Review Article

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Innate immune response to oral bacteria and the immune evasive characteristics of periodontal pathogens

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Periodontitis is a chronic inflammation of periodontal tissue caused by subgingival plaque-associated bacteria. Periodontitis has long been understood to be the result of an excessive host response to plaque bacteria. In addition, periodontal pathogens have been regarded as the causative agents that induce a hyperinflammatory response from the host. In this brief review, host-microbe interaction of nonperiodontopathic versus periodontopathic bacteria with innate immune components encountered in the gingival sulcus will be described. In particular, we will describe the susceptibility of these microbes to antimicrobial peptides (AMPs) and phagocytosis by neutrophils, the induction of tissue-destructive mediators from neutrophils, the induction of AMPs and interleukin (IL)-8 from gingival epithelial cells, and the pattern recognition receptors that mediate the regulation of AMPs and IL-8 in gingival epithelial cells. This review indicates that true periodontal pathogens are poor activators/suppressors of a host immune response, and they evade host defense mechanisms.

Keywords: Epithelial cells, Host-pathogen interactions, Immune evasion, Neutrophils, Perodontitis.

INTRODUCTION

Periodontitis is the inflammation of periodontal tissue caused by subgingival plaque bacteria, leading to alveolar bone destruction [1]. Periodontitis affects about half of the adult population worldwide [2]. Periodontitis is not only a prevalent oral disease but also a risk factor for cardiovascular disease, pulmonary disease, and preterm birth [3,4]. Periodontitis is a complex disease involving microbial, host, and environmental factors in its development [1]. The complexity of microbial factors alone is overwhelming, not to mention the complex host-microbial interactions. Therefore, the pathogenesis of periodontitis is difficult to understand. For decades, periodontitis has been regarded as the result of hyperimmune or hyperinflammatory responses to plaque bacteria [5-7]. In addition, it has been widely accepted that periodontal pathogens induce hyperinflammatory responses, whereas commensal bacteria are well tolerated [8]. However, recent studies indicate that periodontal pathogens are poor activators and/or suppressors of host immune responses, evading host defense mechanisms. The immune-evasive characteristics of periodontal pathogens will be reviewed in this article.

DEFENSE MECHANISMS IN THE GINGIVAL SULCUS

The gingival sulcus is a unique anatomic site surrounded by

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hard tissue on one side and soft tissue on the other side. Approximately 700 bacterial species can colonize the gingival sulcus in varying quantities, from approximately 10^3 in healthy sulci to greater than 10^8 in pathologic pockets [9,10]. Although more than thousands of bacteria continually colonize subgingival sites, most periodontal sites in most individuals do not exhibit new loss of periodontal tissue until 35 years of age [10]. Gingival epithelia form barriers between plaque bacteria and gingival tissue, providing the first line of defense against plaque bacteria.

The epithelial barrier consists of physical, chemical, and immunological barriers [11]. Physical barriers are created by the unique architectural integrity of the stratified gingival epithelia, where epithelial cells are adjoined by tight junction-related structures and adhering junctions [12,13]. Chemical barriers are mainly formed by a variety of antimicrobial peptides (AMPs) [14]. AMPs, referred to as endogenously produced antibiotics, are cationic peptides with an amphipathic structure. They have a broad spectrum of antimicrobial activity; thus, they contribute to controlling the bacterial load in the gingival sulcus [14,15]. They function by associating with the anionic microbial structure, and then aggregate to form pores in the microbial membranes [16]. Defensins and a cathelicidin are major AMPs detected in the oral cavity. Defensins are subdivided into two families, α-defensins (human neutrophils peptides) that are essentially found in neutrophils and human β-defensins (HBDs) that are generally found in association with oral epithelium [17]. LL-37, the only human member belonging to the cathelicidin family, is produced by epithelial cells and neutrophils [17]. The lack of LL-37 in saliva and neutrophils observed in patients with Kostmann syndrome who develop severe periodontitis in young adulthood underscores the importance of chemical barriers [18,19].

