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Immune evasion strategies of *Porphyromonas gingivalis*

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Abstract

Porphyromonas gingivalis is strongly correlated with chronic periodontitis. Its chronic persistence in the periodontium depends on its ability to evade host immunity without inhibiting the overall inflammatory response, which is actually beneficial for this and other periodontal bacteria. Indeed, the inflammatory exudate (gingival crevicular fluid) is a source of essential nutrients, such as peptides and hemin-derived iron. In this review, I discuss how *P. gingivalis* can promote its adaptive fitness through instigation of subversive crosstalk signaling. These interactions involve Toll-like receptor-2, complement receptor 3, C5a anaphylatoxin receptor, and CXC-chemokine receptor 4. Their exploitation by *P. gingivalis* allows the pathogen to escape elimination, obtain nutrients, and collaterally inflict periodontal tissue injury.

Keywords

Porphyromonas gingivalis; complement; Toll-like receptors; immune evasion; inflammation

Introduction

Human periodontitis is likely the most common infection-driven chronic inflammatory disease and is characterized by destruction of the supporting tissues of the teeth, including resorption of alveolar bone [1)]. Periodontal tissue degradation results primarily from unwarranted inflammatory host responses to a group of subgingival gram-negative anaerobic bacteria [2)]. Several oral microbes have been implicated in periodontitis including the so-called 'red-complex' group comprising *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* [3)]. The presence of *P. gingivalis* is strongly correlated with disease and is thought of as a keystone species, *i.e*., an organism that 'serves an essential function for the entire community, similar to a differentiated cell serving a function for an entire tissue' [4,5)]. This review summarizes evidence supporting that *P. gingivalis* can manipulate the host response in ways that can benefit companion species of the same microbial community and, moreover, amplify destructive inflammation. Before presenting the latest advances in this field, it would be necessary to provide a brief background on *P. gingivalis* virulence traits.

P. gingivalis is a gram-negative anaerobic and asaccharolytic rod which, besides being a predominant contributor to human periodontitis, is also implicated as an accessory factor in certain systemic conditions, such as atherosclerosis, aspiration pneumonia, and perhaps rheumatoid arthritis [1,6,7)]. This pathogen is perhaps the most intensively studied oral organism at the molecular level and its pathogenicity is attributed to an array of potential virulence factors, such as cysteine proteinases (gingipains), hemagglutinins,

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lipopolysaccharide (LPS), and fimbriae, *i.e*., adhesive hair-like appendages emanating from the bacterial cell surface [8]. These molecules are thought to facilitate *P. gingivalis* initial colonization, retention, and growth within the gingival crevice [9,10].

However, the capacity of any pathogen to secure an appropriate niche and persist requires more than simply possessing virulence factors for tissue adherence and nutrient procurement. Successful human pathogens have also evolved strategies to escape protective immunity, often by manipulating key components of innate immunity, such as the Toll-like receptor and complement systems [11,12]. Such subversive strategies enable these pathogens to disable the overall host response, since complement and TLRs play instructive roles in the development of adaptive immunity [13,14].

To establish a chronic infection in the hostile host environment of the gingival crevice, it is imperative that *P. gingivalis* find ways to evade or subvert host defense mechanisms aiming to eliminate it. For example, *P. gingivalis* expresses heterogeneous and atypical LPS molecules that either act as potent TLR4 antagonists or are immunologically inert [15]. This allows the pathogen to evade or proactively inhibit a variety of potential TLR4-mediated antimicrobial functions, such as inhibition of expression of antimicrobial peptides (β defensins) in human epithelial cells [4]. Since *P. gingivalis* releases LPS-bearing membrane vesicles that can readily diffuse in the crevice or even penetrate gingival tissue [16], the TLR4 antagonistic LPS of *P. gingivalis* can inhibit TLR4-mediated antimicrobial responses against other bacteria in the same mixed-species biofilm [4]. This is just one example of how *P. gingivalis* can undermine innate immune responses for the microbial community at large.

In contrast to TLR4, *P. gingivalis* cannot antagonize TLR2 at the receptor level. However, this oral pathogen has evolved the ability to instigate subversive crosstalk interactions between TLR2 and other innate receptors for blunting the TLR2 antimicrobial response. These novel mechanisms are the focus of this review. General mechanisms by which *P. gingivalis* may undermine innate immunity are summarized in Table 1. Below we describe how this pathogen specifically exploits crosstalk pathways of innate immunity to promote its survival.

