Porphyromonas gingivalis virulence factors involved in subversion of leukocytes and microbial dysbiosis

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Introduction

Chronic periodontitis is an oral inflammatory disease leading to the destruction of the tissues that support the teeth (periodontium) and is associated with increased risk for certain systemic disorders.\(^1\) Recent metagenomic, metatranscriptomic, and animal model-based mechanistic studies indicate that periodontitis is characterized by polymicrobial synergy and dysbiosis.\(^3\)\(^-\)\(^8\) Porphyromonas gingivalis is a gram-negative asaccharolytic bacterium, which expresses a variety of virulence factors (Table 1) and has long been implicated in periodontitis.\(^9\) In a mouse model of periodontitis, oral inoculation with P. gingivalis leads to low-level colonization of this bacterium as well as to dysbiosis, an elevation of certain populations in the oral bacterial community leading to inflammatory bone loss.\(^10\) Remarkably, P. gingivalis is unable to elicit disease by itself in germ-free mice despite colonizing this host, suggesting that disease pathology requires the presence of a polymicrobial community.\(^1\) This community-wide dysbiotic effect of P. gingivalis, while present at relatively low colonization levels, has led to its characterization as a keystone pathogen, that is, an organism with a disproportionately large effect on its environment relative to its abundance.\(^11\)\(^,\)\(^12\) Contrary to the findings of some of the early culture-based microbiological studies, more recent investigations based on culture-independent molecular methods show that P. gingivalis is a quantitatively minor constituent of human periodontitis-associated biofilms.\(^3\)\(^,\)\(^3\)\(^,\)\(^14\) Whether P. gingivalis can act as a keystone pathogen in human periodontitis has not been specifically addressed, although this would require an interventional study (e.g., to specifically target P. gingivalis and assess its effect on the microbiome and the disease). In this regard, in non-human primates which naturally harbor P. gingivalis in the subgingival biofilm, a gingipain-based vaccine causes a decrease both in P. gingivalis counts and in the total subgingival bacterial load (as well as inhibits bone loss),\(^15\) suggesting that the presence of P. gingivalis benefits the entire microbial community.

The pathogenicity of periodontitis and the virulence of P. gingivalis require a susceptible host. Susceptibility is influenced by host genotype (immunoregulatory defects or immunodeficiencies), stress, diet, or risk-associated behavior such as smoking.\(^16\)\(^-\)\(^21\) Moreover, the virulence of P. gingivalis is influenced by its environment, involving host-related factors or other bacteria (such as accessory pathogens that assist P. gingivalis in terms of colonization and metabolic activities).\(^3\) Consistent with this, several P. gingivalis virulence proteins including gingipains, FimA fibrinect, HtrA protease and lipid A phosphatase have been shown to be regulated by environmental factors such as temperature and hemin.\(^22\)\(^-\)\(^25\) Moreover, the metabolic profiles of P. gingivalis (and other periodontal bacteria) are significantly altered when compared in healthy versus diseased sites from the same patient.\(^4\) For instance, virulence gene expression (e.g., encoding for gingipains, collagenase, and hemagglutinin proteins) is modified, yet the precise expression pattern for the different genes varies from patient to patient suggesting that environmental factors play a role in shaping P. gingivalis virulence. Here, we review strategies utilized by P. gingivalis to compromise host immune function, which in turn can cause compositional and quantitative shifts to the oral microbiome toward a pathogenic phenotype.

P. gingivalis manipulation of leukocytes

The almost exclusive niche of P. gingivalis is in the oral cavity where it hijacks leukocytes altering their migration and defense functions, and elicits inflammation to obtain nutrients from tissue breakdown.\(^26\)\(^,\)\(^27\) The oral cavity is home to approximately...
600 species of bacteria.\textsuperscript{28} Unlike other mucosal tissues, the oral mucosa contains no tight junctions or mucus layer to keep microbes at bay. Instead, the gingival epithelium is charged with a steady gradient of IL-8, a cytokine that signals the migration of neutrophils through the junctional epithelium at an astonishing rate of 30,000 PMNs per minute.\textsuperscript{29,30} The neutrophils create a wall-like defense system, making up the primary cellular defense in healthy oral tissues.\textsuperscript{31} \textit{P. gingivalis} positions itself within the sub-gingival pocket due to the strict requirements of an anaerobic lifestyle. It is here that the bacterium will necessarily encounter a neutrophil. Upon contact with the surrounding epithelium, \textit{P. gingivalis} secretes a serine phosphatase (SerB) which suppresses IL-8 production by dephosphorylating the Ser536 of NF-κB p65 preventing nuclear translocation and transcription\textsuperscript{32}.

