

REVIEW ARTICLE

Dynamic interactions of neutrophils and biofilms

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Background: The majority of microbial infections in humans are biofilm-associated and difficult to treat, as biofilms are highly resistant to antimicrobial agents and protect themselves from external threats in various ways. Biofilms are tenaciously attached to surfaces and impede the ability of host defense molecules and cells to penetrate them. On the other hand, some biofilms are beneficial for the host and contain protective microorganisms. Microbes in biofilms express pathogen-associated molecular patterns and epitopes that can be recognized by innate immune cells and opsonins, leading to activation of neutrophils and other leukocytes. Neutrophils are part of the first line of defense and have multiple antimicrobial strategies allowing them to attack pathogenic biofilms.

Objectivedesign: In this paper, interaction modes of neutrophils with biofilms are reviewed. Antimicrobial strategies of neutrophils and the counteractions of the biofilm communities, with special attention to oral biofilms, are presented. Moreover, possible adverse effects of neutrophil activity and their biofilm-promoting side effects are discussed.

Results/conclusion: Biofilms are partially, but not entirely, protected against neutrophil assault, which include the processes of phagocytosis, degranulation, and formation of neutrophil extracellular traps. However, virulence factors of microorganisms, microbial composition, and properties of the extracellular matrix determine whether a biofilm and subsequent microbial spread can be controlled by neutrophils and other host defense factors. Besides, neutrophils may inadvertently contribute to the physical and ecological stability of biofilms by promoting selection of more resistant strains. Moreover, neutrophil enzymes can degrade collagen and other proteins and, as a result, cause harm to the host tissues. These parameters could be crucial factors in the onset of periodontal inflammation and the subsequent tissue breakdown.

Keywords: *biofilms; neutrophils; periodontitis; host–biofilm interactions*

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Neutrophil granulocytes constitute the majority of white blood cells and are recruited to sites of inflammation and microbial perturbation as the first cellular responders. Invading microorganisms can effectively be eliminated or inactivated by various antimicrobial mechanisms of neutrophils (1). Many microorganisms live within multifarious communities of microbes, embedded in an extracellular polymer matrix attached to host tissue surfaces. These three-dimensional microarchitectural structures are called biofilms, which allow microbes to live and reproduce in a protected environment. Therefore, they present a considerable challenge to the immune system (2, 3). Moreover, long-term synergistic interactions among the mixed community are established enabling biofilms to subsist and facilitating growth of species that require the metabolites of helper organisms

(4, 5). Current research is directed towards understanding biofilm formation, microbial interactions, and host–biofilm communication. This review addresses the latter aspect and focuses on the current understanding of the role of neutrophils in the host response to biofilms and vice versa. In particular, oral biofilms, which are the causative agents of oral diseases such as gingivitis, periodontitis, and caries, will be given special attention. To provide the reader with an overview of some functional characteristics of biofilms, the following paragraph presents a general view of their beneficial and adverse effects on the host.

Biofilms: infection versus protection

Biofilms grow on solid or soft moist surfaces and usually harbor a multitude of microbial species. In the human body, biofilms are naturally found on many mucosal surfaces,

including the gut and oral cavity (6, 7). As opposed to planktonic living microorganisms, those living in biofilms establish an organized three-dimensional structure that becomes widely resistant to many host-derived or exogenous antimicrobials. Biofilms are very heterogeneous and undergo fast genetic shifts, modified gene expression patterns, and morphological changes. These alterations depend on the specific interactions with other species present in the biofilms and environmental conditions such as availability of nutrients, temperature, pH, ion concentration, or oxygen content (8–11). Furthermore, an intermicrobial communication called quorum sensing (QS) takes place and enables the microbial community to optimize these conditions and ensure nutrient supply (12). More than 65% of microbial infections are caused by biofilm-forming microorganisms and commonly affect skin, urinary tract, the lungs, middle ear, prosthetic joint implants, catheters, or heart valves. To date, biofilm-associated infections are difficult to treat and constitute a medical challenge that also leads to high treatment costs particularly for medically indicated explantations of prosthetic devices (13, 14).

