Polymicrobial synergy and dysbiosis in inflammatory disease

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Abstract

Uncontrolled inflammation of the periodontal area may arise when complex microbial communities transition from a commensal to a pathogenic entity. Communication among constituent species leads to polymicrobial synergy among metabolically compatible organisms that acquire functional specialization within the developing community. Keystone pathogens, even at low abundance, elevate community virulence and the resulting dysbiotic community targets specific aspects of host immunity to further disable immune surveillance while promoting an overall inflammatory response. Inflammophilic organisms benefit from proteinaceous substrates derived from inflammatory tissue breakdown. Inflammation and dysbiosis reinforce each other and the escalating environmental changes further select for a pathobiotic community. We have synthesized the polymicrobial synergy and dysbiotic components of the process into a new model for inflammatory diseases.

Periodontitis: An exemplar of polymicrobial synergy and dysbiosis

Recent years have witnessed a sea change in our perception of diseases of microbial origin. It has become apparent that the etiology of many of diseases that initiate on the skin and mucosal membranes does not involve monocultures of bacteria, but rather heterotypic communities of organisms. Organisms within these communities often display polymicrobial synergy (see Glossary) and the communities become dysbiotic, resulting in disruption of tissue homeostasis and normal immune responses. Periodontal diseases are an exemplar of an inflammatory disease that involves the concerted action of polymicrobial communities, and the pathogenicity of periodontal diseases can be explained through a Polymicrobial Synergy and Dysbiosis (PSD) model [1] (Figure 1). In this model, colonizing bacteria first assemble into physiologically compatible communities, and the organisms within these communities communicate through sophisticated signaling mechanisms. Overgrowth and overt pathogenicity are controlled by the host inflammatory response, and

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indeed a controlled immuno-inflammatory state is normal in a healthy gingiva. It is interesting to note here that the oral cavity is not unique in this regard and a similar homeostatic inflammatory state has been described in the gut [2]. In the mouse model of periodontitis, it has been established that pathogenicity is initiated by colonization with keystone pathogens, such as *Porphyromonas gingivalis,* which even in low numbers can elevate the virulence of the entire community [3]. Communication between *P. gingivalis* and organisms that are otherwise commensal, the accessory pathogens, facilitates synergy and the transition to pathogenicity [4]. The dysbiotic community continues to develop and stimulate inflammatory responses; however in susceptible hosts these are poorly controlled and are ineffective at constraining the community. Worse, frustrated and misdirected responses contribute to tissue destruction and shape a modified 'inflammophilic' community which sustains itself through inflammatory tissue breakdown-derived nutrients [5]. Pathobionts in the community become active and further exacerbate the disease process [6].

Interbacterial interactions

The central tenets of the PSD model are that communities of periodontal bacteria exhibit properties that are more than the sum of their constituent organism parts, and that pathogenicity is dictated by a subset of these bacteria. Initial colonizers of the periodontal area adhere to each other through a multiplicity of complementary adhesins, forming spatially distinct polymicrobial consortia [7]. Constituent organisms are generally metabolically compatible, and communities are thus physically and physiologically integrated, and through the progressive action of collective metabolic enzymes are capable of utilizing a wider range of nutritional substrates than possible for individual species [8]. Further development of heterotypic communities involves interspecies communication and adaptive responses which can occur through direct contact, soluble mediators and nutrient transfer (summarized in Figure 1). Within communities, bacteria are thus able to collectively regulate activities and functional specialization can arise. The composition of the communities varies over time, from person to person and even from site to site; however these communities are in a homeostatic equilibrium with the host [1]. Immune responses, characteristic of a healthy gingiva, limit bacterial overgrowth and neutralize toxic products such as proteases [9,10]. The delicately balanced host-microbe interaction changes upon colonization with keystone pathogens such as *P. gingivalis*.

Direct effects of keystone pathogens on other members of the community in combination with targeted subversion of specific components of the host responses, elevates the virulence of the community as a whole, and the now dysbiotic community increases in number causing further disruption of tissue homeostasis and destruction of periodontal tissues, mediated predominantly by pathobionts [6,11–13] (Figure 1). In human periodontitis, pathobionts are likely represented by previously underappreciated species (e.g. *Filifactor alocis*, *Peptostreptococcus stomatis* and species from the genera *Prevotella*, *Megasphaera*, *Selenomonas*, and *Desulfobulbus*) that have now been shown by metagenomic analyses to exhibit as good (or better) a correlation with disease as traditional pathogenic species, such as *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola* [14–17]. Consistent with this notion, a recent metatranscriptomic study revealed that the majority of virulence factors upregulated in the microbiome of periodontitis patients is primarily derived from previously

underappreciated species that were not traditionally implicated in periodontitis [18]. It is possible that pathobionts might outcompete keystone or keystone-like pathogens such as *P. gingivalis* and *T. forsythia* in late stages of periodontal disease pathogenesis. Indeed, the relative abundance of *P. gingivalis* and *T. forsythia* shows a trend for negative correlation with the total bacterial load in human periodontitis, in contrast to certain newly recognized periodontitis-associated organisms probably acting as pathobionts [15]. This notion is reminiscent of the bacterial driver–passenger model for colorectal cancer, in which driver bacteria (i.e. bacteria which initiate the pathogenic process) are outcompeted in late stages of the disease by passenger bacteria (e.g. pathobionts which exacerbate the disease) [19].

Consistent with the PSD model, synergistic pathogenicity of combinations of periodontal organisms in animal models has been documented for some time [20]. Moreover, while considerable evidence implicates *P. gingivalis* as a major pathogen in chronic and severe manifestations of periodontal diseases, it is also clear that pathogenicity is only expressed in the context of a community, and *P. gingivalis* is unable to cause disease by itself in germ-free mice [3]. The full range of interactions between *P. gingivalis* and periodontal microbial community members is yet to be revealed; however, the molecular underpinnings of community pathogenicity are now beginning to emerge. Introduction of *P. gingivalis* into an otherwise health-associated community causes major transcriptomic and proteomic reverberations [21,22]. Post-translational cooperation is also evident as the cysteine protease interpain A (InpA) of *Prevotella intermedia* works in conjunction with the HmuY hameophore of *P. gingivalis* to acquire haem from haemoglobin [23]. *P. gingivalis* and *T. denticola,* which co-localize in subgingival biofilms *in vivo*, also exhibit symbiotic biofilm growth *in vitro* as well as synergistic virulence upon co-infection in animal models of alveolar bone loss. These organisms co-operate metabolically through the generation of a variety of metabolites by each species which can be used as a nutritional substrates by the other [24]. In addition, pathways that lead to the production of mutually beneficial nutrients are upregulated in co-culture of the organisms. Co-culture of the two species also results in increased production of virulence factors including dentilisin by *T. denticola*, and the RgpA, Kgp gingipains along with HagA in *P. gingivalis*. In combination, RgpA, Kgp and HagA increase nutrient acquisition and, through the Hag domains on all of these proteins, enhance binding of *P. gingivalis* to substrates including epithelial cells and *Streptococcus gordonii* [25].

