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Neutrophil Role in Periodontal Disease

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Abstract

Oral tissues are constantly exposed to damage from the mechanical effort of eating and from the invasion of foreign microorganisms such as bacteria, fungi, and virus. In healthy oral tissues, there is a balance between symbiotic bacteria and cells from the innate immune system, mainly neutrophils. When this balance is broken, inflammation appears and more immune cells are recruited to the gingiva. Neutrophils form a barrier against dysbiotic bacteria. However, when neutrophils are insufficient, bacteria thrive causing periodontitis, a chronic inflammatory disease that destroys the tooth-supporting tissues or periodontium. Damage of periodontal tissues leads to tooth loss, and in severe cases, it can also affect systemic health by increasing a person's risk for atherosclerosis, rheumatoid arthritis, diabetes, and even cancer. The mechanisms neutrophil employ to keep a balance with bacteria in order to maintain healthy oral tissues is the focus of this chapter. We discuss how neutrophil antimicrobial functions keep bacteria at check and how some dysbiotic bacteria block neutrophils to promote an inflammatory state. Also, novel therapeutic approaches for periodontitis are discussed.

Keywords: neutrophil, phagocytosis, degranulation, NETs, oral microbiota, dysbiotic microbiota

1. Introduction

Periodontal disease is a major public health problem due to its high prevalence worldwide [1]. Periodontitis is a more advanced inflammatory form of periodontal disease. It is a chronic inflammatory disease that causes tooth loss, by destroying the periodontium. Periodontal destruction may be caused by different factors, including accumulation of dental biofilm, poor oral hygiene, and loss of balance between oral microbiota and immune response. Dysbiosis

(an alteration of oral microbiota) is thought to be the initial trigger for periodontitis [2]. The accumulation of bacteria biofilm leads to an increase in the inflammatory infiltrate, composed mainly by neutrophils into oral tissues. In this chapter, we will discuss the role of neutrophils in periodontal disease.

2. Neutrophil homeostasis

Neutrophils are considered to be the first line of defense during infections and inflammation [3]. They are the most abundant leukocytes in blood and can live for much longer than previously thought. It is estimated that neutrophils half-life is days instead of hours [4]. When microorganisms invade the organism, an inflammatory response is induced. Neutrophils are recruited from the circulation into the tissues where they destroy microorganisms by phagocytosis, by releasing antimicrobial substances, or by NETosis (**Figure 1**). This last mechanism was

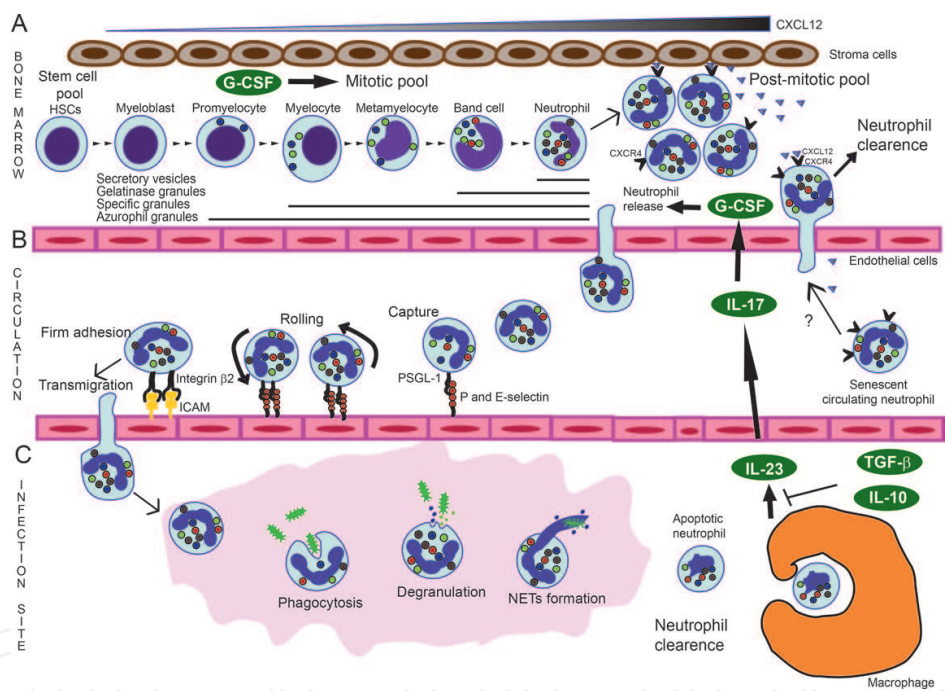


Figure 1. Neutrophil homeostasis involves production, trafficking, and clearance of these cells. (A) Production of neutrophils takes place at the bone marrow. Neutrophils mature in the bone marrow accumulating different granules (arrow heads), azurophil, specific, and gelatinase. Finally, they also produce secretory vesicles. Lines show the moment of appearances of granules and vesicles. Neutrophils are released from the bone marrow to the circulation by interfering with the CXCR4-CXCL12 interaction. (B) Neutrophils mobilization to infection site through a leukocyte adhesion cascade that includes capture, rolling, firm adhesion, and transmigration of neutrophils (thin arrows). Senescent circulating neutrophils increase the expression of CXCR4, and respond to CXCL12 by homing back to the bone marrow. (C) Neutrophils kill bacteria by phagocytosis, degranulation, and NETs formation. Apoptotic neutrophils are cleared by macrophage phagocytosis. The process of “neutrostat” that maintains steady-state neutrophil levels (molecules with green background and green arrows). In an infected site, macrophages produce IL-23, which activates IL-17. IL-17 induces G-CSF that promotes neutrophil differentiation and release from the bone marrow (thick arrows). After macrophages phagocyte apoptotic neutrophils, they downregulate the production of IL-23 and produce IL-10 and TGF-β, this events stop the recruitment of neutrophils. CXC, chemokine receptor; IL, interleukin; G-CSF, granulocyte colony-stimulating factor.

recently discovered and consists on the formation of neutrophil extracellular traps (NETs) [5]. Activated neutrophils produce a variety of chemokines and cytokines, directing the inflammatory and the immune responses [6]. Unfortunately, if there is not a proper clearance of neutrophils after an infection, the proteases released from neutrophils into the surrounding tissue can cause damage to the host [7]. Bacteria biofilm deposited on teeth induces a constant recruitment of neutrophils (>95%) to the gingival sulcus (**Figure 2**) [8, 9]. Therefore, neutrophil homeostasis is important to prevent collateral damage to the host by the potent proinflammatory and antimicrobial effects of these cells. As neutrophils are the most abundant leukocytes, their excess or absence in the mouth leads to periodontal tissue damage. Moreover, neutrophil distribution and numbers are essential in maintaining oral health. Neutrophil homeostasis involves production, trafficking, and clearance of these cells [10].