The immunological barriers of gingival epithelia are provided by neutrophils, T cells, dendritic cells, macrophages, and mast cells distributed within the epithelia, lamina propria, and gingival sulcus [20]. Neutrophils are a particularly predominant cell type observed in the subgingival sulcus and in the gingival crevicular fluid [21]. Neutrophils are guided into the gingival sulcus from the capillary beds of the connective tissue by a gradient of interleukin (IL)-8 that is produced by epithelial cells. Neutrophils recruited to the gingival sulcus actively phagocytose plaque bacteria [21]. The presence of neutrophils in clinically healthy gingival mucosa differentiates it from other mucosa of the body. This infiltration of percolating immune cells has been explained by the theory that particular subgingival bacteria induce the characteristic epithelial cell IL-8 gradient and promote a mild inflammatory infiltrate in clinically healthy gingival tissue [21]. Neutrophils also produce LL-37 and human neutrophil defensins [22]. Various conditions that accompany abnormalities in the number or function of neutrophils, including chronic/cyclic neutropenia, leukocyte adhesion deficiency syndrome, Papillon-Lefevre syndrome, and Chediak-Higashi syndrome, are associated with severe periodontitis [23-28].

SUSCEPTIBILITY OF NONPERIODONTOPATHIC AND PERIODONTOPATHIC BACTERIA TO DEFENSE MECHANISMS IN THE GINGIVAL SULCUS

Resistance to killing by host immune machineries is one of the pathogenic mechanisms of many persistent pathogens. There are several reports on the susceptibility of oral bacteria to AMPs such as HBDs, HNPs, and LL-37, but most studies have focused on periodontopathic and cariogenic bacteria [29-34]. We hypothesized that features differentiating periodontopathic bacteria from nonperiodontopathic bacteria may provide new insights into the pathogenesis of periodontitis. To address the potential role of immune evasion in the pathogenesis of periodontitis, we compared the susceptibility of nonperiodontopathic and periodontopathic bacteria to major defense mechanisms for bacterial clearance in the gingival sulcus: phagocytosis by human neutrophils and inhibition by AMPs LL-37 and HBD-3. Four species of nonperiodontopathic bacteria were selected from members of the genera *Streptococcus*, *Actinomyces*, and *Veillonella*, which are all early colonizers and are thought to be compatible with periodontal health [35]. Nine species of periodontopathic bacteria, including *Eikenella corrodens*, *Fusobacterium nucleatum*, *Prevotella nigrescens*, *Prevotella intermedia*, *Peptostreptococcus micros*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans*, were chosen from the list of bacteria that are known to be associated with periodontitis, that is, bacteria that accumulate with increasing pocket depth. The susceptibilities of oral bacteria to phagocytosis and AMPs were quite variable, depending on the species. Although the periodontopathic group was more resistant to phagocytosis than the nonperiodontopathic group, a significant difference in susceptibility to AMPs was not observed. When the bacteria were grouped by the modified Socransky classification [36] into early colonizers, the orange complex, the red complex, and other (*A. actinomycetemcomitans*), differences in susceptibility to phagocytosis or AMPs were more evident. The late colonizing red complex bacteria were more resistant to both phagocytosis and LL-37 than the others. In addition, two out of three strains of *A. actinomycetemcomitans* presented overall resistance to phagocytosis and AMPs [37]. This study showed that immune evasion is an important feature of true periodontal pathogens

such as the red complex triad and *A. actinomycetemcomitans*. Meanwhile, the increase in the orange complex bacteria observed in periodontal lesions must be the result of disease rather than the cause of disease. In addition, proper control of the orange complex bacteria in the gingival sulcus, particularly, *F. nucleatum* and *P. intermedia* that were highly sensitive to both phagocytosis and AMPs, would be critical to maintaining periodontal health by preventing colonization of resistant periodontal pathogens.