As a strict anaerobe, *P. gingivalis* colonization benefits from the activity of the antecedent host-controlled community to reduce the oxygen tension and also to provide an attachment substratum. The influence of the accessory pathogen *S. gordonii* on *P. gingivalis* extends beyond passive attachment, and when in combination with each other *P. gingivalis* and *S. gordonii* differentially expresses a large proportion of their proteomes, indicating that interspecies communication causes profound phenotypic changes [21]. *P. gingivalis* and *S. gordonii* avidly bind to each other through two sets of adhesin-receptor pairs, namely the FimA and Mfa1 component fimbriae of *P. gingivalis* that interact with streptococcal GAPDH and SspA/B surface proteins respectively (Figure 2). Community development between *P. gingivalis* and *S. gordonii* requires the soluble mediator AI-2 and is controlled by a tightly regulated signal transduction cascade within *P. gingivalis* based on protein

tyrosine (de)phosphorylation [26–29] (Figure 2). In association with *S. gordonii*, the *P. gingivalis* tyrosine kinase Ptk1 acts to limit production of the transcription factor CdhR, which is a negative regulator of *mfa1* and hence coadhesion of the organisms is favored. Over time, however, there is increased expression of a tyrosine phosphatase, Ltp1, which dephosphorylates Ptk1 thus lifting inhibition of CdrR, reducing *mfa1* expression and constraining further community development. Moreover, tyrosine phosphorylation/ dephosphorylation also regulates protease expression and capsule production by *P. gingivalis*, thus influencing pathogenic potential. This is reflected in murine models of alveolar bone loss in which dual infection with *P. gingivalis* and *S. gordonii* results in more bone loss compared to infection with either organism alone [30].

S. gordonii can also increase the virulence of *Aggregatibacter actinomycetemcomitans,* a pathogen more closely associated with Localized Aggressive Periodontitis, an aggressive form of localized periodontitis. *A. actinomycetemcomitans* displays resource partitioning to favor carbon sources such as lactate generated by streptococcal metabolism [31]. Moreover, *A. actinomycetemcomitans* possesses two mechanisms, known as fight or flight responses, to avoid toxicity from H_2O_2, a metabolic by-product of *S. gordonii*. Detection of H_2O_2 induces upregulation of catalase (KatA) which degrades H_2O_2, and upregulation of Dispersin B (DspB) an enzyme that releases *A. actinomycetemcomitans* cells from biofilms [32]. In the murine abscess infection model, both of these responses are required for *A. actinomycetemcomitans* to successfully cross-feed with *S. gordonii*. Study of the spatial organization in a co-infection experiment shows that *A. actinomycetemcomitans* is positioned at an optimal distance (>4 μm) from *S. gordonii*, which allows cross-feeding but reduces exposure to inhibitory levels of H_2O_2 [32]. Growth of *S. gordonii* in abscesses is also favored in the presence of *A. actinomycetemcomitans*, which is known to express leukocyte killing toxins [33]. Such properties establish keystone pathogen credentials of *A. actinomycetemcomitans* in that it can impact the fitness of the community as a whole [32] and disable essential elements of immune surveillance.

Not all interactions among periodontal bacteria are synergistic. *S. cristatus* for example, induces downregulation of fimbrial gene expression in *P. gingivalis* through the signaling action of arginine deiminase [34]. Consequently, the distribution of *P. gingivalis* and *S. cristatus* in human subgingival plaque is negatively correlated, and *S. cristatus* attenuates *P. gingivalis*-induced bone loss in mice [35]. Such antagonistic interaction may lead to a 'Red Queen' effect, as *P. gingivalis* can induce cell death of another oral streptococcus, *S. mitis* [18]. Overall, however, the periodontal microbiota conforms more with the 'Black Queen Hypothesis' [36], whereby functions that are energetically costly and are discarded by 'cheaters', are nevertheless retained by a subset of community members as they benefit the entire community. Furthermore, The Black Queen Hypothesis predicts the emergence of keystone organisms: those that have maintained a function that provides an indispensable public good.

Interactions between bacterial communities and epithelial cells

When considering the multi-species aspects of periodontal microbiology, it is important to remember that microbial communities exist in and on gingival epithelial cells (GECs). In

addition to hosting microbial communities, GECs constitute an interactive interface that senses organisms and signals their presence to the underlying cells of the immune system. The encounter between colonizing bacteria and GECs thus sculpts the early stages of both innate and acquired immunity. Many periodontal bacterial are capable of actively internalizing within GECs, a location that provides a nutrient-rich, generally reducing environment that is partially protected from immune effector molecules [1]. Intracellular bacteria disrupt host cell signaling pathways and gene expression patterns which can have profound effects on immune activity and cellular physiology [37]. Co-operative invasion among periodontal bacteria has been documented, for example consortia of *P. gingivalis* and *F. nucleatum* invade GECs in higher numbers than either organism alone [38]. *Filifactor alocis* and *P. gingivalis* also exhibit synergistic infection of epithelial cells and dual species invasion elicits a distinct pattern of host cell response [39]. Furthermore, transcriptional profiling of epithelial cell responses to several periodontal community members (*P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* and *S. gordonii*) revealed that the common core transcriptional response to all organisms was very limited, and organism-specific responses predominate. Overall, *F. nucleatum* and *S. gordonii* perturbed the transcriptome much less significantly than *A. actinomycetemcomitans* or *P. gingivalis* [40]. This supports the concept that microbial communities associated with health tread more lightly on host cells, as compared with the keystone pathogens that disrupt not only an ecologically balanced microbial community, but also epithelial cell physiology [40].

A major reaction of epithelial cells to bacterial challenge is the production and secretion of cytokines. Regulated recruitment of neutrophils, and to a lesser extent T and B cells, is an important feature of a healthy periodontium. Disruption of normal cytokine signaling is, therefore, a major contributor to microbially-induced dysbiotic host responses. Proteolytic degradation of primary and secondary cytokines by *P. gingivalis*, *T. denticola* and other pathogens is well established. However, *P. gingivalis* can selectively impinge upon the production of specific cytokines and chemokines, creating what is known as a localized chemokine paralysis which will favor the growth of the entire microbial community [12,41]. Indeed, studies in mice have shown that the colonization of *P. gingivalis* is associated with increased total counts and altered composition of the periodontal microbiota [3]. This dysbiotic effect of *P. gingivalis* may in part be attributed to suppression of the neutrophil chemokine CXCL8 (IL-8) production by GECs, even in the presence of otherwise stimulatory organisms such as *F. nucleatum*. Inhibition of CXCL8 production depends upon the capacity of *P. gingivalis* to invade GECs and secrete the bacterial serine phosphatase SerB, which specifically dephosphorylates the S536 residue on the p65 subunit of NF-κB. Dephosphorylation of p65 prevents nuclear translocation and ultimately the transcription of the *IL8* gene which is predominantly controlled by NF-κB p65 homodimers [42] (Figure 3). Endothelial cells are additionally targeted by *P. gingivalis* whereby upregulation of E-selectin by other periodontal bacteria is diminished, thus impacting the leukocyte adhesion and transmigration aspects of neutrophil recruitment [43]. While this immune-evasive action may be transient, it appears to have long lasting consequences for the composition and virulence of the microbial community, and for host tissue damage, as a mutant of *P. gingivalis* incapable of producing SerB induces less bone loss in experimental animals compared to the parental strain [44].

