

## REVIEW ARTICLE

# Periodontal Pathogens' strategies disarm neutrophils to promote dysregulated inflammation

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**Funding information**

National Institute of Dental and Craniofacial Research, Grant/Award Number: DE024509

**Abstract**

Periodontitis is an irreversible, chronic inflammatory disease where inflammophilic pathogenic microbial communities accumulate in the gingival crevice. Neutrophils are a major component of the innate host response against bacterial challenge, and under homeostatic conditions, their microbicidal functions typically protect the host against periodontitis. However, a number of periodontal pathogens developed survival strategies to evade neutrophil microbicidal functions while promoting inflammation, which provides a source of nutrients for bacterial growth. Research on periodontal pathogens has largely focused on a few established species: *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*. However, advances in culture-independent techniques have facilitated the identification of new bacterial species in periodontal lesions, such as the two Gram-positive anaerobes, *Filifactor alocis* and *Peptoanaerobacter stomatis*, whose characterization of pathogenic potential has not been fully described. Additionally, there is not a full understanding of the pathogenic mechanisms used against neutrophils by organisms that are abundant in periodontal lesions. This presents a substantial barrier to the development of new approaches to prevent or ameliorate the disease. In this review, we first summarize the neutrophil functions affected by the established periodontal pathogens listed above, denoting unknown areas that still merit a closer look. Then, we review the literature on neutrophil functions and the emerging periodontal pathogens, *F. alocis* and *P. stomatis*, comparing the effects of the emerging microbes to that of established pathogens, and speculate on the contribution of these putative pathogens to the progression of periodontal disease.

**KEYWORDS**

emerging oral pathogen, *Filifactor alocis*, human neutrophils, periodontitis

**1 | INTRODUCTION**

Periodontal diseases affect the integrity of one or several tissues of the periodontium, which is comprised of the gingiva, periodontal

ligament, cementum, and alveolar bone. Periodontitis is an irreversible, chronic inflammatory disease that causes the loss of connective tissue, alveolar bone, and eventually the loss of teeth (Pihlstrom et al., 2005). Preventative and treatment measures aim to control or

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remove the periodontal biofilm (Douglass, 2006); but unfortunately, their efficacy is transient since the infection almost always returns. Even in cases where inflammation is resolved and some tissue is regenerated, it is impossible for the lost tooth support to be restored.

Under homeostatic conditions, colonizing bacteria initially assemble into physiologically compatible communities that can communicate through sophisticated signaling mechanisms (Plancak et al., 2015). Any overgrowth or pathology is efficiently controlled by the host inflammatory response, and the gingiva will return to its normal, mild inflammatory state called para-inflammation (Borenstein et al., 2018). However, changes in host immune competence, diet, or behaviors like smoking can affect microenvironmental factors including inflammation, pH, redox potential, or nutrient availability, which can drive the selection and enrichment of specific pathogenic bacteria. Under the poly-microbial synergy and dysbiosis (PSD) model, a perturbation of the symbiotic microbial community will result in an increase in the diversity and abundance of oral microbes (Hajishengallis & Lamont, 2012). The microbial community is rendered dysbiotic by the actions of specialized microorganisms, and once a dysbiotic environment is established, it is stabilized by the nature of the microbes present. As the dysbiotic community develops, an inflammatory response occurs, which includes elevated neutrophil recruitment, but which is nonetheless ineffective at confining the bacterial community. Aggravated and dysregulated immune responses, in the context of periodontal inflammation, contribute to tissue destruction that affords a source of nutrients for the microbial community's growth, and thus a continuous cycle of dysbiosis and inflammation ensues.

Neutrophils are the most abundant white blood cell in the gingival crevice or periodontal pocket (Delima & Van Dyke, 2003), where they are indispensable in the innate immune response against bacterial infection and are critical for maintaining homeostasis in the oral cavity (Uriarte et al., 2016). This is best illustrated in patients with neutropenic diseases or genetic defects that affect neutrophil functions, who develop severe periodontitis (Hajishengallis et al., 2016; Silva et al., 2019). Despite their beneficial role in homeostasis, neutrophils are implicated as the main immune cells responsible for the progression of periodontitis (Hernandez et al., 2010; Lee et al., 1995).

This review summarizes the current understanding of the evasion strategies utilized by established and emerging periodontal pathogens to disarm neutrophil effector functions (Tables 1 and 2). Out of the growing list of emerging oral microbes, *Filifactor alocis* and *Peptoanaerobacter stomatis* will be the focus of the second half of this review because they have the most characterized interaction with neutrophils of all emerging periodontal pathogens.

## 2 | A MOVING TARGET: MAKING AN INVENTORY OF ORAL MICROBES IN PERIODONTITIS

The microbial shift from a symbiotic microbiota to a dysbiotic polymicrobial community has been tracked and refined as

culture-independent techniques that have advanced (Berezow & Darveau, 2011; Hajishengallis, 2014; Hajishengallis & Lamont, 2016; Lamont & Hajishengallis, 2015). Together, the pioneer traditional studies and the more advanced sequencing analyses have identified the presence of approximately 700 predominant taxa in the oral microbiome, of which approximately one-third are 'yet-to-be-cultivated' (Krishnan et al., 2017). Plaque analysis from periodontitis-diseased sites has revealed the presence of *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* related with disease severity (Socransky & Haffajee, 2005; Socransky et al., 1998), whereas the presence of *Aggregatibacter actinomycetemcomitans* has been associated with aggressive periodontitis (Feres et al., 2004). *Fusobacterium nucleatum* is considered a commensal species in the oral cavity that increases in abundance in diseased individuals; however, it has been associated with most types of periodontal diseases due to its highly synergistic interactions with *P. gingivalis* and *T. forsythia* (Han, 2015).

The microbes described above are the most well-studied and are therefore well established as periodontal pathogens. However, there are 17 newly identified species that have a moderate association with the etiology of periodontitis. This list is comprised of four species in the not-yet-cultivable group (*Desulfobulbus* sp. oral taxon 041, *Fretibacterium* sp. oral taxon 360, *Fretibacterium* sp. oral taxon 362, TM7 [G-5] sp. oral taxon 356), eight Gram-negative species (*Bacteroidales* [G-2] sp. oral taxon 274, *Porphyromonas endodontalis*, *Treponema lecithinolyticum*, *Treponema medium*, *Treponema vincentii*, *Anaeroglobus geminatus*, *Selenomonas sputigena*, and *Fretibacterium fastidiosum*), and five Gram-positive species (*Eubacterium saphenum*, *Enterococcus faecalis*, *Mogibacterium timidum*, *Peptoanaerobacter stomatis*, and *Filifactor alocis*; Perez-Chaparro et al., 2014). Out of these newly identified species, *P. endodontalis* is known to induce cytokine and chemokine release from neutrophils and intraperitoneal injection of *E. saphenum* can recruit murine neutrophils (Ko & Lim, 2002; Salam et al., 2001).

