DOI: 10.1111/imr.13152

INVITED REVIEW

Immunological Reviews WILEY

Neutrophils in the periodontium: Interactions with pathogens and roles in tissue homeostasis and inflammation

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Funding information
National Institute of Dental and Craniofacial Research, Grant/Award Number: DE014615, DE024509, DE026152, DE028561, DE029436 and DE031206

Summary

Neutrophils are of key importance in periodontal health and disease. In their absence or when they are functionally defective, as occurs in certain congenital disorders, affected individuals develop severe forms of periodontitis in early age. These observations imply that the presence of immune-competent neutrophils is essential to homeostasis. However, the presence of supernumerary or hyper-responsive neutrophils, either because of systemic priming or innate immune training, leads to imbalanced host–microbe interactions in the periodontium that culminate in dysbiosis and inflammatory tissue breakdown. These disease-provoking imbalanced interactions are further exacerbated by periodontal pathogens capable of subverting neutrophil responses to their microbial community's benefit and the host's detriment. This review attempts a synthesis of these findings for an integrated view of the neutrophils' ambivalent role in periodontal disease and, moreover, discusses how some of these concepts underpin the development of novel therapeutic approaches to treat periodontal disease.

KEYWORDS
dysbiosis, immune evasion, inflammation, neutrophils, periodontitis

1 | INTRODUCTION

Periodontitis is best described as a dysbiotic disease that, in susceptible individuals, affects the integrity of the tissues that surround and support the teeth, collectively known as periodontium.[1] The state of periodontal dysbiosis is associated with breaking the "friendly" or homeostatic dialog established between the indigenous ecological microbial environment and the host immune system. As a result, the disease-associated microbial communities develop immune subversion strategies to counteract the host immune response, provoking a state of dysregulated, non-resolving inflammation with collateral tissue damage and periodontal disease progression.

The mutualistic relationship between the host and the indigenous microbial community maintains the immunological tone in homeostatic conditions. Williams et al. performed a single-cell transcriptome atlas of the tooth-associated tissue or gingiva to identify the predominant cell populations in health versus disease.[2] The oral mucosa shares the same four cellular groups, epithelial-endothelial-fibroblast, and immune cells, with other mucosal tissues, however, with differential representation. The epithelial and stromal analysis

This article introduces a series of reviews covering Neutrophils and Friends in Volume 314 of *Immunological Reviews*

of buccal and gingival tissue biopsies in periodontitis patients identified an inflammatory signature wired toward neutrophil recruitment and inflammation.[2] Neutrophils represent the most abundant innate immune cell at oral mucosal tissues and barrier sites such as the gingiva. Defining neutrophil interactions with the microbial environment in health and disease has received particular attention. Neutrophils are armed with lethal anti-microbial responses and capabilities to release inflammatory mediators; interacting with the microbial adversaries tunes the magnitude of neutrophil responses. Under homeostatic conditions, neutrophil responses toward the microbial communities result in self-resolved inflammation. However, these professional phagocytes mount a heightened attack upon encountering a dysbiotic microbial challenge, resulting in a dysregulated, non-resolving inflammatory response driving disease progression.[3]

As alluded to above, the transition of symbiotic, commensal communities to a dysbiotic entity undermines tissue homeostasis resulting in destructive tissue inflammation, involving the participation and crosstalk of cells and molecular systems of both innate and adaptive immunity.[4] These include the complement system, neutrophils, and IL-17-expressing $CD4^+$ T cells (Th17), and the resulting inflammation leads to pathologic activation of osteoclasts and resorption of the tooth-supporting alveolar bone.[5–8] In addition, periodontal inflammation fortifies microbial dysbiosis by creating a nutritionally conducive environment due to the accumulation of inflammatory tissue breakdown products that the bacteria could exploit to derive essential nutrients (e.g., amino acids and iron).[4] The generation of this feed-forward cycle connects inflammation and dysbiosis and leads to the chronification of periodontitis.

Therefore, the chicken or the egg analogy to describe the primary culprit of disease progression, either the microbial community or the immune response elicited by the host, does not apply to periodontitis. Instead, the actual reciprocal interaction between the dysbiotic subgingival biofilm and the dysregulated immune response, which reinforce each other in a feed-forward loop, drives the disease pathology.[1] Based on this premise, this review focuses on discussing how the studies published by our laboratories and others paved the way to describe the interplay between neutrophils and established—as well as newly dominant—periodontal pathogens in the co-development of periodontitis pathology. Furthermore, we discuss how some of these findings underpin the design of novel therapeutic approaches to treat periodontitis and draw attention to potential areas of future research in this field.

2 | TO BE OR NOT TO BE: NEUTROPHILS' PARTICIPATION IN PERIODONTAL TISSUE DESTRUCTION

In periodontitis, the displacement of the composition and abundance of the microbial ecosystem alters the terms of the initial dialog established between the host and the microbial symbionts. The breakdown of these terms results in development of a more powerful attack strategy deployed by the immune system with unwanted consequences to the susceptible host. Within the oral mucosa, neutrophils are a vital component of the innate host response. These cells engage in a dialog with the symbiotic microbial community members to sustain a mutualistic state within the oral mucosal barrier. However, a transformation occurs under dysbiotic conditions, and the microbial partners become adversaries changing the tone of the initial host–microbe relationship. Neutrophils have a vast and diverse arsenal of anti-microbial resources to deal with microbial organisms, which mount sophisticated evasion strategies to survive the encounter.[9,10] Under this new environment supernumerary, hyper-activated neutrophils arrive at the mucosal tissue, where they unleash the toxic cargo contributing to collateral tissue damage. The primordial role neutrophils play in preserving oral health is highlighted by human and mouse studies describing how neutropenia, neutrophilia, and defects in neutrophil effector functions are associated with periodontitis.[11,12]

The primary anti-microbial functions of neutrophils include phagocytosis, respiratory burst response, degranulation, and extrusion of chromatin DNA through neutrophil extracellular trap (NET) formation. Degranulation represents a crucial anti-microbial and immunomodulatory neutrophil function with significant implications for periodontitis progression.[3] The presence or absence of myeloperoxidase within the granule matrix classifies them into two categories: peroxidase positive or negative.[13] Among several matrix components, azurophilic or peroxidase-positive granules contain potent anti-microbial compounds such as α-defensins and several serine proteases such as neutrophil elastase (NE), proteinase 3 (PR3), and cathepsin G. Specific and gelatinase granules, also known as peroxidase negative, contain anti-microbial compounds such as lactoferrin, and lysozyme, as well metalloproteinases (MMPs), such as collagenase and gelatinase. Lastly, smaller intracellular vesicles distributed throughout the neutrophil cytosol with a high propensity for secretion are named secretory vesicles. These vesicle matrices comprise plasma proteins such as albumin.[13,14] The degree of stimulation neutrophils encounters drives the hierarchical process of granule exocytosis. This graded exocytic process aids neutrophils in performing their effector functions during immune surveillance. The neutrophil degranulation sequence occurs opposite to granule formation during maturation in the bone marrow.

Studies performed during the last two decades established that neutrophil participation during inflammation is not restricted to their professional anti-microbial capabilities.[15] Neutrophils possess effector functions ranging from anti-microbial to immunomodulatory. These features position this professional phagocyte with unique qualifications to serve as a liaison between cells from the innate and adaptive arms of the immune response. The response neutrophils mount during a given insult, from infection to tissue injury, needs to be finely tuned and regulated to minimize collateral damage to the host tissue. Priming is a unique reversible state that helps circulating neutrophils fine-tune their activation level.[16] Primed neutrophils are in a high alert state, which allows them to display enhanced responses, such as phagocytosis, respiratory burst, degranulation, NET

formation, and release of inflammatory mediators, upon subsequent stimulation.[17] Several endogenous inflammatory mediators like cytokines, chemokines, growth factors, and pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS) can prime neutrophils. However, when neutrophil priming occurs in the context of chronic systemic inflammation, it might have detrimental consequences.[18] It could be envisioned that priming of blood neutrophils could occur by exposure to low systemic levels of cytokines/chemokines and/or by encountering microbial PAMPs derived from the dysbiotic oral subgingival biofilm that reaches the bloodstream. When those primed circulating neutrophils arrive at the gingival pocket, they encounter secondary stimulation (local dysbiotic microbiota), which induces an exacerbated overall response. Therefore, acquiring a priming state is a double-edged sword for neutrophils. On the one hand, defects in priming are associated with recurrent infections.[19] On the other hand, elevated levels of circulating primed cells are associated with chronic inflammatory diseases such as sepsis, periodontitis, and rheumatoid arthritis.[20–22]