2.1. Production

Thousands of neutrophils are daily produced in the bone marrow and released into the circulation [11]. Three pools of neutrophil population reside in the bone marrow: the stem cell pool, the mitotic pool, and the postmitotic pool (**Figure 1A**). The first pool consists of undifferentiated pluripotent hematopoietic stem cells (HSCs), the second pool consists of committed granulocytic progenitor cells that proliferate and differentiate. Finally, the third pool consists of fully differentiated neutrophils, which form the bone marrow reserve, available for release [12]. HSCs differentiate into myeloblasts, a developmental cell type committed to becoming granulocytes (**Figure 1A**). Granulocyte colony-stimulating factor (G-CSF) regulates both, production or granulopoiesis, and neutrophil release from the bone marrow. G-CSF regulates granulopoiesis by inducing proliferation of granulocytic precursors in the bone marrow [10]. A large postmitotic pool is retained in the bone marrow by the interaction of CXC chemokine receptor 4 (CXCR4) on neutrophils with chemokine CXCL12 (stromal-derived factor-1/SDF-1)

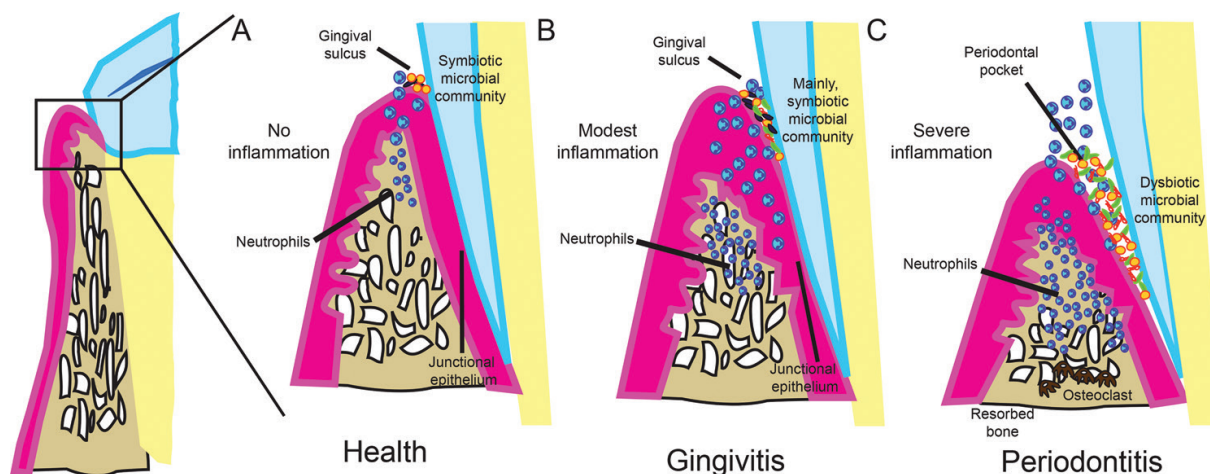


Figure 2. Neutrophil infiltrate and inflammation state. (A) In health conditions few neutrophils are recruited to the gingival sulcus to maintain symbiotic microbial community and the gum is not inflamed. (B) During gingivitis more neutrophils are recruited to the gingival sulcus and the gum is moderate inflamed. The junctional epithelium is starting to detach from the tooth. (C) During periodontitis a mayor neutrophil infiltrate is recruited to the periodontal pocket and the gum is severely inflamed. Inflammatory response activates osteoclasts, which in turn reabsorb bone.

produced by bone marrow stromal cells (**Figure 1A**). G-CSF regulates mature neutrophil release from the bone marrow by interfering with the CXCR4-CXCL12 interaction [12]. In addition, interleukin-17 (IL-17) endorses granulopoiesis and neutrophil release by upregulation of G-CSF (**Figure 1**) [10]. IL-17 builds on an interesting positive loop of neutrophil recruitment. For example, in chronic inflammation sites, neutrophils produce IL-17 and can also attract IL-17-producing CD4⁺ T lymphocytes (Th17 cells) [13]. Neutrophils also release CCL20 and CCL2 chemokines, which are ligands for CCR6 and CCR2 chemokine receptors, respectively, on Th17 cells. This interaction maintains Th17 cells at inflammation sites. Therefore, Th17 cells secrete more IL-17 and more neutrophils are recruited [14].

2.2. Trafficking

Circulating neutrophils can be quickly mobilized to infection or inflammation sites through a systematically controlled process known as the leukocyte adhesion cascade, which achieves neutrophil transmigration (**Figure 1B**) [15]. The process initiates when endothelial cells get activated and upregulated the expression of adhesion receptors such as E- and P-selectins. Neutrophils recognize these selectins and begin rolling on endothelial cells. This rolling depends on transient interactions of selectins with glycoprotein ligands on neutrophils. Next, neutrophils get activated by chemokines, which induce a high affinity state in integrins, another group of adhesion receptors. Interaction of both selectins and integrins with their corresponding ligands leads to slow neutrophils rolling followed by a firm adhesion that brings neutrophils to a full stop. Finally, neutrophils crawl on the endothelium and transmigrate into infection or inflammation sites. This last process is regulated mainly by β 2 integrins. Integrins are heterodimeric receptors formed by a unique α (CD11) and a common β (CD18) subunit that interact with adhesion ligands such as intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 on endothelial cells (**Figure 1B**). This leukocyte adhesion cascade is positive regulated by tissue-derived cytokines and by tissue-derived chemokines. Cytokines control the expression of endothelial adhesion molecules and chemokines induce integrins to change conformation into a high affinity state [16]. Once neutrophils move into tissues, they follow chemoattractant gradients to reach infection or inflammation sites. Some chemoattractants for neutrophils are activated by complement components, such as the anaphylatoxin C5a, and bacterial components, such as formyl-methionyl-leucyl-phenylalanine (fMLF). Recently, it has been discovered that the leukocyte adhesion cascade is also negatively regulated by endogenous inhibitors such as Del-1 (developmental endothelial locus-1), pentraxin 3, and growth-differentiation factor 15 [17].