\textbf{Table 1. Virulence factors of \textit{P. gingivalis} involved in immune subversion.}

| Virulence factor | Action and consequences | Refs. |
|------------------|------------------------|-------|
| Gingipains (HRgpA, RgpB, Kgp) | Degradation of host defense molecules including antimicrobial peptides and the central complement component C3; generation of nutrient peptides from host protein degradation | 76-78 |
| Arg-specific gingipains (HRgpA and RgpB) | CS convertase-like activity generates C5a, involved in subversive C5aR-TLR2 crosstalk; inflammation and evasion of leukocyte killing. | 26,79 |
| HRgpA gingipain | Captures C4b-binding protein, a negative regulator of complement; prevention of complement activation | 80 |
| Lys-specific gingipain (Kgp) | Proteolytic shedding of CD46 complement regulatory protein from epithelial cells; renders host cells susceptible to complement attack, potential for tissue damage and inflammation. | 81 |
| Lipid A 1- and 4'-phosphatases | Lipid A modifications; generation of LPS structures that evade or antagonize TLR4 and are resistant to cationic antimicrobial peptides | 49,53,82 |
| SerB (serine phosphatase) | Suppresses IL-8 production by dephosphorylation of the Ser536 of NF-κB p65 preventing nuclear translocation and transcription | 32 |
| Peptidylarginine deiminase | Host and bacterial protein citrullination; alteration of host protein function disrupting tissue homeostasis; generation of neopeptidases leading to induction of autoantibodies in rheumatoid arthritis | 83,84 |
| Nucleoside diphosphate kinase | ATP hydrolysis; suppression of ATP-induced epithelial cell apoptosis; enhanced intracellular persistence | 85,86 |
| FimA fimbriae | Binds CXCR4 and induces CXCR4-TLR2 crosstalk; cAMP signaling and inhibition of nitric oxide-dependent killing | 44,87 |
| | Required for \textit{P. gingivalis} oral colonization and β1 integrin-mediated invasion of and survival within gingival epithelial cells | 73,88 |
| Mfa1 fimbriae | Binds DC-SIGN for invasion of and survival within dendritic cells. | 55,89 |
| Hemagglutinins | Nonfimbrial adhesins that agglutinate erythrocytes and promote adherence to host tissue including endothelial cells; induce platelet aggregation (hemagglutinin A). | 90 |

unproven possibility. Once a mature pathogenic biofilm develops that is capable of resisting neutrophil defenses, the recruitment of neutrophils can promote inflammation thereby contributing to the escalating dysbiosis.\textsuperscript{26} Extensive \textit{in vivo} microscopy studies have revealed hierarchical chemokines that facilitate neutrophil migration to a pinpoint locale containing bacteria or microbe-associated molecular patterns.\textsuperscript{35} In gingival tissues, IL-8 directs neutrophils to the leading edge of the junctional epithelium, far away from the depths required for growth of \textit{P. gingivalis}. Although transmigrating neutrophils initially follow the IL-8 gradient, they then have to move toward gradients existing in the infected or inflamed tissue. Such gradients involve chemotacticants derived from bacteria (\textit{e.g.}, N-formyl-methionyl-leucyl-phenylalanine) or from local activation of complement (C5a fragment).\textsuperscript{36} Intriguingly, \textit{P. gingivalis} expresses Arg-specific gingipains (cysteine proteases) that cleave C5 and release biologically active C5a, independently of the canonical activation of the complement cascade.\textsuperscript{37} This activity enables \textit{P. gingivalis} to induce a subversive crosstalk between the C5a receptor (C5aR; CD88) and Toll-like receptor (TLR)-2 (Fig. 1B).\textsuperscript{26} This C5aR-TLR2 crosstalk causes degradation of the signaling adaptor MyD88, thereby allowing decoupling of microbicidal activity from the inflammatory response which is mediated by an alternative pathway involving the MyD88-adapter-like (Mal) molecule and phosphoinositide 3-kinase (PI3K). The same Mal-PI3K pathway also causes inhibition of the small GTPase RhoA, thereby blocking actin polymerization and phagocytosis of bacteria.\textsuperscript{26} Inhibition of TLR2 or C5aR counteracts \textit{P. gingivalis} control of the neutrophil allowing the cell to regain effective immune clearance of the bacteria. This very precise manipulation of the neutrophil
bequests *P. gingivalis* a safe niche rich with food, yet the subversion of the neutrophil causes the disruption of host protective mechanisms that benefits the entire oral bacterial community (more below).