## 3 | FILIFACTOR ALOCIS AND PEPTOANAEROBACTER STOMATIS: FINDING THEIR PLACE IN DYSBIOTIC PERIODONTAL COMMUNITY

*Filifactor alocis* is a slow-growing, non-spore forming, obligate anaerobic rod that has been classified as Gram positive and asaccharolytic, due to its metabolic preference for specific amino acids like arginine (Aruni et al., 2015; Jalava & Eerola, 1999; Uematsu et al., 2003). It was identified from the gingival sulcus in gingivitis and periodontitis patients, and named *Fusobacterium alocis* (Cato et al., 1985), but was later reclassified under the genus *Filifactor* (Jalava & Eerola, 1999). Multiple studies show a stark difference in colonization of healthy versus diseased tissues by *F. alocis*. While there is a high incidence of *F. alocis* in periodontitis patients, there is a complete absence or low number detected in healthy patients, designating *F. alocis* as a crucial marker for periodontitis along with *P. gingivalis*, *T. denticola*, and *T.*

TABLE 1 Established and emerging Periodontal Pathogens' effects on neutrophil antimicrobial functions

	Phagocytosis	ROS Generation	Granule Exocytosis	NET formation	Bacterial Killing
<i>T. denticola</i>	Resisted by Td (Shin et al., 2008) Inhibited by MSP (Puthengady Thomas et al., 2006)	Induced (Shin et al., 2008; Yamazaki et al., 2006)	MMP9 (Godovikova, Goetting-Minesky, & Fenno, 2011; Sela, 2001; Yamazaki et al., 2006)	?	?
<i>T. forsythia</i>	Ingested (Moriguchi et al., 2017)	Induced (Moriguchi et al., 2017)	?	?	?
<i>F. nucleatum</i>	Ingested (Ding et al., 1997; Polak et al., 2013; Shin et al., 2008)	Induced (Katsuragi et al., 2003; Shin et al., 2008)	No MMP9 & Robust Elastase (Ding et al., 1997; Shin et al., 2008)	Induced (Alyami et al., 2019)	Killed (Mangan et al., 1989)
<i>A. actinomycetem-comitans</i>	Resisted (Permanich et al., 2006)	Induced (Katsuragi et al., 2003)	MMP8 by Aa & Lactoferrin, Elastase by Leukotoxin (Claesson et al., 2002; Johansson, 2011)	Induced (Hirschfeld et al., 2016; Palmer et al., 2016)	Not Killed (Guentsch et al., 2009; Lai et al., 2015) Killed (Permanich et al., 2006)
<i>P. gingivalis</i>	Resisted (Maekawa et al., 2014; Makkawi et al., 2017; Stobernack et al., 2018) Ingested (Jayaprakash et al., 2015; Lenzo, O'Brien-Simpson, Cecil, Holden, & Reynolds, 2016)	Minimally induced (Hirschfeld et al., 2017)	MMP9 (Ding et al., 1997)	No effect (Hirschfeld et al., 2017) Induced (Bryzek et al., 2019)	Not killed (Odeil & Wu, 1992) Killed (Jayaprakash et al., 2015)
<i>F. alocis</i>	Ingested (Edmisson et al., 2018)	Minimally induced (Edmisson et al., 2018)	Secretory vesicles, Gelatinase & Specific granules (Armstrong et al., 2018; Edmisson et al., 2018)	No effect & inhibits PMA-induced (Armstrong et al., 2018)	Not killed (Edmisson et al., 2018)
<i>P. stomatis</i>	Resisted (Jimenez Flores et al., 2017)	Robust induction (Jimenez Flores et al., 2017)	Robust, all 4 granule subtypes (Jimenez Flores et al., 2017)	Induced (Armstrong et al., 2018)	Killed (Jimenez Flores et al., 2017)

**TABLE 2** Periodontal Pathogens' effects on neutrophil non-antimicrobial functions

	Chemotaxis	Apoptosis	Release of inflammatory mediators
<i>T. denticola</i>	Decreased by MSP (Jones et al., 2017; Magalhaes et al., 2008; Puthengady Thomas et al., 2006; Visser et al., 2013)	No effect (Ding et al., 1997; Puthengady Thomas et al., 2006; Shin et al., 2008)	Minimal IL-1 $\beta$ (Shin et al., 2008)
<i>T. forsythia</i>	No Effect (Gosling, Gemmell, Carter, Bird, & Seymour, 2005)  Increased by Mirolysin (Jusko et al., 2015)	?	?
<i>F. nucleatum</i>	Decreased by <i>F. nucleatum</i> extract (Van Dyke et al., 1982)	No effect (Ding et al., 1997; Shin et al., 2008)  Accelerates (Jewett et al., 2000; Kurgan et al., 2017)	IL-1 $\beta$ , IL-8 & TNF $\alpha$ (Kurgan et al., 2017; Ling et al., 2015; Polak et al., 2013; Shin et al., 2008)
<i>A. actinomycetem-comitans</i>	Decreased by <i>A. actinomycetemcomitans</i> extract (Van Dyke et al., 1982)  Increased by leukotoxin (Ashkenazi et al., 1992; Hirschfeld et al., 2016)	Accelerated by Aa (Permpnich et al., 2006)  Lysed by leukotoxin (Johansson, 2011)	IL-1 $\beta$ , TNF $\alpha$ and IL-8 (by Aa LPS) (Yoshimura et al., 1997)
<i>P. gingivalis</i>	Extract from <i>P. gingivalis</i> was chemotactic, but inhibited chemotaxis to other stimuli (Van Dyke et al., 1982)	No effect (Ding et al., 1997; Galicia et al., 2009; Hiroi et al., 1998; Preshaw et al., 1999)	IL-1 $\beta$ , TNF $\alpha$ , IL-8, and CCL2 (Polak et al., 2013; Vashishta et al., 2019; Yoshimura et al., 1997)
<i>F. alocis</i>	<i>F. alocis</i> infected neutrophils had enhanced chemotaxis toward IL-8 (Armstrong et al., 2016)	?	Minimal IL-1 $\beta$ , TNF $\alpha$ , IL-8, High levels IL-1ra, CCL4 (Vashishta et al., 2019)
<i>P. stomatis</i>	Conditioned supernatants from <i>P. stomatis</i> infected neutrophils induced migration of neutrophils and monocytes (Vashishta et al., 2019)	?	IL-1 $\beta$ , TNF $\alpha$ , IL-1ra, CXCL1, CCL2, CCL3, CCL4 (Vashishta et al., 2019)

*for sythia* (Ahmed et al., 2009; Deng et al., 2017; Ikeda et al., 2019; Kumar et al., 2003, 2006; Schlafer et al., 2010; Schulz et al., 2019). In the oral cavity, *F. alocis* forms biofilms in close proximity to the soft tissue where some traditional pathogens like *T. denticola* are also found (Liu et al., 2020; Schlafer et al., 2010). Thus, *F. alocis* is strongly associated with periodontitis as a key player in biofilm formation (Aruni et al., 2011; Chen et al., 2015; Naginyte et al., 2019; Wang et al., 2013).