Specifically, I will discuss the ability of *P. gingivalis* to promote its adaptive fitness through instigation of crosstalk interactions between TLR2 and three important innate receptors, the complement anaphylatoxin C5a receptor (C5aR), the complement receptor 3 (CR3), and the CXC-chemokine receptor 4 (CXCR4).

Induction and exploitation of the TLR2-CR3 crosstalk pathway

The TLR system senses *P. gingivalis* primarily through TLR2 both in vitro and in vivo, whereas TLR4 is not playing an important role [17,18]. As alluded to above, this is because the pathogen expresses atypical lipopolysaccharide molecules. Specifically, the bacterium utilizes lipid A 1-and 4-phosphatases and a deacylase which in concert generate a tetra-acylated and dephosphorylated lipid A structure that is biologically inert [15]. At the same time, this modification confers protection against polymyxin B and perhaps other cationic anti-microbial peptides [15]. Moreover, high concentrations of hemin (as can be found in inflamed periodontal sites) suppress the lipid A 1-phosphatase activity and lead to generation of a mono-phosphorylated lipid A, which acts as a TLR4 antagonist [15]. Although *P. gingivalis* does not directly antagonize TLR2, it has evolved strategies to exploit TLR2 signaling to its own advantage.

Following activation by *P. gingivalis*, TLR2 induces two distinct downstream signaling cascades [19]. One of the pathways is MyD88-dependent and leads to induction of mostly nuclear factor-κB-dependent proinflammatory and antimicrobial responses. The other is a

proadhesive pathway that leads to the induction of the high-affinity conformation of CR3 [20]. Specifically, *P. gingivalis* induces TLR2 inside-out signaling, which proceeds through Rac1, phospatidylinositol-3-kinase (PI3K), and cytohesin-1, which acts as the ultimate effector that transactivates CR3 [19,21] (Fig. 1).

P. gingivalis can then bind transactivated CR3 by means of its fimbriae and induces its phagocytic uptake by macrophages [22]. However, CR3 is not linked to vigorous microbicidal mechanisms, possibly because this receptor is heavily committed with phagocytosis of iC3b-coated apoptotic cells, which are not normally recognized as danger [14]. Consistent with this, CR3-mediated phagocytosis does not promote the killing of *P. gingivalis* [23]. In fact, *P. gingivalis* enhances its in vivo survival by exploiting CR3; conversely, pharmacological inhibition or genetic ablation of CR3 greatly facilitate its killing [24].

Additional CR3-dependent mechanisms, however, may contribute to this in vivo evasion of immune clearance. In this regard, CR3 ligation by *P. gingivalis* induces outside-in signaling and extracellular-signal-regulated kinase 1/2 activation, which in turn selectively suppresses IL-12 production through inhibition of mRNA expression of the IL-12 p35 and p40 subunits [24]. In vivo, CR3-deficient mice elicit higher levels of IL-12 (and secondarily increased interferon-γ production) and clear infection with *P. gingivalis* more efficiently than wild-type controls [24]. In this evasion strategy, *P. gingivalis* appears to have co-opted a natural immunosuppressive mechanism, since induction of IL-12 is similarly inhibited during phagocytosis of apoptotic cells by macrophages [14]. From a translational viewpoint, pharmacological blockade of CR3 suppresses *P. gingivalis*-induced periodontal bone loss in a mouse model [24]. In the context of the periodontitis-atherosclerosis connection and the observation of viable *P. gingivalis* in atherosclerotic plaques [1,7], it is intriguing to hypothesize that the intracellular persistence of *P. gingivalis* in macrophages [23] might allow this organism to exploit these cells as ‘Trojan horses’ to relocate to systemic tissues and subsequently infect permissive cells (*e.g.*, endothelial cells). Although this remains to be tested, the capacity of *P. gingivalis* for cell exit and infection of new host cells has been demonstrated [25].

Hijacking of complement-TLR2 crosstalk signaling

Although *P. gingivalis* cannot antagonize TLR2 at the receptor level as it does with TLR4 (see above), it has evolved the ability to intercept and undermine a subset of TLR2 signaling events for corrupting innate immunity [23,24,26,27]. This section will discuss how this oral bacterium exploits complement and its capacity to crosstalk with the TLR system for inhibiting specific aspects of TLR2 immunity.

