory vesicles are the easiest granule to undergo exocytosis, followed by gelatinase granules, then specific granules, and finally azurophil granules which will require a very strong stimuli to induce its mobilization.<sup>24</sup> The diverse repertoire of proteins and receptors present at the membrane of each granule subtype as well as within the granule lumen highlights the important role each granule plays in the different neutrophil responses.<sup>36–38</sup> The azurophil granule, also called peroxidase positive due to the presence of myeloperoxidase (MPO) on its lumen, possesses most of the ‘heavy artillery’ to combat microorganisms. The  $\alpha$ -defensins, the different serine proteases like neutrophil elastase (NE), proteinase 3 (PR3), cathepsin G, and neutrophil serine protease 4 (NSP4) are stored in this granule subtype.<sup>35,39</sup>

Papillon-Lefèvre syndrome (PLS), is a rare genetic disorder caused by a defect on the gene that encodes for the cysteine protease cathepsin C (CTSC). Clinically, it manifest as hyperkeratosis and severe periodontitis early on in the lives of affected individuals.<sup>10,22</sup> This is partially because the affected protein, CTSC, is an important enzyme that cleaves the N-terminal on the different NSPs, a key step that is required for the activation and proper biological function of the NSPs.<sup>40,41</sup> Thus, neutrophils from PLS patents lack all the serine proteases normally stored in azurophil granules; which will compromise some of the neutrophil antimicrobial activity, but ultimately does not result in more susceptibility to bacterial infection besides the severe periodontitis.<sup>40</sup> This raises the question, why is bacterial susceptibility limited to the periodontium of those affected by PLS? It has been

suggested that one of the causes is the minimal activity of PR3, which is one of the four serine proteases present in neutrophil azurophil granules. PR3 is an indispensable serine protease that cleaves hCAP18 to generate an important host-defense peptide LL-37, which has a strong antimicrobial activity toward oral bacterium such as *Aggregatibacter actinomycetemcomitans*,<sup>42</sup> and also toward the keystone periodontal pathogen *P. gingivalis*.<sup>43</sup> High levels of hCAP18, the precursor of LL-37, are detected in the crevicular fluids of PLS patents but not the mature antimicrobial peptide.<sup>44</sup> Although, the absence of the NSPs and of LL-37 in PLS patents is correlated with the severity of periodontal disease, the disease progresses because of several factors.<sup>43–45</sup> When neutrophils from PLS patents are challenged ex vivo with PMA, or opsonized *S. aureus*, or the heat-killed oral pathogen *A. actinomycetemcomitans* significantly higher levels of pro-inflammatory cytokines, as well as of matrix metalloprotease-9, are detected in comparison to healthy controls.<sup>46</sup> Also, PLS neutrophils show a higher respiratory burst response upon PMA stimulation compared to control healthy donors, with deficient chemotactic activity toward both MIP-1 $\alpha$  and the formylated bacterial peptide formyl- methionyl-leucyl-phenylalanine (fMLF).<sup>46</sup> This led Roberts et al.<sup>46</sup> to propose that the hyperactive neutrophil phenotype present in PLS patents, together with the deficient chemotactic ability, will halt neutrophils in the connective tissue and contribute to gingival tissue damage and promote inflammation—both hallmarks of periodontal disease.

CGD and PLS are often compared as examples of rare genetic diseases affecting different neutrophil antimicrobial defense mechanisms but with opposite manifestations in relation to periodontal disease.<sup>13,47</sup> Despite the fact that both conditions share suboptimal neutrophil responses, CGD patents have no predisposition to periodontal disease, whereas PLS patents manifest severe periodontitis. Neutrophils from both CGD and PLS patents will induce NET formation when cells are exposed to saliva.<sup>28</sup> This provides an important, basal antimicrobial defense response against symbiotic microbiota that ultimately helps maintain the homeostatic environment in the oral mucosa. When the conditions in the oral cavity change from symbiotic to a dysbiotic microbiota, the hyperactive neutrophil phenotype described in PLS patents in combination with the absence of serine proteases, lack of an active LL-37, and an inefficient chemotactic capacity prolongs their stay in the connective tissue contributing to tissue destruction and progressing to severe periodontitis.<sup>46</sup> In contrast, neutrophils from CGD patents have impaired ROS generation, but intact oxygen-independent antimicrobial responses and normal chemotactic response, allowing them to mount a more effective fight toward the dysbiotic oral microbiota and cause less tissue destruction.

Overall, several host-derived mediators contribute to a healthy periodontium. The specialized junctional epithelium participates in the release of antimicrobial peptides, like  $\beta$ -defensins and LL-37, which helps to maintain less diverse bacterial community that has been associated with a healthy periodontium. Bacterial presence constantly stimulates the release of IL-8 from the gingival epithelium, which generates a chemotactic gradient that continually guides neutrophils to the gingival sulcus (Fig. 2A). In addition, the local oral symbiotic microbial community induces the production of IL-1 $\beta$ ,<sup>48</sup> which can prime the immune system and generates a state of mild inflammation which is linked to promoting immune fitness.<sup>2,49</sup>

### 3 | Neutrophils and Periodontal Disease: The Result of a Discordant Coexistence

#### 3.1 | Periodontitis: a chronic, dysbiotic, polymicrobial inflammatory disease

It is estimated that approximately 46% of the adult population in the United States who are at age 30 or older suffer from periodontitis with Hispanics showing the highest prevalence when compared to non-Hispanics individuals.<sup>50</sup> Periodontitis is a complex chronic inflammatory disease of the periodontium, the tissue that surrounds and supports the teeth. If left unattended, periodontitis results in an increase rate of bone resorption around the tooth root area, with consequential loss of the tooth.<sup>9,51</sup> Treatment for this chronic inflammatory disease is targeted to remove the dysbiotic biofilm and includes scaling, root planning and curettage (to remove the biofilm formed in the periodontal pocket), a regimen of broad spectrum antibiotics and surgery in severe cases. Unfortunately, these treatments are only a temporary solution to control and prevent the disease progression into a massive destruction of the periodontium leading to bone loss (Fig. 1, inset). Moreover, periodontal disease and periodontal pathogens have also been linked to increased risk for serious systemic conditions such as atherosclerosis, diabetes, and rheumatoid arthritis.<sup>9</sup>

In the oral cavity, the tooth surface offers a suitable ecological niche for bacteria colonization and biofilm formation resulting in a varied polymicrobial community.<sup>51</sup> Specifically, the diverse environment present in the oral cavity allows the microbial communities to find the best ecological niche that fits their growth requirements, resulting in the formation of unique microbial biofilm communities associated to the oral location.<sup>52</sup> Yet until recently, research into periodontal disease has focused primarily on a relatively small group of putative pathogens including *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola*.<sup>11,53</sup> However, a reappraisal of microbial etiology has marked a turning point in the field due to the development of culture-independent techniques that allow oral biologists to identify bacterial species directly from nucleic acids. Due to this, several newly recognized/appreciated organisms have been found to be elevated in periodontal disease as compared to health.<sup>54-56</sup> Aas et al.<sup>57</sup> show that a diverse and complex bacterial community is part of the healthy oral cavity after characterizing the bacterial profile of five clinically healthy individuals at nine different oral sites including the buccal epithelium, the maxillary anterior vestibule, the dorsum and lateral tongue surface, the hard and soft palate, the tonsils, the tooth surfaces, and the subgingival plaque. Another report using 16S pyrosequencing and a higher number of patents (29 healthy controls and 29 individuals with chronic periodontitis) shows a differential bacterial community composition between healthy controls and disease patents with 100-300 bacterial species detected in a single individual.<sup>58</sup> Some of the newly identified uncultivated bacterial species are clearly associated with disease such as *Filifactor alocis*,<sup>59</sup> and some of the health-associated bacterial species are still present in periodontitis patents, however in lower numbers.<sup>58</sup> The use of next-generation sequencing techniques provides sophisticated tools to achieve a more precise characterization of the complex composition of the inhabitants of the oral microbiome. Similarly, sequencing enables the identification of low abundance microorganisms and helps to understand their role in the progression from oral health to disease.<sup>58,60</sup>