INDUCTION OF TISSUE-DESTRUCTIVE MOLECULES FROM HUMAN NEUTROPHILS BY NONPERIODONTOPATHIC AND PERIODONTOPATHIC BACTERIA

The neutrophil is often referred to as a double-edged-sword: crucial for defense against sub-gingival microbes but also involved in periodontal tissue destruction. The infiltration of neutrophils increases in periodontal lesions, and activated neutrophils release a variety of tissue-damaging molecules including elastase, matrix metalloproteinases (MMPs), reactive oxygen species (ROS), and inflammatory cytokines [21,38-40]. The induction of the release of tissue-destructive molecules from neutrophils by periodontal pathogens was repeatedly reported as one of the pathogenic mechanisms that leads to periodontal destruction [41-47]. However, when we compared the ability of periodontopathic bacteria (*F. nucleatum* and *T. denticola*) to induce the release of tissue-destructive molecules, including ROS, MMP-8, and IL-1β, from neutrophils, with that of a nonperiodontopathic species (*S. sanguinis*), *S. sanguinis* was the most potent inducer and *T. denticola* was the least [48]. We also found that the levels of tissue-destructive molecules produced by neutrophils are positively correlated with the degree of phagocytosis. Therefore, the ability of oral bacteria to induce tissue-destructive molecules from neutrophils is associated with the extent of phagocytosis rather than with the pathogenicity of the bacteria, and it is not an inherent characteristic of periodontopathic bacteria [48]. Considering the resistance of periodontal pathogens to phagocytosis by neutrophils [37], not only *T. denticola* but also other periodontal pathogens are expected to induce relatively low levels of tissue-destructive molecules from neutrophils.

INNATE IMMUNE RESPONSE OF GINGIVAL EPITHELIAL CELLS TO NONPERIODONTOPATHIC AND PERIODONTOPATHIC BACTERIA

Gingival epithelium actively participates in innate immune protection by secreting AMPs and IL-8, a chemoattractant to neutrophils [49]. In addition, gingival epithelial cells secrete inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1α, and IL-1β. The epithelia of many body sites express HBD-2 and -3 only under conditions of an infection or inflammation [50]; however, clinically healthy gingival epithelium is characterized by the presence of HBD-2 and a gradient of IL-8 that guides the transmigration of neutrophils through the junctional epithelium, presumably due to the constant exposure to oral bacteria [50,51].

Periodontal pathogens may also present differences in their ability to induce immune responses from the host; thus, we evaluated the effects of various oral bacteria on the expression of AMPs and IL-8 by gingival epithelial cells. Nonperiodontopathic early colonizing bacteria (*S. sanguinis*, *S. gordonii*, and *V. atypica*) up-regulated some AMPs without affecting the levels of IL-8. Among the periodontopathic bacteria, the orange complex bacteria *F. nucleatum* and *P. intermedia* induced AMPs and IL-8 most efficiently, whereas the red complex triads rather suppressed the expression of HBDs and IL-8, again presenting immune-evasive characteristics [52]. The down-regulation of IL-8 by *P. gingivalis* at both the mRNA and protein levels, a so-called "chemokine paralysis," has been well characterized [53,54]. In our study, the red complex bacteria down-regulated not only IL-8 but also HBD-3 [52]. Through down-regulation of IL-8 and HBD-3, red complex bacteria are expected to perturb the immunological and chemical barriers. In addition to killing microorganisms, HBDs affect the adaptive immune response by selectively recruiting immature dendritic and memory T cells to the site of microbial invasion [55] and the down-regulation of HBD-3 may affect the overall innate and adaptive immune responses.

Taken together with the result of susceptibility of oral bacteria to AMPs and phagocytosis by neutrophils [37,52], early colonizers induced AMPs but not IL-8 from gingival epithelial cells and had susceptibility to those peptides and phagocytosis at intermediate levels. Therefore, the early colonizers seem to be in balance with the host defense system. Bridging colonizers that belong to the orange complex efficiently induced both AMPs and IL-8, and these bacteria were highly susceptible to the two killing mechanisms. The bridging colonizers may be sensed as a threat by the host and induce active clearance by the host. In contrast, late colonizing periodontal pathogens suppressed the expression of AMP and IL-8 and were often resistant to AMPs and phagocytosis; thus they may easily secure their niches once they colonize and provide a favorable environment for other anaerobes.