The dysbiotic influence of *P. gingivalis* on chemokines is not limited to CXCL8, and the organism also inhibits production of the T-helper 1 (Th1) chemokines CXCL10 (IP-10), CXCL9 (Mig) and CXCL11 (ITAC) from GECs, through downregulation of IRF-1 and Stat1 [45]. As with CXCL8, this inhibition supersedes the ability of pro-inflammatory organisms such as *F. nucleatum* to incite production of these chemokines. *P. gingivalis* may therefore reduce Th1 development, which arguably dictates cell-mediated immunity against periodontal bacteria [46], and allow Th17-mediated inflammation to flourish, as suggested by its ability to induce Th17-promoting cytokines (IL-6 and IL-23, but not the Th1-related IL-12) in antigen-presenting cells [47]. Th17 cells are abundant in periodontitis lesions where they can function as an osteoclastogenic subset that links T-cell activation to inflammatory bone loss.

Similar to *P. gingivalis, T. denticola* can incapacitate *F. nucleatum*-driven GEC IL-8 responses. *T. denticola* suppresses the fusion of internalized *F. nucleatum* with lysosomes, which may inhibit TLR9-dependent IL-8 secretion. In addition, *T. denticola* can reduce the induction of intracellular reactive oxygen species (ROS) in response to *F. nucleatum* which, besides inhibiting a killing mechanism, will also impede TLR responses that control IL-8 production [48].

Subversion of host immunity by dysbiotic microbial communities

In periodontitis, the periodontal pockets become a 'breeding soil' for periodontal bacteria that successfully escape host defense mechanisms. However, evasion of immune-mediated killing is not sufficient to ensure the persistence of periodontitis-associated microbial communities. Growth of these communities depends on nutrients derived from inflammatory tissue breakdown, for example degraded collagen peptides and haem-containing compounds [5]. The inflammophilic community, therefore, needs to subvert the host response in ways that interfere with bacterial killing while promoting inflammation. Several illustrative examples are given below.

Subversion of complement

The activation of complement proceeds via distinct initiation mechanisms (classical, lectin, or alternative) which converge at the third complement component (C3), leading to the generation of effector molecules that mediate recruitment and activation of inflammatory cells (anaphylatoxins C3a and C5a), microbial opsonization and phagocytosis (opsonins such as C3b), and direct lysis of targeted microbes (C5b-9 membrane attack complex) [49]. Components of the complement system can be found in the gingival crevicular fluid (GCF) at up to 80% of their concentration in serum [50]. Periodontitis-associated bacteria, including *P. gingivalis*, *T. forsythia,* and *P. intermedia*, express proteases that effectuate avoidance of complement-mediated phagocytosis and killing. Whereas *P. gingivalis* gingipains and *P. intermedia* InpA degrade the central component C3 [51,52], *T. forsythia* karilysin degrades upstream components such as C4 or pattern-recognition molecules (mannose-binding lectin or ficolins) [53] (Figure 4). As a consequence of complement inhibition, the deposition of opsonins or the membrane attack complex on the bacterial surface is suppressed. Importantly, the gingipains act synergistically with karilysin or interpain A in inhibiting complement [51–53], suggesting that these synergistic proteases

can interfere with complement activation even after their release and diffusion within the biofilm, thereby protecting the entire the microbial community.

Intriguingly, at low concentrations the gingipains of *P. gingivalis* and the InpA of *P. intermedia* activate, rather than inhibit, complement [51,52]. As alluded to above, complement-mediated inflammation is beneficial for the bacteria as it increases the flow of GCF and hence the amount of GCF-derived nutrients. Moreover, these organisms have alternative mechanisms for protection against activated complement. Indeed, both *P. gingivalis* and *P. intermedia* can capture and co-opt physiological soluble inhibitors of the complement cascade, such as the C4b-binding protein [53,54] (Figure 4). In a similar context, *T. denticola* expresses a protein which binds and exploits factor H, a major soluble inhibitor of complement [55].

An interesting hypothesis synthesizing these findings is the following: *P. gingivalis* and *P. intermedia* activate complement when present at low numbers (such as in the initial stages of colonization), resulting in a nutritionally favorable inflammatory response that does not eliminate them (or other bacteria that can similarly hijack host-derived complement inhibitors). However, complement activation can suppress complement-sensitive bacteria, which could otherwise compete for space and nutrients (these might be species associated with periodontal health). At advanced stages of biofilm development, the concentration of proteases is sufficient to inhibit complement activation by all three initiation mechanisms, thereby promoting the survival of the entire biofilm [56,57]. Notably, even under conditions that suppress the canonical activation of the complement cascade, *P. gingivalis* Arg-specific gingipains and *T. forsythia* karilysin were shown to act directly on C5 to release biologically active C5a (the most proinflammatory component of the entire cascade), thereby stimulating inflammatory serum exudate for acquisition of hemin and other nutrients [52,53,58]. Interestingly, the same proteases readily destroy the C5b component of C5, thus preventing the generation of the membrane attack complex [52,53] (Figure 4).

Manipulation of neutrophils

Neutrophils comprise the overwhelming majority (95%) of phagocytes recruited to the gingival crevice or periodontal pocket [59,60]. Biofilms used to be thought of as intrinsically resistant to phagocytosis because phagocytes were assumed to be unable to penetrate biofilms. However, biofilms are actually more like a porous hydrogel than a solid and rigid structure, and thus do not constitute an impermeable physical barrier to phagocytes [61,62]. Therefore, phagocytes do penetrate biofilms and if they fail to engulf the bacteria within, this may be attributed to mechanisms such as the operation of immune evasive strategies within the biofilm [61,62]. In periodontitis, neutrophils largely fail to control bacterial growth and prevent dysbiosis, despite maintaining viability and the capacity to elicit immune responses [6,63]. A number of studies have shown that *P. gingivalis*, *T. denticola, T. forsythia* and certain other periodontal bacteria can resist neutrophil killing through various mechanisms including degradation or inactivation of antimicrobial molecules or granular proteases (for review see [60]). However, it should be noted that *in vitro* resistance of periodontal bacteria to antimicrobial molecules might not necessarily apply to *in vivo* conditions, at least not with equal potency. This is because the activity of antimicrobial

molecules and their susceptibility to bacterial proteases can be modified by other host molecules expected to be found at sites of infection or inflammation [64,65].