The macrophage is also amenable to *P. gingivalis* exploitation (Fig. 2A). While few are found in healthy gingival tissues, macrophages are most notable for their rapid response to inflammatory insult and as such are thought to play a principal role mediating an immune response as well as inflammation resolution in oral tissues.38,39 The macrophage is a professional phagocyte, well known for its efficient uptake of cellular debris or invading pathogens. Normally, a bacterium is sensed by the macrophage via pattern recognition receptors and quickly phagocytosed into an acidic phagolysosome capable of killing the bacteria; during this process, an inflammasome can form leading to the induction and release of inflammatory cytokines IL-1β and IL-18.40 Instead of waiting for macrophage phagocytosis to occur, *P. gingivalis* takes the initiative to hijack lipid rafts that form on the macrophage cellular membrane.41,42 With its fimbriae and a complex of accessory proteins, *P. gingivalis* causes co-association of CXCR4 and TLR2; the resulting signaling crosstalk inhibits nitrogen oxide production in a manner dependent upon cAMP-dependent protein kinase A activation (Fig. 2A).43,44 Fimbriae

**Figure 1.** Manipulation of neutrophil function by *P. gingivalis*. (A) Model of chemokine paralysis. Under homeostatic conditions, oral bacteria are kept at bay by steady recruitment of neutrophils following a gradient of IL-8 production by the gingival epithelium. *P. gingivalis* can manipulate the IL-8 gradient by secreting SerB, an enzyme that dephosphorylates the p65 subunit of NF-κB thereby inhibiting translocation into the nucleus and preventing IL-8 transcription. The result is chemokine paralysis that disrupts the recruitment of neutrophils into the junctional epithelium and control of the outgrowth of oral bacteria. (B) Model of Neutrophil subversion by *P. gingivalis* that leads to dysbiotic inflammation. Due to C5a ligand generation by Arg-specific gingipains coupled with potent TLR2 agonists (e.g., lipoproteins), *P. gingivalis* is able to co-activate C5aR and TLR2 resulting in Smurf1-dependent MyD88 degradation thus preventing an antimicrobial response. This signaling event also induces Mal- and PI3K-dependent inhibition of RhoA, thereby preventing phagocytosis while the same subversive pathway mediates inflammatory responses. In total, *P. gingivalis* can successfully decouple antimicrobial killing from a nutritionally favorable inflammatory response in neutrophils. This mechanism provides bystander support to neighboring bacteria.
activation of CXCR4 also leads to induction of the high-affinity conformation of complement receptor 3 that \textit{P. gingivalis} exploits for safe intracellular entry.\textsuperscript{45,46} Once inside the macrophage, \textit{P. gingivalis} has additional methods to disrupt cellular processes that mediate bacterial killing. In this regard, it was recently shown that the lipid A moiety of the LPS structure can elicit TLR4-independent, noncanonical activation of the inflammasome.\textsuperscript{47} \textit{P. gingivalis} LPS is heterogeneous and contains several different lipid A moieties which can cause differential signaling through TLR4.\textsuperscript{48} Remarkably, a \textit{P. gingivalis} mutant fixed in its expression of a lipid A moiety that functions as a TLR4 antagonist was found to evade activation of the noncanonical inflammasome, thereby enhancing its intracellular survival in macrophages (Fig. 2A).\textsuperscript{49} In contrast, a \textit{P. gingivalis} mutant expressing only the TLR4 agonist lipid A predictably induces noncanonical inflammasome activation characterized by production of IL-1\(\beta\) and loses the ability to survive in the macrophage.\textsuperscript{49} Noncanonical activation of the inflammasome by the LPS of gram-negative bacteria involves the participation of caspase 11.\textsuperscript{50} In the noncanonical mechanism, intracellular LPS directly binds and activates caspase 11.\textsuperscript{51} In contrast, TLR4 antagonist lipid A binds caspase 11 but does not induce caspase 11 oligomerization required for its activation.\textsuperscript{51} This explains why \textit{P. gingivalis} equipped with antagonistic or evasive lipid A interferes with the activation of both TLR4 and the caspase 11-dependent non-canonical inflammasome. Importantly,
the lipid A phosphatase activity responsible for lipid A alterations was shown to be critical for the oral colonization capacity of *P. gingivalis*. Moreover, the regulation of the lipid A moiety also shapes the community of oral bacteria as discussed below.