2.3. Clearance

Neutrophils are mostly cleared in tissues (**Figure 1C**) and possibly also in the bone marrow (**Figure 1A**). In tissues, once neutrophils have completed their antimicrobial duty, they undergo apoptosis. Resident phagocytes, for instance, macrophages and dendritic cells, clear neutrophils locally. Phagocytosis of apoptotic neutrophils reprograms macrophages to initiate an anti-inflammatory response, characterized by the synthesis of tumor growth factor (TGF)- β and IL-10, and by a reduction in IL-23 synthesis (**Figure 1**) [18]. IL-23 cytokine induces IL-17 synthesis; thus, the reduced IL-17 levels lead to less

G-CSF production and in consequence, less neutrophil production. This process is a control loop that has been described as a “neutrostat” (neutrophil rheostat), and maintains steady-state neutrophil levels (**Figure 1**) [14]. Senescent circulating neutrophils are recruited for clearance in the bone marrow. These neutrophils increase the expression of CXCR4, and respond to CXCL12 by homing back to the bone marrow (**Figure 1**) [19]. Apoptosis and proper removal of apoptotic cells are key aspects of inflammation resolution. Neutrophils death is influenced by environmental conditions including hypoxia and presence of inflammatory mediators, such as granulocyte/monocyte colony-stimulating factor (GM-CSF) and lipopolysaccharides (LPS). Neutrophil clearance depends on signals that apoptotic neutrophils express on their surface. These signals allow macrophages to recognize and ingest the neutrophils (**Figure 1C**) [20]. Failure to clear these apoptotic cells results in secondary necrosis and release of products that generate proinflammatory signals. Neutrophils express molecules that regulate their survival. Some of these molecules are survivin, cyclin-dependent kinases and proliferating cell nuclear antigen (PCNA). Survivin is expressed more highly in immature neutrophils than in mature ones, but its expression can be reestablished in mature cells by inflammatory signals, for instance, GM-CSF or G-CSF [21]. Similarly, cyclin-dependent kinases function as prosurvival factors. Their inhibition induces caspase-dependent apoptosis. PCNA in neutrophils associates with procaspases in the cytosol and is thought to prevent their activation. During apoptosis, PCNA is targeted for proteosomal degradation, which correlates with an increase in caspase-3 and caspase-8 activities [11].

3. Neutrophils in oral health

Commonly, it is thought that microbes are harmful to our health. Contrary to this thought, there are plenty of microbes that harmoniously live within our bodies and form our microbiota. Homeostasis between the host and its symbiotic microbiota is a key factor to understand and maintain our health [22, 23]. Nevertheless, we are constantly exposed to microbes not belonging to our microbiota through the things we touch, the food we eat, and the air we inhale. Fortunately, our innate immune system protects us from this constant threat. The oral cavity is a special place, where the microbiota is constantly changing. Yet, homeostatic mechanisms exist that keep the oral microbiota in balance with the immune system. Neutrophils are actively recruited into the gingival sulcus by an interleukin (IL)-8 gradient continuously secreted by the junctional epithelium, as this tissue is in close contact with the oral biofilm bacterial community (**Figure 2**). Neutrophils are mostly responsible for ensuring periodontal health by keeping this biofilm at check [24]. However, during gingivitis a moderate inflammatory response is generated. If this inflammation is not controlled, for example, in situations of poor oral hygiene, gingivitis can lead to periodontitis, a chronic inflammatory disease. In this condition, microbial pathogens cannot be eliminated or controlled by neutrophils. In response, more neutrophils are recruited to the periodontal tissue. Neutrophil accumulation, instead of protecting, favors periodontal tissue damage and even bone loss (**Figure 2**). Thus, a close balance between neutrophil function and microbe challenge must be maintained to ensure periodontal health.

4. Antimicrobial mechanisms of neutrophils

Neutrophils are equipped with different antimicrobial mechanisms, which help them to fight a broad spectrum of bacteria, fungi, and protozoa. These mechanisms include phagocytosis, degranulation, and neutrophil extracellular traps (NETs) (**Figure 1**).

4.1. Phagocytosis

Phagocytosis is a receptor-mediated process during which a particle is internalized by the cell into a vacuole called the phagosome. Neutrophils recognize pathogens through pattern-recognition receptors (PAMPs), or opsonins (antibody molecules or complement components). Opsonized pathogens are efficiently phagocytosed when they bind with antibody receptors (Fc receptors) or complement receptors on the neutrophil (**Figure 3**). After engulfment, the nascent phagosome matures by fusing with lysosomes. This brings antimicrobial molecules into the phagosomal lumen. The vesicle is now called phagolysosome. Concurrently, reactive oxygen species (ROS) production starts by the assembly of the NADPH oxidase on the phagosomal membrane, and the pH inside the phagosome drops to 4.5–5. Also, potassium ions (K^+) are pumped into the phagolysosome; this K^+ influx mediates the release of serine proteases. In addition, hydrogen peroxide (H_2O_2) is also converted into hypochloric acid (HOCl) in a reaction catalyzed by myeloperoxidase (MPO) [25]. Granules content and ROS create an environment toxic to the pathogen. Unfortunately, not all pathogens are killed inside the phagosome. Moreover, some have advanced strategies to survive inside neutrophils. These strategies include interfering with engulfment, modulating phagosome maturation, and creating a more hospitable intraphagosomal environment.