Our general idea is that infectious diseases are caused or triggered by the action of a single foreign pathogen; however, it is clear that many human diseases with an infectious etiology, like periodontitis, are originated by the complex association and interaction of a diverse polymicrobial community.<sup>4,11,61,62</sup> The initial paradigm of periodontitis, established more than 20 years ago already contemplated the idea that the disease was not originated by a single pathogen and explained the progression and severity of the disease by a shift from a predominant oral gram-positive aerobic microbial community toward a gram-negative anaerobic community.<sup>11,63</sup> In addition, the data obtained from oral biofilm studies using checkerboard DNA-DNA techniques link the different stages of the disease to a specific bacterial group or complex with the presence of the triad of bacteria composed by *P. gingivalis*, *T. forsythia* and *T. denticola* strongly associated with increase severity of periodontitis.<sup>64,65</sup> The advance of new technology has revealed that the etiology of periodontitis is more complex than the initial paradigm. For example, high numbers of the gram-positive bacterium *F. alocis* are present in periodontal disease sites, whereas increased levels of the gram-negative uncultivated bacterium *Veillonella* sp. oral clone X042 are associated with healthy periodontal sites and classified as a beneficial bacterial species.<sup>66</sup> Based on the current evidence, the development of periodontitis is associated with a shift in the symbiotic oral microbiota into a dysbiotic polymicrobial community.<sup>11,53,61,67</sup> In a healthy gingival tissue, the local symbiotic bacterial community is less diverse and rich, with neutrophil recruitment to clear the infection and resolving the inflammation with no collateral damage to the host<sup>4,61,68</sup> (Fig. 2A). The progression from health to periodontitis is now explained as the transition from a symbiotic microbial community that due to several risks factors, such as smoking, changes in the diet, an immunocompromised host, tissue injury, or the colonization of the oral cavity by pathogenic bacteria such as the oral keystone pathogen *P. gingivalis*, can modify the oral ecosystem resulting in a dysbiotic polymicrobial community<sup>61</sup> (Fig. 2B). As a result, the host response toward the highly rich and diverse dysbiotic microbial challenge is more robust and dysregulated, transitioning from a controlled/stable immune response into a non-resolving chronic inflammatory response<sup>4,61,68</sup> (Fig. 2B).

The stability of the local oral microbial community reveals important information to understand the transition from health to disease. Over a 2-year period, 75% of the subgingival oral bacterial community was conserved for individuals grouped under a healthy stable periodontal status; however, a significant change to that local or indigenous bacterial community with loss or gain of bacterial species was seen in patients whose periodontal status worsen.<sup>66</sup> However, none of those subjects who showed an increase in periodontitis severity within the 2 years period had a complete replacement of their bacterial community.<sup>66</sup> It is clear that modifications in the bacteria-bacteria relationship, resulting in differential expression of bacterial genes or in the composition of the oral community associated with periodontal health, will lead to disease.<sup>69</sup> The shift in the community can be explained by the combined effort of bacteria acting as ‘accessory pathogens’ helping ‘keystone pathogens’ to manipulate both the bacterial and host interactions providing an optimal niche for ‘pathobionts’ to flourish and outgrow in the new ecological system<sup>61,70</sup> (Fig. 2B). The new paradigm to describe the etiology of periodontitis introduces a new concept of ‘nososymbiocity’ in which some members of the local or indigenous microbial



community can participate and/or facilitate the establishment of exogenous keystone bacterial members leading to a synergistic dysbiotic community (reviewed in 61).

### 3.2 How human neutrophils cope with the 'nosymbiocity' of periodontitis

In the periodontal tissue, neutrophils are mainly found in the junctional epithelium and in the crevicular fluids where it has been described that neutrophils can form a protective 'wall' facing the oral plaque and protecting the epithelium.<sup>7</sup> A study done almost 30 years ago demonstrated that different ultrastructural neutrophil morphology was seen in cells isolated from the crevicular fluid of patients with severe periodontitis.<sup>71</sup> At that time, the transmission electron micrographs reveal neutrophils with no evidence of undergoing phagocytosis, cells with high number of phagocytic vacuoles, and cells with 'ghost' phagocytic vacuoles. Both cells which contain phagocytic vacuoles show less granule content overall.<sup>71</sup> Moreover, high content of immunoglobulins, predominantly IgG, and complement component 3 is found in the crevicular fluid, forming immune complexes detectable inside neutrophils.<sup>71</sup> This study indicates that not only the phagocytic clearance of the oral bacteria can induce neutrophil activation, but also the presence of immune complexes in the crevicular fluid is a potent cell activator. Furthermore, analysis of neutrophils collected from the periodontal pockets from 11 adult patients with periodontitis, without periodontal treatment for a year and with evidence of alveolar bone loss shows that necrosis, and not apoptosis, is the predominant form of cell death, which contributes to the tissue damage and prolongs the inflammation seen in periodontitis.<sup>72</sup> A more recent study, which analyzed gingival biopsies and purulent crevicular exudates from patients with chronic periodontitis, reveals that bacteria in the subgingival plaque is constantly dispersed as a way to colonize new surfaces, and that the abundance of NETs in the crevicular exudates is more effective than phagocytosis in preventing bacteria adhesion and invasion of the gingival epithelium.<sup>73</sup>

To protect against the collateral damage that neutrophil antimicrobial serine proteases such as NE and PR3 may cause the host tissue, endogenous protease inhibitors are present in the gingival tissue such as secretory leukocyte protease inhibitor (SLPI) and elafin. Analysis of crevicular fluids from chronic periodontitis patients reveals significantly lower levels of SLPI with concomitant higher activity of both NE and PR3 activity compared to periodontal healthy controls.<sup>74</sup> Moreover, the keystone periodontal pathogen, *P. gingivalis*, has several virulence factors that tip the balance in favor of dysbiosis and host tissue damage. An exemplary type of virulence factor is the secreted cysteine proteolytic enzymes called gingipains.<sup>75</sup> One of the many properties that makes *P. gingivalis* gingipains so successful is the ability to inactivate the endogenous protease inhibitors.<sup>76</sup> Crevicular fluids from periodontitis patients have a positive correlation of high NE and PR3 activity with the presence of *P. gingivalis* and a negative correlation with the levels of SLPI.<sup>74</sup> Furthermore, in crevicular fluids collected from periodontitis patients, the levels of elafin, which is normally anchored to the extracellular matrix to protect it from degradation and to assure the enzyme activity to guard the host tissue integrity, show a positive correlation with *P. gingivalis* arginine-specific gingipain, Rgps.<sup>74</sup> These findings suggest that the shedase activity of *P. gingivalis* gingipains on elafin will compromise the protective function of the inhibitor and leave the host tissue more susceptible to degradation by neutrophil active proteases present in the gingival tissue.