A novel mechanism for escaping human or mouse neutrophil killing was recently described for *P. gingivalis.* The pathogen was shown to block an antimicrobial Toll-like receptor (TLR)2-MyD88 pathway via proteasomal degradation of MyD88, whereas it activates a proinflammatory TLR2-Mal-phosphoinositide 3-kinase (PI3K) pathway that additionally blocks phagocytosis by interfering with RhoA GTPase-dependent actin polymerization [66] (Figure 5). Both subversive pathways require an intimate crosstalk between TLR2 and C5aR [66], which is directly controlled by *P. gingivalis* through the C5 convertase-like activity of its Arg-specific gingipains (HRgpA, RgpB) [52,58]. Moreover, the survival of the otherwise neutrophil-susceptible *F. nucleatum* is dramatically enhanced in the presence of *P. gingivalis*, unless C5aR, TLR2, or PI3K signaling in neutrophils is genetically or pharmacologically ablated [66]. Consistent with the *in vitro* findings, local treatments of *P. gingivalis*-colonized mice with inhibitors of C5aR, TLR2 (but not TLR4), or PI3K essentially eliminate *P. gingivalis*, reverse dysbiosis, and inhibit development of periodontitis [3,66,67]. Therefore, by dissociating inflammation from immune clearance that is disarmed, *P. gingivalis* is able to protect itself and also bystander community bacteria.

Whereas opsonization of bacteria with complement fragments (e.g. with C3b) or specific antibodies can enhance phagocytic killing by neutrophils, *P. gingivalis* was shown to effectively degrade both C3 and IgG [52,68–71]. Conversely, hyperimmune antisera to *P. gingivalis* proteases reverses their capacity to degrade C3 and IgG, thereby suppressing the organism's resistance to neutrophil killing [68]. In principle, therefore, specific antibodies induced in the course of periodontitis against *P. gingivalis* could be protective, although in fact periodontitis can proceed despite the presence of antibodies to *P. gingivalis* and other periodontal bacteria [46]. In this regard, naturally induced antibodies to periodontal bacteria are thought to be of low affinity and poor functionality [10,46].

Inhibition of macrophage responses

Although macrophages are minimally present in the gingival crevice (<3%), they can readily encounter periodontal bacteria upon their invasion into the gingival connective tissue [72]. In this context, nitric oxide is a major effector of the intracellular killing capacity of macrophages [73]. *P. gingivalis* can counteract this antimicrobial function by inhibiting the expression of the inducible nitric oxide synthase (iNOS) via a cAMP-dependent mechanism [74]. Maximal induction of the cAMP response requires the concurrent activation of TLR2, C5aR, and CXCR4 by *P. gingivalis* (Figure 6), whereas antagonistic blockade of either C5aR or CXCR4 diminishes the intracellular cAMP levels and promotes the killing of the microbe [74].

The expression of iNOS can also be induced by TLR4 signaling, although *P. gingivalis* can prevent the activation of this receptor (Figure 6). In this regard, the organism can enzymatically modify the lipid A moiety of its lipopolysaccharide (LPS) to either evade or antagonize TLR4 activation [9,12]. The shifting of the lipid A activity from TLR4 agonist (mono-phosphorylated penta-acylated lipid A) to TLR4 evasive (non-phosphorylated tetra-acylated lipid A) or antagonist (mono-phosphorylated tetra-acylated lipid A) is dependent

upon lipid A phosphatase activity [75]. Genetic ablation of 4′-phosphatase activity leads to synthesis of lipid A with TLR4 agonist activity, whereas ablation of 1-phosphatase activity leads to lipid A that functions as a TLR4 antagonist [75,76]. In experiments using wild-type *P. gingivalis* and isogenic phosphatase mutants with a 'locked' lipid A profile, the expression of antagonistic or evasive (inert) lipid A was linked to increased bacterial survival in macrophages, as compared to the expression of TLR4 agonist lipid A [77]. Although it is likely that less nitric oxide is produced in macrophages in response to *P. gingivalis* strains expressing antagonistic or evasive lipid A, a documented mechanism associated with protection of these strains involves the suppression of non-canonical inflammasome activation [77] (Figure 6). The non-canonical activation of the inflammasome, which requires the participation of caspase 11 and can be triggered by the LPS of gram-negative bacteria independently of TLR4, is thought to discriminate cytosolic from vacuolar bacteria that can be detected by canonical inflammasome consisting of the NLRP3 protein (NOD-like receptor family pyrin domain-containing-3), the adaptor molecule ASC (Apoptosis-associated speck-like protein containing CARD), and caspase 1 [78]. In the non-canonical mechanism, cytosolic LPS directly binds and activates caspase 11 [79]. Although TLR4 antagonist lipid A structures can similarly bind caspase 11, they fail to induce caspase 11 oligomerization that is required for its activation [79], thereby explaining why *P. gingivalis* expressing antagonistic or evasive lipid A can prevent the activation of both TLR4 and the caspase 11-dependent non-canonical inflammasome. Importantly, the ability of *P. gingivalis* to modulate the lipid A structure of its LPS was recently shown to contribute to its oral colonization and augmentation of the commensal bacterial load in a rabbit model of experimental periodontitis [76].

In addition to blocking the non-canonical pathway, *P. gingivalis* can inhibit canonical inflammasome activation by other bacteria. Indeed, *P. gingivalis* was shown to suppress endocytic events required for *F. nucleatum*-induced NLRP3 inflammasome activation in macrophages [80]. This mechanism is consistent with the capacity of *P. gingivalis* to modulate actin cytoskeletal rearrangements [66,81] and can potentially promote the virulence of the entire microbial community.

Concluding remarks and future perspectives

Emerging trends in periodontal disease have established that no single microbe is adequately pathogenic by itself; what drives disease is the properties of the community as a whole. According to the PSD model, the periodontal host response is initially subverted by keystone pathogens, the colonization and metabolic activities of which are assisted by accessory pathogens, and is subsequently over-activated by pathobionts. This concept is increasingly acknowledged to be pertinent to other chronic diseases, such as inflammatory bowel disease or colon cancer, where no single or selected few species have been consistently associated with disease but, rather, microbial communities work in concert to cause pathology [19,82–84]. Moreover, keystone or keystone-like pathogens are considered as potentially important players in the gut ecosystem [82,85,86]. Due to inherent limitations and ethical considerations, human studies cannot normally address cause-and-effect relationships and, therefore, addressing the validity of the keystone-pathogen concept may be quite challenging. However, useful insights can be obtained by interventional studies that