Since many antibiotics are no threat to biofilms, researchers have, in recent years, paid increasing attention to probiotics and intermicrobial suppression mechanisms. As a result, the fact that different species share the same ecological niche can lead to competition and monolateral displacement. For instance, some lactobacilli segregated pathogenic gastrointestinal bacteria *in vivo* and produced antimicrobial and anti-inflammatory factors *in vitro*, whereas *Staphylococcus epidermidis* stabilized cutaneous defense by superseding other species (15, 16). Also some fungi were shown to inhibit pathogenic yeasts and to have anti-inflammatory traits (17, 18). Since many of these bacterial species and fungi are able to grow in biofilms, it is possible that biofilms can exhibit host-protective properties depending on their microbial composition. As another benefit for the host, many microorganisms produce essential nutrients, participate in host metabolism or control pH balance (19, 20). In the oral cavity, several Gram-positive members of early biofilms are thought to be associated with dental and periodontal health, such as *Streptococcus salivarius* or *Lactobacillus* species (21, 22). In a beagle dog model of periodontitis, oral cocci administered into periodontal pockets were able to suppress regrowth of periopathogens, reduced inflammation, and improved bone density (23, 24). Moreover, some lactobacilli were found to suppress the growth of periodontal and cariogenic pathogens and have been in use as therapeutic and preventive agents for several years (25, 26). Taken together, biofilms containing health-associated bacteria can protect the host from pathogenic overgrowth, but at the same time, pathogenic biofilms are the causative agents of inflammation and severe disease.

Neutrophils: powerful (h)arms of innate immunity

Neutrophils exert microbial killing by phagocytosis, which is the process of internalizing microbes and digesting them in the phagolysosome, or by degranulation and generation of reactive oxygen species (ROS), where these bactericidal components are secreted into the phagolysosome or to the extracellular environment (27–29). A more recently discovered mechanism is the generation of neutrophil extracellular traps (NETs). Here, nuclear and mitochondrial DNA is released to the extracellular space in an active process that involves the activation of NADPH oxidase, histone hypercitrullination, and decondensation of chromatin (30–32). These NETs are comprised of DNA strands and filaments containing high local concentrations of formerly intracellular antimicrobial proteins (33). By undergoing this process, often referred to as NETosis, neutrophils can immobilize vast amounts of microorganisms that would otherwise overwhelm their phagocytosis capacity and at the same time prevent their further spread into the environment and blood stream. NETosis triggers include bacterial cell wall components that activate complement receptors, Fc receptors, or Toll-like receptors on neutrophil surfaces (34). While several groups could show that NETosis leads to cell death, others have suggested that neutrophils remain viable and functional, specifically after mere release of mitochondrial DNA (35–37).

Neutrophils are crucial to the host's integrity, and impaired neutrophil function or neutropenia leads to severe disorders such as Chédiak-Higashi syndrome or Kostmann syndrome (38). In the oral cavity, neutrophils are signaled to the sites of bacterial invasion by a multitude of chemoattractants such as interleukin 8 (IL-8), complement fragment C5a, or chemokine CXCL5 (39–41). They migrate through the junctional epithelium, where they are abundantly found and appear in the gingival sulcus and in gingival crevicular fluid (GCF). In saliva, they were found to retain their phagocytic function, and their ability to generate ROS. However, phagocytosis seems to be reduced in saliva, whereas hydrogen peroxide production is increased (42–44). NETs containing trapped bacteria have been described within the gingival crevice, in purulent periodontal pockets and on the surface of gingival epithelial cells. Here, they presumably have a role in protecting epithelial cells from being harmed and invaded by bacteria and their proteases (45–47). On condition that neutrophils and NETs do not occur excessively and are rapidly cleared, relatively little damage to the adjacent tissues is induced (48, 49).