Since neutrophils are equipped with a lethal arsenal of antimicrobial compounds, their response to control infection is finely tuned to diminish collateral damage to the host tissue. It would be very detrimental to the host to have circulating professional phagocytes, like neutrophils, which will respond and become fully activated independent of what type of enemy or threat they have to contain and control. Hence, neutrophils can fine tune the degree of response they mount upon a given dangerous situation. The level of cell activation is the result of a range of increasing degree of activation states instead of an on/off switch mechanism, with priming being a reversible step that modulates neutrophils graded response to an insult. Priming of neutrophils is understood as the enhanced functional response of the cell to a stimulus by previous exposure of the cell to a priming agent. Priming agents can be host-derived, like different cytokines or chemokines, such as TNF $\alpha$ , IL-1 $\beta$ , IFN $\alpha$ , IL-8, growth factors like GM-CSF, or bacterial-derived compounds like lipopolysaccharide (LPS).<sup>77</sup> The regulation of priming is key in the host-defense response since it prepares neutrophils to mount an appropriate level of response when encountering a particular stimulus. Exposure of neutrophils to priming agents like TNF $\alpha$  will enhance not only the cells respiratory burst response, but also the release of neutrophil granules upon a subsequent stimulation. Neutrophil granule exocytosis contributes to TNF $\alpha$  priming of the respiratory burst response by increasing the expression of the NADPH oxidase membrane components at the neutrophil plasma membrane.<sup>78</sup>

Several studies have shown that circulating neutrophils isolated from patients diagnosed with community acquired pneumonia, or who suffered from trauma, or from a chronic inflammatory disease like rheumatoid arthritis have a primed phenotype and can mount an enhanced respiratory burst response upon exposure to a second stimulus.<sup>79–81</sup> In neutrophils, the respiratory burst response induced by different stimuli can trigger the assembly of the NADPH oxidase complex at the phagosomal membrane or at the cell plasma membrane, and, upon enzyme activation, superoxide radicals are released either to the phagosomal lumen or outside the cell into the extracellular space. Neutrophils isolated from peripheral blood of chronic periodontitis patients, have higher basal respiratory burst activity when compared to healthy matched individuals. Patient neutrophils present a primed phenotype determined by a significantly higher extracellular ROS production when challenged *ex vivo* with opsonized *S. aureus*, or non-opsonized oral pathogens such as *Fusobacterium nucleatum* or *P. gingivalis*.<sup>82</sup> *F. nucleatum* is a gram-negative oral bacterium that acts as an important ‘accessory pathogen’ involved in the progression from a symbiotic to a dysbiotic oral community.<sup>83–85</sup> A transcriptome analysis of human neutrophils challenged with *F. nucleatum* for 3 hours resulted in the differential regulation of 208 genes including some genes involved in the respiratory burst response as well as upregulation of ‘stress-response’ genes HSPA1A, HSPH1, and HSPA8, along with oxidative stress response genes like superoxide dismutase 2 and metallothioneins, which could be a cell protective mechanism to counterbalance the high ROS generation by the bacterial challenge.<sup>85</sup> Another study shows that peripheral blood neutrophils isolated from patients with severe chronic periodontitis when challenged with IgG-opsonized *S. aureus*, or with complement opsonized zymosan or with a soluble stimulus like PMA have a 32% higher extracellular ROS production, measured by isoluminol, compared to healthy age and gender-matched individuals.<sup>86</sup>

The enhanced respiratory burst response by peripheral blood neutrophils isolated from periodontal disease patients when exposed to a second stimulation like *S. aureus* or PMA is an indication that those circulating cells are primed. High plasma levels of IL-8, GM-CSF, and IFN $\alpha$  are detected in periodontitis patients compared to age-matched healthy individuals. Both IL-8 and GM-CSF are responsible for the enhanced baseline release of ROS generation upon challenge of neutrophils from healthy individuals with the plasma collected from those periodontitis patients.<sup>87</sup> Furthermore, when healthy neutrophils are incubated with plasma from periodontitis patients, the elevated levels of IFN $\alpha$  contribute to both the enhanced basal respiratory burst response and the priming of fMFL-stimulated superoxide release.<sup>87,88</sup>

Cytokine release by peripheral blood neutrophils isolated from healthy individuals, or from patients with chronic periodontitis before and after a successful non-surgical periodontal treatment, were determined after 18 hours of no challenge or cells challenged with *Escherichia coli* LPS, or opsonized *S. aureus*, or *F. nucleatum*, or with *P. gingivalis*. The release of IL-8, IL-6, TNF $\alpha$ , and IL-1 $\beta$  from unstimulated cells after 18 hours is similar between all the groups; however, a significantly higher release of all cytokines is seen by patient neutrophils upon the different bacterial challenge when compared to the response of neutrophils from healthy controls.<sup>89</sup> There are no differences in the bacteria-stimulated cytokine response by neutrophils isolated from postperiodontal therapy patients compared to the levels detected previous to the periodontal therapy, which might indicate that the priming state of the circulating cells is still present after periodontal therapy or that neutrophils hyper-reactivity is imprinted or constitutive.<sup>89</sup>

Some periodontitis patients who do not respond to periodontal treatments, both performed at the dentist office and after a good home care, are classified as refractory periodontitis patients.<sup>90</sup> The respiratory burst response was analyzed from oral neutrophils isolated from 13 patients with refractory periodontitis upon PMA stimulation and two groups are distinguished within the cohort, one group as high and another as low responders based on the percent of ROS production. The refractory periodontitis patients within the cohort classified as high responders also present more severe bone loss than the low responders; however, the bacterial plaque index and the age are not significantly different between these two groups in this patient cohort. The magnitude of ROS generation by the oral neutrophils is correlated with the disease severity and larger longitudinal studies might confirm if a hyperactive oral neutrophil phenotype could predict if patients would be refractory to treatment and more susceptible to disease progression.<sup>90</sup>

Significantly higher number of neutrophils are recruited into the oral cavity of chronic periodontitis patients when compared to healthy individuals, and a transcriptome analysis reveals significant changes in gene expression between the two groups.<sup>91</sup> Oral neutrophils from chronic periodontitis patients show increased expression of Bcl-2, a pro-survival marker, and a decreased expression of Bax, a pro-apoptotic marker, both by transcriptome analysis and validation by immunoblot and flow cytometry compared to oral cells isolated from healthy controls.<sup>91</sup> Furthermore, oral neutrophils from chronic periodontitis patients have a differential enhanced surface expression of CD11b, CD63, CD66b, which is associated with an active neutrophil phenotype.<sup>92</sup> All three surface markers are present in different neutrophil granule subtypes, and indicate that oral neutrophils are undergoing active granule

exocytosis. The exocytosis of azurophil granule content into the extracellular space is tightly regulated to prevent tissue damage. The significant increase of CD63 surface expression, an azurophil granule marker, observed in oral neutrophils, not on peripheral blood neutrophils, from chronic periodontitis patents highlights the magnitude of cell activation and the contribution of it to inflict tissue damage in sites of chronic unresolved inflammation such as what occurs in the disease periodontium. Moreover, peripheral blood neutrophils from chronic periodontitis patents display reduced chemotactic ability toward IL-8 and fMLF when compared to cells isolated from age and gender-matched control individuals. Blood neutrophils isolated from those chronic periodontitis patents 3 months after non-surgical therapy show partial recovery with similar velocity and accuracy migration toward IL-8 but not to fMLF<sup>93</sup> The reduced chemotactic ability paired with the hyperactive phenotype described for both circulating as well as oral neutrophils from chronic periodontitis patents further supports the role that these dysfunctional innate immune cells might have in collateral tissue damage and periodontal disease progression. The presence of primed neutrophils both in circulation and in the oral cavity described in periodontitis patents might shed some light to understand the proposed association between periodontal disease as well as periodontal pathogens with systemic inflammatory conditions such as atherosclerosis, diabetes, and rheumatoid arthritis.