The other Gram-positive emerging periodontal pathogen, *Peptoanaerobacter stomatis*, is an obligate anaerobic rod, with a diameter of 0.5–0.7  $\mu\text{m}$  and a length of 1.0–2.3  $\mu\text{m}$ , often forming chains (Sizova et al., 2015). It is highly motile due to peritrichous flagella (Downes & Wade, 2006; Sizova et al., 2015), and it represents the first known cultivable member of the human oral taxon O81. In 2015, it was classified as a novel genus and species within the *Peptostreptococcaceae* family (Sizova et al., 2015). The newly appreciated oral bacterium is not readily detected in the biofilms of healthy patients but was found in high numbers in the biofilms of patients with periodontal diseases (Kumar et al., 2005; Murphy & Frick, 2013; Sizova et al., 2015). In addition to periodontitis, *P.*

*stomatis* is associated with dentoalveolar abscesses and endodontic infections (Downes & Wade, 2006).

## 4 | NEUTROPHILS IN THE PROGRESSION OF PERIODONTITIS

Many studies comparing neutrophils from periodontitis patients to those of healthy controls have found that, under periodontal disease conditions, neutrophils are supernumerary, hyperactivated, and/or display dysregulated functions. Compared to healthy controls, periodontitis neutrophils had augmented phagocytic capacity and increased expression of degranulation markers; showed defects in chemotaxis, apoptosis; and released significantly higher quantities of reactive oxygen species (ROS), anti-bacterial enzymes, neutrophil extracellular traps (NETs), and pro-inflammatory cytokines (Allen et al., 2011; Dias et al., 2011; Fine et al., 2016; Guentsch et al., 2009; Ling et al., 2015). Together, these aberrant functions result in inefficient killing of microbial pathogens, unresolved inflammation, and the destruction of tooth supporting tissues by

releasing tissue-degrading enzymes or inflammatory and toxic molecules (Chapple & Matthews, 2007; Eskin et al., 2012; Ryder, 2010). Neutrophil effector functions are likely altered in periodontitis through products from the chronic inflammatory environment that contribute to a primed and hyperactive phenotype (Miralda et al., 2017; Sochalska & Potempa, 2017) or through the manipulating periodontal pathogens.

## 5 | NEUTROPHIL EFFECTOR FUNCTIONS: BEFORE AND DURING THE DYSBIOTIC STORM

### 5.1 | Chemotaxis

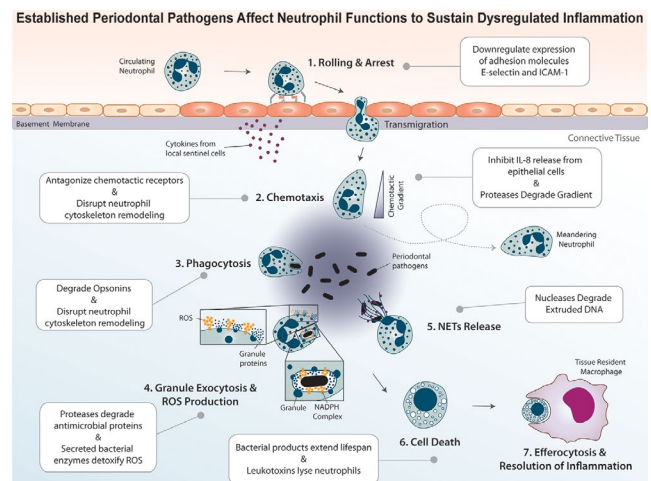
Circulating neutrophils exist in a basal state, characterized by non-adherence, a round morphology, minimal transcriptional activity, and a limited capacity to respond to activating stimuli. However, microbial invasion or tissue injury will release pathogen-associated molecular pattern (PAMPs) or damage-associated molecular pattern (DAMPs) molecules that modify the adhesion molecules on endothelial cells to facilitate the capture of circulating neutrophils, followed by transmigration to enter the extravascular space (Filippi, 2019; Reichel et al., 2008). Once there, neutrophils detect the intensity of chemotactic gradients and move with efficient directionality toward intermediate chemoattractants (interleukin (IL)-8, leukotriene B<sub>4</sub>, and platelet activated factor) first, and then toward end target chemoattractants (bacterial formylated peptides and complement fragments C5a and C3a) (Majumdar et al., 2014; Parent, 2004).

As the bacterial burden increases during periodontitis progression, neutrophil recruitment and accumulation also magnify (Figure 1.1). In fact, neutrophil counts correlate with the level of inflammation, the increasing depth of the periodontal pocket, and the severity of chronic periodontitis (Bender et al., 2006; Fine et al., 2016; Landzberg et al., 2015; Rijkschroeff et al., 2016). Defects in chemotaxis greatly contribute to the high presence of neutrophils in tissues, and pathogenic oral bacteria are known to modulate this neutrophil function (Roberts et al., 2015). One of the most effective ways pathogenic oral bacteria restrict neutrophil chemotaxis is indirectly, by interfering with the chemotactic gradient. *T. denticola* can degrade IL-8 through the action of dentilisin, a major outer membrane protease (Jo et al., 2014). Additionally, *T. denticola* and *P. gingivalis* can both suppress IL-8 production by gingival epithelial cells to cause local chemokine paralysis (Brissette et al., 2008; Darveau et al., 1998).

Alternatively, oral bacteria can disarm neutrophil migration by acting on the neutrophil itself. The supernatant from sonicated organisms like *F. nucleatum*, *A. actinomycetemcomitans*, and *P. gingivalis* can bind and antagonize neutrophil chemotactic receptors to prevent the detection of the chemotactic gradient and thus, inhibit neutrophil migration toward known chemoattractants (Ashkenazi et al., 1992; Van Dyke et al., 1982). Microbial products like the Major Sheath Protein (MSP) of *T. denticola* can also interfere with

cytoskeletal signaling pathways that mediate cell movement (Jones, Vanyo, & Visser, 2017, 2019; Magalhaes et al., 2008; Puthengady Thomas et al., 2006; Visser et al., 2013). Pathogenic oral bacteria also employ misdirection mechanisms: *P. gingivalis* can downregulate the expression of cell adhesion molecules like E-selectin and intracellular adhesion molecule (ICAM)1 that are required for leukocyte extravasation (Figure 1.2) (Darveau et al., 1995; Huang et al., 1998, 2001; Madianos et al., 1997), while leukotoxin from *A. actinomycetemcomitans* enhances random migration of neutrophils (Hirschfeld et al., 2016).