Nevertheless, it has been widely reported that neutrophils can be responsible for both host defense and host tissue damage. Various experimental and clinical studies have demonstrated a role of neutrophils in periodontal tissue destruction, which is the leading symptom of

periodontitis. This injury occurs through the release of proteolytic and collagenolytic enzymes as well as ROS within or near host tissues, often leading to extracellular matrix degradation and lingering inflammation (50–56). Connective tissue degradation might be understood as a mechanism of loosening up dense connective tissues and extracellular matrix, allowing for fast transmigration of leukocytes and cells involved in wound healing (57–59). On the other hand, periodontitis is a chronic inflammatory disease triggered by persisting biofilms. Hence, inflammation overweighs resolution, and host tissue destruction becomes progressive, eventually resulting in pathological osteolysis and tooth loss.

Neutrophil strategies against biofilms

Contrary to earlier presumptions, biofilms are not inherently protected from neutrophil assault. Neutrophils are attracted towards biofilms through chemotactically active molecules released by several immunocompetent cells adjacent to biofilms, such as epithelial cells or neutrophils themselves (60, 61). Moreover, small QS molecules of the *N*-acyl homoserine lactone (AHL) family as well as bacteria-derived formyl-Met–Leu–Phe act as potent chemoattractants for neutrophils (62, 63). The recognition of biofilms by neutrophils is mediated by a wide array of receptors to lipopolysaccharides, peptidoglycans, microbial DNA, and other pathogen-associated molecular patterns (PAMPs) (64–66). Subsequently, activated neutrophils migrate towards attached microbes and have been localized within, on, and around biofilms in several *in vitro* and *in vivo* studies. Here, neutrophils accumulate and react towards biofilms with phagocytosis, degranulation, and NETosis. Moreover, the generation of extracellular ROS is a strategy for attacking biofilms (67–74).

There have been a number of studies investigating neutrophil–biofilm interactions focusing on *Pseudomonas aeruginosa* and staphylococcal biofilms, as they are clinically relevant in several human infections and widely used as biofilm models (75, 76). Jesaitis et al. found that neutrophils settled on *P. aeruginosa* biofilms, where they underwent degranulation, became immobilized by loss of their pseudopodia, and showed membrane rearrangement on the side adjacent to bacteria. Whilst attached to the biofilms, they became enveloped by planktonic bacteria but still retained their phagocytic activity. At the same time, oxygen consumption within the biofilm was increased due to neutrophil oxidative burst (77). A further group demonstrated that collectin surfactant protein D, a soluble pattern recognition receptor present on mucosal surfaces, assists bacterial binding of NETs by microagglutinating *P. aeruginosa* and binding to NET fragments simultaneously. This NET opsonization also led to enhanced clearance of extracellular DNA by macrophages and reduced the generation of anti-DNA autoantibodies (78, 79).

Phagocytosis is a very effective strategy of neutrophils to eliminate single bacteria or small bacterial aggregates. Also, 2–6 days old *Staphylococcus aureus* biofilms were shown to be cleared by neutrophil phagocytosis as they moved over and into the biofilms (80). In a follow-up study by the same group investigating *S. epidermidis* biofilms, neutrophils failed to move over them and adhered less compared to *S. aureus*. Accordingly, *S. epidermidis* had little susceptibility to phagocytic destruction by neutrophils, supposedly due to higher mechanical biofilm stability (81). These findings were enhanced by data confirming neutrophil adherence to *S. aureus* biofilms and their accumulation in the creases and channels of the biofilms, followed by neutrophil penetration. However, they could not provide evidence for phagocytosis (68). Stroh et al. reported that *S. aureus* biofilm clearance by neutrophils and ROS production was significantly enhanced when biofilms had been opsonized by IgG, whereas Meyle et al. observed degranulation as a response to *S. aureus* biofilms as confirmed by measurement of released lactoferrin and elastase (72, 82). Also, these biofilms as well as biofilms formed by oral bacteria and *P. aeruginosa* could be reduced in several studies by applying the AMPs lactoferrin and LL-37, both of which are present in the secondary granules of neutrophils (83–87).

When biofilm masses form, however, phagocytosis seems to be limited. Instead, neutrophils form a physical barrier on the surface of biofilms, as was shown by means of electron microscopy. At these sites of neutrophil accumulation and frustrated phagocytosis, neutrophil intracellular contents such as AMPs, ROS, and proinflammatory substances are often released, harming the superficial layers of the biofilm, but as well host tissues (88).