It has been firmly established that *P. gingivalis* degrades C3 and inhibits the complement cascade regardless of the initiation pathway involved [28]. Strikingly, however, the gingipain enzymes of this bacterium (specifically HRgpA and RgpB) act in a C5 convertase-like manner to generate biologically active C5a. In fact, *P. gingivalis* can rapidly generate high levels of C5a (> 30 nM) in heat-inactivated human serum [27]. This seems counterproductive for the survival of this pathogen, since C5a is probably the most potent effector of the complement cascade and generally promotes host defense. For example, C5a induces chemotactic recruitment and activation of leukocytes [14]. Stunningly, however, *P. gingivalis* exploits C5a to undermine TLR2 immunity: Mechanistically, upon C5aR binding, C5a stimulates Gαi-dependent intracellular Ca^{2+} signaling which synergistically enhances an otherwise weak cAMP response induced by *P. gingivalis*-induced TLR2 activation alone. In this crosstalk pathway, sustained elevated production of cAMP leads to the activation of the cAMP-dependent protein kinase A (PKA) which inactivates the glycogen synthase kinase-3β (GSK3β) and impairs nitric oxide-dependent killing of *P. gingivalis* in macrophages [27] (Fig. 1).

The above discussed in vitro evasion mechanism is supported by in vivo observations. Mice with genetic deficiency of C5aR, or with pharmacological inhibition of the same receptor, elicit higher levels of nitric oxide and clear *P. gingivalis* more effectively than untreated normal controls [27]. Moreover, the *P. gingivalis*-induced C5aR-TLR2 crosstalk also regulates cytokine production in favor of the pathogen [29]. Specifically, this oral bacterium proactively and selectively inhibits TLR2-induced IL-12p70, whereas the same C5aR-TLR2 crosstalk upregulates other inflammatory and bone-resorptive cytokines (IL-1β, IL-6, and TNF-α). In vivo, the ability of *P. gingivalis* to manipulate TLR2 activation via the C5a-C5aR axis allows it to escape IL-12p70-dependent immune clearance and to cause inflammatory bone loss in a murine model of experimental periodontitis [29]. Therefore, *P. gingivalis* targets C5aR not only to promote its adaptive fitness but also to cause periodontal disease. This has profound implications for the treatment of periodontal disease given the current availability of safe and effective C5aR antagonists [14]. Specifically, C5aR antagonists have the potential to both promote the killing of *P. gingivalis* and to inhibit the host inflammatory response.

Because the C5aR-TLR2 crosstalk inhibits only a subset of TLR2 signaling events, C5aR was characterized as a 'TLR modulatory receptor' to differentiate it from 'TLR inhibitory receptors'. Activation of such receptors, for example the IL-10 receptor and the TGF-β receptor, can block most, if not all, inflammatory responses [30]. In fact, it would not be in *P. gingivalis* 'best interest' to induce a generalized immunosuppression. This is because inflammation brings in nutrients (present in the gingival crevicular fluid) and thus contributes to its growth and survival [10]. In this regard, *P. gingivalis*-generated C5a may cause vasodilation and stimulation of inflammatory exudate for acquisition of essential nutrients like hemin [31]. Interestingly, unlike C5a, the C5b remnant is readily degraded by *P. gingivalis* gingipains to ostensibly prevent activation of the terminal complement pathway and formation of the membrane attack complex.

On the basis of these novel findings, there is sufficient rationale for the use of C5aR antagonists to control *P. gingivalis* infections and to also inhibit periodontal inflammation.

Instigation of CXCR4-TLR2 subversive crosstalk

In the absence of C5aR signaling, *P. gingivalis* uses an alternative mechanism to interfere with TLR2-dependent antimicrobial responses in macrophages. In this case, the pathogen induces a crosstalk between TLR2 and CXCR4 [26]. Specifically, the concomitant activation of CXCR4 and TLR2 by the fimbriae of *P. gingivalis* induces cAMP-dependent PKA signaling, which in turn suppresses TLR2-dependent nitric oxide in response to the pathogen [26] (Fig. 1).

The biological relevance of the CXCR4-TLR2 crosstalk was confirmed in vivo. Specifically, mice treated with a selective CXCR4 antagonist display increased production of nitric oxide and enhanced ability to clear infection with *P. gingivalis* compared to untreated control mice [26]. Intriguingly, CXCR4-TLR crosstalk interactions have been recently described also in zebrafish. Here, CXCR4 interfered with toxic effects of LPS activation of TLR4 [32].