### 3.3 | Neutrophil interaction with the terrible threes of periodontitis: the unresolved contest

**3.3.1 | The first kid of the periodontal triad—***T. denticola* is an anaerobic, gram-negative motile spirochete that can be detected, albeit in low numbers, in the gingival plaque of healthy individuals; however, it is present in very high numbers in the subgingival periodontal pocket and is considered one of the major microorganisms associated with the dysbiotic biofilm formation in periodontal lesions.<sup>84</sup> Moreover, *T. denticola* may contribute to the bacterial colonization of periodontal tissues by limiting neutrophil chemotaxis.<sup>94,95</sup> IL-8 secretion by periodontal epithelial cells is an important mechanism by which neutrophils are recruited to the periodontal pockets, and epithelial cells fail to secrete IL-8 as a response to *T. denticola* exposure.<sup>94</sup> Additionally, this pathogen is able to degrade IL-8 that is already present at the infection site, which disables the neutrophil chemotactic gradient. Combined with the impaired neutrophil chemotactic phenotype described for periodontitis patents, this discovery might help to understand the described neutrophil meandering and halt in the gingival tissue associated with the collateral tissue damage.<sup>15</sup>

Other studies suggest that different virulence factors of *T. dent-cola* may modulate key innate immune responses, contributing to the development of periodontal diseases.<sup>95–97</sup>

Several studies agree that the major outer sheath protein (Msp) of *T. denticola* is one of its most important virulence factors in contributing to the disease progression. This membrane protein modulates neutrophil signaling pathways involved in cytoskeletal dynamics that are relevant in chemotaxis and phagocytosis.<sup>98–100</sup> Magalhães et al.<sup>100</sup> determined that pretreatment with Msp from *T. denticola* has an inhibitory effect on murine neutrophil movement and polarity even when they were exposed to the potent chemoattractant fMLF in vivo. Similarly, Msp pretreatment impairs human neutrophil chemotaxis toward fMLF and phagocytosis of immunoglobulin G-coated polystyrene microspheres in vitro, by perturbing

actin remodeling and reorganization.<sup>97</sup> Puthengady et al.<sup>97</sup> observed that pretreatment with *T. denticola*'s Msp reduces transient cytosolic calcium concentrations in neutrophils. The presence of calcium is fundamental during the actin filament modifications that lead to cell motility; thus, by reducing cytosolic calcium, the bacteria can disrupt neutrophil movement.<sup>97</sup>

Another way Msp controls neutrophil cytoskeletal functions like migration, adhesion, and cell shape is by preventing the activation of Rac1, a small G protein from the Rho-GTPases family. Rac1 is critical for the formation of lamellipodia and the modulation of the phosphatidylinositol-3 kinase in neutrophils, required for adequate cell polarization and chemotaxis.<sup>100,101</sup> *T. denticola* Msp Rac1 inhibition is selective as indicated by an experiment in which under the same conditions the activation of other small G proteins, such as Rac2, Cdc42, and RhoA, is not affected by the Msp challenge.<sup>100</sup> These data further support the concept that the disruption of cytoskeletal dynamics may serve as this pathogen's evasion mechanism.<sup>100</sup> Lastly, *T. denticola* Msp also causes extracellular matrix degradation by stimulating the release of activated MMPs from neutrophils, an effect that might lead to periodontal tissue damage.<sup>96,102</sup>

Dentilisin, a surface lipoprotein-protease complex of *T. denticola*, is another important virulence factor of this periopathogen.<sup>102</sup> Yamazaki et al.<sup>103</sup> found that dentilisin activates the alternative complement pathways by hydrolyzing host proteins, such as complement component 3 (C3), and generate iC3b, which can bind to complement receptor 3 present on neutrophils' plasma membrane and result in cell activation. Challenge of human neutrophils with *T. denticola*, but not with the dentilisin-deficient mutant, results in ROS generation and release of MMP-9.<sup>103</sup> Induction of neutrophils respiratory burst response with the high generation of ROS is a potent and effective antimicrobial weapon; however, despite the fact that *T. denticola* is recognized as a strict anaerobe, this bacterium is able to survive in the presence of ROS generated by the host.<sup>96</sup> Treatment of *T. denticola* with the protease inhibitor chymostatin prevented complement activation, the bacteria-induced ROS generation and MMP-9 release by human neutrophils. *T. denticola* dentilisin-induced neutrophil activation contributes to periodontal tissue damage and the chronic inflammation of the periodontium.<sup>103</sup>

**3.3.2 | The second kid of the periodontal triad—*Tannerella forsythia*** is a gram-negative anaerobic rod that, together with *P. gingivalis* and *T. denticola*, form the triad of putative periodontal pathogens strongly associated with the severity of the disease. The presence of *T. forsythia* in the oral biofilm is considered a marker of periodontal disease progression, since it is strongly associated with the advanced and refractory forms of periodontitis and it is rarely cultured from healthy sites.<sup>104,105</sup> Yet, despite being one of the main constituents in the pathogenesis of periodontal diseases, *T. forsythia* and its interaction with the immune response is arguably under-studied, probably due to its unique growth requirements.<sup>106–108</sup>

Gosling et al.<sup>106</sup> showed that after subcutaneous immunization of mice with *T. forsythia*, lesions consist mainly of infiltrated neutrophils, granulomatous tissue, and peroxidase positive granules associated with neutrophil exocytosis. Neutrophil recruitment peaked at

day 1 after infection and decreased by day 7. These results suggest that unlike the other two putative periodontal pathogens, *T. forsythia* does not suppress neutrophil recruitment to the infected site.<sup>106</sup>

The complement system is another key innate immune response for host immune protection from microorganisms.<sup>109</sup> Activation of any of the three complement pathways will result in deposition of an active complement component C3b on bacterial surfaces leading to opsonization of the microorganism which facilitates its recognition by professional phagocytes like neutrophils. Besides contributing to a more efficient bacterial recognition, complement activation at terminal stages results in the formation of membrane attack complexes and the lysis of microbes.<sup>109</sup> *T. forsythia* utilizes the metalloproteinase virulence factors karilysin and mirolysin to modulate and evade the complement arm of the innate immune response. Incubation of *T. forsythia* with human serum proteins leads to the activation of only one of the three complement pathways, resulting in some opsonization of the bacteria.<sup>104,110</sup>