When the neutrophil directed migration pattern is altered, they continue to accumulate at the periodontal pocket and adjacent connective tissue but are unable to effectively track target microbes. Meandering neutrophil behavior is beneficial to the microbial community because it decreases the chances of neutrophil–bacteria interaction while increasing the chances for bacterial colonization (Figure 1). Paradoxically, the same periodontal bacteria can contribute to the recruitment of neutrophils to the gingival tissue through the actions of their proteases. Mirolysin and karilysin from *T. forsythia* and arginine-specific gingipain (RgpA) from *P. gingivalis* can cleave complement component 5 (C5) to generate the anaphylatoxin C5a, which strongly recruits neutrophils (Jusko et al., 2012, 2015; Wingrove et al., 1992). It has been shown that the concentration of gingipains released by *P. gingivalis* is tailored to promote bacterial survival. At early infection stages gingipains can be found at low concentrations, promoting cleavage of C5 and generation of C5a, which stimulates neutrophil recruitment that will enhance tissue



**FIGURE 1** The strategies of established periodontal pathogens to disarm neutrophil functions. To effectively clear invading organisms, neutrophils must be capable of mounting rapid, vigorous responses to activating stimuli; however, uncontrolled or prolonged neutrophil activation and antimicrobial responses result in injury to normal host cells, leading to pathologic changes. Numbers indicate the different functions that neutrophils perform from circulation to the site of infection and how established pathogens, including *T. forsythia*, *T. denticola*, *F. nucleatum*, *A. actinomycetemcomitans*, and *P. gingivalis* can manipulate these functions (shown in white boxes) to drive inflammation. Refer to the text for additional details [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

destruction and provide nutrients for the dysbiotic community. However, when their concentration increases and the biofilm has been formed in deeper periodontal pockets, they inhibit the complement pathway to protect *P. gingivalis* and bystander bacteria from complement and neutrophil killing (Popadiak et al., 2007).

## 5.2 | Phagocytosis

As professional phagocytes, neutrophils will rapidly and readily undergo the receptor-mediated process of phagocytosis (Levin et al., 2016). Phagocytosis occurs most efficiently by the recognition of complement or IgG-opsonized particles, and while non-opsonized phagocytosis can occur it is less efficient since neutrophils possess fewer non-opsonic receptors than macrophages (Allen & Criss, 2019). Phagocytosis initiates a series of events that typically result in the killing and degradation of the ingested cargo. However, the manipulation of neutrophils by periodontal pathogens continues at the level of phagocytosis and intra-phagosomal killing (Figure 1.3).

*Treponema denticola* and *P. gingivalis* efficiently block neutrophil phagocytic events by interfering with cytoskeleton pathways, which provide protective advantages to other bacteria in the oral cavity (Maekawa et al., 2014; Makkawi et al., 2017; Puthengady Thomas et al., 2006). Neutrophils treated with MSP from *T. denticola* had diminished incorporation of actin monomers during de novo filament assembly, which resulted in decreased uptake of IgG-coated spheres (Puthengady Thomas et al., 2006). Alternately, by modulating signaling cascades, *P. gingivalis* inhibits the formation and extension of lamellipodia necessary to form the phagocytic cup (Maekawa et al., 2014; Makkawi et al., 2017). While *T. denticola*, *A. actinomycetemcomitans*, and *P. gingivalis* naturally avoid phagocytosis by neutrophils, the presence of complement and antibodies can overcome this resistance (Guentsch et al., 2009; Lingaas et al., 1983; Shin et al., 2008). However, *T. denticola* and *P. gingivalis* proteases can degrade C3 or other upstream components of the complement pathway to inhibit complement opsonization and killing (Jusko et al., 2012; Popadiak et al., 2007). Additionally, despite the finding that patients with periodontitis have high levels of *P. gingivalis*-specific IgG in serum, the lysine-specific gingipain K (Kgp) is able to cleave IgG1 and IgG3 at the hinge region, which separates the antigen binding region of the antibody from the effector fragment, resulting in the inactivation of IgG-mediated opsonization (Guentsch et al., 2013; Kobayashi et al., 2001; Vincents et al., 2011). When neutrophils are not able to engulf the bacteria, they resort to extracellular killing mechanisms that fuel the ongoing inflammation.

## 5.3 | Reactive oxygen species generation

Once a bacterial particle is engulfed, production of reactive oxygen species (ROS) and fusion of antimicrobial granules will contribute to the phagosome's maturation and lethality (Levin et al., 2016). The complementary actions of the granule proteases and ROS

production will create a highly toxic environment that few microbes can survive. ROS are generated through the conversion of molecular oxygen to superoxide by the multi-component NADPH oxidase complex. Spatial separation of the NADPH membrane and cytosolic components maintains enzymatic inactivity in resting neutrophils. Upon stimulation, the cytosolic components translocate to the membrane bound components to form the catalytically active enzyme complex (el Benna et al., 1994, 1996; Groemping et al., 2003). Neutrophils have an unparalleled ability to rapidly form ROS, and they can tailor their response depending on the type of stimuli they encounter. Activation of neutrophils by a soluble stimulus, such as fMLF, triggers assembly of the NADPH oxidase at the plasma membrane and release of superoxide anions toward the extracellular space. In contrast, if neutrophils encounter a particulate stimuli, for example a bacterium, assembly and activation of the NADPH oxidase will take place at the membrane of the bacteria-containing phagosome with release of superoxide anions inside the phagosome (Babior et al., 2002; Nauseef, 2007, 2014, 2019). Generally, ROS produced from neutrophils contributes to bacterial killing and intracellular signaling; however, excessive ROS can have cytotoxic effects on periodontal tissues through oxidative damage to DNA and proteins, interference with cell growth and cell cycle progression, and induction of apoptosis of gingival fibroblasts (Chang et al., 2013; Esterbauer et al., 1991; Kanzaki et al., 2017; Wells et al., 2009; Yu et al., 2012). Indirectly, ROS can also induce alveolar bone resorption through their role as intracellular signaling molecules in osteoclastogenic pathways (Ha et al., 2004).

Like other effector functions, normal ROS generation by neutrophils is compromised in the context of periodontitis (Figure 1.4). When tested against individual periodontal bacteria in vitro, neutrophils can mount a ROS response of varying degrees against *T. denticola*, *T. forsythia*, *F. nucleatum*, *A. actinomycetemcomitans*, and *P. gingivalis*, although the extent of ROS production heavily depends on the strain used, multiplicity of infection, and type of opsonization (Hirschfeld et al., 2017; Katsuragi et al., 2003; Kurgan et al., 2017; Moriguchi et al., 2017; Shin et al., 2008; Yamazaki et al., 2006). Once generated, ROS do not discriminate between host and pathogen cells, and contribute heavily to disease progression. This is most clearly demonstrated by patients with hyperactive ROS response that are more susceptible to periodontitis (Aboodi et al., 2011; Johnstone et al., 2007), and periodontitis patients that were treated with the antioxidant, lycopene. Lycopene-treated patients had reduced oxidative stress and improved clinical parameters that could be observed up to 4 months after discontinuing treatment. Notably, patients with Chronic Granulomatous Disease (CGD), who cannot mount a respiratory burst response, are not more susceptible to periodontitis, but suffer from recurrent aphthous ulcers and severe gingivitis (Nussbaum & Shapira, 2011). More studies are highlighting the immunomodulatory role of ROS, by showing how both ends of the spectrum, absence or excess, contribute to dysregulated inflammation (Dinauer, 2019; Zeng et al., 2019).