specifically target a keystone species such as *P. gingivalis*. In this regard, in non-human primates where *P. gingivalis* is a natural inhabitant of the subgingival biofilm, a gingipain-based vaccine causes a reduction both in *P. gingivalis* numbers and in the total subgingival bacterial load (as well as inhibiting bone loss) [87]. These findings suggest that the presence of *P. gingivalis* benefits the entire biofilm, as predicted by the keystone-pathogen hypothesis [88]. Indeed, periodontitis is an attractive study model of dysbiotic diseases, as it is readily accessible for obtaining both microbial and host tissue samples for longitudinal studies. For instance, study of the host-microbe interaction is facilitated by the availability of organisms recovered directly from specific microenvironments in the mouth, rather than those that have flowed through the ecosystem. The identification of organisms present in discrete clinical conditions at defined times has allowed the molecular dissection of interbacterial interactions, pathogenic mechanisms and host responses. What has emerged is a picture of an intricate synergistic network among organisms that maximizes persistence and metabolic activity through induction of a dysfunctional immune response. Moreover, ineffective immune responses actually stimulate the growth of the periodontal microbial community through provision of proteinaceous and haem-containing substrates derived from inflammatory tissue breakdown. Clearly the periodontal microbiota is well-adapted to the human host, and disease can been regarded as a side-effect of survival and long-term nutritional strategies. Of course, not all individuals are equally susceptible to disease, and although not addressed in this review, genetic and epigenetic variations along with risk factors such as smoking, make a major contribution to the initiation and progression of disease.

Although key questions still remain (Box 1), the PSD model has a number of sequelae that are consistent with clinical and microbiological observations of periodontal disease. For example, immunological rebound and the moderating influence of some community members on *P. gingivalis* will constrain the pathogenic activity of the microbial community and ameliorate tissue destruction, in concurrence with clinical reports of bursts of disease activity. Additionally, decades of microbiological study have failed to produce an inviolable association between any organism or group of organisms and periodontitis. According to the PSD model, however, the identities of individual organism are irrelevant so long as the appropriate genes and associated functions are operational. This notion is supported by a recent metatranscriptomic study which showed that disease-associated microbial communities in periodontitis patients exhibit highly conserved metabolic gene expression profiles, despite high inter-patient variability in terms of microbial composition [89]. Finally, the PSD model has implications for the development of novel therapeutics. Hitherto, most strategies are based on the elimination of *P. gingivalis,* which is difficult to achieve as the organism is embedded in biofilms and within epithelial cells. Instead, community manipulation may be a more viable option. Theoretically at least, favoring the growth of organisms that are antagonistic to *P. gingivalis* and reducing the levels of accessory pathogens would impede the transition to a pathogenic community. Another potentially effective measure could involve targeted modulation of immunity to limit destructive inflammation and reverse the microbial immune evasive strategies that fuel dysbiosis (e.g. chemokine paralysis, subversion of complement-TLR crosstalk, and TLR4 antagonism). For example, antagonizing complement pathways in the gingival tissues could reset host

immunity to a state that is nonresponsive to the subversive activities of *P. gingivalis*, and help restore periodontal tissue homeostasis.

BOX 1

Outstanding questions

- In complex multispecies communities, how do organisms decipher and sort multiple, possibly conflicting, signals from different community members?

- Can epithelial cell signaling be reprogrammed and 'locked' by colonization with health-associated organisms such that they are resistant to dysbiotic communities?

- Can key targets (receptors, signaling molecules) of microbial immune subversion be confirmed by genetic association studies?

- Can host modulation to counteract microbial immune subversion, or bacterial modulation to establish a dominant health-associated community, contribute to the treatment of human periodontitis?

Acknowledgments

Glossary

Accessory pathogen	an organism that, while commensal in a particular microenvironment, nonetheless supports or enhances the virulence of another organism
Co-adhesion	binding between different bacterial species mediated by specific adhesin-receptor interactions
Dysbiosis	a state of imbalance in the relative abundance or influence of species within a microbial community associated with inflammatory disease
Gingival crevicular fluid (GCF)	serum exudate originating in the gingival capillaries and flowing into the gingival crevice containing also locally produced immune and inflammatory mediators
Homeostasis	a condition of equilibrium or stability in a system which is maintained by adjusting physiological processes to counteract external changes; a balanced relation between a host tissue and the resident microbiota that prevents destructive inflammation or disease

Keystone pathogen	a pathogen with a disproportionately large effect on its environment relative to its abundance (e.g. low-abundance *P. gingivalis* remodels a commensal microbial community into a dysbiotic and disease-provoking microbiota)
Pathobiont	an organism that promotes pathology under conditions of disrupted host homeostasis
Periodontal diseases	group of microbially induced inflammatory disorders of the tissues that surround and support the tooth including the gingiva (gum), periodontal ligament and alveolar bone. Uncontrolled periodontal disease leads to tissue destruction and eventually tooth loss
Periodontal pocket	the pathologically deepened gingival crevice (narrow space between the tooth surface and the free gingiva)
Polymicrobial synergy	the process by which one organism enhances the colonization and/or virulence of another
Red Queen effect	an hypothesis in evolutionary biology which holds that organisms must continually adapt in order to survive in an environment with continuously changing competing organisms

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Highlights

- Polymicrobial synergy and dysbiosis drives periodontitis in a susceptible host

- Dysbiosis involves specialized accessory and keystone pathogens and pathobionts

- Microbial immune subversion is central to the persistence of dysbiotic communities

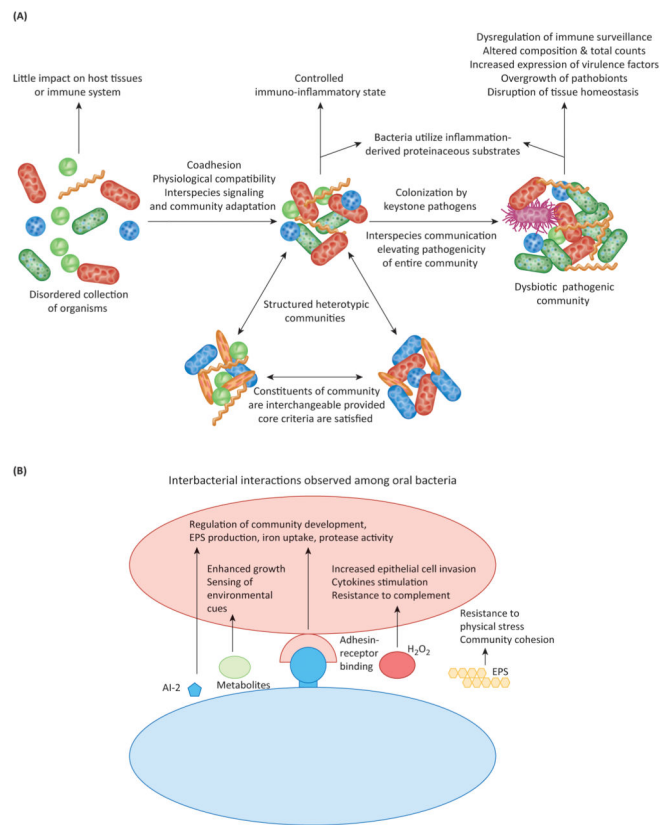- The dysbiotic microbiota sustains itself by feasting on the 'inflammatory spoils'