Circulating neutrophils isolated from periodontitis patients show enhanced reactive oxygen species (ROS) production upon exposure to either a soluble stimulus, like phorbol myristate acetate (PMA) or bacterial stimulation, like *Porphyromonas gingivalis*, when compared to the response elicited by cells from a healthy control.[21] These and similar findings from related studies[23–28] support the hypothesis that circulating neutrophils from periodontitis patients have a primed or trained phenotype. Priming shares some similarities with trained innate immunity in that both can lead to enhanced secondary responses; however, there are also important differences. In terms of active transcription, the altered immune state of a primed cell does not return to its basal level before the secondary stimulus, which could thus be additive (or synergistic) with the original stimulus. In contrast, in a trained cell, the altered immune state phenotypically returns to its basal state before the secondary stimulus is applied.[29] What persists in trained innate immune cells after the removal of the initial stimulus are epigenetic changes that underlie sustained innate immune memory associated with the induction of trained immunity.[29] Briefly stated, priming refers to the short-term enhancement of the immune response, whereas training refers to the long-term imprinting of enhanced immune responsiveness. Besides epigenetic rewiring, the induction of trained immunity involves cellular metabolic adaptations.[30,31]

Besides ROS production, many other neutrophil functional responses are enhanced upon secondary stimulation in primed or trained cells.[17] For example, the enhanced degranulation in TNF-primed neutrophils contributes to the increase of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase membrane components resulting in high ROS production.[32] In addition, oral neutrophils from periodontitis patients show enhanced plasma membrane expression of several clusters of differentiation (CD) markers present in neutrophil granules compared to cells from healthy individuals. This increase in granule markers, such as CD11b, CD66b, and CD63, indicates that neutrophils undergo an exocytic process. Both circulating and oral neutrophils from periodontitis patients share a primed or trained phenotype.[33] In this regard, it should be noted that enhanced responses upon secondary stimulation of peripheral blood neutrophils cannot be safely attributed to priming or innate immune training simply based on functional assays (e.g., induction of cytokine or ROS release). Intriguingly, periodontitis patients often retain the neutrophil-primed or trained phenotype despite appropriate treatment to mitigate the disease.[25,34,35] In a recent study, Fine et al.[36] showed that ligature-induced periodontitis (LIP) in mice provoked enhanced neutrophil counts in both the bone marrow and the oral cavity. In addition, LIP followed by peritonitis resulted in enhanced neutrophil counts and activation compared to mice with only peritonitis or LIP. These results indicate that gingival inflammation primed neutrophils in these animals, enhancing their effector functions upon a second inflammatory challenge.

Similarly, neutrophils from individuals subjected to experimental gingivitis showed a primed phenotype upon secondary stimulation with bacterial-derived *N*-formylated peptide, fMLF, which was reversed once those individuals resumed oral hygiene.[36] In contrast, periodontal treatment is often ineffective in reversing the hyperresponsiveness of circulating primed or trained neutrophils.[25,27,35] The same can be observed for peripheral blood leukocytes in general, which often maintain their hyper-responsiveness (as assessed by ex vivo restimulation) even if the treatment involved full-mouth tooth extraction.[37,38] This begs the question of whether the neutrophil priming state is readily reversible during gingivitis but becomes potentially irreversible during inflammatory periodontitis.

A recent study by the Hajishengallis and Chavakis groups[39] showed that 21 days of LIP-induced periodontitis in mice caused a sustained myeloid-differentiation bias in hematopoietic stem and progenitor cells (HSPC) that was predominantly evident at the epigenetic level (essentially absent at the phenotypic or transcriptomic levels), hence revealing induction of trained immunity in the bone marrow. The trained HSPC gave rise to increased production of monocytes and neutrophils with enhanced immune responsiveness to secondary stimulation. Using a competitive bone marrow transplantation approach with sorted long term-hematopoietic stem cells (LT-HSC) from LIP-trained or -untrained mice, the same study showed that LIP-trained LT-HSC not only transmitted inflammatory epigenetic memory (myeloid bias and generation of hyper-responsive myeloid cells) to transplanted mice but also lead to exacerbated joint inflammation (characterized by increased neutrophil infiltration) and arthritis in recipient mice, as compared to mice that received untrained LT-HSC.[39] Using mice with HSPC-specific deletion of the IL-1 receptor and controls, the study showed that this LIP-induced maladaptive trained immunity critically depends on IL-1 signaling in HSPC.[39] The acquisition of maladaptive innate immune training in bone marrow progenitors proved bidirectional in that maladaptive trained myelopoiesis induced by collagen antibody-induced arthritis rendered mice more susceptible to periodontitis.[39] Therefore, the bone marrow can sense periodontitis-associated systemic inflammation, which induces epigenetic rewiring of HSPC and leads to enhanced production of myeloid cells, including neutrophils, with enhanced

inflammatory responsiveness; these maladaptively trained cells infiltrate not only oral but also extra-oral tissues (such as inflamed joints) and thereby exacerbate comorbid inflammatory conditions.[39] The notion that innate immune training of myeloid cells may occur in the setting of human periodontitis is consistent with clinical observations that peripheral blood neutrophils and monocytes from periodontitis patients exhibit hyper-responsive phenotypes upon ex vivo stimulation that are often not reversed by successful treatment (reviewed in Ref. [18]). Intriguingly, in this regard, even 32 months after full-mouth tooth extraction, peripheral blood leukocytes from a former periodontitis patient mostly retained their hyper-responsive phenotype in ex vivo LPS-stimulated whole blood cell cultures. Indeed, in this longitudinal study (0, 3, 9, 20, and 32 months), only two (IL-8 and MCP-1) of the twelve investigated cytokines displayed reduced levels over time, whereas the rest (IL-1β, IL-6, TNF, IL-12p70, MIP-1α/CCL3, and MIP-1β/CCL4 among others) remained at high levels during the entire period.[37]

In general, trained immunity can be central, that is, induced in hematopoietic progenitors in the bone marrow, or peripheral, that is, induced in mature innate immune cells in the blood or tissue sites. Acquiring trained immunity enables neutrophils to respond faster and stronger upon subsequent stimulation, which has both beneficial (e.g., increased resistance to infections or tumors) and destructive effects (aggravation of chronic inflammatory diseases; Figure 1). Cellular metabolism, which is linked with epigenetic changes,[31] is another critical driver of trained immunity. Understanding neutrophil immune activation status, from priming to trained immunity, in health versus disease would significantly move the periodontitis field forward.

3 | IN THE CONTEXT OF DYSBIOSIS: WHO SETS THE RULES, NEUTROPHILS OR ORAL PATHOGENS?

Established and newly dominant members of the oral dysbiotic community develop different survival strategies to endure as well as capitalize on the host's immune and inflammatory response (Figure 2). For example, some community members evade anti-microbial responses, while others utilize neutrophil-derived molecules to survive.[3] In active periodontitis sites, the collateral tissue damage due to the non-resolving inflammation offers a nourishing environment for the inflammophilic oral pathogens to grow. The keystone oral pathogen *P. gingivalis* best characterizes this statement.[40,41] *P. gingivalis* is a Gram-negative, asaccharolytic, strict anaerobe oral pathogen, that inhabits mainly the subgingival crevice.[41] For *P. gingivalis*, colonization of the oral mucosa combines the best of both worlds: evading neutrophil and macrophage anti-microbial responses, while promoting their inflammatory responses allows the organism to sustain a nutritionally favorable inflammatory environment (e.g., inflammatory degradation of collagen provides a rich source of amino acids) to prevail in the gingiva.

Neutrophils, as critical host defenders, can recognize microbial organisms through pattern recognition receptors (PRRs). An

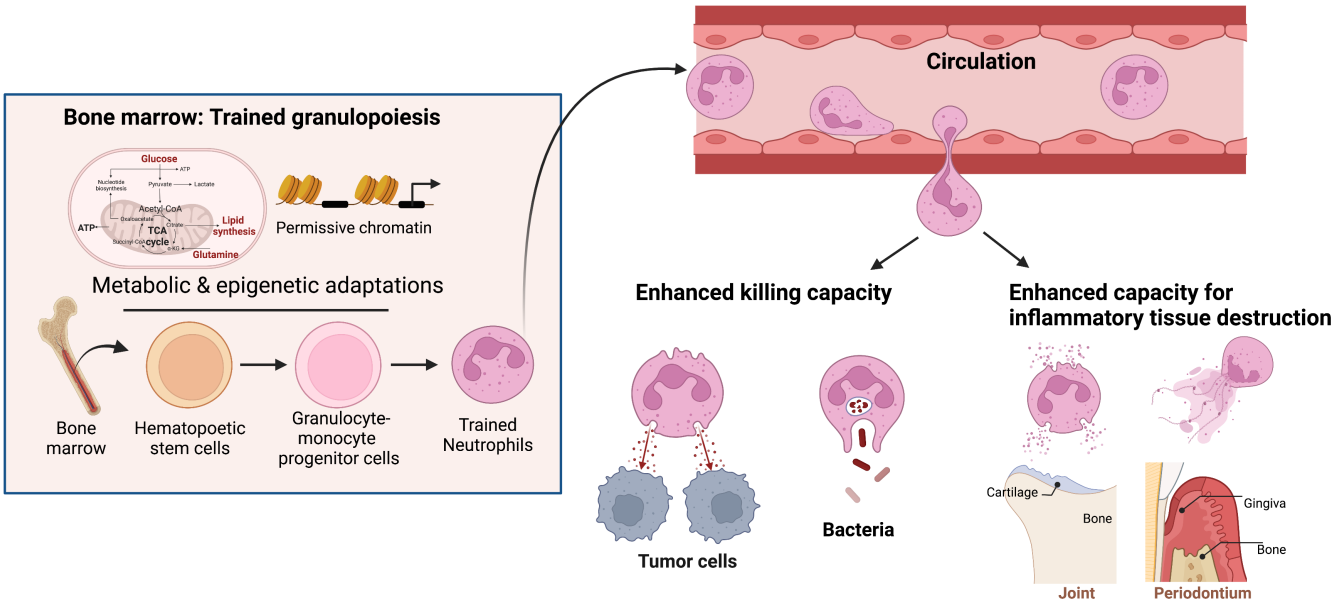

FIGURE 1 Innate immune training of neutrophils and potential outcomes. Trained innate immunity can be initiated at the level of the bone marrow hematopoietic stem and progenitor cells and represents a form of epigenetic memory associated with metabolic adaptations induced by certain inflammatory or microbial stimuli.[180,179] Trained progenitor cells give rise to increased numbers of neutrophils with enhanced immune and inflammatory preparedness for future challenges ("trained granulopoiesis").[181,179] Trained immunity-associated epigenetic adaptations in granulocyte-monocyte progenitors are passed on to progeny neutrophils, which have an increased capacity to kill tumor cells[181] or microbial pathogens,[182] thereby conferring increased resistance against cancer and infections. On the flip side, the enhanced inflammatory responsiveness of trained neutrophils may contribute to increased inflammatory tissue pathology in inflammatory disorders, such as periodontitis and arthritis[132,39]