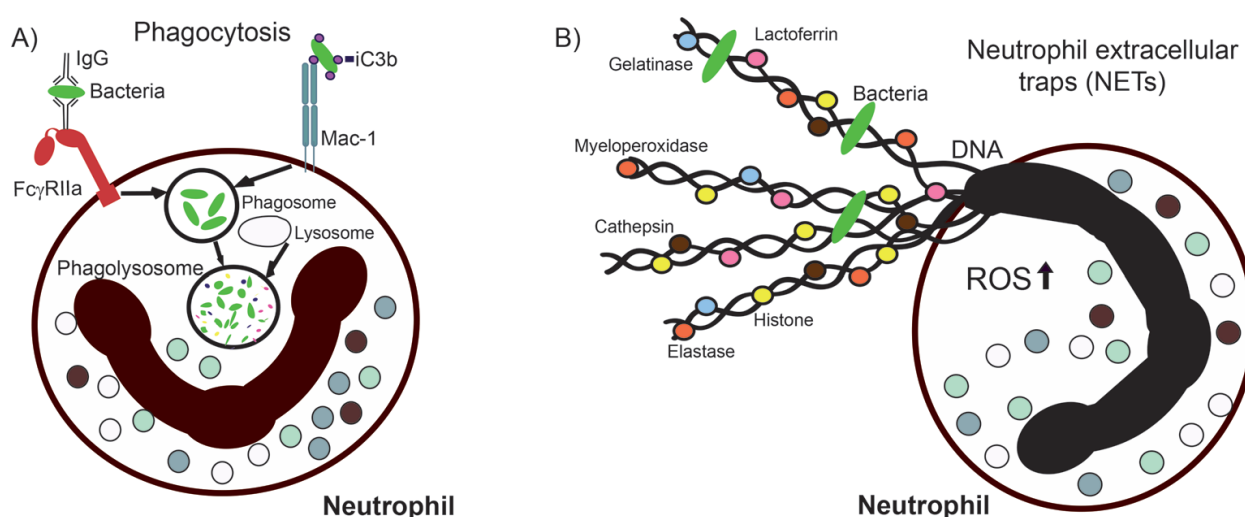


Figure 3. Phagocytosis and NETs. (A) Neutrophils recognize opsonized pathogens through Fc Receptors (FcγRIIa) or complement receptors (Mac-1) on their membrane. The pathogen is internalized into a nascent phagosome, which matures by fusing with lysosomes forming a phagolysosome. (B) Neutrophil extracellular traps (NETs) are formed when neutrophils release decondensed chromatin decorated with antimicrobial molecules, into the extracellular space. ROS, reactive oxygen species.

4.2. Degranulation

In the bone marrow, as precursor cells mature into neutrophils, they synthesize proteins that are sorted into different granules [26]. Granules formation begins in early promyelocytes and continues throughout the various stages of myeloid cell development. The granules are arbitrarily subdivided into three different classes based on their resident cargo molecules: azurophilic, specific, and gelatinase granules (Figure 1A, Table 1). Neutrophils also form secretory vesicles until the last step of their differentiation (Figure 1A, Table 1). Granule heterogeneity is explained by regulated expression of the granule protein genes. This regulation is mediated by the combination of myeloid transcription factors that express at specific stages of neutrophil development. Vesicle availability and exocytosis depends on mobilizable intracellular compartments of the neutrophil. Mature neutrophils are released into the circulation and, in response to infection, they leave the circulation and migrate toward the inflammatory site. Exocytosis of granules and secretory vesicles plays a crucial role in most neutrophil functions from early activation to the destruction of phagocytosed microorganisms. Secretory vesicles have the highest propensity for extracellular release, followed by gelatinase granules, specific granules, and azurophil granules [27, 28]. For example, neutrophil stimulation with phorbol myristate acetate (PMA) induces complete release of gelatinase granules, restrained release of specific granules, and minimal exocytosis of azurophil granules. In a different way, neutrophil stimulation with fMLF induces release of mostly secretory vesicles without

Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles
Azurocidin	CD11b/CD18	Acetyltransferase	Alkaline phosphatase
Bacterial/permeability-increasing protein	Cathelicidin	CD11b/CD18	CD11b/CD18 (CR3)
	Collagenase	Cytochrome b558	CD14
Cathepsins	Cytochrome b558	Gelatinase	CD16
Defensins	fMLP-R	Leukolysin	CR1
Lysozyme	Lactoferrin	Lysozyme	Cytochrome b558
Myeloperoxidase	Leukolysin	Natural-resistance-associated macrophage protein 1 (NRAMP1)	FRP
Natural serine proteases	Lysozyme		fMLF-R
Cathepsin G	Neutrophil gelatinase-associated lipocalin (NGAL)		
Neutrophil elastase			
Proteinase 3			

Note: CR, complement receptor; FPR, formyl peptide receptor; NGAL, neutrophil gelatinase-associated lipocalin; NRAMP1, natural-resistance-associated macrophage protein 1.

Table 1. Cytoplasmic granules of neutrophils [24–26, 28].

significant release of granules. The hierarchical mobilization of neutrophil granules and secretory vesicles depends on intracellular Ca^{2+} level. Gradual elevations in intracellular Ca^{2+} are induced by ligation of L-selectin, CD11b/CD18, and the fMLP receptors [26].

4.3. Neutrophil extracellular traps (NETs)

Neutrophil stimulation can also undergo a mechanism called NETosis. Although NETosis has previously been described as a special form of programmed cell death, there are forms of NET production that do not end with the demise of neutrophils. NETosis leads to the release of decondensed chromatin into the extracellular space. The chromatin forms a trap for pathogens that looks like a net, which is why they are called neutrophil extracellular traps (NETs). NETs also contain histones, cytoplasmic proteins, and antimicrobial granular molecules. NETs formation mechanisms are still unknown, nevertheless, NADPH oxidase activation, reactive oxygen species (ROS) production, myeloperoxidase (MPO), and neutrophil elastase (NE) release (**Figure 3**) are required [25].