The macrophage is not the only leukocyte type which *P. gingivalis* may call home; the dendritic cell is also susceptible to proactiv *P. gingivalis* internalization and manipulation. Dendritic cells in healthy gingival tissues are scarce but very effective at sampling the environment and responding rapidly to bacterial stimuli (for a review see). Once stimulated, the dendritic cell normally becomes activated and matures into effector cell capable of eliciting a polarized T-cell response. *P. gingivalis* uses its Mfa1 fimbriae to interact with a C-type lectin in dendritic cells, specifically the DC-specific ICAM-3 grabbing nonintegrin (DCSIGN) (Fig. 2B). This interaction is followed by *P. gingivalis* internalization and survival within the dendritic cell which is further manipulated in ways that appear to contribute to an athrogenic phenotype and the systemic dissemination of *P. gingivalis*. Although the association of *P. gingivalis* with systemic disease is beyond the scope of the present review, it is of interest to note that *P. gingivalis* has been detected in blood myeloid dendritic cells of patients along with several other species including *Helicobacter pylori*, *Pseudomonas spp.*, *Moraxella catarrhalis*, *Klebsiella pneumonia*, and *Salmonella enterica*. Although uncertain, it is tempting to hypothesize that the manipulation of dendritic cells by *P. gingivalis* might contribute to the intracellular survival of the other detected species. *P. gingivalis* causes suboptimal maturation of dendritic cells and modulates the effector response from a T helper 1 (Th1)-biased to a Th2-biased response, which may have local consequences in periodontitis. This is because the Th1 response directs effective cell-mediated immunity against periodontal bacteria. However, this notion does not represent a consensus given the overall uncertainty regarding the precise roles of T helper subsets in periodontal disease pathogenesis.

As alluded to above, *P. gingivalis* may manipulate the adaptive immune response, although it has not been conclusively determined whether it can directly subvert lymphocytes. A microarray analysis in mice systemically exposed to *P. gingivalis* revealed a predominant downregulatory effect on the expression of immune response-related genes in CD4+ and CD8+ T cells. A more recent study by an independent group provided a possible mechanism by which *P. gingivalis* may subvert T cell function: Upon systemic injection in mice, *P. gingivalis* causes the production of high levels of IL-10 by CD11b+ cells and CD4+ as well as CD8+ T cells. IL-10, in turn, potently inhibits IFN-γ production by CD8+ T cells and CD4+ Th1 cells, which arguably (see above) mediate protective cell-mediated immunity against periodontal bacteria.

**Community-wide effects**

*P. gingivalis* is unmistakably adept at intercepting host immune function for its own benefit, but, as alluded to above, the surrounding bacterial community can also benefit. To be precise, only those species that can both endure and exploit the inflammatory environment can really take advantage of *P. gingivalis*‘ company. Many of these species behave as pathobionts that further exacerbate inflammatory tissue destruction.