Biofilm strategies against neutrophils

In reverse, microbes in biofilms have an arsenal of self-defense mechanisms, which can be either directed against neutrophils or camouflage the biofilm. Jensen et al. described in 2007 that QS molecules promote the production of bacterial surfactants (rhamnolipids) by *P. aeruginosa* biofilms, which then cause rapid cell death in neutrophils (89). Similar events were shown in planktonic cultures of *S. aureus* and *Aggregatibacter actinomycetemcomitans*, where bacterial toxins induced lysis and degranulation of neutrophils (90–92). Besides directly attacking neutrophils, bacteria in biofilms can render themselves resistant to neutrophil-mediated killing by disguising their immunogenicity. This was reported for *in vivo* and *in vitro* *Haemophilus influenzae* biofilms containing NETs, in which these biofilms persisted without being harmed. This is presumably due to their expression of certain lipooligosaccharide glycoforms, which shield PAMPs and thus inhibit recognition and opsonization and can provide protection against antimicrobial peptides (93–96). Also *S. epidermidis* biofilms expressing

poly-*N*-acetylglucosamine seem to be protected against opsonic phagocytosis and can reduce IgG and complement component C3b binding, allowing these bacteria to escape from neutrophil assault (97, 98). Experimental results published by Bjarnsholt et al. provided evidence for QS molecules being controllers of neutrophil ROS response and penetration into *P. aeruginosa* biofilms (99). In this study, QS-deficient biofilms were more susceptible than wild-type biofilms to ROS and to phagocytic clearance *in vitro* and *in vivo*, suggesting that QS molecules can dampen immune response. *P. aeruginosa* biofilms also showed mutations in the *mucA* gene upon stimulation with neutrophils and ROS, which led to enhanced mucus production in these biofilms, providing protection against ROS (100).

Further evasion strategies have been shown in multiple studies, although these are often referring to planktonically grown bacteria. Nevertheless, these data can be equally useful for understanding possible mechanisms of host–biofilm interactions as it is likely that some of these interactions occur in biofilms as well. A suitable example is the alteration of gene expression in host cells caused by bacteria, for instance by *Fusobacterium nucleatum*, which is present in early and mature dental biofilms (101). In this study by Wright et al., *F. nucleatum* upregulated the expression of stress-response genes and those encoding enzymes involved in removal of ROS. As suggested by the authors, the latter may be the neutrophils' response to its own bacteria-induced ROS production, which was detected by ROS assays in these experiments. However, the induction of cell-protective molecules is also likely to thwart injury of microorganisms. One important microbial defense mechanism is the evasion of phagocytosis. This was demonstrated for biofilm-forming *Prevotella* strains, which were recognized by neutrophils but not phagocytosed, depending on whether they produced mannose-rich exopolysaccharides as part of their extracellular matrix (102). Moreover, serotypes expressing capsules were more protected against being trapped by NETs than non-capsulated strains in this study. Another effective defense mechanism was demonstrated by a Swedish group for pneumococci (103). Here, the bacteria were able to add a positive charge to their cell surface and thus to protect themselves from being lysed by cationic AMPs, which are thought to intrude bacteria by interacting with their more negatively charged membrane (104). Despite the highly cytotoxic content of neutrophils, some bacteria are even able to survive within neutrophils after being phagocytosed, like *S. aureus* (105). Also *Escherichia coli* expressing antigen 43, which allows them to form tight multicellular aggregates and biofilms, was found to survive within neutrophils in clusters (106, 107). However, *S. aureus* and other bacteria discussed in this chapter are known to be potent triggers

for NETosis and degranulation. Therefore, it can be assumed that implementation of such survival strategies coexists with elimination of bacteria by neutrophils.

After all, it is important to be aware of the fact that notably periodontal non-saccharolytic and proteolytic bacteria profit from increased nutrient availability in GCF, saliva, and soft tissues as a consequence of the inflammatory response (108). It seems reasonable that they have developed strategies to manipulate host immune cells in order to maintain inflammation. For instance, *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 (109–112). 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 (113). Fig. 1 summarizes the above-mentioned reciprocal interactions between neutrophils and biofilms and gives an overview of the factors involved.