5. Neutrophil interactions with symbiotic oral bacteria

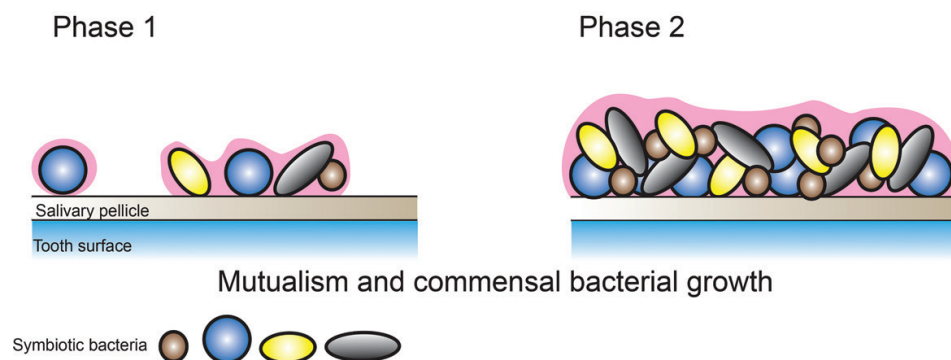
In periodontal health, the interaction between symbiotic microbial community and neutrophils is strongly controlled to prevent tissue damage. This interaction has been evaluated in studies with germ-free mice and specific pathogen-free mice. Results of these studies showed that oral symbiotic commensal microbiota has no impact on the structure of gingival tissue of germ-free mice, while gut commensal microbiota is fundamental on the structural formation of the intestinal tissue [29]. Periodontal tissue recruits neutrophils by means of the chemotactic receptor CXCR2. This receptor has two ligands CXCL1 and CXCL2. Both ligands are expressed in the junctional epithelium of germ-free and specific pathogen-free mice, but there is a significant increase on CXCL2 in the epithelium of specific pathogen-free mice. Therefore, oral bacterial community induces an increase in neutrophil recruitment via CXCL2 [29]. Neutrophils play a key role in preserving oral health, since low neutrophil counts as well as deficiency in neutrophil functional responses have been associated with periodontal disease. As mentioned before, neutrophils kill pathogens by phagocytosis, degranulation, or NETs formation (**Figure 1C**). Neutrophils are very efficient phagocytic cells and have a very efficient antimicrobial mechanism to do so, the respiratory burst response. In this response, high consumption of oxygen results in the production of reactive oxygen species (ROS), through the activation of the NADPH oxidase complex (**Figure 3**) [5]. Patients with chronic granulomatous disease, a rare genetic disorder that consist on mutations in the NADPH oxidase, are inefficient in mounting a respiratory burst response. As a consequence, these patients present early in life recurrent infections [30]. These patients present higher bacteria colonization and gingivitis; however, they do not present periodontitis [31].

6. The evolution from a healthy periodontium to periodontitis

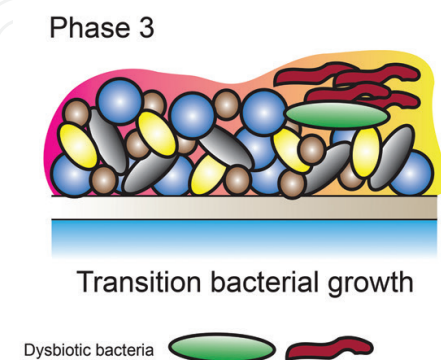
In the oral cavity, the tooth surface offers a niche for bacteria colonization and biofilm formation resulting in a varied polymicrobial community. A healthy environment is maintained if the

multiplication of symbiotic microbiota is regulated. Periodontal diseases are related to a shift from symbiotic microbiota to dysbiotic microbiota, and this shift is related with the accumulation of dental plaque or biofilm. Biofilm elaboration consists of four sequential phases. Phase 1 consists of the adsorption of different molecules to a surface to condition the biofilm formation. Phase 2 consists of single organism adhesion. Phase 3 consists of growth of extracellular matrix production and multiplication of adhering bacteria and phase 4 consists of sequential adsorption of further bacteria to form a more complex and mature biofilm (**Figure 4**) [32]. The microbial etiology of gingivitis and periodontitis has been established for several decades. In 1994, Haffajee and Socransky adapted Koch's postulates to be used in the identification of periodontal pathogens. In 1996, at the World Workshop in Periodontics three species of pathogens were identified as causative factors of periodontitis *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia*; however, these three species cannot be considered to be the only causative pathogens of periodontitis, but we are certain that they participate in the disease [32].

Health



Gingivitis



Periodontitis

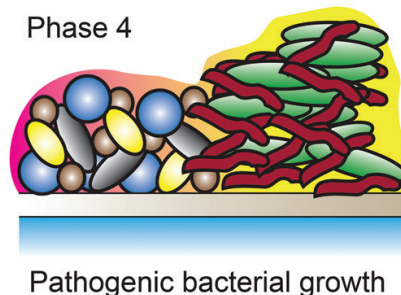


Figure 4. Biofilm elaboration consists of four sequential phases. Phase 1 consists of the adsorption of different molecules to a surface to condition the biofilm formation. Phase 2 consists of single organism adhesion. Phase 3 consists of growth of extracellular matrix production and multiplication of adhering bacteria and phase 4 consists of sequential adsorption of further bacteria to form a more complex and mature biofilm. The first two phases are representative of health, phase 3 of gingivitis, and phase 4 of periodontitis.

6.1. Balanced inflammation

Neutrophils are the main leukocytes recruited to the gingival sulcus. Neutrophils exit the gingival blood vessels and travel through the gingival junctional epithelium until they reach the sulcus [8]. In the sulcus, neutrophils create a barrier against the growing bacteria biofilm to prevent bacteria from invading the underlying tissues. Neutrophil migration from vessels toward the gingival sulcus requires CXCR2 binding to CXCL2. Migration is controlled by gradients of chemokines and adhesion molecules such as IL-8, ICAM-1, and E-selectin [29]. Neutrophil presence in the sulcus is necessary to preserve oral health since patients with altered neutrophil production and distribution develop severe periodontitis at early ages [33]. Chédiak-Higashi syndrome, Papillon-Lefèvre syndrome, neutropenias, and leukocyte adhesion deficiency (LAD) are some examples of neutrophil diseases. In Papillon-Lefèvre syndrome neutrophils have defective chemotaxis, as a consequence, they are not efficiently recruited to the sites of inflammation and infection [34]. Neutropenia, a persistent reduction of neutrophil numbers in circulation, is frequently associated with susceptibility to infections. In many neutropenic conditions, severe periodontal disease is recurrently seen since primary dentition eruption [35]. CXCR2-deficient mice cannot recruit neutrophils to oral tissues. These mice also experience periodontitis and periodontal bone loss early in life [36]. Leukocyte adhesion deficiency is a group of inherited disorders, in which neutrophils fail to transmigrate from the circulation to the site of inflammation or infection. Neutrophils of patients with leukocyte adhesion deficiency have defective expression and function of adhesion molecules like integrins. Therefore, neutrophils cannot adhere firmly to the vascular endothelium to transmigrate. Even though the presence of neutrophils is necessary to control infections, plenty of neutrophils on a site of infection is not always protective. In fact, neutrophil numbers in inflamed periodontal tissues correlate with the severity of the lesions [37], and tissue destruction seems to be a collateral damage of hyperactive neutrophils [38].