Other bacterial species may be outcompeted and disappear from the escalating inflammatory environment. Consistent with the requirement of intact C5aR signaling for successful evasion of neutrophil killing by *P. gingivalis* in vitro, the organism fails to colonize the periodontium of C5aR-deficient mice, whereas local treatment of *P. gingivalis*-colonized mice with a C5aR antagonist essentially eliminates *P. gingivalis*, reverses dysbiosis, and inhibits development of periodontitis. The C5aR-dependent evasive mechanism, as established in vitro, strictly requires a crosstalk with TLR2 and activation of downstream PI3K signaling; consistently, local inhibition of TLR2 or PI3K in the periodontium of *P. gingivalis*-colonized mice similarly leads to near elimination of this keystone pathogen and counteracts its earlier effect to increase the total microbial load. It should be noted, however, that additional cell types in the periodontal environment also express the implicated molecules (C5aR, TLR2, and PI3K); their inhibition, therefore, in cells other than neutrophils might contribute to effects on *P. gingivalis* and the dysbiotic microbiota. For instance, *P. gingivalis* induces and exploits PI3K signaling also in gingival epithelial cells, where it inhibits apoptosis in a PI3K-dependent mode to promote its intracellular persistence. In contrast to the dysbiotic effects of wild-type *P. gingivalis*, oral inoculation of mice with a gingipain-deficient mutant that cannot generate C5a has no influence on the microbiota. Additional *P. gingivalis* virulence factors, such as the LPS lipid A moiety, can also cause alterations to the microbiota composition and bone loss in animal models of periodontal disease. Indeed, in contrast to wild-type *P. gingivalis*, mutant strains that are unable to modify the lipid A moiety fail to colonize the periodontal tissue in a rabbit model of periodontitis.

**Conclusion**

Decades of research have identified a plethora of virulence factors of *P. gingivalis*, some of which are shown in Table 1. However, only recently have we started to understand how *P. gingivalis* integrates its virulence properties into the collective pathogenicity of the oral polymicrobial community. The emerging role of *P. gingivalis* involves the subversion of the host immune response in ways that enhance the fitness of the community in a nutritionally favorable inflammatory environment. It should be noted, however, that the presence of *P. gingivalis* does not necessarily prompt a pathological transition toward periodontal disease; rather, this bacterium signifies a risk factor for disease. Indeed, periodontally healthy individuals may also harbor *P. gingivalis* albeit with reduced frequency relative to periodontitis patients. The most likely explanations, which are not mutually exclusive, involve changes in the status of the bacterium or the host. For instance, there is considerable strain and virulence diversity within the population structure of *P. gingivalis* and, as alluded to above, at least some of its key virulence factors (e.g., gingipains and lipid A phosphatases) are regulated by local environmental conditions that are likely different among...
different individuals. From a broader point of view, a susceptible host is necessary for the development of periodontitis as implied by cases of individuals who do not develop periodontitis despite considerable biofilm accumulation at dentogingival sites. 

In this context, there might be individuals who can resist the capacity of P. gingivalis to convert a symbiotic microbiota into a dysbiotic one by virtue of their intrinsic immune status (e.g., alterations in signaling pathways required for immune subversion by P. gingivalis). Although this review focused on the manipulation of leukocytes by P. gingivalis, it should be noted that the capacity of this pathogen to subvert additional aspects of host immunity and homeostasis (e.g., gingival epithelial cells, complement, antimicrobial molecules) is also important and the reader is referred to other reviews. Moreover, it should be noted that P. gingivalis may additionally influence the periodontal biofilm also through host-independent effects; for instance, its gingipains were shown to qualify to and quantitatively affect the composition of polymicrobial biofilms in vitro. The elucidation of mechanisms by which P. gingivalis promotes dysbiosis has important translational implications. In this regard, host-modulation strategies aiming to block receptors or signaling pathways by which P. gingivalis elevates the pathogenicity of the dysbiotic microbial community may offer promising options for the treatment of human periodontitis.

Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

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