Figure 1. The polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology
A. Model overview. Community formation driven by co-adhesion and physiological compatibility initially leads to a balanced interaction between host immunity and the metabolism of the inflammophilic microbiota. The identities of individual species are less important than the presence of the appropriate complement of genes. As established in mouse models, colonization by keystone pathogens such as *P. gingivalis* enhances community virulence through interactive communication with accessory pathogens such as *S. gordonii* and disruption of immune surveillance. The dysbiotic community increases in number, pathobionts (green) overgrow and become more active, and tissue destruction ensues. B. Summary of synergistic interbacterial interactions that have been documented among oral bacteria. Both direct contact, primarily through adhesin-receptor binding, and sensing of compounds in solution can effect signaling and modulate the phenotypic properties of partner organisms (e.g. induction of adhesin/invasin, protease or complement-resistance genes). Interspecies signaling can also increase levels of extracellular polymeric substances (EPS) which contribute to community cohesion and resistance to physical stresses.
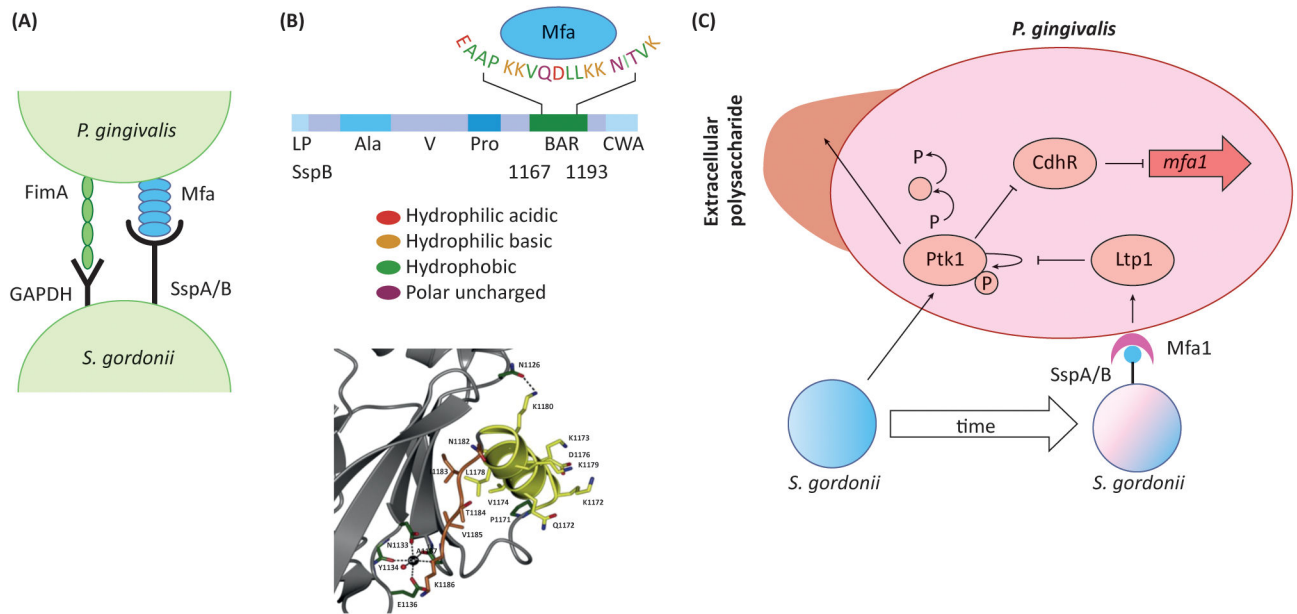
Figure 2. The molecular basis of synergy between *P. gingivalis* and *S. gordonii*
A. The physical association between *P. gingivalis* and *S. gordonii* involves FimA–GAPDH and Mfa1–SspA/B adhesin–receptor pairs on the surfaces of the organisms. B. The upper panel shows the domain structure of the SspB protein and the amino acid residues involved in recognition of Mfa1. BAR spans aa residues 1167–1193, and the EAAP, KKVQDLLKK and NITVK sequences are involved in Mfa1 recognition. The lower panel shows the structure of the SspB C-terminal region with the protruding BAR domain stabilized by a calcium ion, and coordinated by three main chain and two side-chain oxygen atoms and a water molecule. Reproduced from [4] with permission. C. Signaling interactions between *S. gordonii* and *P. gingivalis*. *S. gordonii* induces autophosphorylation of the Ptk1 tyrosine kinase. Ptk1 activates a signaling cascade which converges on inactivation of the transcriptional repressor CdhR. As a result, expression of the minor fimbrial adhesin subunit Mfa1 is elevated and *P. gingivalis* is 'primed' for attachment to *S. gordonii*. Ptk1 is also a key component of the machinery for secretion of extracellular polysaccharide, and active Ptk1 increases the amount of polysaccharide material on the surface of *P. gingivalis*. Over time, however, direct contact mediated by Mfa1-SspA/B binding increases expression of the tyrosine phosphatase Ltp1, which dephosphorylates Ptk1, ultimately relieving repression of ChdR. Expression of the Mfa1 fimbrial adhesin is reduced and community development is constrained.

Abbreviations: Ala, alanine-rich repeats; BAR, SspB Adherence Region, the Mfa1-interacting domain; CWA, cell wall anchor; LP, leader peptide; Pro, proline-rich repeats; V, variable region.
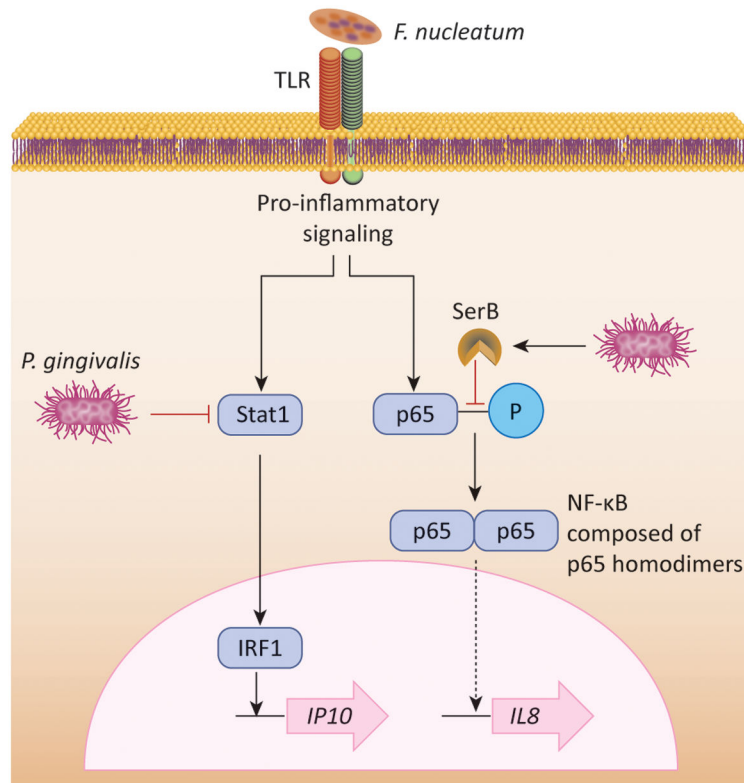
Figure 3. Mechanisms of localized chemokine paralysis