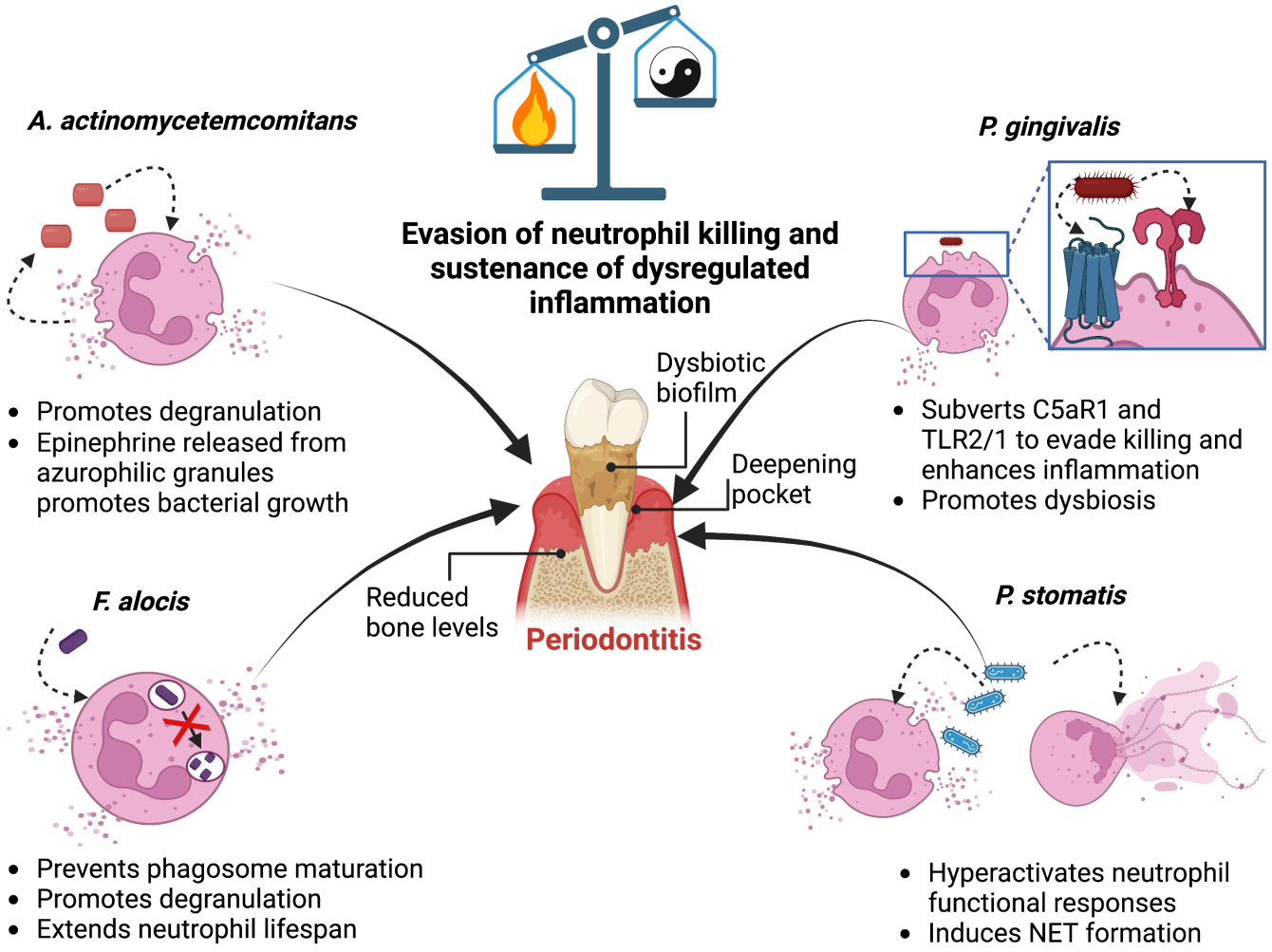

FIGURE 2 Evasion of neutrophil killing by established and newly dominant periodontal pathogens contributes to dysregulated inflammation. All four depicted periodontal pathogens, *A. actinomycetemcomitans*, *P. gingivalis*, *F. alocis*, and *P. stomatis*, promote neutrophil degranulation, contributing to tissue damage and fostering a nutritional environment for these assacharolytic microbial pathogens and their communities. Top left panel: *A. actinomycetemcomitans* interaction with neutrophils leading to the release of epinephrine, which promotes bacterial growth.[62] Top right panel: *P. gingivalis* suberts C5aR1 and TLR2/1 to evade neutrophil killing while promoting inflammation and dysbiosis.[49,52] Bottom left panel: *F. alocis* strategy to evade neutrophil killing by preventing phagosome maturation, while extending the neutrophil lifespan to promote a sustained neutrophil activation status.[3,79,83,86] Bottom right panel: *P. stomatis* strategy to induce an excessive neutrophil activation phenotype, release of inflammatory mediators, and NET formation to sustain gingival inflammation[3,87,88]

important family of these microbial sensing receptors is composed of the Toll-like receptors (TLRs), formed by several family members with the ability to sense distinct microbial structures. Except for the intracellular TLR3 and TLR7, neutrophils express other TLR family members.[42] The ability of TLRs to form homotypic or heterotypic receptor complexes jeopardizes microbial anonymity. However, being recognized by the host does not preclude the survival of some microorganisms. Another important surveillance arm of the innate response is the complement system. This intricate immune network system comprises nearly 50 protein members.[43] The classical, alternative, and lectin pathways are three separate mechanisms that induce complement activation. A central complement component, C3, is the converging point of those three complement initiation mechanisms.[44,45] Both complement and TLR systems are activated during infection and inflammation and work synchronously to optimize the host response. The keystone periodontal pathogen, *P. gingivalis*, takes advantage of the established dialog between complement and TLR to thrive in the gingival tissue.[44]

P. gingivalis major fimbriae (FimA), possibly the first factor of this pathogen to engage in close contact with the innate immune cells, binds to CD14, a vital co-receptor for TLR2 activation. As a result of this interaction, activation of PI3K and cytohesin-1 induces an "inside-out signaling" that promotes a high-affinity conformational change in the β2 integrin [Mac-1/Complement Receptor 3 (CR3)] in both neutrophils and monocytes.[46] *P. gingivalis* binds to the high-affinity CR3 configuration to ensure a "safe pass" entry into macrophages.[41,44]

Another subversive TLR2-complement crosstalk strategy deployed by *P. gingivalis* involves complement receptor 5 (C5aR1) and its ligand C5a. *P. gingivalis* expresses a family of cysteine proteases

called gingipains, which confer this organism with a decisive virulence factor to evade the host anti-microbial response.[47,48] *P. gingivalis* gingipains can cleave complement component 5 (C5), generate biologically active C5a, a potent neutrophil chemotactic molecule, and degrade C5b to prevent terminal complement activation. In this manner, *P. gingivalis* can co-activate C5aR1 (via the gingipain-generated C5a) and TLR2/1, the TLR complex that detects this oral pathogen. The co-activation of TLR2/1 and C5aR1 by *P. gingivalis* triggers the proteasomal degradation of a critical TLR adapter protein, MyD88, thereby blocking neutrophils' anti-microbial response.[49] In the absence of MyD88, another TLR2 adapter protein, Mal, takes the lead with concomitant activation of the PI3 kinase pathway. The collaborative interaction between Mal and the PI3K signaling pathway triggers the induction of proinflammatory mediators by neutrophils. Furthermore, activation of the PI3K signaling pathway by *P. gingivalis* prevents actin polymerization and limits phagocytosis of the organism by neutrophils.[49] In other words, by disengaging the TLR2-MyD88 axis and promoting the TLR2-Mal-PI3K axis, *P. gingivalis* evades killing by neutrophils.[1,44] In vivo studies show that all the pieces of this intricate puzzle are required for *P. gingivalis* to evade neutrophil killing and contribute to supporting the survival of other microbial community members.[49] For instance, blocking C5aR1 or genetic ablation of this receptor diminishes *P. gingivalis* colonization of the periodontal tissue and renders mice resistant to *P. gingivalis*-induced bone loss.[50] Similarly, mice deficient in TLR2, but not TLR4, are resistant to *P. gingivalis*-induced alveolar bone loss.[51] On the other hand, MyD88-deficient mice are susceptible to *P. gingivalis*-induced alveolar bone loss, similar to wildtype controls.[52] These studies collectively indicate that *P. gingivalis* exploits the crosstalk between complement receptors and TLR2 to evade the host anti-microbial response while promoting destructive inflammation. Furthermore, *P. gingivalis* encounters other host cells besides neutrophils within the gingiva. The oral pathogen uses similar or different mechanisms to hijack or exploit the host. For the microbial aficionado, we encourage reading several recent reviews which provide additional descriptions of the organisms' modus operandi within the subgingival biofilm environment.[1,41]