TOLL-LIKE RECEPTOR 2 IS A MAJOR PATTERN RECOGNITION RECEPTOR THAT MEDIATES ORAL BACTERIA-INDUCED REGULATION OF HBD EXPRESSION IN GINGIVAL EPITHELIAL CELLS

At the receptor level, induction of HBD-2 expression is mediated via pattern recognition receptors (PRRs) and proinflammatory cytokine receptors that respond to cytokines such as TNF-α, IL-1, and IL-17 [56]. The regulation of HBD expression by gingival epithelial cells in response to bacteria, which is a part of the innate immune response, must be initiated by the recognition of unique microbial molecular patterns by PRRs. An array of PRRs is distributed on the surface, in the cytoplasm, and in the endosomal compartments of host cells. Toll-like receptors (TLRs) are expressed either on the cell surface (TLR1, 2, 4, 5, 6, and 10) or in the endosomal compartments (TLR3, 7, and 9) [57,58]. In contrast, new families of PRRs, the nucleotide-binding oligomerization domain (NOD)-like receptors and retinoic acid-inducible gene-I-like receptors, have been characterized as cytoplasmic microbe sensors [48]. TLR1, 2, 4, 5, 6, and 9 are involved in the recognition of bacterial components such as lipoproteins, lipopolysaccharide, flagellin, and DNA [58]. NOD1 and NOD2 recognize peptidoglycan. NACHT, LRR, and pyrin domain-containing protein (NALP) 3 is activated in response to bacterial pore-forming toxins and bacterial mRNA [59]. In addition, the cytoplasmic DNA sensors, AIM2 and DAI, are involved in the recognition of bacterial dsDNA in the cytoplasm [60,61]. Compared to peripheral blood mononuclear cells, unstimulated gingival epithelial cells express a limited repertoire of PRRs, and predominantly express NALP2 and TLR2 [62].

We characterized the PRRs and regulatory mechanisms involved in the regulation of HBD-2 and -3 expression by *F. nucleatum* in gingival epithelial cells. Lipopolysaccharide from *F. nucleatum*, a ligand to TLR4, was known to be a poor inducer of HBD-2 from cultured human oral keratinocytes [63], excluding the role of TLR4 in HBD-2 induction. The knockdown of NALP2 by RNA interference (RNAi) significantly reduced the *F. nucleatum*-induced upregulation of HBD-3 but not HBD-2 or IL-8. In addition, knockdown of TLR2 RNA reduced the *F. nucleatum*-induced upregulation of HBD-2 and -3, but not IL-8. These data showed that TLR2 and NALP2 mediate the induction of HBDs by *F. nucleatum* in gingival epithelial cells [62]. The TLR2-mediated up-regulation of HBD-2 and -3 was confirmed in another laboratory using *F. nucleatum* cell wall extracts [64]. Using blocking antibodies, Lu et al. [65] showed that up-regulation of HBD-2 by *P. gingivalis* LPS_{1690} with a penta-acylated lipid A structure involves both TLR2 and TLR4. The authors proposed cooperation of TLR2 and TLR4 in the modulation of HBD-2 by *P. gingivalis* LPS_{1690}. Although *P. gingivalis* LPS had been shown to activate both TLR2 and TLR4 [66], a lipoprotein contaminant in the LPS preparation from *P. gingivalis* turned out to be a principal component for TLR2 activation [67]. Therefore, TLR2 seems to be a major PRR that mediates bacteria-induced upregulation of HBDs in gingival epithelial cells. In addition to PRRs, protease activated receptor 2 has been shown to be partially involved in the up-regulation of HBD-2 by *P. gingivalis* [68].