Many oral bacteria such as *F. nucleatum* engage TLRs on epithelial cell surfaces and activate pro-inflammatory signaling pathways. However, production of the neutrophil chemokine IL-8 (CXCL8) and the T-cell chemokine IP-10 (CXCL10) from epithelial cells is suppressed by *P. gingivalis*. Invasive *P. gingivalis* inactivates Stat1 which in turn reduces expression of *IP10* promoted by the IRF1 transcription factor. Intracellular *P. gingivalis* also secretes the serine phosphatase SerB which specifically dephosphorylates the serine 536 residue of the p65 NF-κB subunit, and prevents the formation of p65 homdimers. Translocation of NF-κB into the nucleus is impeded, and transcription of the *IL8* gene is reduced.
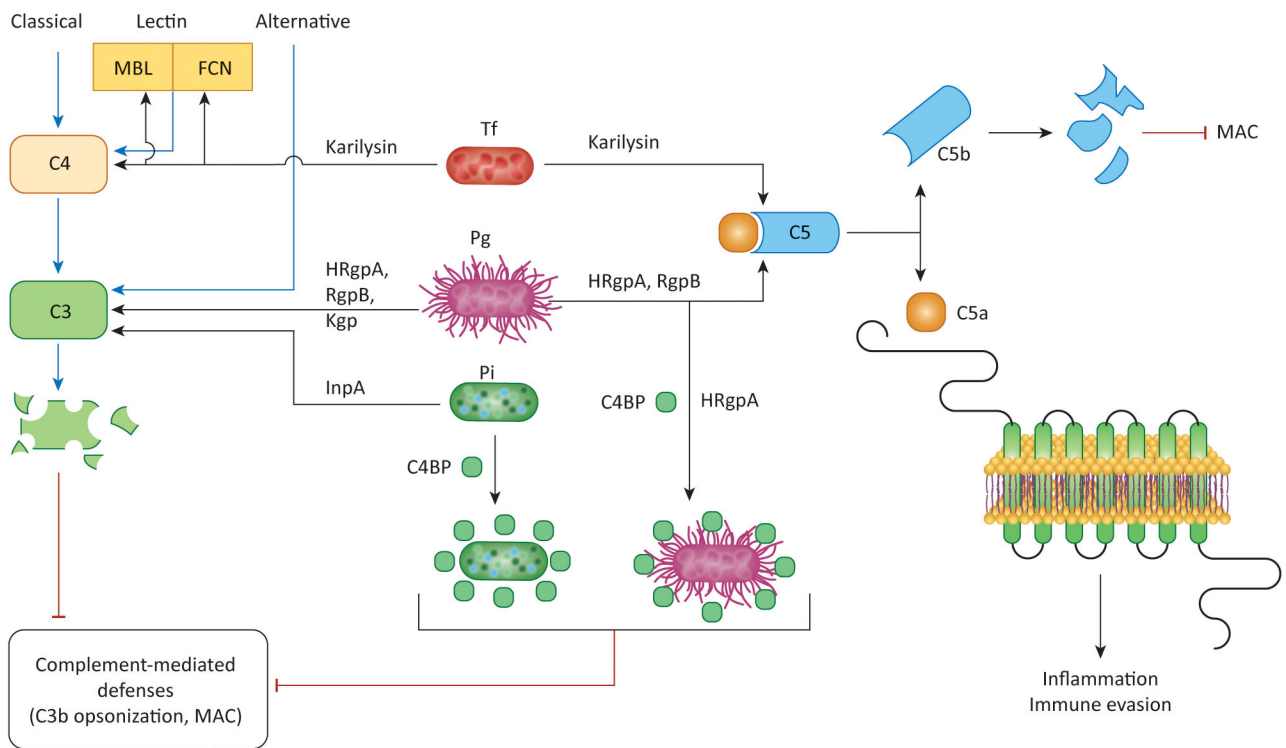
Figure 4. Synergistic inhibition of complement-dependent antimicrobial activities

P. gingivalis, *P. intermedia,* and *T. forsythia* can inhibit the classical, lectin, and alternative pathways of complement activation by degrading C3, C4, mannose-binding lectin (MBL), or ficolins (FCN) through the action of proteases, as indicated. These activities are synergistic and prevent the deposition of C3b opsonin or the membrane attack complex (MAC) on the surface of these pathogens as well as bystander bacterial species. Moreover, *P. gingivalis* and *P. intermedia* protect themselves against complement also by using surface molecules (HRgpA gingipain for *P. gingivalis*, undefined molecule for *P. intermedia*) to capture the circulating C4b-binding protein (C4BP), a physiological negative regulator of the classical and lectin pathways. Furthermore, *P. gingivalis* (via its Arg-specific gingipains HRgpA and RgpB) and *T. forsythia* (via its karilysin) can release biologically active C5a from C5, thereby stimulating inflammation through the activation of the C5a receptor (C5aR). Abbreviations: InpA, interpain A; Kgp, Lys-specific gingipain.
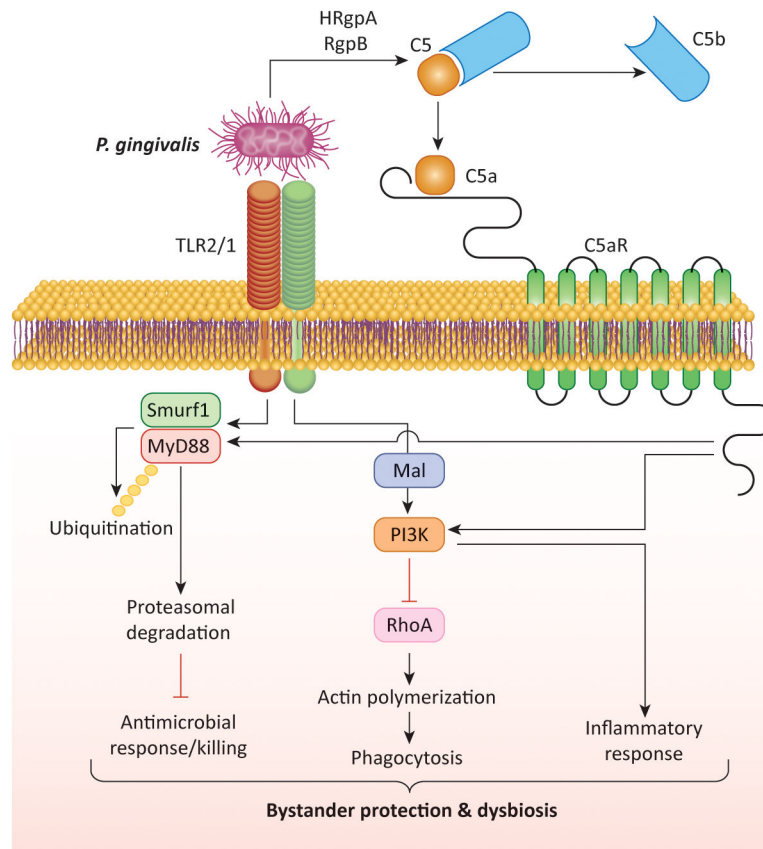
Figure 5. Subversion of neutrophil function and dysbiosis