Additional studies by the Hajishengallis and Lambris groups further suggest that the activation of complement promotes rather than restrains the growth of the periodontal microbiota,[49,53] many of which have evolved to counteract the anti-microbial effects of the complement system.[54,55] Accordingly, mice genetically lacking C3 and subjected to experimental periodontitis display significantly diminished periodontal bacterial load compared to wildtype littermate controls, as revealed by quantitative real-time PCR of the 16S rRNA gene.[53] As inflammation is a major ecological factor driving the selective expansion of periodontitis-associated bacteria,[4,56] the diminished periodontal inflammation seen in C3-deficient mice is likely to explain the reduced microbial load.[53]

Another established periodontal pathogen, *Aggregatibacter actinomycetemcomitans*, utilizes different strategies to evade the host's innate immune response to survive in the oral mucosa.[3] *A. actinomycetemcomitans* is a Gram-negative opportunistic oral pathogen, facultative anaerobe, and non-motile coccobacillus of the *Pasteurellaceae* family.[57] In addition, *A. actinomycetemcomitans* possess several virulence factors that allow this organism to survive in the dysbiotic microbial environment.[58] The host environment's metal acquisition and other nutrients are essential for bacterial growth. *A. actinomycetemcomitans* expresses a two-component system named QseBC, which allows the organism to acquire iron and catecholamines, such as epinephrine, from the environment.[59–61] A recent novel finding by the Uriarte and Demuth groups[62] identifies neutrophils as a source of catecholamines. This study shows that neutrophils store epinephrine within azurophilic granules. Furthermore, *A. actinomycetemcomitans* induces degranulation of all neutrophil granule subtypes. The bacterial-induced azurophilic granule exocytosis results in the release of active epinephrine (Figure 2). Supplementation of chemically defined media with iron and epinephrine from human neutrophils promoted *A. actinomycetemcomitans* growth and the induction of the qseBC operon.[62]

A. actinomycetemcomitans embarks on a different strategy from *P. gingivalis* when encountering neutrophils since the former organism appears to use predominantly frontal rather than stealth attack. As alluded to above, triggering neutrophil degranulation allows *A. actinomycetemcomitans* to obtain epinephrine, a critical nutrient to facilitate bacterial growth in anaerobic conditions. The release of neutrophil-derived epinephrine by *A. actinomycetemcomitans* might benefit other bacterial members in the subgingival pocket. For example, epinephrine and norepinephrine also increase the growth of *Fusobacterium nucleatum*,[63] as well as *Lactobacillus* spp., which express transporter systems for uptake of catecholamines[64] and other catecholamine-responsive species such as *Prevotella* spp. and *Leptotrichia*.[65–67] Furthermore, similar to *A. actinomycetemcomitans*, some of these bacteria cannot produce siderophores.[68,69] Based on these findings, the release of neutrophil-derived epinephrine by the encounter with *A. actinomycetemcomitans* could contribute to the growth of other microbial members at the subgingival pocket. In brief, *A. actinomycetemcomitans* obtains critical nutrients while sustaining collateral tissue damage by inducing neutrophil degranulation and contributing to the non-resolving inflammatory loop that drives periodontitis.

The gingiva's microbial composition is complex and comprises more than 700 microbial species. The development of high throughput technology allows to define and characterize with more precision the complex microbial ecological system that inhabits the gingival tissue in both health and disease. For example, the analysis of active periodontitis sites by 16S ribosomal RNA revealed the presence of not only the well-characterized established periodontal pathogens, like *P. gingivalis* and *A. actinomycetemcomitans* but also newly dominant members of the community, such as *Filifactor alocis* and *Peptoanaerobacter stomatis*.[70–75] Both *F. alocis* and *P. stomatis* are Gram-positive, anaerobic rods, highly abundant in subgingival communities associated with periodontitis. The challenge is to characterize and define what role these newly dominant members play in periodontitis pathology.

F. alocis was identified from the gingival sulcus in gingivitis and periodontitis patients and was initially named *Fusobacterium alocis*[76] before being reclassified under the genus *Filifactor*.[77] In the oral cavity, *F. alocis* forms biofilms close to the apical and middle thirds of the gingival pocket, which is close to soft tissue.[78] The massive neutrophil infiltration highly surveils this tissue location. Hence, what are *F. alocis* evasion strategies to overcome neutrophils' potent anti-microbial artillery? In contrast to other periodontal pathogens, resisting phagocytosis is not how *F. alocis* evades neutrophils' first anti-microbial response. Studies by the Uriarte and Lamont groups show that phagocytosis of heat-killed *F. alocis* occurs at a higher rate than the viable bacterium; however, after 1 h, nearly 50–60% of the viable organism is internalized independently of serum opsonization.[79] Phagosome maturation in neutrophils does not occur through the endosomal pathway like in macrophages. Instead, phagosome maturation occurs by rapidly recruiting specific and azurophilic granules to the phagosome. In addition, a robust respiratory burst response by activating the NADPH oxidase enzyme complex generates high levels of ROS, creating an inhabitable environment for the microbial invader.[9,80] In that regard, certain bacterial pathogens developed evasion strategies to modulate the respiratory burst response to minimize ROS production and prevent phagosome maturation.[81] For example, limiting the recruitment of specific and azurophilic granules to the bacteria-containing phagosome is how *F. alocis* prevents phagosome maturation.[79] Furthermore, *F. alocis*, but not a heat-killed bacterium, induces minimal respiratory burst response in human neutrophils.[79] Some pathogens like *Francisella tularensis* and *Neisseria gonorrhoeae* modulate ROS production by inhibiting the activity of the NADPH oxidase, rendering neutrophils unable to mount an appropriate response upon subsequent stimulation.[81] However, *F. alocis*'s modulation of ROS production seems to be targeted to its phagosome since it does not prevent neutrophils from mounting an appropriate response toward a secondary stimulation. Preventing phagosome maturation and inducing minimal ROS production allows *F. alocis* to remain viable inside human neutrophils for extended periods[79] (Figure 2).

Neutrophil granules and the degranulation process represent a powerful non-oxidative anti-microbial strategy to kill microbes. However, releasing potent anti-microbial compounds and matrix metalloproteases contained within neutrophil granules comes with a cost for the host.[82] Degranulation can kill extracellular microbes and cause collateral tissue damage. Periodontal pathogens like *P. gingivalis* and *A. actinomycetemcomitans* induce neutrophil degranulation.[3] Similarly, *F. alocis* induces exocytosis, secretory vesicles, gelatinase, and specific granules, albeit not azurophilic granules. The degranulation induced by *F. alocis* depends on TLR2 and MAPK activation.[83] Furthermore, degranulation enhanced *F. alocis* chemotactic activity toward interleukin (IL)-8, a potent neutrophil chemoattractant.[83] By promoting the release of neutrophil granule components, such as matrix metalloproteinases, *F. alocis* sustains collateral tissue damage and promotes the growth of inflammophilic bacteria.

The Uriarte and Lamont group took an unbiased systems biology approach to characterize how *F. alocis* might undermine neutrophil responses at the gene transcription level. This study revealed significant changes in the expression of genes involved in various effector functions. The RNA-based next-generation sequencing (RNAseq) screen revealed that the most significant changes in neutrophil transcriptome occurred early in the time course, with 624 genes differentially expressed at 1 h and a significant increase in the number of differentially expressed genes by 3 and 6 h postbacterial challenge.[84] This interaction affected several neutrophil biological processes, with the highest percent changes related to inflammation, signal transduction, immune response, and apoptosis. In addition, this study revealed that TNF-induced release of IL-8 is transiently impaired by previous exposure of neutrophils to live *F. alocis*. In contrast, cells exposed to the heat-killed bacterium or a TLR2/6 ligand showed a normal TNF response.