Do neutrophils promote or curb biofilm infections?

Biofilms often cannot be fully eliminated, leading to persistence of neutrophils at sites of biofilm adherence, where they continue to release granule contents that eventually cause host tissue damage. As proposed in several publications, it is likely that the extent of neutrophil (hyper)reactivity determines whether host tissues are injured and whether repair mechanisms can compensate for such damage (33, 114). Nonetheless, it has become clear that neutrophils and NETs have a distinct role in promoting inflammation and supporting biofilms. In cystic fibrosis, neutrophils may even serve as a source of extracellular biofilm matrix enhancing *P. aeruginosa* biofilm development, as neutrophil-derived F-actin and DNA polymers are promoters of bacterial attachment (115). Neutrophils could also mediate competitive interactions between bacterial species colonizing mucosal surfaces, thus leading to species selection and result in the establishment of persisting biofilms (70, 116). As some biofilm-associated inflammations occur in loci with little oxygen flow, it is important to raise the question whether neutrophils are able to perform under anaerobic conditions. For instance, polymicrobial biofilms in periodontitis often colonize low-oxygenated niches within periodontal pockets, whereas neutrophils require oxygen for many of their physiological functions. However, already in the 1980s, it was shown that neutrophils can carry out bacterial killing by oxygen-independent mechanisms involving the activity of AMPs, which may be leveraged in such environments (117–120). Later on, Jensen et al. argued that neutrophils may even promote the formation of