Jusko et al.<sup>104</sup> showed that *T. forsythia* inhibits the classical and lectin pathways by degrading the initial complement components, such as the mannose-binding lectin, ficolin, and C4, whereas the classical pathway is inactivated at a later stage by degrading C5 molecules. Therefore, *T. forsythia* avoids complement-mediated killing by neutralizing the activated classical pathway, which ultimately allows the bacteria to remain viable after human complement exposure. Interestingly, the interaction of the bacterial virulence metalloproteinases, karilysin and mirolysin, with the host complement pathways still promotes the release of active C5a, an anaphylotoxin that forms chemotactic gradients to recruit neutrophils to the site of infection. C5a stimulates neutrophil chemotaxis into sites of infection, but exposure to high concentrations of C5a causes neutrophil activation and inefficient migration.<sup>104,110</sup> *P. gingivalis* and *T. forsythia* are both found in the periodontal disease sites and there is a synergistic effect between both of the bacterial proteases, gingipains and karilysin, to prevent complement deposition.<sup>7</sup> These two putative periodontal pathogens join forces to evade complement attack and promote high concentration of the complement anaphylotoxin C5a, which will induce neutrophil granule exocytosis and ROS which results in tissue disruption and leads to the progression of periodontal diseases.<sup>110</sup>

LL-37 is an important antimicrobial peptide secreted by neutrophils, usually present in high numbers in the gingival crevicular fluid (discussed above). It plays an important role in preventing the colonization of gingival tissue by pathogenic species.<sup>111</sup> Incubation with purified karilysin results in LL-37 cleavage and the subsequent reduction of this peptide bactericidal activity—suggesting that this mechanism may contribute to protect not only *T. forsythia* but also other periodontal pathogens, such as *P. gingivalis*, against bacterial degradation by LL-37.<sup>111</sup> Besides being bactericidal, LL-37 has potent anti-inflammatory activity; it binds to LPS, preventing both the stimulation of macrophages and the consequent release of pro-inflammatory mediators. Koziel et al.<sup>111</sup> found that treatment with karilysin inactivates LL-37 capacity to neutralize LPS. Moreover, incubation with karilysin results in cleavage of the LL-37-LPS complex, causing the release of active LPS and therefore the secretion of pro-inflammatory cyto-kines such as TNF- $\alpha$ .<sup>111</sup> This finding suggests that the

inactivation of LL-37 by karilysin interferes with the ant-inflammatory function of this peptide, preserving local inflammation and promoting periodontal tissue damage.<sup>111</sup>

*T. forsythia* possesses a glycosylated S-layer on its cell surface that has been linked to bacterial resistance to innate immune clearance.<sup>106–108</sup> In an oral gavage model, mice were infected either with a glycosylation deficient mutant or with a *T. forsythia* wildtype strain. Settem et al.<sup>108</sup> found a significant increase in neutrophil recruitment to the infected gingival space, as well as a limited Th17 response in mice inoculated with the glycosylation deficient mutant. Normally, the Th17 response is required for an efficient recruitment of neutrophils; so, these findings suggest that the antigenic *T. forsythia* cell exterior favors bacterial survival in the periodontal pocket by blocking Th17-mediated neutrophil infiltration.<sup>108</sup>

The third most studied virulence factor of *T. forsythia* is serpins, which are serine protease inhibitors that are usually expressed in eukaryotic cells, but occasionally can also be found in prokaryotes.<sup>112</sup> In the context of *T. forsythia* infection, serpins play an important role in maintaining homeostasis by regulating mechanisms like inflammation, complement activation and the inhibition of the neutrophil-derived proteases released for bacterial degradation. Specifically, the newly discovered serpin, miropin, inhibits the enzymatic activity of several neutrophil proteases present in the gingival crevicular fluid such as elastase and cathepsin G. This interference may mitigate bacterial clearance, promoting *T. forsythia* survival in the periodontal pocket.<sup>112</sup>

Illustrating yet another virulence factor of this periodontal pathogen to elude the innate host immune response is the finding that *T. forsythia* cultures contained significant levels of bacterial DNase as noted by Palmer et al.<sup>113</sup> after finding zones of DNA hydrolysis surrounding *T. forsythia* colonies on plate cultures supplemented with mammalian DNA. It is known that some pathogens secrete DNase, an enzyme able to degrade nucleic acids, as a survival mechanism to escape NETs. It is tempting to speculate that *T. forsythia* could evade NETs antimicrobial and/or entrapping effect by use of its DNase to degrade the chromatin and of its karilysin to inactivate the antimicrobial peptide, LL-37, normally present in NETs.<sup>113,114</sup> It is also possible that the presence of bacterial DNase can benefit *T. forsythia* in biofilm remodeling and contribute to bacteria dispersal and colonization of new surfaces.

**3.3.3 | The third and most mischievous kid of the periodontal triad—*P. gingivalis*** is a gram-negative anaerobic rod that has been called the ‘keystone pathogen’ in the onset of periodontitis.<sup>115</sup> In this model, a low abundance of *P. gingivalis* will upset the balance between the oral microbiome and the immune system.<sup>115</sup> Immune subversion must be acquired either through environmental stresses, diet or through the activity of *P. gingivalis* itself as previously discussed. The interactions between this species and the host immune system have been extensively studied.

To survive in the periodontal cavity, *P. gingivalis* must impair the neutrophil antimicrobial responses to prevent its killing while directing the neutrophils' many potent weapons at the host. *P. gingivalis* requires nutrients that can be taken from the host via the degradation of

tissue.<sup>51,116</sup> *P. gingivalis* is a very resourceful periodontal pathogen that makes use of enzymes, LPS and even other host cells to modulate neutrophil behavior.

As stated, *P. gingivalis* must be able to withstand the chronic inflammation that is characteristic of periodontal disease.<sup>116</sup> Early studies on neutrophil-*P. gingivalis* interactions show neutrophils unable to effectively kill the bacteria intracellularly.<sup>117</sup> Neutrophils isolated from periodontal disease patients demonstrate an even further reduced killing efficacy.<sup>118</sup> Within the biofilm, *P. gingivalis* enjoys some camouflage from the immune system, but *P. gingivalis* also exists in the crevicular fluid in a planktonic or free-floating state.<sup>116</sup> In this space, neutrophils can actively invade and phagocytose the bacteria.<sup>119</sup> In periodontitis, recent studies have shown that the neutrophils are hyper-activated and undergo frequent NETosis in the crevicular fluid.<sup>119</sup> Other than the crevicular fluid, the actual dental plaques are the main home for *P. gingivalis*.<sup>116</sup> Neutrophils can mount a defense even against these biofilms by forming the previously mentioned 'wall' of neutrophils against the plaque.<sup>120</sup> The neutrophils are highly impaired in their ability to phagocytose bacteria within the biofilm, and they turn to extracellular killing mechanisms instead.<sup>51</sup> They undergo exocytosis of their different granule subtypes resulting in the release of antimicrobial peptides while generating ROS. The best way for *P. gingivalis* to avoid clearance by the neutrophils is to invade host cells such as gingival epithelial cells.<sup>121</sup> Within these cells, *P. gingivalis* inhibits apoptosis and occupies its own replicative niche within autophagosomes.<sup>121,122</sup>

One of the major ways that *P. gingivalis* manipulates the neutrophils is through the process of local chemokine paralysis.<sup>123</sup> Neutrophils are recruited to sites of infection by following a gradient of chemical signals generated from the resident tissue cells via a process called chemotaxis.<sup>9</sup> In the case of periodontitis, gingival epithelial cells (GECs) secrete the chemoattractant IL-8 to recruit neutrophils.<sup>124</sup> *P. gingivalis* inhibits the secretion of IL-8 from GECs with the ultimate effect of lowering the number of neutrophils that are called to the area.<sup>123</sup> The mechanism for inhibition of IL-8 secretion is dependent on the *P. gingivalis*' serine phosphatase SerB following invasion of GECs by the pathogen.<sup>125</sup> The synthesis and eventual secretion of IL-8 is mediated by the NF- $\kappa$ B transcription factor.<sup>126</sup> SerB dephosphorylates the p65 subunit of the NF- $\kappa$ B RelA/p65 homodimers that allow for IL-8 transcription-suppressing IL-8 synthesis<sup>125</sup> (Fig. 2B).