The inefficacy of ROS against specific oral pathogens may be due, in part, to bacterial virulence factors that confer protection

for the entire bacterial community. Several oral pathogens like *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis* express superoxide dismutase (SOD), which catalyzes the dismutation of superoxides into hydrogen peroxide and produces molecular oxygen (Balashova et al., 2007; Choi et al., 1991; Diaz, Zilm, & Rogers, 2000, 2002). *P. gingivalis*, also expresses rubrerythrin (Rbr) and alkyl hydroperoxidase reductase (Ahp), which detoxify hydrogen peroxide (Diaz et al., 2004; Johnson et al., 2004; Sztukowska et al., 2002). Additionally, through the proteolytic action of gingipains, *P. gingivalis* can acquire heme deposits on its cell surface, which act as an oxidative sink to further protect bacteria against the deleterious effects of ROS (Rangarajan et al., 2017; Smalley & Olczak, 2017). However, other periodontal bacteria may also be acting directly on neutrophils to control the ROS response, for example *F. nucleatum* inhibits fMLF-induced superoxide generation, although the mechanism is still unknown (Kurgan et al., 2017).

## 5.4 | Granule exocytosis

Neutrophil granules are divided into four subtypes based on granule density and contents (Borregaard & Cowland, 1997; Lominadze et al., 2005; Sengelov et al., 1993, 1995). Like ROS production, the different neutrophil granule subtypes can either be recruited to the bacteria-containing phagosome or stimulated to undergo exocytosis and release their matrix content extracellularly (Niels Borregaard et al., 2007). Neutrophil granule subsets undergo an ordered release based on stimulus intensity, termed graded exocytosis (Sengelov et al., 1993, 1995). A weak stimuli induces mobilization of secretory vesicles, and increasing stronger stimulation is required to mobilize gelatinase, specific, and azurophil granules, respectively (Nauseef & Borregaard, 2014). 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 during inflammation (Lominadze et al., 2005; Rørvig et al., 2013; Uriarte et al., 2008). However, the killing efficacy of neutrophils against intracellular and extracellular microorganisms is enhanced by combining the activity of antimicrobial granule contents and ROS production (Figure 1.4).

The resistance of certain periodontal pathogens to bactericidal activity of neutrophil microbicidal peptides can be partially explained through the action of bacteria-derived proteolytic peptides. From *T. forsythia*, miropin, and karilysin efficiently inhibit the activity of a broad range of proteases (LL-37, neutrophil and pancreatic elastases, cathepsin G, subtilisin, and trypsin) (Koziel et al., 2010; Ksiazek et al., 2015), and gingipains from *P. gingivalis* directly cleave multiple host antimicrobial proteins, which confer resistance against extracellular, ROS-independent killing mechanisms (Carlisle et al., 2009; Gutner et al., 2009; Kuula et al., 2008; Odell & Wu, 1992). Interestingly, the expression of these gingipains is increased when *P. gingivalis* undergoes oxidative stress, suggesting there is a feed forward mechanism of suppression of neutrophil mechanisms (Shelburne et al., 2005).

While neutrophil granule contents can be released into the extracellular matrix or into a microbe-containing phagosome, most studies related to periodontal pathogens have focused on the release of neutrophil granule contents in the context of tissue degradation. *A. actinomycetemcomitans*, *F. nucleatum*, *T. denticola*, and *P. gingivalis* have all been reported to induce the release of matrix metalloproteinase 8 (MMP8), matrix metalloproteinase 9 (MMP9), neutrophil elastase, and lysozyme (Claesson et al., 2002; Ding et al., 1997; Gursoy et al., 2018; Sela, 2001; Sela et al., 1997; Sheikhi et al., 2000; Yamazaki et al., 2006). The exocytosis of neutrophil granules has also been confirmed in neutrophils isolated from the oral cavity, since neutrophils from periodontitis patients express increased levels of degranulation markers on their surface (Fine et al., 2016; Nicu et al., 2018). Elevated levels of neutrophil elastase, plasminogen, and MMP9 were detected in periodontal ligament from chronic periodontitis patients (Ujiiie et al., 2007), but zymographic analysis of these three proteinases showed that elastase was the only proteinase involved in the degradation of collagen fibrils of periodontal ligaments in vitro. Notably, the morphological features from the in vitro system were similar to that of the periodontal ligament in chronic periodontitis, which directly implicates this neutrophil enzyme in the early destructive stages of periodontal disease (Ujiiie et al., 2007). Similarly, bacterial proteases can also directly induce tissue degradation. Karilysin from *T. forsythia* shares structural homology to human matrix metalloproteinases and can cleave elastin, fibrinogen, and fibronectin (Karim et al., 2010).

## 5.5 | NET formation

NETs represent an immune defense mechanism deployed by neutrophils to immobilize and kill invading microbes or contain biofilms from disseminating into other sites of the body (Sollberger et al., 2018). However, as with all other neutrophil mechanisms, this function is a double-edged sword that has been implicated in inflammation and induction of auto-immunity by providing a source of autoantigens (White et al., 2016). Confocal and electron microscopy studies confirmed there was NET formation in the oral cavity of chronic periodontitis patients (Vitkov et al., 2009), and a later study found that neutrophils are attracted to the supragingival biofilms, where they release NETs (Hirschfeld et al., 2015). When individual periodontal bacteria were tested against neutrophils, *A. actinomycetemcomitans* and *F. nucleatum* both induced NET formation. *F. nucleatum* activated neutrophils through nucleotide oligomerization domain (NOD) 1 and 2 and induced a time-dependent, robust release of NETs (Alyami et al., 2019), which was independent of TLR stimulation and ROS production (Hirschfeld et al., 2017). Challenge of neutrophils with *A. actinomycetemcomitans* or its leukotoxin induced NETosis (Hirschfeld et al., 2016); a process that was enhanced by the presence of serum and signaling through complement receptor (CR)1 (Palmer et al., 2016). Contrasting reports have shown that *P. gingivalis* does (Bryzek et al., 2019; Jayaprakash et al., 2015) and does not (Hirschfeld et al., 2017) induce NET formation, though this

conflicting data may be due to bacterial strain differences. No studies have directly tested whether *T. forsythia* or *T. denticola* can induce NET release from neutrophils.

Regardless of whether NETosis was induced by a particular oral pathogen, both *A. actinomycetemcomitans* and *P. gingivalis* can be trapped by HOCl-produced NETs (Hirschfeld et al., 2017). However, several periodontal microbes like *P. gingivalis*, *Prevotella intermedia*, and *F. nucleatum*, but not *A. actinomycetemcomitans*, express nucleases with differing DNA degradation capacities (Doke et al., 2017; Palmer et al., 2012). Additionally, *P. gingivalis* expresses *Porphyromonas* peptidylarginine deiminase (PPAD), an enzyme that citrullinates histone H3, thereby facilitating the bacterial escape from NETs (Aliko et al., 2019; Cooper et al., 2013; Stoberneck et al., 2018; Vitkov et al., 2018). When neutrophils were cultured with *P. gingivalis* or purified Rgp gingipains, both stimulants induced NETs that lacked bactericidal activity and actually stimulated the growth of bacteria species that normally are susceptible to killing by NETs (Bryzek et al., 2019). This protection was mediated by the proteolysis of bactericidal components on NETs. Taken together, some periodontal pathogens may be playing a dual role in NET formation. They are the potent direct inducers of NETs formation but simultaneously prevent bacterial entrapment and subsequent killing by degrading NETs or the antimicrobial proteins embedded in the extruded DNA (Figure 1.5).