Furthermore, the same study showed that TNF-induced phosphorylation of p38 MAPK and the two downstream effectors, AKT and S6 ribosomal protein, was significantly impaired in *F. alocis*-infected neutrophils. Interestingly, however, *F. alocis* did not affect TNF-induced ERK1/2 activation.[84] Since activation of ERK1/2 by TNF is critical to delaying neutrophil apoptosis., these findings suggest that by keeping ERK1/2 activation intact in TNF-stimulated neutrophils, *F. alocis* selectively protected the phagocyte's survival pathway. The molecular regulation of neutrophil apoptosis is a sophisticated process, but generally, whether a neutrophil lives or dies is determined by the ratio of pro-survival or pro-apoptotic regulatory factors.[85] A recent Miralda et al.[86] study shows that *F. alocis* significantly extends neutrophil lifespan. This oral pathogen induces a significant increase in both the gene and protein expression of XIAP, a member of the inhibitor of the apoptosis family, which inhibits caspase activation. Neutrophils exposed to *F. alocis* had minimal activation of the executioner caspase −3 and two other critical caspases, 8 and 9, involved in the extrinsic and intrinsic pathways, respectively.

Furthermore, the delay of neutrophil apoptosis required contact with *F. alocis* via TLR2/6. Moreover, *F. alocis* extended not only the lifespan of neutrophils but also the ability of the phagocytic cell to perform other effector functions such as phagocytosis and ROS production upon subsequent stimulation.[86] Extending neutrophil lifespan in the gingival tissue will preclude clearance by macrophages and aid in maintaining a dysregulated, non-resolving inflammatory environment.

Another newly dominant member of the dysbiotic subgingival community, *P. stomatis* activates TLR2/6.[87] A study by the Uriarte and Lamont groups compared the inflammatory response by human neutrophils exposed to *P. gingivalis*, *F. alocis*, and *P. stomatis*. It showed that among the three oral pathogens, *P. stomatis* promoted the release of significantly larger amounts of neutrophil-derived cytokines and chemokines.[87] Furthermore, only the conditioned supernatant collected from *P. stomatis*-infected neutrophils displays chemotactic activity for both naive neutrophils and monocytes. This putative periodontal pathogen ignites neutrophils by inducing degranulation of all the granule subtypes, high intracellular ROS production, and priming neutrophil responses upon subsequent stimulation.[88] *P. stomatis*, strain CM2, has peritrichous flagella and

display a low phagocytic uptake by human neutrophils. The low percent of internalized bacteria suffer a delayed killing by neutrophils which is ROS-independent.[88]

In addition to degranulation and ROS production, neutrophils can release their chromatin DNA decorated with granule components through a process described as NETs.[89,90] NET formation, degranulation, and ROS generation formed the triad of neutrophil effector functions associated with undesired collateral tissue damage. In addition to triggering robust degranulation and ROS production, *P. stomatis* induces NET formation, which depends on the multiplicity of infection[91] (Figure 2). *P. stomatis* induces the triad of neutrophil effectors contributing to tissue damage and likely periodontitis pathology. In contrast, *F. alocis* does not induce NET formation but can inhibit PMA-induced NETs.[91] In summary, in active periodontitis sites, a tug of war occurs between the host and the dysbiotic microbial ecosystem. Established and newly dominant oral pathogens pursue a common goal: to sustain a dysbiotic environment by developing an array of strategies to subvert neutrophil responses to promote their adaptive fitness.

4 | NEUTROPHIL HOMEOSTASIS IN PERIODONTAL HEALTH AND DISEASE

Although excessive numbers and/or activity of neutrophils play a major role in periodontal disease pathogenesis, the presence of neutrophils is integral to periodontal tissue homeostasis. This concept becomes evident by observations that different congenital deficiencies in neutrophil numbers and/or function are associated with developing aggressive periodontitis early in life.[12] Papillon-Lefèvre syndrome (PLS) and leukocyte adhesion deficiency type 1 (LAD1) are genetic disorders causing periodontitis and bone loss early in life in affected individuals due to impaired neutrophil functional responses. These examples highlight neutrophils' crucial role in maintaining a balanced immunological tone at the mucosal barrier and are discussed below.

PLS represents a genetic defect in the cathepsin C gene, a key enzyme involved in the activation and function of neutrophil serine proteases stored within azurophilic granules. This genetic defect results in the generation of neutrophils with azurophilic granules lacking all the serine proteases.[92,93] The clinical manifestation of this genetic neutrophil defect is PLS individuals' susceptibility to developing severe periodontitis early in life.[12,94] PLS individuals face a paradox related to susceptibility to periodontitis but not other infectious diseases. The lack of PR3 activity, a serine protease typically stored in azurophilic granules, from PLS neutrophils jeopardizes the generation of a potent anti-microbial peptide LL-37 due to the inability to cleave the precursor hCAP18. The small peptide, LL-37, has anti-microbial activity toward two established periodontal pathogens, *A. actinomycetemcomitans*[95] and *P. gingivalis*.[96] Besides lacking serine proteases, ex-vivo stimulation of neutrophils from PLS individuals shows an enhanced response compared to cells isolated from healthy controls. Stimulation of PLS neutrophils with a potent neutrophil activator, PMA, or with a more physiological stimulus, heat-killed *A. actinomycetemcomitans*, show enhanced gelatinase granule exocytosis and release of proinflammatory mediators when compared to stimulated cells from healthy controls.[97] Further characterization of PLS neutrophil functional responses shows that upon stimulation, these phagocytes display enhanced respiratory bursts with high ROS production but defective chemotactic activity.[97] The differential responses triggered by PLS neutrophils, a hyperactive phenotype with defective chemotaxis, result in a toxic combination, extending the time of the cell in the tissue contributing to further tissue damage, thereby preventing the resolution of the inflammation.[97]

LAD1 is an exemplar of the significance of neutrophils in maintaining a healthy periodontium. LAD1 is an autosomal recessive primary immunodeficiency owing to mutations in the *ITGB2* gene, which encodes for the common CD18 subunit of the β2 integrins[98] that mediate neutrophil adhesion to the endothelium, a prerequisite step to extravasation.[99,100] Consequently, patients with LAD1 have a severe paucity of neutrophils in the periodontium (and other tissues) and, besides aggressive periodontitis, typically present with recurrent bacterial infections and pathological inflammation in mucosal surfaces and the skin.[101,102] Although the etiology of LAD1-associated periodontitis was traditionally thought to involve defective neutrophil surveillance of the periodontal infection, the Hajishengallis and Moutsopoulos groups have shown that the underlying cause involves a dysregulated host response, namely the overproduction of IL-23 and IL-17[101] (Figure 3). Furthermore, inhibition of either cytokine with locally administered neutralizing antibodies in LFA-1-deficient or CD18-deficient mice, which mimic the periodontal LAD1 phenotype, resulted in protection from inflammatory bone loss.[101,103] Consistent with these preclinical findings, systemic administration of ustekinumab (a monoclonal antibody that blocks the shared p40 subunit of IL-23 and IL-12) in a human LAD1 patient resulted in attenuated expression of IL-17 in the gingiva and resolved inflammatory periodontal lesions.[104]

The question as to why the absence or paucity of neutrophils causes a dysregulated IL-23/IL-17 response (or conversely, why the neutrophils are required for physiological IL-23/IL-17 responses in the gingival tissue) may not have been completely addressed yet. However, the unraveling of the underlying mechanism(s) has been facilitated by the "neutrostat" concept developed by Ley and colleagues.[105] The "neutrostat" is a homeostatic mechanism that coordinates the recruitment and tissue clearance (via efferocytosis) of neutrophils with their production in the bone marrow.[105] In this regard, the efferocytosis of apoptotic neutrophils by tissue macrophages does not solely serve as a clearance mechanism that prevents secondary necrosis but also reprograms the efferocytic macrophage to inhibit the expression of IL-23 (and other inflammatory cytokines) and upregulate the expression of pro-resolving mediators.[105–109] The efferocytosis-associated inhibition of IL-23 production leads to downstream inhibition of the expression of IL-17 by $CD4^+$ T cells (Th17), diminishing the IL-17-dependent production of G-CSF, thereby limiting a key factor for the generation of neutrophils in the