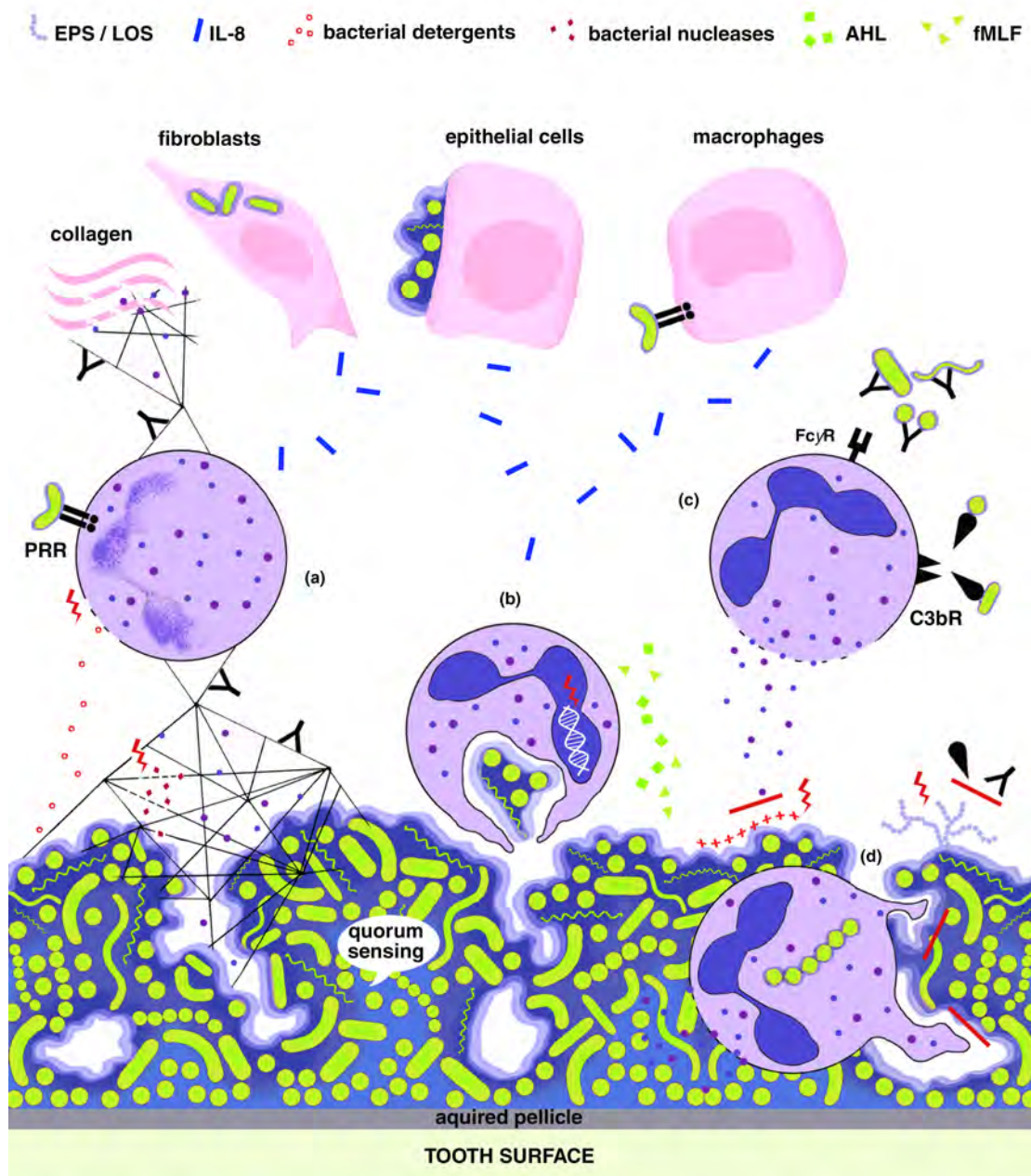


Fig. 1. A depiction of some important interaction mechanisms between neutrophils and biofilms in the oral cavity. Microbial adhesion to tooth surfaces and to epithelial cells, the invasion of fibroblasts and recognition by innate immune cells lead to the release of different chemotactic factors, e.g. IL-8, whereas biofilms produce chemotactically active molecules [exemplified with *N*-acyl homoserine lactone (AHL) and *N*-formyl-Met-Leu-Phe (fMLF)] as well. Recognition of PAMPs and antigens by neutrophils is mediated by various receptors that bind to opsonized or unopsonized biofilm components (PRR = pattern recognition receptor, Fc γ R = immunoglobulin G receptor, C3bR = complement component 3b receptor). Biofilms, however, are able to shield themselves from being recognized by expressing certain exopolysaccharides (EPSs) or lipooligosaccharides (LOSs). Upon stimulation, neutrophils respond by NETosis (a), phagocytosis (b) or degranulation (c,d), although biofilms are often protected against phagocytosis and some microbes can render themselves unsusceptible to cationic AMPs by adding positive charge to their surfaces. Instead, several bacteria are able to invade neutrophils and survive intracellularly (d) or may influence gene expression (b). Adversely, NETs are thought to stabilize biofilm structures on the one hand, but are cleaved by bacterial nucleases on the other hand. Autoantibodies against NETs presumably promote inflammation, whereas NET clearance by other phagocytes could be facilitated at the same time. Neutrophil antimicrobial granule contents and ROS (blue and purple dots) frequently also lead to collagen degradation and subsequent host tissue injury, enhanced by microbial detergent molecules that rapidly lyse neutrophil membranes (a).

anaerobic habitats by oxygen exhaustion during oxidative burst (121). Hence, this originally considered bactericidal property may become biofilm-promoting in periodontal pockets. This underlines the adaptive forces and the successful survivalist responses of microbes as they interact with host cells and fluids.

The host–biofilm interface is an extremely complex functional entity with reciprocal actions carried out that depend on numerous environmental factors. In this review, antimicrobial mechanisms of neutrophils against biofilms and the opposing biofilm defense strategies were summarized. It has become apparent that neutrophils act against biofilms by phagocytosis, NET release and degranulation after chemotactically migrating into them. If in the hyperactive phenotype phase or present in excessive numbers, they can cause considerable harm to the host. On the other hand, the antineutrophil strategies of biofilms are manifold and very effective. For instance, biofilms contain bacterial nucleases that may help microorganisms to release themselves from NETs and survive (122, 123). Interestingly, this can be useful for the host likewise, since efficient clearance of NETs is highly important for tissue integrity. By helping to recruit neutrophils to the sites of biofilm attachment through QS molecules, biofilms can profit from the increased nutrient supply brought along by inflammation, but they also promote their own elimination. At the same time, neutrophil-derived serine proteases such as elastase and cathepsin G seem to have a role in controlling inflammation, as many proinflammatory cytokines are inactivated by these broad-spectrum enzymes (124, 125).