## 5.6 | Intra-phagosomal bacterial killing

Blocking of any of the killing mechanisms previously described (NETs, phagocytosis, ROS, granules) can result in defective bacterial killing. Neutrophils isolated from the crevicular fluid of periodontitis patients showed decreased intracellular killing against *A. actinomycetemcomitans* and *P. gingivalis* compared to neutrophils from healthy controls, indicating that there is pervasive inhibition of bacterial killing during periodontitis (Eick et al., 2000). *P. gingivalis* is also resistant to killing by neutrophil granule contents (Odell & Wu, 1992; Yoneda et al., 1990), which was later shown to be dependent on the gingipain activity (Kadowaki et al., 2004). On the other hand, neutrophils are able to carry out significant killing of *F. nucleatum* and *A. actinomycetemcomitans* strain Y4 within an hour (Guentsch et al., 2009; Lai et al., 2015; Mangan et al., 1989). Other strains of *A. actinomycetemcomitans* were able to resist intracellular microbicidal mechanisms after an hour of challenge, which correlated with the levels of leukotoxin expression (Permpnich et al., 2006). Notably, exposure of neutrophils to nicotine severely dampens their ability to kill *F. nucleatum* and *A. actinomycetemcomitans*, (Pabst et al., 1995), which presents one possible mechanism for increased periodontal disease in smokers. To the best of our knowledge, no studies have examined whether neutrophils can directly kill *T. denticola* or *T. forsythia*.

*P. gingivalis*' survival around neutrophils is the best characterized and depends on the cross-talk between two receptors on the surface of neutrophils in vivo (Hajishengallis, 2020; Maekawa et al., 2014). While *P. gingivalis* directly activates TLR2/1 receptor, the C5a formed

by the gingipain-dependent cleavage of C5 will activate the C5a receptor (C5aR1). The co-stimulation of these receptors results in the degradation of the TLR adaptor protein MyD88 (Burns et al., 2010; Maekawa et al., 2014). This reroutes signaling through another adaptor protein, MyD88 adaptor-like (Mal, also known as TIRAP). This is significant because activation of the MyD88-dependent signaling pathway is associated with initiation of antimicrobial responses while Mal-dependent pathways activate PI3K and block phagocytosis, while still resulting in the release of pro-inflammatory cytokines. Thus, by redirecting signaling through Mal instead of MyD88, *P. gingivalis* dismantles the killing mechanisms of neutrophils, but not their proinflammatory activity (Hajishengallis et al., 2016). Nonetheless, little to no literature is available to explain how other established oral pathogens can survive within the hostile environment of the neutrophil phagosome.

In the case of the periodontal dysbiotic community, the protective effects from one species could benefit others that do not have any virulence factors of this type. For example, succinic acid, a metabolic, fatty acid byproduct of *P. gingivalis* and other *Bacteroides* species, abolished neutrophil killing of *Escherichia coli* by decreasing neutrophils' ability to produce ROS (Rotstein et al., 1985, 1987). Similarly, when neutrophils were incubated with short chain fatty acids derived from anaerobic bacteria, their ability to undergo granule exocytosis and produce ROS was also diminished (Eftimiadi et al., 1987). Characterizing the periodontal pathogens' individual and community protective mechanisms provide valuable knowledge to better understand the complex interactions that take place between neutrophils and the dysbiotic microbial ecosystem.

## 5.7 | Cytokine production

The role of neutrophils as regulators of the immune response has gained increasing recognition because of their capacity to transcribe, perform de novo synthesis, and release different cytokines and chemokines (Cassatella, 1999; Nicola Tamassia et al., 2018; Cristina Tecchio & Cassatella, 2016). Depending on the type of stimulation they encounter, neutrophils can produce and release an array of different cytokines and chemokines, which is significant in the amplification loop of the local immune response (Cassatella, 1999; Cassatella et al., 2019; Scapini & Cassatella, 2014; Tamassia et al., 2018; Tecchio & Cassatella, 2016). Although each individual neutrophil may not produce quantities comparable to other immune cells, in periodontitis, where neutrophils accumulate in great numbers, the collective release of cytokines and chemokines by neutrophils can play a significant role in amplifying the immune response. It is unknown whether *T. forsythia* or *A. actinomycetemcomitans* induce direct cytokine production by neutrophils, but reports show that *T. denticola*, *F. nucleatum*, and heat-killed *P. gingivalis* can induce neutrophils to release the pro-inflammatory cytokine IL-1 $\beta$  (Polak et al., 2013; Shin et al., 2008). *F. nucleatum* and its LPS also induce robust release of IL-8 and TNF $\alpha$  from neutrophils (Kurgan et al., 2017; Ling et al., 2015). LPS from *A. actinomycetemcomitans* stimulated the



release of significantly greater amounts of IL-1 $\beta$ , TNF $\alpha$ , and IL-8 than the response elicited by *P. gingivalis*-LPS (Yoshimura et al., 1997). *P. gingivalis* does, however, stimulate neutrophil production of TNF $\alpha$ , IL-8, and CCL2, possibly through an LPS-independent mechanism (Vashishta et al., 2019; Yoshimura et al., 1997). A noteworthy distinction is that the best practice for measuring neutrophil-derived cytokines is to test a neutrophil population that is as pure as possible since contamination of even 0.01% of monocytes can skew the cytokine and chemokine populations significantly (Calzetti et al., 2017; Tecchio et al., 2014).

## 5.8 | Apoptosis

Under homeostatic conditions, neutrophils are programmed to undergo apoptosis after ~12–24 hr, and are cleared by macrophages in the liver, spleen, and bone marrow (Savarymuttu et al., 1985). In tissues, neutrophil lifespan is prolonged by cytokines (IL-1 $\beta$ , TNF $\alpha$ , GM-CSF, G-CSF, and interferon (IFN)  $\gamma$ ), microbial components, and the local environment (Kennedy & DeLeo, 2009). Moreover intracellular pathogens can promote neutrophil viability as a way to protect their replicative niche, whereas other microbes accelerate apoptosis, trigger neutrophil lysis, or redirect cell death toward necrosis to evade intracellular killing (Allen & Criss, 2019). Apoptosis is a critical step to minimize tissue damage by downregulating the phagocytic and proinflammatory capacity of neutrophils and preventing release of neutrophil cytotoxic components into the extracellular space (Fox et al., 2010; Kobayashi, Voyich, Braughton et al., 2003; Kobayashi, Voyich, Somerville, et al., 2003). Additionally, timely apoptosis and clearance of neutrophil corpses by macrophages (Figure 1.6 & 1.7) minimizes tissue damage because this process dampens pro-inflammatory cytokine production and reprograms macrophages to a pro-resolution phenotype that favors restoration of tissue homeostasis (Fadok et al., 1998; Korn et al., 2011; Voll et al., 1997).