*P. gingivalis* also utilizes very direct mechanisms against the neutrophil to hamper its chemotactic function.<sup>127</sup> Bacterial products recovered from culture of *P. gingivalis* have an immobilizing effect on human neutrophils which may be due to the binding of non-chemotactic formylmethionyl peptides to the fMLF receptor.<sup>127,128</sup> Interestingly, the inhibitory effect on neutrophil chemotaxis is not solely for the fMLF receptor, but C5a-mediated chemotaxis is also blocked.<sup>127,128</sup> This could suggest two independent blocking agents or one with a more global effect on the neutrophil's chemotactic integrity.<sup>127</sup> Succinic acid is a metabolic by-product of the *Bacterioides* species under which *P. gingivalis* used to be classified, and it could be responsible for the previously described culture product effects.<sup>129</sup> Succinic acid has been shown to inhibit chemotaxis and even reduce neutrophil respiratory burst by decreasing intracellular pH.<sup>129,130</sup> Although succinic acid is produced by *P. gingivalis*, its major short-chain fatty acid metabolic by-product is butyric acid which



has similar inhibitory effects in animal models, but has not yet been tested in human neutrophils.<sup>131</sup>

*P. gingivalis*' LPS also has a possible role in the control of cytokines and chemokines released.<sup>132</sup> Inflammatory cytokine (IL-1 $\beta$ , TNF $\alpha$ , and IL-8) release from human neutrophils is significantly lower when stimulated with LPS from *P. gingivalis* compared to other gram-negative bacteria.<sup>132</sup> The release of the IL-1 inhibitory molecule, IL-1ra, is significantly enhanced by neutrophils stimulated with *P. gingivalis*' LPS.<sup>132</sup> Both TLR-2 and TLR-4 have been identified as possible receptors for LPS from *P. gingivalis*.<sup>133</sup> Current evidence shows that LPS from *P. gingivalis* has an antagonistic role with TLR-4, and this may explain how the stimulation with LPS lowers the amount of inflammatory cytokines.<sup>133</sup> There has been some evidence to the contrary showing that LPS from *P. gingivalis* actually increases IL-8 synthesis.<sup>134</sup> It is possible that the IL-8 transcription is increased, but secretion is still inhibited. However, the study showing lower cytokine secretion does not show inhibition, but just less secretion than the others tested. The most likely explanation is that *P. gingivalis*' LPS has less affinity for the pattern recognition receptors than LPS from *A. actinomycetemcomitans* or *E. coli*. The LPS from *P. gingivalis* has also been implicated in bone resorption which is one of the primary symptoms of periodontal disease.<sup>135</sup>

Aside from the LPS effect on cytokine and chemokine secretion, it also delays apoptosis of differentiated HL60 cells.<sup>136</sup> However, LPS has not yet been tested for this inhibition with isolated human neutrophils. Inhibition of apoptosis is a mechanism employed by other bacteria as well to induce further inflammation and tissue damage.<sup>137</sup> The relationship between impairment and activation is complex because pathogens like *P. gingivalis* require inflammation to thrive, but they must also keep the inflammation under some control.<sup>115</sup> If they do not impair neutrophil killing mechanisms or invoke their own defense mechanisms, then the exorbitant inflammation would result in its own clearance.

Other *P. gingivalis* produced products have also been shown to manipulate neutrophil function.<sup>138</sup> These bacterial products recovered from culture lowered neutrophils ability to agglutinate which may correspond to control of granule exocytosis.<sup>138</sup> Interestingly, there has been some evidence that *P. gingivalis*' products recovered from its growth culture, even alter neutrophil morphology.<sup>139</sup> One bacterial product, trypsinlike protease (TLPase), plays a role in inhibition of phagocytosis.<sup>140</sup> This inhibitory effect is more profound in neutrophils from periodontitis patients than healthy controls which may point to added effects from other pathogens and/or systemic alteration to the host's neutrophils.<sup>118,140</sup> Neutrophils from periodontal disease patients are impaired in their ability to phagocytose bacteria, and TLPase from *P. gingivalis* may be one of the causes of this.<sup>140,141</sup> Additionally, TLPase has been shown to increase the generation of superoxide anions and cleave the neutrophil formyl peptide receptor.<sup>142</sup> Increasing the superoxide concentration in the extracellular environment will contribute to damage for both the host tissue and any sensitive bacteria.

Endogenous lipoxin A<sub>4</sub> (LXA<sub>4</sub>) appears to inhibit the oxidative burst response and overall activation of human neutrophils when challenged by *P. gingivalis*.<sup>143</sup> LXA<sub>4</sub> is a eicosanoid that is heavily involved in the resolution of inflammation.<sup>144</sup> Its presence in the crevicular fluid of periodontal disease patients and relative absence in asymptomatic patients has made it

of particular interest.<sup>145</sup> The generation of LXA4 occurs in response to the chronic inflammation found in the periodontal pocket.<sup>146,147</sup> Further research in this area could explain how *P. gingivalis* takes advantage of host chemical mediators.

The Triggering Receptor Expressed on Myeloid cells 1 (TREM-1) is another endogenous factor that is believed to be manipulated by *P. gingivalis*.<sup>148</sup> TREM-1 has been much more actively studied with human neutrophils. It is a membrane bound protein whose binding results in activation of the neutrophil.<sup>148</sup> *P. gingivalis* affects TREM-1 in both a gingipain-dependent and independent manner. Gingipains (cysteine proteases) are discussed in more depth below, but they function in this case to cleave membrane bound TREM-1 to sTREM-1 (soluble). The whole bacteria increase the expression of TREM-1 as indicated by increased TREM-1 mRNA production. After longer incubation, the membrane expression of TREM-1 decreases while sTREM-1 increases due to Arg-gingipain cleavage of TREM-1 from the membrane.<sup>148</sup> The increase in sTREM-1 is thought to increase the inflammatory signaling by increasing IL-8.<sup>148</sup> *P. gingivalis* can also lower the sTREM-1 by Lys-gingipain activity.<sup>148</sup> It has been hypothesized that the decision to upregulate Arg- vs Lys-gingipains may be due to nutrient availability. *P. gingivalis* would increase Arg-gingipain to promote inflammation associated tissue damage, but upregulate Lys-gingipain if the inflammation becomes deleterious to the bacteria.<sup>127,148</sup> However, it could also be a time-dependent regulation; sTREM-1 accumulates at late time points. The local chemokine paralysis and other neutrophil impairing functions of *P. gingivalis* tend to occur shortly after infection while the inflammation promoting functions occur later in the infection. *P. gingivalis* could be lowering inflammation initially to disturb the biofilm undetected and then promote inflammation once it has set itself up in the protected niches of the GECs and biofilms (Fig. 2B).