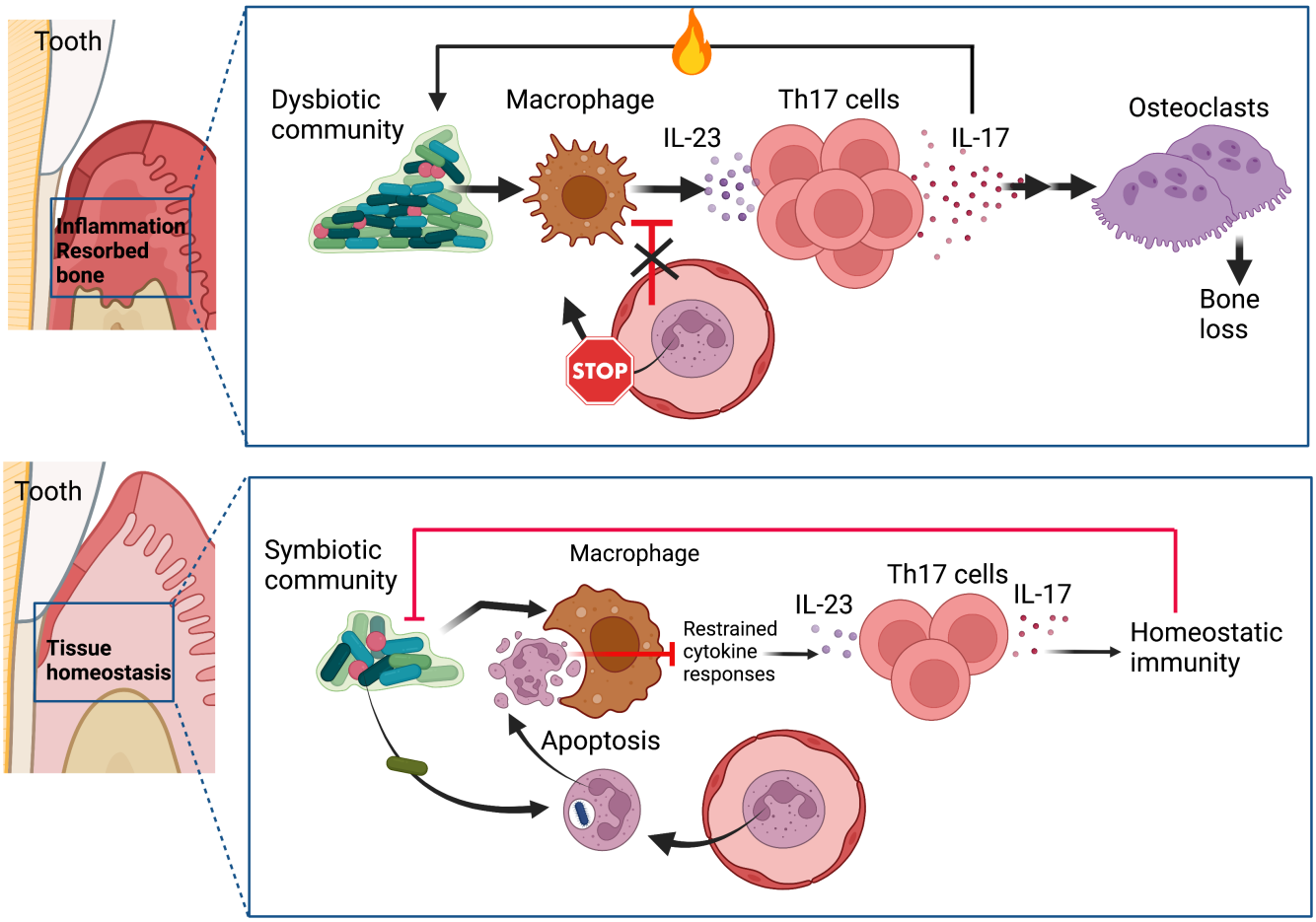

FIGURE 3 Neutrophils are required for homeostatic IL-17 responses, whereas their absence leads to IL-17-driven bone loss in LAD1 periodontitis. (Top panel) In LAD1, β2 integrin-deficient neutrophils fail to transmigrate to sites of infection or inflammation. The paucity of recruited neutrophils in the periodontium and the local microbiome lead to unrestrained production of IL-23 (by local macrophages) and hence of IL-17 (e.g., by Th17 cells). The elevated IL-17 drives inflammatory bone loss.[101] (Bottom panel) In normal (β2 integrin-proficient) individuals, the recruited neutrophils are eventually (after they undergo apoptosis) cleared by macrophages through efferocytosis. The efferocytosis process reprograms the macrophage toward a pro-resolving phenotype with reduced expression of IL-23 (and other proinflammatory cytokines), leading to attenuated production of IL-17,[103] which likely mediates homeostatic immunity and contributes to maintaining a symbiotic microbiome

bone marrow.[105] Given that in LAD1, neutrophils fail to transmigrate to the periodontium, the regulatory (inhibitory) signals that could restrain IL-23 and IL-17 production are missing (or severely decreased); consequently, the local expression of IL-23 and IL-17 (in response to the local microbial challenge) remains unrestrained and drives destructive tissue inflammation and bone loss, as seen in LAD1 patients and preclinical mouse models of LAD1[101,103,104] (Figure 3).

Intriguingly, NADPH oxidase activation was shown to regulate the efferocytosis of apoptotic neutrophils by macrophages and hence efferocytosis is defective in mice with inactivation of X-linked Cybb ($p91^{phox}$ subunit of the NADPH oxidase).[110] These mice serve as a model for chronic granulomatous disease (CGD), a rare condition driven by mutations in the genes encoding for components of the NADPH oxidase.[111] Since defective neutrophil clearance leads to exacerbation of inflammation, it would be reasonable to expect that CGD patients are susceptible to periodontal diseases, also in part because CGD neutrophils have impaired production of ROS and thus ability to control pathogens that are susceptible to oxidative killing.[113,112] However, CGD patients do not display increased susceptibility to periodontal diseases (gingivitis and/or periodontitis) relative to the general population,[114,115,11] suggesting the operation of complex or even compensatory mechanisms. In the latter regard, it should be noted that CGD neutrophils cannot readily form NETs,[117,116] which might contribute to periodontal disease pathogenesis.[119,118]

Integral to the process of efferocytosis, the macrophage displays activation of liver X receptors (LXR; LXRα and LXRβ) and peroxisome proliferator-activated receptors (PPAR), which are ligand-activated transcription factors of the nuclear receptor superfamily that link efferocytosis to the resolution of inflammation.[120,121,109] Sterol lipids and polyunsaturated fatty acids, derived from the plasma membrane of apoptotic neutrophils, can respectively activate LXRs and PPARs.[122] Efferocytosis-associated activation of LXR signaling also upregulates the expression of c-Mer tyrosine kinase

(Mer), a major efferocytic receptor, thereby further augmenting efferocytosis.[121,123,106]

Consistent with the notion that the absence of neutrophils from the periodontal tissue causes IL-23/IL-17-driven inflammation due to lack of (or diminished) efferocytosis, antibody-mediated blockade of Mer leads to excessive IL-23 and IL-17 expression in the gingiva of wildtype mice, thereby mimicking the LAD1 phenotype of CD18-deficient mice.[103] Further support for the concept that the presence of neutrophils is required for periodontal tissue homeostasis was obtained in adoptive transfer experiments. Indeed, the expression of IL-23 and IL-17 was restored to normal levels in CD18-deficient mice that were given wildtype (transmigration-competent) neutrophils.[103] Moreover, pharmacologic induction of regulatory signals that are normally induced by efferocytosis (when the neutrophils are present in the gingiva) resulted in the restoration of periodontal tissue homeostasis in CD18-deficient mice. Specifically, when these LAD1 mice were locally administered a combination of synthetic LXR and PPAR agonists (GW3965 and GW0742, respectively), they exhibited diminished expression of IL-23 and IL-17 and improved levels of alveolar bone.[103]

It appears, therefore, that too many or too few neutrophils are incompatible with periodontal health. Intriguingly, both the excessive presence and the absence of neutrophils are associated with the overproduction of IL-17 in the periodontal tissue. The periodontal phenotype associated with excessive infiltration of the periodontal tissue with neutrophils was studied using mice deficient in developmental endothelial locus-1 (DEL-1), an endothelial-secreted protein that interacts with β2 integrins, such as LFA-1, on neutrophils and prevents their firm adhesion to the endothelium.[125,124] As a result, DEL-1-deficient mice show unrestrained neutrophil recruitment to the periodontium and develop spontaneous inflammation and bone loss.[126] Moreover, DEL-1-deficient mice display defective inflammation resolution because macrophage-secreted DEL-1 promotes the efferocytosis of apoptotic neutrophils and the LXR-dependent pro-resolution reprogramming of the efferocytic macrophage.[124,109] Specifically, DEL-1 engages the "eat-me" signal phosphatidylserine on apoptotic neutrophils and the αvβ3 integrin on macrophages, thereby bridging the apoptotic cells to the macrophages.[109] Although DEL-1-deficient and CD18-deficient mice exhibit the opposite phenotype in terms of neutrophil infiltration of the gingiva, both genotypes display elevated expression of IL-23 and IL-17 and IL-17-driven bone loss.[126,103] This phenotype is because both DEL-1-deficient and CD18-deficient mice fail to generate efferocytosis-associated regulatory signals (hence leading to overexpression of IL-23 and IL-17, as explained above); the former genotype because it lacks DEL-1 and the latter genotype because it lacks apoptotic neutrophils.