Although cell death pathways in epithelial cells and macrophages have been well studied after interaction with periodontal pathogens, there are few publications that have addressed neutrophil viability. Transcriptionally, neutrophils from chronic periodontitis patients have significantly upregulated pro-survival pathways (Lakschevitz et al., 2013); however, most studies on periodontal bacteria and neutrophil lifespan have only measured cell death at very short timepoints (3 hr or less) post-bacterial challenge. For example, *F. nucleatum*, *P. gingivalis*, and *T. denticola* did not induce lactate dehydrogenase (LDH) release from neutrophils after 1-hr challenge with neutrophils (Ding et al., 1997). Incubation of neutrophils with *T. denticola* for an hour resulted in a mild increase in cell death only detected in neutrophils that phagocytosed antibody coated *T. denticola* at a multiplicity of infection (MOI) of 100 bacteria per neutrophil (Shin et al., 2008). Under the same experimental set-up, *F. nucleatum* induced cell death in a MOI and antibody opsonization-dependent manner (Jewett et al., 2000; Shin et al., 2008). The induction of apoptosis persisted when neutrophils were treated with multiple strains of *F. nucleatum* (Kurgan et al., 2017). Uptake of antibody-opsonized

*A. actinomycetemcomitans* after 1 hr of challenge also resulted in the rapid cell death of neutrophils (Permpnich et al., 2006). The induction of apoptosis after such short time points suggests that neutrophils challenged with *A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum*, and *T. denticola* underwent phagocytosis-induced cell death (PICD), which is linked to the production of ROS (Coxon et al., 1996; Kobayashi et al., 2002); however, since periodontal bacteria can resist neutrophil phagocytosis, the probability of PICD may not occur very often in vivo.

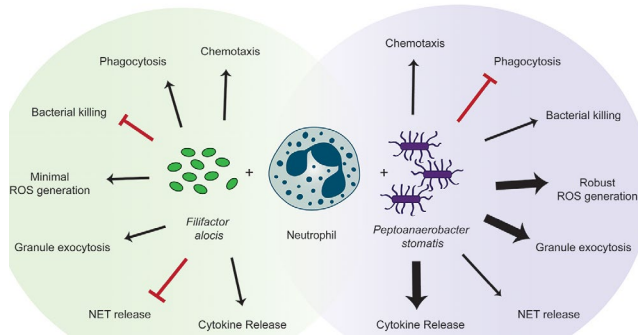
Compounding the length of neutrophil lifespan is the presence of several pro-inflammatory mediators that promote neutrophil longevity at sites of inflammation, such as LPS, lipoteichoic acids (LTA), TNF $\alpha$ , C5a, IL-1 $\alpha$ , granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and IFN $\gamma$ , and render these granulocytes resistant to extrinsic ligand-induced apoptosis (FS-7-associated surface antigen (FAS) and TNF) (Gamonal et al., 2003; Hotta et al., 2001). Additionally, the products of periodontal bacteria can also lead to changes in neutrophil survival. Neutrophil challenge for 1–3 hr with MSP from *T. denticola* does not induce cell death (Puthengady Thomas et al., 2006) and culture of neutrophils with the LPS from three different *P. gingivalis* strains delayed apoptosis in a dose-dependent fashion (Galicia et al., 2009; Hiroi et al., 1998; Preshaw et al., 1999), but leukotoxin from *A. actinomycetemcomitans* directly lyses neutrophils (Johansson, 2011; Permpnich et al., 2006). Notably, it is unknown whether direct interaction with *T. forsythia* or products from the bacteria will affect neutrophil lifespan.

Induction of neutrophil apoptosis is beneficial to bacteria because it will prevent neutrophil phagocytosis and microbicidal mechanisms from eliminating the bacterial burden. However, when neutrophil apoptosis is delayed, the inflammophilic bacteria still profit because inflammation worsens by two mechanisms: (a) collateral tissue damage increases because neutrophils will accumulate and continue to release degradative neutrophil enzymes and ROS, and (b) resolution of inflammation is delayed because clearance of apoptotic cells through efferocytosis cannot take place, which is an essential step in initiating tissue restoration.

## 6 | NEUTROPHIL RESPONSE TOWARD EMERGING ORAL PATHOGENS *F. ALOCIS* AND *P. STOMATIS*

Characterization of *F. alocis* and *P. stomatis* interaction with neutrophils has demonstrated that these putative oral pathogen impact neutrophil effector functions, albeit with opposing consequences (Figure 2). Starting with chemotaxis, neutrophils challenged with either live or heat-killed *F. alocis*, showed enhanced random and directed migration toward IL-8 (Armstrong et al., 2016). It is unknown whether *P. stomatis* has a direct effect on neutrophil chemotaxis. However, in vitro transwell assays show that the bacteria-free conditioned supernatant collected from neutrophils challenged with *P. stomatis*, but not from *P. gingivalis* or *F. alocis*, contained active

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**FIGURE 2** *Filifactor alocis* and *Peptoanaerobacter stomatis*: Two newly identified species that target neutrophils. When human neutrophils respond to *F. alocis* and *P. stomatis*, two distinct neutrophil functional signatures can be observed. In the case of *F. alocis*, neutrophils readily phagocytize the bacterium; however, the oral pathogen is able to persist within neutrophils by inducing minimal production of intracellular ROS and delaying the fusion of antimicrobial granules to its phagosome. In contrast, *P. stomatis* prevents phagocytosis by human neutrophils as an evasion strategy. At the same time, *P. stomatis* induces a strong pro-inflammatory neutrophil response. Ultimately, both of these putative oral pathogens are able to disrupt neutrophil functional mechanisms to promote bacterial survival and/or dysregulate inflammation. Refer to text for additional details [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

chemotactic factors that significantly recruited both neutrophils and monocytes. These results indicate that *P. stomatis* could be playing an important role in the recruitment of inflammatory cells to the oral cavity (Vashishta et al., 2019).

Unlike many periodontal pathogens, *F. alocis* does not block phagocytosis by human neutrophils (Edmisson et al., 2018). In fact, most neutrophils challenged with *F. alocis* rapidly internalized the bacteria independent of viability or opsonization with complement. This is likely because viable *F. alocis* can disrupt the neutrophil antimicrobial response. After 4 hr of challenge, >50% of *F. alocis* remain viable inside neutrophils, and electron microscopy analysis of *F. alocis*-infected neutrophils showed that electron dense bacteria could still be found inside neutrophil phagosomes after 20 hr of co-culture, suggesting a defect in the degradation and clearance of engulfed bacteria. Despite the toxic capacity of neutrophils, *F. alocis* remains viable intracellularly by disrupting the maturation of neutrophil phagosomes. First, challenge with viable *F. alocis* induces minimal ROS response (Edmisson et al., 2018). Viable *F. alocis* did not suppress the respiratory burst induced by a secondary stimulus like heat-killed *Staphylococcus aureus*; suggesting that inhibition of ROS production is not a global effect, but rather a local phagosomal mechanism geared toward promoting survival. Intracellular killing is also mediated through the fusion of pre-formed granules with the bacteria-containing phagosome; however, *F. alocis* phagosomes had decreased co-localization with key antimicrobial proteins lactoferrin and elastase (Edmisson et al., 2018). The phenotype of minimal ROS and decreased co-localization was not observed when neutrophils were challenged with heat-killed *F. alocis*, indicating that *F.*

*alocis* actively enhances its survival by interfering with phagosome maturation.