There is adequate evidence that *P. gingivalis* can integrate the CXCR4-TLR2 and C5aR-TLR2 crosstalk pathways for enhanced immune subversion. In this regard, confocal microscopy and fluorescence resonance energy transfer analysis indicates that all three receptors, CXCR4, TLR2, and C5aR, co-associate in the lipid rafts of *P. gingivalis*-challenged macrophages [27]. Although the C5aR-TLR2 crosstalk can proceed independently of CXCR4 and potently upregulate cAMP, maximal cAMP induction requires cooperation of all three receptors [27]. The following integrative model is proposed to describe combined CXCR4- and C5aR-mediated manipulation of TLR2 by *P. gingivalis*: The bacterium

induces a weak cAMP response by acting on TLR2 alone, whereas activation of CXCR4 or C5aR signaling alone fails to induce cAMP. On the other hand, *P. gingivalis*-induced TLR2 signaling with concomitant activation of C5aR and CXCR4 synergistically enhances cAMP-PKA signaling that inactivates GSK3β and impairs iNOS-dependent killing (Fig. 1).

Summary and conclusions

In the course of evolution successful pathogens have 'learned' to breach innate defense systems such as complement and TLRs [11,33] but also, as exemplified here with *P. gingivalis*, to exploit their communication hubs [34]. These subversive strategies of *P. gingivalis* (Table 1 & Fig. 1) may explain, at least in part, its ability to persist and establish chronic infections in the periodontium. A keystone pathogen is expected to modify the immune selective pressure in ways that stabilize the microbial community in which it resides. In this regard, *P. gingivalis*' tactics to undermine innate immunity may promote the survival of other members of the periodontal biofilm community [5,10]. This capacity is reinforced by the fact that *P. gingivalis* releases easily diffusible membrane vesicles that contain key virulence factors like gingipains, LPS, and fimbriae, which can thus become available to other bacteria within the same biofilm [4,16]. Stimulation of inflammatory responses that do not kill (e.g., C5a-induced inflammation) can moreover result in acquisition of essential nutrients (*e.g.*, gingival crevicular fluid-derived peptides and hemin, a source of iron) [31]. Therefore, it is reasonable to suggest that periodontal pathogens have evolved in ways that allow them to not only endure inflammation but also exploit it for promoting their survival and, collaterally, inflicting periodontal tissue damage. In conclusion, pathogen manipulation of periodontal innate immunity may perturb otherwise homeostatic host-bacterial interactions, thereby leading to non-protective and non-resolving chronic inflammation in the periodontium. On the other hand, elucidation of the mechanisms by which periodontal bacteria interfere with immune clearance mechanisms and induce nonproductive inflammation, would facilitate the rational design of therapeutic interventions in periodontitis.

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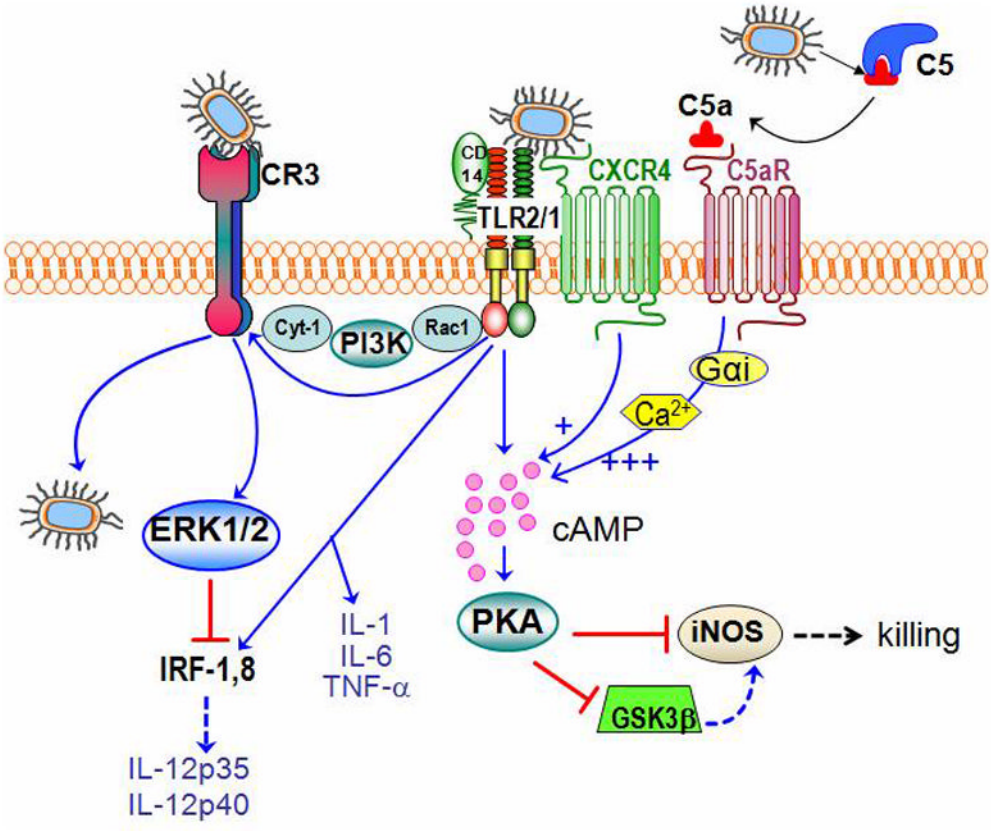