The above-discussed phenotypes associated with the paucity of neutrophils in the periodontal tissue may not be relevant only in the context of LAD1 but might also apply to additional conditions associated with poor or no gingival tissue infiltration with neutrophils due to defective chemotaxis (e.g., Chediak-Higashi syndrome) or neutropenic states (e.g., HIV-associated neutropenia, congenital or autoimmune neutropenia, or neutropenia in cancer patients under radiation therapy or chemotherapy). Unless the low neutrophil counts are appropriately dealt with, all these patients present with uncontrolled inflammation and rapidly advancing alveolar bone loss at a very early age.[127–129,102,94]

Even in individuals with transmigration-competent neutrophils, neutrophil homeostasis may be compromised under conditions of metabolic dysregulation, which may exacerbate their inflammatory destructive activities in periodontitis. Mechanistically, hypercholesterolemia and hyperglycemia can cause alterations in neutrophil production, activation, and function, thereby promoting chronic neutrophil-mediated inflammation.[131,130] Consistently, hypercholesterolemia and hyperglycemia are not only risk factors for cardiometabolic disorders but are also implicated in periodontitis, which is epidemiologically associated with both obesity and diabetes.[131–133]

In hypercholesterolemia, impaired cholesterol efflux and high cholesterol content in the plasma membrane of HSPCs lead to augmented cell surface abundance and signaling via the common β-subunit of the receptor for IL-3 and GM-CSF, designated CD131[130] (Figure 4). Consequently, the proliferation and myeloid-biased differentiation of HSPCs is increased, giving rise to elevated production of neutrophils.[130] In hypercholesterolemia, moreover, neutrophils display increased mobilization from the bone marrow and circulate in a "primed" state, characterized by elevated expression of CD11b and enhanced responsiveness (in terms of ROS and myeloperoxidase) to secondary stimuli.[134–136] In hyperglycemia, neutrophils release enhanced amounts of S100A8 and S100A9, members of the S100 calcium-binding family of proteins, which act as danger signals and activate the receptor for advanced glycation end products (RAGE) on macrophages in the bone marrow; this interaction in turn causes increased production of macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Subsequently, M-CSF and GM-CSF can stimulate granulopoiesis by acting on granulocyte-monocyte progenitors (GMP)[137] (Figure 4). The neutrophils generated under these hyperglycemic conditions are primed for increased production of ROS and release of NETs.[139,138] These systemically primed neutrophils can exacerbate inflammatory tissue destruction when recruited to the periodontium and thereby mediate, at least in part, the association of metabolic dysregulation (obesity, diabetes) with periodontal disease.[140,133] In an analogous manner, as alluded to above, neutrophils, generated in the context of maladaptive training of the myelopoiesis, are in a state of enhanced inflammatory preparedness and, once recruited to inflamed tissues, they aggravate chronic diseases such as periodontitis and arthritis[39] (Figure 1).

5 | TARGETING NEUTROPHIL-MEDIATED INFLAMMATION

Knowledge of the mechanisms whereby neutrophils are recruited to and activated in peripheral tissues has provided options for therapeutic interventions in inflammatory diseases, including periodontitis. As alluded to above, the β_2 integrin LFA-1 (CD11a/CD18) is crucial for

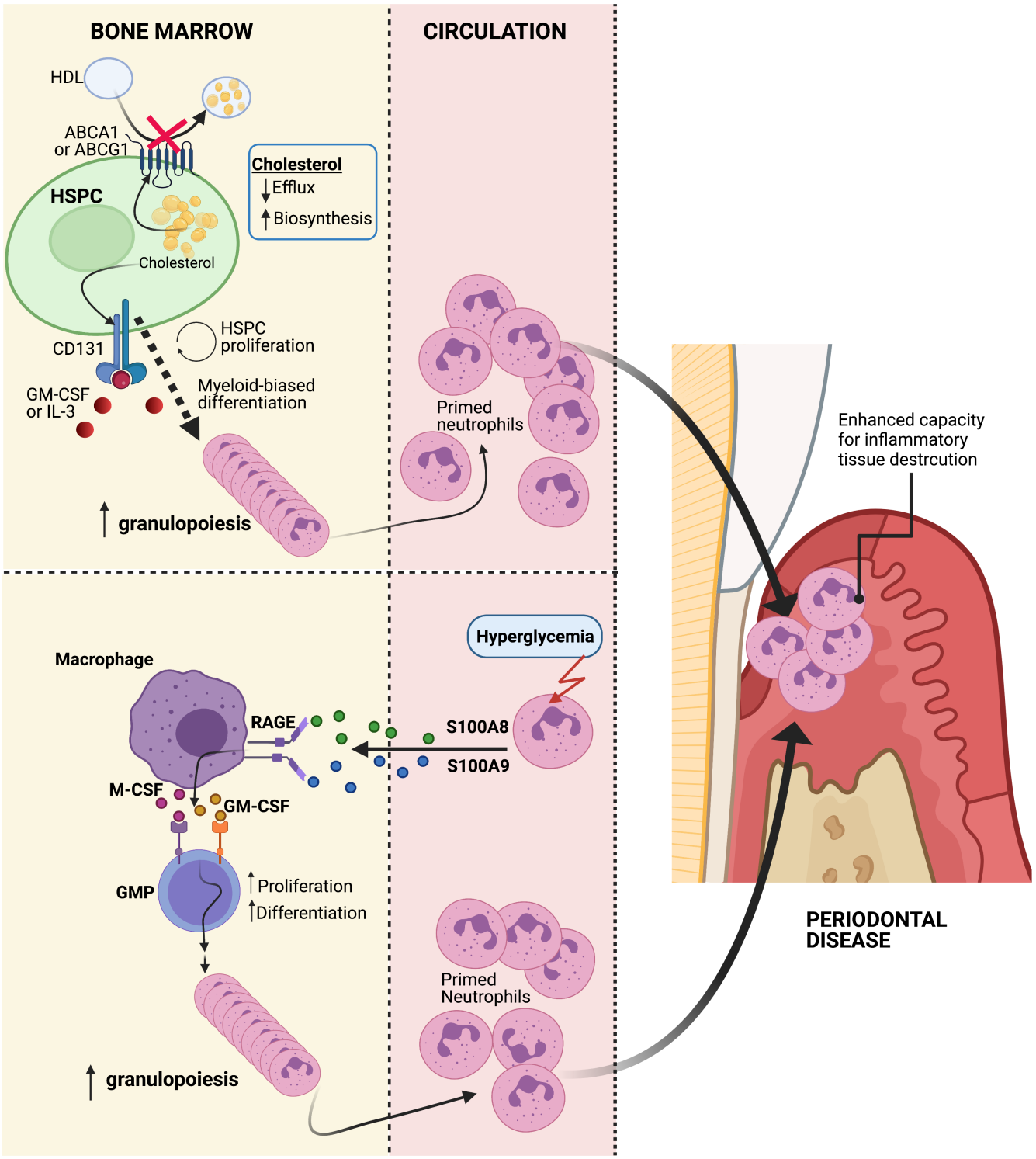

FIGURE 4 Neutrophil dysfunction under conditions of metabolic dysregulation. The bone marrow production and activation state of neutrophils are enhanced under hypercholesterolemic (top panel) or hyperglycemic conditions (bottom panel), as depicted here and detailed in the text.[130,134,136–139] The generated neutrophils are in a primed state in that they can respond faster and stronger to future infectious or inflammatory stimuli. Thus, when such systemically primed neutrophils are recruited to the periodontal tissue, they are likely to exacerbate periodontal inflammation, consistent with the association of metabolic dysregulation (obesity, diabetes) with periodontitis. ABCA1/ABCG1, ATP binding cassette transporter A1/G1; GMP, granulocyte-monocyte progenitor; HDL, high-density lipoprotein; HSPC, hematopoietic stem and progenitor cell

the ability of neutrophils to firmly adhere to the endothelium and extravasate to sites of infection and/or inflammation.[141,99] Endothelial cell-derived DEL-1 binds to LFA-1 and antagonizes its interaction with intercellular adhesion molecule-1 (ICAM-1), thereby restraining neutrophil transmigration.[126,125] Treatment of non-human primates subjected to ligature-induced periodontitis with recombinant

DEL-1 inhibits neutrophil recruitment to the periodontal tissue and protects the animals from inflammatory bone loss.[142] In principle, neutrophil recruitment in periodontitis could also be controlled by inhibitors that block other steps of the leukocyte adhesion cascade. Pentraxin-3 (PTX-3) inhibits neutrophil recruitment in a model of lung inflammation by interfering with neutrophils rolling along the endothelium; specifically, PTX3 binds to P-selectin on endothelial cells and blocks its interaction with P-selectin glycoprotein ligand-1 on neutrophils.[143] The neutrophil-derived protein annexin A1 and the TGFβ-related growth differentiation factor-15 both can inhibit chemokine-triggered "inside-out" activation of LFA-1 for adopting its high-affinity binding state. Mechanistically, both molecules block the activity of the Rap1-GTPase that mediates chemokine-induced activation of β_2 integrins, and thereby restrain neutrophil recruitment in models of cardiovascular inflammation.[145,144]

A recent study in mice by the Moutsopoulos and Bugge groups suggested that increased neutrophil recruitment is insufficient to precipitate immune pathology. Specifically, the authors showed that fibrin deposition in the periodontium is obligatory for the full activation of recruited neutrophils and their ability to mediate tissue destruction via the release of ROS and NETS.[146] The β_2 integrin Mac-1 (CD11b/CD18) is required for fibrin-induced neutrophil activation, which is counteracted by plasmin-induced fibrinolysis.[146] Consistent with the findings in mice, genetic variations in plasminogen (the inactive proenzyme of plasmin) are associated with severe periodontitis.[146] It is thus likely that pharmacological blockade of the fibrin interaction with neutrophils could control their destructive inflammatory activities.