When neutrophils are cultured with *P. stomatis* in suspension, the bacteria avoids phagocytosis, likely as a result of its flagellar motility (Jimenez Flores et al., 2017). However, the low percentage of engulfed *P. stomatis* resulted in robust intracellular ROS at the *P. stomatis* phagosome, and more than 50% of the phagosomes co-localized with lactoferrin and elastase, indicating that granule fusion with the phagosome was not impaired. Thus, due to the normal development of the phagosome, the ingested *P. stomatis* was rapidly eliminated by neutrophils. Notably, despite the considerable ROS production at the phagosome, only the antimicrobial granule proteins were responsible for the antimicrobial effect against *P. stomatis* since treating neutrophils with the NADPH oxidase inhibitor, diphenyl-eneiodonium (DPI), did not dampen their killing capacity against *P. stomatis* (Jimenez Flores et al., 2017). Thus, unlike *F. alocis*, *P. stomatis* does not directly interfere with neutrophils' antibacterial functions.

As inflammophilic bacteria, both *F. alocis* and *P. stomatis* still drive pro-inflammatory processes to secure a source of nutrients for replication. Recognition of *F. alocis* by neutrophils through ligation of TLR2 triggers the exocytosis of three of the four neutrophil granule subtypes through activation of both p38 MAPK and ERK1/2 (Armstrong et al., 2016). *P. stomatis*, on the other hand, induces robust exocytosis of all four granule types and strongly activates both p38 and ERK1/2 MAP kinases (unpublished data, Uriarte's lab). The release of toxic granules has implications for tissue degradation, which could be directly observed through gelatin zymography of supernatants of *F. alocis*- and *P. stomatis*-stimulated neutrophils (Edmisson et al., 2018; Jimenez Flores et al., 2017). Adding to the pro-inflammatory phenotype is the fact that when *F. alocis* and *P. stomatis* are recognized by TLR2/6 heterodimers, they promote the release of neutrophil-derived cytokines and chemokines, although *P. stomatis* induces a substantially larger amount of cytokines and chemokines compared to both *F. alocis* and *P. gingivalis* (Vashishta et al., 2019). Another notable difference between these two Gram-positive periodontal pathogens is that *P. stomatis* elicits significant NET release, while *F. alocis* does not induce NET formation at any bacterial dose or time tested, and pre-treatment of neutrophils with *F. alocis* significantly inhibits PMA-induced NETs (Armstrong et al., 2018). Although it is unknown what effect *P. stomatis* has on neutrophil apoptosis, there is some data that indicate that *F. alocis* prolongs neutrophil lifespan (Miralda et al., 2020). Not only were apoptosis pathways a top hit in the RNAseq screen of *F. alocis* challenged neutrophils, but Annexin V/7-AAD staining showed that more *F. alocis*-challenged neutrophils remain viable at 24 hr compared to media-cultured neutrophils.

Recently, we published a human neutrophil transcriptome study which reveals that several signal transduction pathways are significantly downregulated by *F. alocis* (Miralda et al., 2020). One of the major findings of the RNA-seq screen is that *F. alocis* affects the neutrophils' expression of components in both the TNF $\alpha$  and MAP kinase signaling pathways (Miralda et al., 2020). Functionally, cells pre-treated with *F. alocis* had decreased p38 MAPK activation by

secondary stimuli TNF $\alpha$ , but not by fMLF. The decreased p38 activation in *F. alocis*-pretreated cells resulted in a transient decline in TNF $\alpha$ -stimulated production of IL-8. However, other TNF- $\alpha$ -mediated effector functions, such as priming of the respiratory burst or pro-survival responses were not affected by *F. alocis* challenge. This phenotype was only observed when neutrophils were pretreated with viable *F. alocis*, which further demonstrates this is one of the mechanisms actively induced by the oral pathogen to control neutrophil functional responses.

## 7 | CONCLUSION AND PERSPECTIVES

Neutrophils mount a robust fight against the dysbiotic microbial targets they encounter on the periodontitis battlefield. Despite using unique virulence factors, both established and emerging oral pathogens share the same end goal: to manipulate neutrophils' antimicrobial mechanisms to evade killing while promoting inflammation (Tables 1 and 2). With the exception of *P. gingivalis*, neutrophils' encounter with established periodontal pathogens results in strong production of reactive oxygen species, which could contribute to bacterial killing as well as exacerbate tissue damage. As a common virulence strategy, established periodontal pathogens also stimulate neutrophil granule exocytosis. The release of antimicrobial components, such as matrix metalloproteinases and serine proteases by neutrophils results in collateral tissue damage, a scenario that benefits nutrient acquisition by periodontal pathogens. Despite the many years of work on established periodontal pathogens and neutrophils, the effects of these microbes on several neutrophil functions remain unknown and hold great therapeutic promise.

The complex interplay that takes place in the subgingival pocket between neutrophils and the dysbiotic microbial community provokes a dysregulated inflammatory environment that contributes to disease progression. Characterization of the interaction between neutrophils and emerging oral pathogens is needed to shed some light into the contribution of these organisms to disease severity. Both *F. alocis* and *P. stomatis*, are found in high numbers in periodontitis diseased sites, which suggests that these organisms developed survival strategies to withstand inflammation. Like the response elicited by established periodontal pathogens, interaction of *F. alocis* and *P. stomatis* with neutrophils induces release of neutrophil granule proteins as well as cytokines and chemokines, which fuel into the dysregulated inflammation. However, while *F. alocis* is easily internalized by neutrophils, the organism takes control over the host cell and survives intracellularly by preventing phagosome maturation. In contrast, *P. stomatis* resists phagocytosis but hyper-activates neutrophils. The findings from these studies strengthen the classification of *F. alocis* and *P. stomatis* as periodontal pathogens, but the remaining challenge is to define the precise molecular mechanism that *F. alocis* uses to evade the neutrophil machinery, and how *P. stomatis* hyper-activates the immune cell. Although periodontitis is an infectious disease caused by a community of bacterial species, defining how each pathogenic organism modulates neutrophil

functional responses to survive in the inflamed environment has significant value. The information provided by this type of studies helps to understand the potential contribution that each bacterial member of the community "brings to the table" to sustain the dysbiotic microenvironment. Furthermore, there is great need to define the pathogenic potential of both *F. alocis* and *P. stomatis* by testing the organisms' ability to colonize and induce bone loss using the established murine models of periodontitis. Understanding these interactions will lay the foundation for development of novel therapeutic approaches to combat periodontitis.

## ACKNOWLEDGEMENTS

The authors' research is supported by the NIH-National Institute of Dental and Craniofacial Research (NIDCR) DE024509 (S.M.U). The authors declare no conflict of interest.

## AUTHOR CONTRIBUTION

IM and SMU wrote the manuscript. IM made the tables and figures.

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**How to cite this article:** Miralda I, Uriarte SM. Periodontal Pathogens' strategies disarm neutrophils to promote dysregulated inflammation. *Mol Oral Microbiol*. 2021;36:103–120. <https://doi.org/10.1111/omi.12321>