P. gingivalis co-activates TLR2 and C5aR in neutrophils, and the resulting crosstalk leads to E3 ubiquitin ligase Smurf1-dependent ubiquitination and proteasomal degradation of MyD88, thereby inhibiting a host-protective antimicrobial response. Moreover, the C5aR-TLR2 crosstalk activates PI3K, which prevents phagocytosis through inhibition of RhoA activation and actin polymerization, while stimulating an inflammatory response. In contrast to MyD88, another TLR2 adaptor, Mal, is involved in the subversive pathway and acts upstream of PI3K. The integrated mechanism provides 'bystander' protection to otherwise susceptible bacterial species and promotes polymicrobial dysbiotic inflammation *in vivo*. Reproduced from [61] with permission.
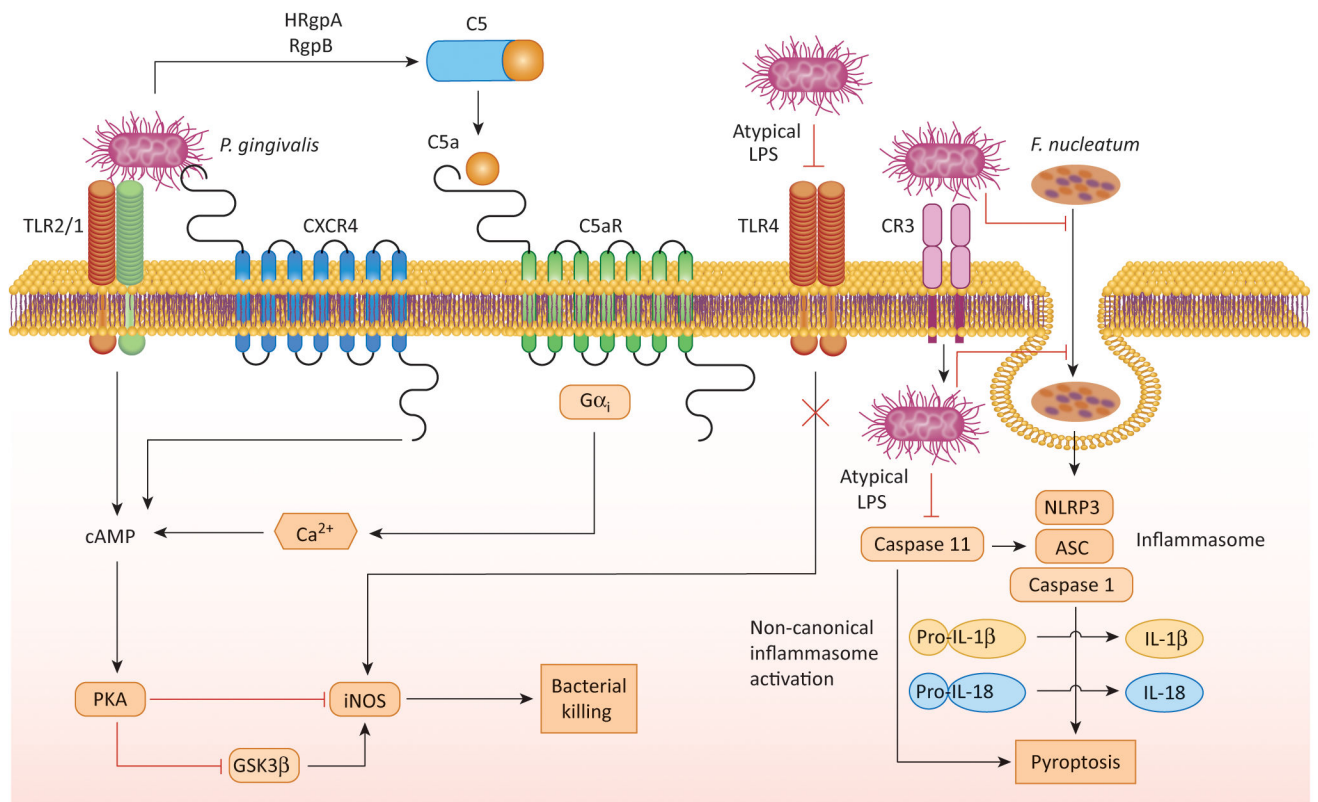
Figure 6. Mechanisms of inhibition of macrophage intracellular killing

P. gingivalis interacts with the TLR2/1 receptor complex, whereas it evades or antagonizes TLR4 by expressing atypical lipopolysaccharide structures (with non-phosphorylated tetra-acylated lipid A or mono-phosphorylated tetra-acylated lipid A, respectively). The TLR2 response is proactively modified through crosstalk with other receptors that are under *P. gingivalis* control, C5aR and CXCR4. *P. gingivalis* induces C5aR activation by virtue of its Arg-specific gingipains (HRgpA and RgpB) which attack C5 and release biologically active C5a. C5a stimulates Gαi-dependent intracellular Ca^{2+} signaling which synergistically enhances the otherwise weak cAMP responses induced by TLR2 activation alone. Maximal cAMP induction is achieved through the participation of CXCR4, which is activated directly by the pathogen's FimA fimbriae and coassociates with both TLR2 and C5aR in lipid rafts. 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. The expression of evasive or antagonistic lipid A moreover allows internalized *P. gingivalis* (possibly entering via complement receptor 3 [CR3] which promotes its intracellular survival) to prevent caspase 11–dependent non-canonical activation of the inflammasome. This mechanism is normally triggered by LPS of intracellular gram-negative bacteria and leads to pyroptosis, a proinflammatory mode of lytic cell death that protects the host against bacterial infection. *P. gingivalis* also inhibits *F. nucleatum*-induced NLRP3 inflammasome activation and cytokine secretion by

inhibiting its endocytosis, although it is uncertain whether this is mediated by extracellular or intracellular *P. gingivalis*.