These examples show that both sides have ambivalent roles. However, pathogenic biofilms such as in periodontitis or in prosthetic joint infection are adverse to the host and require elimination. Importantly, bacterial virulence factors as well as host factors like genetic predisposition and environmental aspects determine whether dynamic interactions between neutrophils and biofilms become imbalanced. Neutrophils constitute indispensable biofilm controllers that, along with other defense mechanisms, can protect the host from severe infections and prevent biofilm overgrowth on tissue surfaces. Nevertheless, they may contribute to biofilm stability and natural selection of more resistant biofilms. Favorably, neutrophils will either help control and defeat biofilms or they may shift the biofilms towards a more symbiotic and protective form, where microorganisms elicit a lesser immune response and express fewer PAMPs and antigens.

Conclusion

Although neutrophils have been a subject of research for more than a century, novel functions and pathways continue to be discovered (126, 127). There are, however, still unresolved issues such as the identification of neu-

trophil subsets and their possible functional consequences (128). In biofilm-related research, great efforts and advances have been made in the past years (129). Nevertheless, to date, not only are inter-biofilm processes weakly understood, but also the interaction patterns between hosts and biofilms. The investigation of host–biofilm interactions constitutes a multifaceted challenge, due to the complexities of both the biofilms and the host responses (130). The issue is further complicated by their reciprocal interplay, which appears to be largely heterogeneous *in vivo*, as individual host factors as well as environmental influences presumably play a role in host–biofilm communication. Moreover, many pathogens have an optimal growth temperature and gene expression patterns only in their natural niche (131). In general, biofilms are more difficult to grow under laboratory conditions than planktonic cultures of bacteria. Thus, current biofilm models reach their limits when addressing complex questions of immune responses in biofilm infections (132). Especially chronic biofilm infections cannot be properly studied using short-duration experiments, which are commonly applied. The outcomes of *in vitro* experiments are also often limited by laboratory techniques that do not allow for the creation of physiologically relevant microhabitats (133). Besides, about 35% of oral bacteria have not been cultivated yet in the laboratory as they are highly fastidious and have complex environmental requirements (134).

In order to fully understand the specific role of neutrophils in biofilm formation and microbial colonization of host surfaces, light needs to be shed on different aspects of neutrophil-biofilm crosstalk. For example, gene expression alterations in neutrophils evoked by biofilms and vice versa or possible effects of signaling molecules on neutrophil function and biofilm composition would be important research target areas. Especially for investigations of oral host–biofilm interactions, advanced methods are needed that allow for *in vitro* cocultures of host cells and complex multispecies biofilms, ideally including bacteria, fungi, and viruses, as only their interplay may evoke those biological reactions found in the oral cavity. Also, aspects of evolution are important to consider in these investigations, because chronicity of infections may result from long-lasting interactions and co-evolution of microorganisms and hosts (135). It can be speculated that the biofilm-promotive and tissue-destructive features of neutrophils discussed in this review may be part of an ongoing evolutionary adaption process, where host and microbes are still striving for a functioning co-existence. The health-threatening biofilm infections as well as the observation of differences between individuals in susceptibility to chronic and acute biofilm infections may be a result of this continuous adaption (136).

Even though neutrophils can have important interactions with (oral) biofilms, they are not the only host cells

attempting to control biofilms. Other cell types, including epithelial cells, fibroblasts, macrophages, and effector cells of acquired immunity are equally important in maintaining homeostasis (137, 138). It seems likely that neutrophils play a co-controlling part and that only the synergy of the host's defense arsenal can provide a peaceful co-existence of the host and its commensal biofilms. Chronic infections driven by pathogenic biofilms indicate, however, that the immune system fails to fully protect the host.

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