The actual mechanism for surviving the antimicrobial effect of ROS by *P. gingivalis* has not yet been fully determined.<sup>116</sup> In the deep periodontal pocket, the environment is fairly anoxic which may in itself preclude neutrophils to mount an appropriate respiratory burst response.<sup>149</sup> *P. gingivalis* lacks catalase, an enzyme which can consume the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) produced in the phagosome as a result of the respiratory burst response and detoxify reactive oxygen radicals and protect the bacterium from oxygen-dependent damage, but it does express superoxide dismutase which catalyzes the dismutation of superoxide into H<sub>2</sub>O<sub>2</sub> and provides some protection from oxygen-dependent damage.<sup>150</sup> Another enzyme of *P. gingivalis*, alkyl hydroper-oxide reductase (Ahp), can metabolize H<sub>2</sub>O<sub>2</sub> and provide some protection against the respiratory burst.<sup>151,152</sup> However, it is still uncertain how much protection is really afforded to each of these enzymes against the copious amounts of ROS generated by neutrophils during an infection.

One of the major virulence factors produced by *P. gingivalis* is gingipains.<sup>153</sup> Gingipains have been called the principal virulence factor created by *P. gingivalis* and are involved in modulation of leukocyte responses.<sup>153</sup> For example, gingipain R cleaves proteinase-activated receptor on neutrophils resulting in neutrophil activation.<sup>154</sup> These endopeptidases are also associated with tissue damage and heme acquisition.<sup>153</sup> The heme acquired is used to form a black deposit on the bacteria which may be an oxidative sink to protect the bacteria from leukocyte-generated ROS.<sup>153,155</sup> Both types of gingipains, the lysine-specific (Kgp) and the

arginine-specific (RgpA), are primarily implicated in heme acquisition and deposition.<sup>153</sup> Studies on gingipain activity and the presence of this heme layer have shown that they are both very important to the bacteria's survival against ROS.<sup>156</sup> Mutants that lack this layer show higher degrees of 8oxo-G DNA damage and subsequent attempts by the cell to repair the damage than the wild-type bacteria.<sup>156</sup>

Beyond the oxygen-dependent mechanisms employed by the neutrophil, they also utilize antimicrobial enzymes such as lysozyme and elastase to curb infection.<sup>157</sup> Investigations of the exocytosis of neutrophil granules have shown no major azurophil granule exocytosis due to low levels of extracellular elastase.<sup>157</sup> They also show a minimum induction of gelatinase granule exocytosis by measuring extracellular matrix metalloprotease-9.<sup>157</sup> Additionally, gingipains are also involved in the breakdown of the neutrophils granule contents.<sup>158</sup> Especially in the anoxic environment of the periodontal crevice, the degradation of oxygen-independent antimicrobial peptides contributes greatly to *P. gingivalis*' survival.

Another function of gingipains (in this case gingipain-1) produced by *P. gingivalis* is the cleavage of complement protein.<sup>159</sup> Complement protein is important to both the opsonization process and the chemotaxis of neutrophils. The generation of complement component 5a (C5a) by gingipain cleavage of C5 results in C5a-mediated neutrophil recruitment.<sup>159</sup> Manipulation of complement and TLR-2 has been shown to occur in *P. gingivalis* mouse infection and modeled in human neutrophils to inhibit bacterial killing while simultaneously increasing the inflammation.<sup>160</sup> Blocking the TLR2 and/or the C5aR in human (HL60) neutrophils results in an inhibition of bacterial killing.<sup>160</sup> *P. gingivalis* acts to specifically uncouple the TLR2-MyD88 pathway which facilitates phagocytosis and subsequent bactericide from the TLR2-Mal-PI3K pathway which increases inflammation.<sup>160</sup> This work has not yet been replicated using primary human neutrophils.

### 3.4 | Neutrophils and their new challenge: coping with the oral pathobionts

The inflamed periodontal tissue is an ideal environment for the growth of asaccharolytic periodontal pathogens because it is a constant source of essential nutrients coming from damaged tissue. Moreover, the inflamed environment with broken homeostasis favors the overgrowth of some bacterial species called 'inflammophilic' or pathobionts which have a fitness advantage and will try to preserve inflammation at all cost.<sup>70</sup> One such emerging pathogen is *F. alocis*, a gram-positive anaerobic rod. Although cultivable, this organism is slow-growing and difficult to detect by conventional culture-based methodologies. Nonetheless, it is becoming increasingly apparent that the presence of *F. alocis* is indicative of oral disease, as the organism has been detected significantly more frequently and in higher numbers at periodontal disease sites compared with healthy sites.<sup>59,66,161–165</sup> Moreover, it has even been proposed that *F. alocis* should be included as a diagnostic indicator of disease.<sup>166</sup> To date, studies on the pathogenic potential of *F. alocis* have established that the organism can form biofilms in vivo, preferentially colonizing the apical parts of the gingival pocket in close proximity to the soft tissues.<sup>167</sup> Characterization of the different oral bacteria composition of different oral sites like saliva, supragingival and subgingival area, found several bacterial groups with strong co-occurrence pattern increasing together in disease sites and decreasing together in healthy sites implying synergism within

the community.<sup>168</sup> One of the co-occurrence group formed by eight members is center on *F. alocis* and some of the group members include two of the putative periodontal pathogens, *P. gingivalis* and *T. forsythia*, as well as some of the newly recognized periopathogen *Peptoanaerobacter stomatis*.<sup>168</sup>

In vitro, *F. alocis* can produce trypsin-like proteases<sup>169</sup> and is resistant to oxidative stress.<sup>170</sup> Study of the interaction with gingival epithelial cells has demonstrated that *F. alocis* is invasive, both in monoculture and synergistically with *P. gingivalis*, and it is pro-inflammatory by promoting the release of TNF $\alpha$ , IL-1 $\beta$ , and IL-6.<sup>171</sup> Furthermore, *F. alocis* induces apoptosis in gingival epithelial cells by suppression of MEK1/2 and activation of caspase 3.<sup>171</sup> In addition, we have recently shown that *F. alocis* is able to interact with other oral biofilm bacteria and accumulates in sites rich in *F. nucleatum*.<sup>172</sup> *F. alocis* can establish colonization in a mouse subcutaneous chamber model, resulting in a quick acute inflammatory response with a peak of neutrophil infiltration 2 hours after infection with concomitant increase of proinflammatory cytokines such as TNF $\alpha$ , IL-6 and IL-1 $\beta$ , and control of the local infection with decreasing bacterial numbers by 2 hours which were completely reduced by 72 hours after infection.<sup>173</sup> In addition, heat-killed *F. alocis* induces CXCL1 expression through NOD1 in mesothelial cells.<sup>174</sup> We are beginning to understand the in vivo pathogenic properties of *F. alocis* and its ability to cause disease may not be solely restricted to the periodontal pocket. Mouse models have also shown the bacteria can spread to other tissues such as the spleen, lung, and kidney tissues with acute kidney damage observed resulting from inflammation.<sup>173</sup>

*F. alocis* survives in human neutrophils after 6 hours after challenge, preventing granule recruitment to the bacteria-containing phagosome and failing to induce the respiratory burst response (J.E. Edmison, C.L. Armstrong, S. Tian, A. Vashistha, J. Le, R.J. Lamont and S.M. Uriarte, unpublished observations). The studies published thus far begin to establish the pathogenic potential of this newly appreciated oral pathogen.