When the ability of several oral bacterial species to activate TLR2 was examined by using the CHO/CD14/TLR2 reporter cell line, nonperiodontopathic early colonizing bacteria (*S. sanguinis*, *S. gordonii*, and *V. atypica*) and two bridging colonizers, *F. nucleatum* and *P. intermedia*, substantially activated TLR2 in a dose-dependent manner. However, late colonizing periodontal pathogens, *P. gingivalis*, *T. forsythia*, and *T. denticola*, did not activate TLR2 [69]. These results coincide with the previous observation that these bacteria did not induce, but rather suppressed the expression of HBDs [52].

The mechanism(s) involved in the suppression of HBD expression in gingival epithelial cells were investigated using *T. denticola*, the most prominent suppressor. In contrast to live *T. denticola*, which suppressed the expression of HBD-2 and -3, heat-killed bacteria did not produce a suppressive effect but instead slightly upregulated the levels of HBD-2 and -3 [69]. In addition, heat-killed *T. denticola* or bacterial lysate, but not live bacteria, could activate TLR2 in CHO/CD14/TLR2 reporter cells, suggesting that live *T. denticola* contains a heat-labile inhibitor(s) of TLR2 in addition to ligands recognized by TLR2. Live *T. denticola*, but not *T. denticola* lysate, was able to inhibit TLR2 activation by Pam3CSK, indicating that the heat-labile inhibitor(s) are not TLR2 antagonists. Knockdown of TLR2 via RNAi abolished the suppressive effect of *T. denticola* on the expression of HBD-2 and HBD-3 [69,70]. Collectively, *T. denticola* suppresses the expression of HBDs in gingival epithelial cells through a heat-labile inhibitor(s) of TLR2-signaling axis. The reversal of suppressive effects of *T. denticola* by TLR2 RNAi suggests that there is an endogenous TLR2 ligand in the control culture without bacteria. HBD-3 is known as a ligand for TLR1/TLR2 [71]. Thus, the interaction of HBD-3 and TLR2 in gingival epithelial cells is expected to form a positive feedback loop. Indeed, the basal level of HBD-3 expression rapidly increased within 3 hours of culture in the control cells [69]. Peyret-Lacombe et al. [64] suggested a role of TLR2 in the down-regulation of HBD-3, IL-8, and MMP-9 induced by *S. sanguinis* extracts. Whether *S. sanguinis* contains antagonist(s) to TLR2 or inhibitor(s) of the TLR2-signaling axis is not clear.

Signaling pathways involved in the expression of HBDs have also been studied. The promoter region of HBD-2 con-

tains numerous regulatory elements, including the binding sites for nuclear factor-kappaB (NF-κB), activator protein (AP)-1, AP-2, and NF–IL-6, whereas the promoter of HBD-3 contains no discernible NF-κB binding elements [72]. Chung and Dale [73] reported that commensal and periodontopathic bacteria utilized different signaling pathways in the induction of HBD-2. In contrast to commensal *S. gordonii* that used Jun N-terminal kinase (JNK) and p38 mitogen activated protein kinases (MAPKs), but not NF-κB, to induce HBD-2 from gingival epithelial cells, periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* used both MAPKs and NF-κB. HBD-3 induction by *Staphylococcus aureus* in skin keratinocytes was shown to involve p38 and AP-1 [74]. In contrast to live *F. nucleatum* that upregulated both HBD-2 and -3, heat-killed bacteria upregulated only HBD-3 at a reduced level [62]. Although heat-killed bacteria activated NF-κB, p38, and JNK, the activation of JNK was significantly reduced compared to activation by live bacteria [62]. Krisanaprakornkit et al. [75] reported that p38 and JNK, but not NF-κB, are involved in the induction of HBD-2 by *F. nucleatum*. Therefore, the reduced JNK activation may be responsible for the inability of heat-killed *F. nucleatum* to induce HBD-2, suggesting a higher threshold of JNK activation for HBD-2 induction than for that of HBD-3. It should be emphasized that in our previous observation, only two out of eight bacterial species induced HBD-2, whereas five species induced HBD-3 [52]. The differential regulation of HBD-2 and -3 may contribute to their different locations in the oral epithelia: the differentiated granular layers versus the basal and spinous layers [76].