6.2. Periodontitis

Periodontal diseases cause the destruction of the tooth supporting tissues, gingiva, periodontal ligament, cement, and alveolar bone and may eventually lead to tooth loss. Severe periodontitis affects approximately 10% of the global population [39]. Periodontal disease is the consequence of a shift in oral microbiota population from a symbiotic to a dysbiotic microbial community in the mouth. Periodontal disease begins when some factors that promote the growth of selected symbiotic bacteria, induce host inflammatory pathways [40, 41]. Periodontitis not only severely deteriorates people's quality of life by impairing the dentition but also adversely affect systemic health. A clear correlation between periodontal disease and atherosclerosis has been established in clinical observations and in animal models. In particular, polymicrobial infection with *Treponema denticola*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Fusobacterium nucleatum* has been shown to promote progression of atherosclerosis [42]. Another correlation between periodontitis and diabetes also has been well documented. Higher plaque levels and higher incidence of chronic gingivitis are both found in adults and in children with diabetes [43, 44]. Periodontal treatment showed a beneficial effect on metabolic control of type 2 diabetic patients. Other various systemic diseases such as diabetes, cardiac disease, low birth weight, renal diseases, metabolic

syndrome, obesity, Parkinson's disease, and Alzheimer's disease have been also proposed to be linked with periodontal disease on the basis of systemic inflammation [40, 41, 45].

6.3. Inflammation in periodontitis

Periodontitis is associated with a change in oral microbiota from symbiotic bacteria to dysbiotic anaerobic microorganisms, which have adapted to succeed in an inflammatory environment (**Figure 4**). Pathogenic bacteria, such as *Porphyromonas gingivalis*, induce changes in the normal microbiota of the gingival crevicular fluid, leading to increased biofilm deposition in the gingival sulcus. The gingival sulcus is the space between the tooth surface and the free gingiva. Pathogenic bacteria also induce moderate inflammation known as gingivitis (**Figure 4**). When this moderate inflammation is not well resolved, a chronic inflammatory state is established, which results in the formation of pathologically deepened gingival sulcus also called periodontal pockets, followed by extensive tissue destruction, including bone loss (**Figures 2 and 4**). These last events are induced by the accumulation of dysbiotic bacteria in the periodontal pockets and are thought to be the initial trigger for periodontitis [46]. Accumulation of dysbiotic bacteria biofilm leads to an increase in the inflammatory infiltrate, composed mainly by neutrophils into oral tissues. There, neutrophils form a barrier that prevents bacteria from invading deeper tissues and are essential for maintaining healthy oral tissues. In the case of neutrophil deficiencies, severe periodontitis appears with a concomitant inflammation state. On the contrary, excess numbers of neutrophils induces a chronic inflammatory state. Thus, inflammation is an important element in periodontitis that is deregulated when neutrophil homeostasis is altered. Periodontitis in the absence of neutrophils has traditionally been explained by the lack of neutrophil control on bacterial infections. Patients with leukocyte adhesion deficiency present frequent infections and develop early severe periodontitis. However, this type of periodontitis does not usually respond to treatment with antibiotics or mechanical removal of bacteria biofilm, suggesting that other mechanisms are at work. Recently it was shown that the driving force for this type of periodontitis involves the production of IL-23 and IL-17. In leukocyte adhesion deficiency type 1 patients, T cells were identified as the main producers of IL-17 [47]. IL-17 not only stimulates fibroblasts to produce G-CSF but also promotes inflammation and stimulates osteoclasts, leading to bone loss. These findings are in agreement with the neutrostat mechanism discussed above. When apoptotic neutrophils are phagocytosed by macrophages, anti-inflammatory signals are produced that lead to less IL-23 production, which is a strong inducer for IL-17 production. IL-17 in turn induces G-CSF production (**Figure 1**).

Neutrophils can be found in large numbers in inflamed periodontal tissues, and their presence correlates with the severity of the periodontal destruction. Therefore, this destruction seems to be collateral damage of hyperactive neutrophils [48, 49]. Neutrophil recruitment is at least in part regulated by Del-1 and LFA-1 interactions. Del-1 blocks LFA-1 binding to its ligand ICAM-1 and prevents neutrophil transmigration [50]. Neutrophil recruitment is also triggered with elevated IL-17 levels, which resulted to be responsible for the tissue damage, because antibodies against IL-17 prevented inflammation and bone loss. High levels of IL-17 could be responsible for the bone loss in chronic periodontitis, by stimulating osteoblast expression of RANKL, an important osteoclastogenesis factor.

6.4. Dysbiotic bacteria

Diverse environments present in the oral cavity allow symbiotic and dysbiotic microbiota to find the best niche that fits their growth requirements, resulting in the formation of unique microbial biofilm communities. Periodontal disease microbiota includes a large number of microorganisms including *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola* [51]. Fortunately, nucleic acid screening and 16S pyrosequencing techniques have made more efficient finding changes in microbiota of healthy and of periodontal disease patients [52]. Screenings have been made at nine different oral sites including the oral epithelium, the maxillary anterior vestibule, the dorsum and lateral tongue surface, the hard and soft palate, the tonsils, the tooth surfaces, and the subgingival plaque [53, 54]. There are between 100 and 300 bacterial species in a single individual. Our general idea is that infectious diseases are caused by the action of a single foreign pathogen. However, periodontitis is originated by the complex association and interaction of a diverse polymicrobial community [37, 51, 55]. Data obtained from oral biofilm studies using checkerboard DNA-DNA techniques link the different stages of the disease to a specific bacterial group or complex with the presence of the triad of bacteria composed by *P. gingivalis*, *T. forsythia*, and *T. denticola*, which are strongly associated with increased severity of periodontitis [56]. Other microorganisms have also been identified such as *F. alocis*, a Gram-positive bacterium, which is present in periodontal disease sites, while *Veillonella sp.*, a Gram-negative uncultivated bacterium, is associated with healthy periodontal sites. This data indicates that the general idea of Gram-negative anaerobic bacteria being the pathogen population is not completely correct.

In a healthy gingival tissue, the local symbiotic microbiota is less diverse and rich, with neutrophil recruitment to clear the infection and resolving the inflammation with no collateral damage to the host (**Figures 4 and 5**). The progression from health to periodontitis is now explained as the transition from a symbiotic microbiota to a polymicrobial dysbiotic microbiota. Several risks factors, such as smoking, tissue injury, diet changes, an immunocompromised host, or the colonization of the oral cavity by pathogenic bacteria can modify the oral ecosystem resulting in a dysbiotic polymicrobial community. In consequence, the host's response toward the polymicrobial dysbiotic microbiota challenge is more robust and not regulated, transitioning from a controlled/stable immune response into a nonresolving chronic inflammatory response [57].