Figure 1. *P. gingivalis* subverts crosstalk pathways between TLRs and other innate immune receptors

In macrophages, *P. gingivalis* is recognized by the CD14/TLR2/TLR1 receptor complex [17]. This interaction activates inside-out signaling, propagated by Rac1, PI3K, and cytohesin-1 (Cyt-1), which induces the high-affinity conformation of CR3 [20,35]. CR3 then binds and internalizes *P. gingivalis*; this is a relatively safe portal of entry since CR3 is not linked to vigorous microbicidal mechanisms. The CR3-*P. gingivalis* interaction also leads to induction of ERK1/2 signaling. This in turn downregulates IL-12 p35 and p40 mRNA expression [24], possibly through suppression of a critical transcription factor (the interferon regulatory factors 1; IRF-1), required for IL-12 expression [14]. This suppressive effect is specific for IL-12 and does not affect induction of other proinflammatory cytokines (*e.g.*, IL-1β, IL-6, and TNF-α). Inhibition of bioactive IL-12 by this mechanism results in impaired immune clearance of *P. gingivalis* in vivo [24]. Moreover, *P. gingivalis* uses its gingipains to attack C5 and release biologically active C5a [27,29]. Upon C5aR binding, C5a stimulates Gαi-dependent intracellular Ca^{2+} signaling which synergistically enhances the otherwise weak cAMP responses induced by TLR2/TLR1 activation alone. Maximal cAMP induction is achieved by the participation of another G protein-coupled receptor, the CXCR4, which interacts directly by *P. gingivalis* and coassociates with both TLR2 and C5aR in lipid rafts [26,27]. The ensuing activation of the cAMP-dependent protein kinase A (PKA) pathway inactivates glycogen synthase kinase-3β (GSK3β) and impairs the inducible nitrogen synthase (iNOS)-dependent killing of the pathogen in macrophages in vitro and in vivo [27].

Table 1

Subversion of innate immunity by *P. gingivalis*

Mechanism		Effector molecules	Refs.
1	Inhibition of complement activation through digestion of the central complement component (C3)	Gingipains, especially HRgpA and RgpB	36,37)
2	Inherent resistance to complement-mediated lysis	LPS with anionic polysaccharide repeat units (A-LPS)	38,39)
3	Hijacking complement regulatory proteins (C4b-binding protein)	HrgpA	40)
4	Shedding and proteolysis of complement regulatory protein CD46 from oral epithelial cells	Kgp	41)
5	TLR4 evasion by expressing dephosphorylated and tetra-acylated lipid A	Lipid A 1-deacylase and 4′-phosphatases and deacylase	15)
6	TLR4 antagonism by expressing monophosphorylated tetra-acylated lipid A	Lipid A 4′-phosphatase and deacylase (lipid A 1-phosphatase suppressed by hemin)	15,42)
7	Upregulation of negative regulators of TLR signaling (IRAK-M) in monocytes	LPS	43)
8	Degradation of TLR coreceptors (CD14), cytokines (IL-12, IL-1β, IL-6, IFN-γ), or antimicrobial peptides (*e.g.*, LL-37)	Gingipains	28)
9	Inhibition of phagocyte killing via instigation of C5aR-TLR2 crosstalk	HRgpA, RgpB	27,29)
10	Inhibition of phagocyte killing via instigation of CXCR4-TLR2 crosstalk	Fimbriae	26)
11	Suppression of TLR2-induced IL-12 via CR3 binding	Fimbriae	24)
12	Promotion of intracellular survival via CR3-mediated entry	Fimbriae	23)
13	Counteraction of oxidative damage; resistance to environmental oxidative stress and oxidative killing by phagocytes	Rubrerythrin (nonheme iron protein), alkyl hydroperoxide reductase, FeoB2 (ferrous iron transport protein)	44,45)