#### 4 Concluding Remarks

The current evidence indicates that upon changes in the environment, such as smoking, diabetes, stress, antibiotic therapy, or colonization by keystone periodontal pathogens, the majority of the microbial composition identified in health will encounter a hostile niche and be outcompeted by the pathobionts, which have a fitness advantage and will flourish to promote inflammation that will sustain the dysbiotic community (Fig. 2B). Neutrophils are the most abundant leukocyte patrolling the oral cavity and the principal cell involved in preserving the host homeostasis by using its sophisticated ant-microbial artillery toward the constant microbial burden present in the mouth. A century has passed since the death of Elie Metchnikof, whose seminal discoveries of the importance of phagocytosis, inflammation, and the balance between homeostasis and disease, are some of the impressive legacies he left as the father of innate immunity. Moreover, his remarkable discoveries of the role of the professional phagocytes, macrophages and ‘microphages’, as he initially referred to neutrophils, in phagocytosis and immunity are the steppingstone to the current interest in the gut microbiome.<sup>175</sup>

Exciting times are nigh since much more research is needed to understand the fascinating interplay between human neutrophils and the oral microbial community. By characterizing this interaction in health and disease, it will be possible to develop new therapeutic strategies to control what triggers the ‘nososymbiocity’ of periodontal disease.

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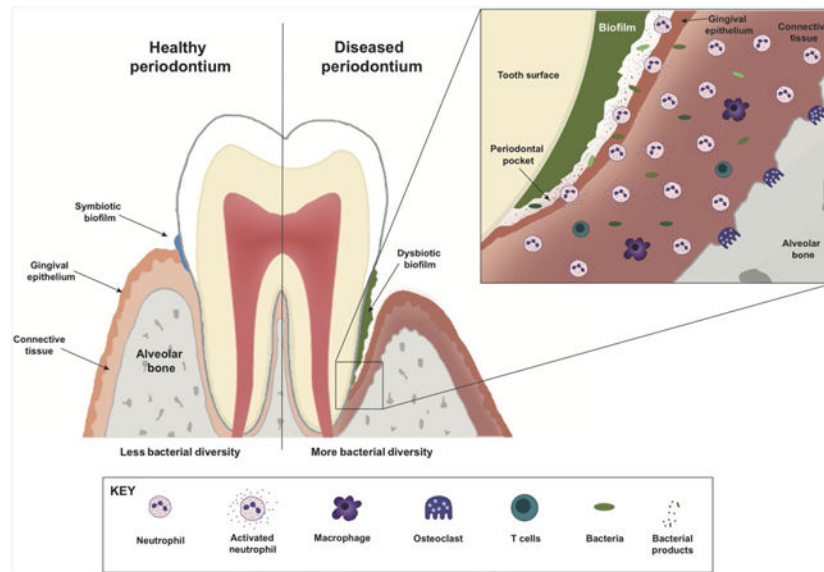
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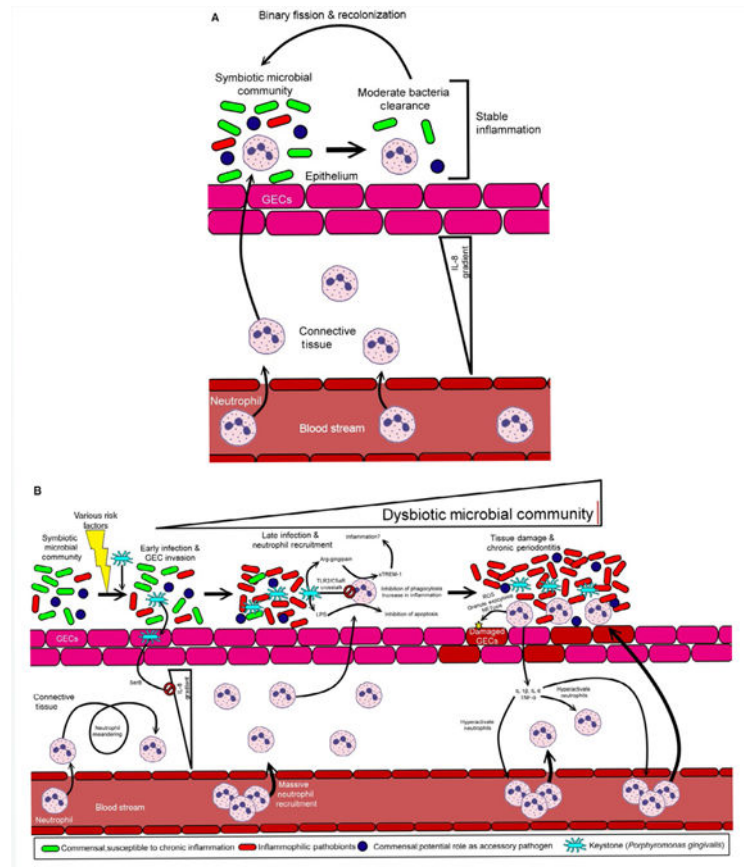
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**Figure 1.**

A schematic of the host response in a healthy and diseased periodontium. The local oral microbiota of a healthy periodontal site (left) is characterized by low bacterial diversity and quantity. In this homeostatic scenario, the symbiotic biofilm enhances the recruitment of neutrophils which mount a controlled acute inflammatory response. However, in the case of periodontitis (right) genetic predisposing conditions of the host, and/or environmental risk factors favor the colonization of exogenous pathogens, which results in an increase in bacterial diversity, formation and overgrowth of a dysbiotic bacterial biofilm, with massive recruitment and neutrophil infiltration invading the gingival epithelium, and the crevicular fluid. The result of this chronic inflammation involves both the innate and adaptive immune cells which results in periodontal lesions (right, and inset) with connective tissue and alveolar bone destruction



**Figure 2.** Neutrophil response in periodontal health and disease. (A) A symbiotic microbial community adheres to the gingival epithelial cells (GECs). As the bacterial burden increases, neutrophils regularly exit the blood stream entering the connective tissue layer beneath the epithelium. Some of these neutrophils will transverse the epithelium to kill some of the associated microbes—reducing the bacteria concentration. This process occurs without inflammation or tissue damage. (B) Following environmental stresses (smoking, poor diet, injury etc.) the keystone pathogen, *Porphyromonas gingivalis*, can colonize the symbiotic microbial community. Following colonization, the GECs are invaded by *P. gingivalis* which shuts down the IL-8 production via SerB resulting in local chemokine paralysis. Neutrophils enter the connective tissue, but are not initially directed to the infection. This causes many neutrophils to accumulate in the connective tissue. As some neutrophils traverse the epithelium in late infection, their killing mechanisms are hindered by multiple mechanisms including TLR2/C5aR crosstalk. Lipopolysaccharide (LPS) from *P. gingivalis* inhibits apoptosis of neutrophils leading to excessive inflammation, and *P. gingivalis*' gingipains may increase inflammation by accumulation of sTREM-1. Neutrophil degranulation, reactive oxygen species (ROS) production and NETosis damage GECs resulting in cytokine (TNF- $\alpha$ , IL-6, and IL-1 $\beta$ ) secretion by the GECs and the activated neutrophils. This further activates circulating neutrophils and results in large neutrophil chemotaxis to the periodontal plaque. A cycle of tissue damage followed by neutrophil activation/recruitment followed by

more tissue damage ensues. Inflammophilic pathobionts take advantage of the inflammation while susceptible commensals are eradicated

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