TLR 9 MEDIATES ORAL BACTERIA-INDUCED IL-8 EXPRESSION IN GINGIVAL EPITHELIAL CELLS

A barrier formed by neutrophils against plaque-associated bacteria in the gingival sulcus plays a critical role in the maintenance of periodontal health. The migration of neutrophils into the gingival sulcus is guided by IL-8, the chemokine produced by gingival epithelial cells [21]. We already mentioned that various oral bacteria have different abilities to induce IL-8 production in gingival epithelial cells [52]. It is important to dissect molecular mechanisms for the regulation of IL-8 expression in response to bacterial challenge. Asai et al. [77] showed that TLR2 mediates IL-8 induction by *S. aureus* peptidoglycan, N-acetylmuranyl-L-alanyl-D-isoglutamine, and *P. gingivalis*, utilizing a monoclonal antibody against TLR2. However, it is now known that peptidoglycan and N-acetylmuranyl-L-alanyl-D-isoglutamine are recognized by NOD1/2 and not by TLR2 [58,59]. Furthermore, IL-8 induction by *F. nucleatum* was not affected by the knockdown of the TLR2 protein [62]. To characterize the PRR(s) that mediate bacteria-induced IL-8 expression, we tested several ligands that mimic bacterial microbe-associated molecular patterns for their ability to induce IL-8 in human oral keratinocytes (HOK-16B) cells. In repeated experiments, only a TLR9 ligand, CpG oligonucleotide, significantly induced IL-8 [78]. An endosomal acidification blocker or a TLR9 antagonist inhibited the IL-8 induction by two potent strains, *F. nucleatum* ATCC 25586 and *P. gingivalis* ATCC 49417. As TLR9 is located in the endosomal compartments of gingival epithelial cells, to induce IL-8 production by TLR9 located in epithelial cells, oral bacteria must be able to invade epithelial cells and they must have DNA with immunostimulatory activity. The ability of eight strains of four oral bacterial species to induce IL-8 expression in HOK-16B cells showed a strong positive correlation with their invasion index and also with the immunostimulatory activity of their bacterial DNA [78]. Therefore, the differential ability of bacteria to induce IL-8 depends on a combination of the invasive ability and the immunostimulatory capacity of the bacterial DNA. Dependence on bacterial invasion for IL-8 induction suggests that gingival epithelia are alarmed by tissue-invading bacteria and recruit neutrophils to defend against them. Tissue-invading but IL-8-degrading bacteria, such as *P. gingivalis* and *T. denticola* [79,80], may thus threaten periodontal health. In addition, *T. denticola* can also evade the TLR9-mediated antimicrobial response by resisting endosomal degradation after invasion into gingival epithelial cells [81].

DISCUSSION

Successful human pathogens have evolved strategies to escape protective immunity, often by manipulating key components of innate immunity, such as TLRs and complement receptors [82,83]. Molecular mechanisms for evasion of various TLR2 antimicrobial responses by *P. gingivalis* in macrophages have been extensively studied. *P. gingivalis* inhibits phagocyte killing via instigation of the complement anaphylatoxin C5a receptor (C5aR)-TLR2 crosstalk [84] and of the CXC-chemokine receptor 4-TLR2 crosstalk [85], and suppresses IL-12 induction via the complement receptor 3-TLR2 or C5aR-TLR2 crosstalk [86]. Because IL-12 is a key cytokine in Th1 differentiation and cell-mediated immunity, it may prevent or attenuate possible intracellular killing of *P. gingivalis* [84]. Although both the orange complex and the red complex bacteria are associated with periodontitis, a review of host-microbe interactions of these bacteria with innate immune components clearly reveals that the two groups of bacteria have different characteristics. The orange complex bacteria are relatively potent immune stimulators and susceptible to host defense. In contrast, the red complex bacteria are poor

activators/suppressors of host immune response, evading host surveillance. Such characteristics of the red complex bacteria define them as true periodontal pathogens and allow them to thrive in subgingival sites.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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