In addition to β_2 integrins, neutrophils are equipped with multiple other receptors for sensing pathogen-derived or host-derived ligands that act as inflammatory and/or stress signals, leading to their activation and release of anti-microbial and inflammatory mediators. Such neutrophil receptors include complement receptors,[148,147] Toll-like receptors,[149] the leukotriene B4 receptor (BLT1),[150] extracellular nucleotide-sensing purinergic receptors (e.g., P2Y2 and P2X),[151] the Triggering Receptor Expressed on Myeloid cells-1,[152] and the Formyl Peptide Receptor 1 which senses *N*-formylated mitochondrial and bacterial peptides.[153] Pharmacological blockade of these receptors using specific antagonists confers protection in several preclinical models of inflammation, such as inflammatory arthritis, lethal shock-like syndrome, cardiovascular inflammation, and periodontal disease.[7,53,154-158] In the context of periodontitis, the most extensively investigated system is a complement, which is discussed in detail below.

Complement plays a crucial role in almost every aspect of neutrophil-mediated inflammation, from tissue recruitment to effector functions. Neutrophils respond to complement activation fragments, C3a and C5a, via specific G-protein coupled receptors, C3aR and C5aR1, respectively.[148] Deposition of C5a on the endothelial cells can activate LFA-1 integrin-dependent neutrophil adhesion and transmigration.[158,147] Moreover, besides following chemokine gradients, transmigrating neutrophils can also follow gradients formed deeper into an infected tissue due to complement activation, namely a C5a chemoattractant gradient.[141] Consistent with the importance of complement in neutrophil activation and the role of the latter in periodontal disease pathogenesis,[159] studies by the Hajishengallis and Lambris groups showed that local inhibition of C3, the central hub of complement activation, blocks periodontal inflammation and bone loss in non-human primates (NHPs).[160,7,53] The C3-targeted drug used in the aforementioned studies was AMY-101 (aka Cp40) of the compstatin family of peptidic C3 inhibitors.[161] Samples of gingival crevicular fluid (GCF; the local periodontal tissue exudate)

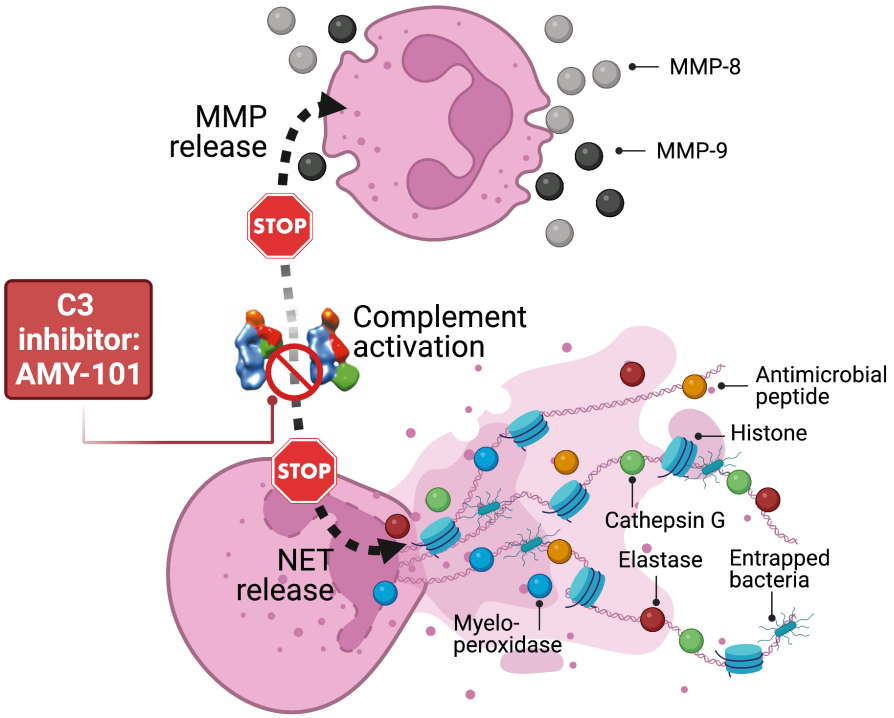

FIGURE 5 Inhibition of complement activation restrains neutrophil release of NETs and matrix metalloproteinases. Studies in humans have shown that the blocking of complement activation by the C3-targeted drug AMY-101 leads (through as yet unidentified pathways) to effective inhibition of the release of neutrophil extracellular traps (NET) and matrix metalloproteinase (MMP)-8 and MMP-9.[167,169] These activities may be responsible, in part, for the ability of AMY-101 to inhibit gingival inflammation in human patients.[169]

collected from NHPs of one of these studies[7] were subjected to proteomics analysis.[162] Mapping of the proteomic fingerprint changes in the GCF of NHP periodontitis in response to C3 inhibition identified the "alternative pathway of complement activation" and "leukocyte degranulation" as main targets, hence likely to have important roles in the pathogenesis of periodontitis.

The findings by Bostanci et al[162] suggest that complement inhibition by AMY-101 may regulate neutrophil exocytosis in the setting of periodontitis. Whereas the exocytosis of granules with pre-formed molecules (e.g., proteases, lipases, and inflammatory mediators) is a key element of neutrophil-mediated anti-microbial defense, the same activity can cause tissue damage in inflammatory conditions such as periodontitis.[163,159] Thus, pharmacological inhibitors of neutrophil exocytosis may be promising in clinical conditions driven by neutrophil-mediated inflammation.[165,164] Similarly, inhibition of NET release in response to neutrophil stimulation by pathogenic organisms or host-derived danger signals (e.g., immune complexes)[166] may also contribute to the treatment of neutrophil-instigated tissue damage. In this context, pharmacological inhibition of complement activation by AMY-101 blocks the release of NETs in the plasma of patients with COVID-19.[167] Therefore, the inhibitory effect of AMY-101 on NET release could contribute to the ability of the same drug to block periodontal inflammation in NHPs and human patients.[169,168] Moreover, by blocking complement, AMY-101 also caused, probably indirectly, a significant decrease in the gingival crevicular levels of matrix metalloproteinase-8 (MMP-8) and MMP-9, key neutrophil-derived proteases involved in periodontal tissue destruction[169] (Figure 5).

In this regard, AMY-101 showed efficacy in a recent phase 2a trial in patients with periodontal inflammation.[169,168] Specifically, AMY-101 caused a significant and sustainable reduction in gingival inflammation (as evidenced by measuring the modified gingival index and bleeding on probing) without any associated adverse events.[169] Moreover, this therapeutic outcome persisted for at least 3 months following treatment. Furthermore, in the same study, AMY-101 significantly reduced the GCF levels of matrix metalloproteinases MMP-8 (collagenase) and MMP-9 (gelatinase).[169] MMP-8 and MMP-9 are key neutrophil-derived proteases associated with periodontal tissue destruction and are considered biomarkers of periodontal tissue breakdown.[170–172] Particularly, MMP-8 is used in point-of-care testing and is highly predictive of severe periodontitis.[173–176] The significant reductions in the levels of both MMP-8 and MMP-9 suggest that AMY-101 treatment may act as a therapeutic agent that can modulate neutrophil function in a manner that benefits patients with periodontal disease.

6 | CONCLUDING REMARKS

Research on neutrophils in the 21st century has come a long way since these first responders of the immune system were considered merely effector cells that release their armamentarium of cytotoxic substances when challenged by infectious or inflammatory agents. Neutrophils are now appreciated for their critical roles in chronic tissue inflammation, a common denominator of different chronic disorders, including periodontitis. Whereas neutrophils were once considered the initiators of acute inflammation that characterized the initial periodontal lesions, they are now regarded as essential drivers of the progression and chronicity of the periodontal disease.[177] In part, this destructive role of neutrophils could be attributed to their subversion by periodontal pathogens that can undermine their anti-microbial function while promoting their destructive inflammatory potential.[49,163] At the same time, neutrophils play non-redundant roles in periodontal immune surveillance and tissue homeostasis, as evidenced by aggressive forms of periodontal disease in young individuals with genetic defects in the generation, trafficking, or function of neutrophils.[178] The accumulating in-depth knowledge on the mechanisms that underlie the production, priming or training, trafficking, activation, and interactions of neutrophils can facilitate their targeted pharmacologic treatment, leading to improved host modulation in inflammatory disorders. The observation that complement-targeted inhibition of periodontal inflammation in a recent clinical trial was associated with reducing the levels of key neutrophil-derived proteases involved in tissue destruction (MMP-8 and MMP-9) further underscores the importance of neutrophils as a pharmacological target in this oral disease.

AUTHOR CONTRIBUTIONS

S.M.U. and G.H. contributed equally to the conceptualization and writing of the manuscript.

ACKNOWLEDGEMENTS

The figures were created using Biorender.com.

FUNDING INFORMATION

The authors' research is supported by grants from the US National Institutes of Health (DE029436, DE031206, DE026152, and DE028561 to G.H. and DE024509 and DE014615 to S.M.U.).

CONFLICT OF INTEREST

G.H. is the inventor of a patent that describes the use of complement inhibitors in periodontal disease ("Methods of Treating or Preventing Periodontitis and Diseases Associated with Periodontitis"; patent no. 10668135). S.M.U. has no conflict to disclose.

DATA AVAILABILITY STATEMENT

There are no data in this manuscript.

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How to cite this article: Uriarte SM, Hajishengallis G. Neutrophils in the periodontium: Interactions with pathogens and roles in tissue homeostasis and inflammation. *Immunol Rev*. 2022;00:1-18. doi: 10.1111/imr.13152