Polymicrobial dysbiotic microbiota has an arsenal of self-defense mechanisms, which can be directed to attack against neutrophils or camouflage the biofilm (**Figure 5**) [58]. Microbiota has an intermicrobial communication called quorum sensing, that enables the dysbiotic microbiota to optimize the biofilm conditions and ensure nutrient supply. Among the defense mechanisms, the production of bacterial surfactants by *P. aeruginosa* biofilms causes rapid cell death in neutrophils [59]. Additionally, quorum sensing molecules control neutrophil ROS response and penetration into *P. aeruginosa* biofilms [60]. Similarly, *Aggregatibacter actinomycetemcomitans* and *S. aureus* produce bacterial toxins that induce neutrophils lysis and degranulation [61–63]. In addition to directly attacking neutrophils, dysbiotic microbiota in biofilms can render themselves resistant to neutrophil-mediated killing by disguising their immunogenicity. NET formation within *Haemophilus influenzae* biofilms does not harm the biofilm. This is presumably due to their expression of certain lipooligosaccharide glycoforms, which shield pathogen-associated molecular patterns (PAMPs) and thus inhibit recognition and

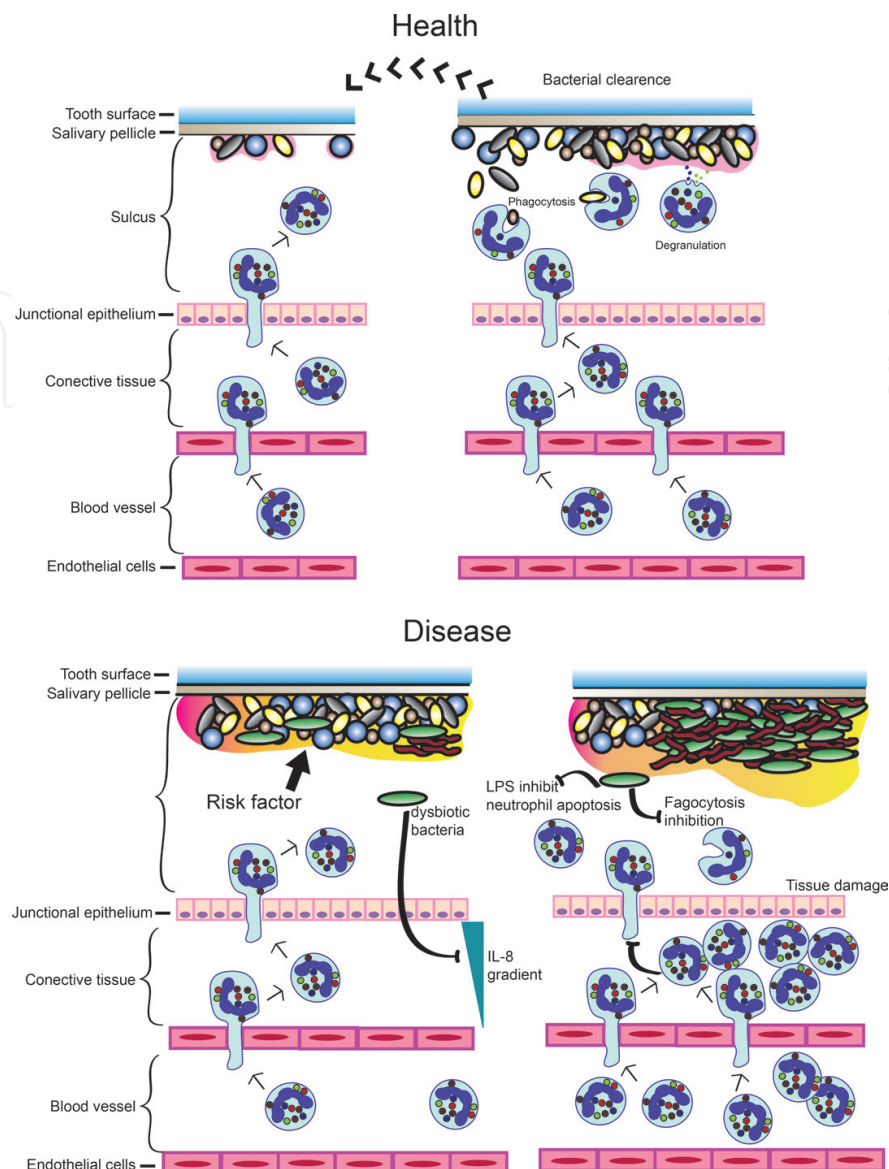


Figure 5. Neutrophil response in periodontal health and disease. Health: Symbiotic bacteria community adheres to molecules of the salivary pellicle that are bound to the tooth surface. Few neutrophils patrol the gingival sulcus, and as the bacterial burden increases, neutrophils regularly exit the blood stream entering the connective tissue layer beneath the junctional epithelium and the tooth and kill some of the associated microbes (thin arrows). Neutrophils maintain bacterial concentration so there is no inflammation or tissue damage (arrow heads). Disease: Following the presence of a risk factor (smoking, poor diet, injury, etc.; thick arrow) dysbiotic bacteria (big oval) can colonize the symbiotic microbial community. Following colonization, the sulcus is invaded by dysbiotic bacteria which shut down the IL-8 production. Neutrophils enter the connective tissue, but do not get to the sulcus. This causes many neutrophils to accumulate in the connective tissue. As some neutrophils transmigrate to the dysbiotic biofilm increase inflammation is conducted by neutrophil degranulation, reactive oxygen species (ROS) production, and NETosis that damages the host tissue.

opsonization. This molecule can provide protection against antimicrobial peptides [64]. One important microbial defense mechanism is the evasion of phagocytosis. *Prevotella* strains were recognized by neutrophils but not phagocytosed, depending on whether they produced mannose-rich exopolysaccharides as part of their extracellular matrix [65]. *S. aureus* is able to survive after being phagocytosed by neutrophils [66]. *S. aureus* is known to be potent triggers

for NETosis and degranulation. Therefore, it can be assumed that implementation of such survival strategies coexists with the elimination of bacteria by neutrophils. Finally, inflammation and tissue destruction mediated by neutrophils evoke frequent gingival bleeding, which these bacteria may use as an additional source of nutrients, such as iron and vitamin K.

7. *Porphyromonas gingivalis*

Porphyromonas gingivalis are anaerobic, Gram-negative, nonmotile, asaccharolytic rods that usually exhibit coccal or short rod morphologies. It is part of the black-pigmented Bacteriodes group [32]. *P. gingivalis*, even in low colonization levels, can induce the shift from symbiotic microbiota to dysbiotic microbiota followed by inflammatory bone loss. This bacteria uses different mechanisms to destabilize neutrophil homeostasis, inhibition of phagocytic killing, resistance to granule-derived antimicrobial agents and to the oxidative burst, impaired recruitment and chemotaxis, promote inflammatory response, and delay of neutrophil apoptosis. *P. gingivalis* has a number of virulence factors related to the subversion of the innate immune system. This ability is what often characterizes a successful pathogen, as it tends to disable the overall host response while simultaneously enhancing the pathogenicity of a polymicrobial community. *P. gingivalis* are resistant to oxidative killing [67] and recruit hyperactive neutrophils with an enhanced response, which is characterized by the release of reactive oxygen intermediates, several cationic peptides, and enzymes such as matrix metalloproteinases (MMPs). All this responses increased tissue damage [48]. *P. gingivalis* also can manipulate both complement and TLR signaling to induce bacterial persistence.

Porphyromonas gingivalis gingipains are able to trigger the expression of proinflammatory surface receptor TREM-1 on neutrophils, and several periodontopathogenic species can induce IL-8 gene expression in gingival epithelial cells and fibroblasts [68, 69].

8. *Treponema denticola*

Treponema denticola is an anaerobic, Gram-negative, motile, spirochete that can be poorly detected in the gingival plaque of healthy individuals. However, it is present in very high numbers in the subgingival periodontal pocket and is associated with the dysbiotic microbiota biofilm formation in periodontal lesions. *T. denticola* limits neutrophil chemotaxis, and inhibits junctional epithelial cells to secrete IL-8. Additionally, this pathogen is able to degrade IL-8 that is already present at the infection site, which disables the neutrophil chemotactic gradient. *T. denticola* major outer sheath protein (Msp) is one of its most important virulence factors in contributing to the disease progression. This membrane protein modulates neutrophil signaling pathways involved in cytoskeletal dynamics that are relevant in chemotaxis and phagocytosis [70]. Msp controls neutrophil cytoskeletal functions like migration, adhesion, and cell shape. It also causes extracellular matrix degradation by stimulating the release of activated MMPs from neutrophils.

9. Neutrophil persistence and chronic inflammation

Neutrophils are recruited to infection and inflammation sites by different chemoattractants such as interleukin 8 (IL-8), complement fragment C5a, or chemokine CXCL5. They migrate through the junctional epithelium and finally arrive in the gingival sulcus and in gingival crevicular fluid. Neutrophils in saliva retain their phagocytic function, and their ability to generate ROS [71, 72]. NETs containing trapped bacteria have been described within the gingival sulcus, in purulent periodontal pockets, and on the surface of gingival epithelial cells. On condition that neutrophils and NETs do not occur excessively and are rapidly cleared, relatively little damage to the adjacent tissues is induced. Nevertheless, it has been widely reported that neutrophils can be responsible for both host defense and host tissue damage. Release of proteolytic and collagenolytic enzymes as well as ROS within host tissues, often lead to extracellular matrix degradation and persistent inflammation, are the main causes of tissue damage. Normally, connective tissue is degraded to allow fast transmigration of neutrophils and other cells involved in wound healing but during periodontitis it produces a chronic inflammatory disease. Hence, inflammation overweighs resolution, and host tissue destruction becomes progressive, eventually resulting in pathological osteolysis and tooth loss [58].

10. Therapeutic approaches

As it was discussed along this chapter, it is clear that both, lack of neutrophils and excess of neutrophils in the periodontium, can lead to periodontal disease. Because both situations involve IL-17-mediated inflammation and bone loss, it is conceivable that IL-17 or IL-17R inhibitors may be promising targets for treatment of human periodontitis. By blocking IL-17 actions, neutrophil recruitment to the periodontium would be reduced and in consequence, the inflammation state would also be reduced. This should prevent tissue damage and loss of bone.

In chronic periodontitis, periodontal bacteria activate neutrophil subversion pathways that allow bacteria to escape neutrophil killing. For example, *P. aeruginosa* biofilms produce bacterial surfactants that induce rapid neutrophil death [59], and *Aggregatibacter actinomycetemcomitans* and *S. aureus* produce bacterial toxins that induce neutrophil lysis and degranulation [61–63]. These bacterial products could be neutralized with antibodies or novel pharmaceutical drugs to prevent their negative effects on neutrophils. Also, *P. gingivalis* can manipulate complement to induce bacterial persistence. Thus, complement components are also good therapeutic targets. In fact, in preclinical models of periodontitis the use of complement inhibitors has led to a reduction of the inflammatory state [73, 74]. In addition, several bacteria including *P. gingivalis* can induce cells such as fibroblasts to produce IL-8 and recruit more neutrophils to the inflamed periodontal tissues [68, 69]. Blocking IL-8 is another interesting therapeutic strategy for reducing periodontitis. Several anti-IL-8 blocking antibodies are available. Their potential benefit in periodontal disease should be evaluated in the near future.

Del-1 is another promising candidate molecule to be used therapeutically to prevent neutrophil recruitment and bone loss associated with periodontal inflammation [17]. Since Del-1

blocks LFA-1 binding to its ligand ICAM-1 and prevents neutrophil transmigration [50], it could be administered to inflamed tissues, to reduce neutrophil recruitment, and to reduce inflammation. In fact, this is exactly what was found in a model of periodontitis with nonhuman primates [75]. Local administration of Del-1 also prevented inflammatory bone loss [75]. Preclinical studies for the use of Del-1 are now underway.

All these potential therapeutic approaches promise a relief from periodontitis and perhaps other inflammatory disorders in the future.

11. Conclusion

Neutrophils are specialized phagocytes that coordinate and execute inflammation. Neutrophils constantly surveil oral tissues in order to guarantee oral health. Alterations in the neutrophil homeostasis (defects in recruitment and proper function) lead to periodontal diseases. Also, hyperactive neutrophils can exacerbate and even cause autoimmune and inflammatory diseases. Chronic infections driven by pathogenic biofilms indicate that the immune system fails to fully protect the host. New potential therapeutic approaches have been identified. They promise a relief from periodontitis and perhaps other inflammatory disorders.

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