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Complementary Tolls in the periodontium: How periodontal bacteria modify complement and Toll-like receptor responses to prevail in the host

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Abstract

The complement and the Toll-like receptors are rapidly activatable systems which, in concert, provide first-line innate defense against infection and act as mediators between the innate and the adaptive immune response. The ability of periodontal bacteria to persist and establish chronic infections in the periodontium suggests that they may have evolved strategies to evade, disarm, or subvert these defense systems to their own advantage. Indeed, accumulating evidence indicates that at least some of the major periodontal pathogens utilize ingenious mechanisms to not only undermine each system separately, but also exploit crosstalk points between the complement and the Toll-like receptor pathways. It is conceivable that immune subversive activities by certain keynote periodontal pathogens, such as those comprising the so-called “red complex”, may be critical for the persistence of the entire mixed-species biofilm community in the diseased periodontium. This review summarizes and synthesizes recent discoveries in this field, which offers important insights into the pathology associated with the complex periodontal host-microbe interplay.

Introduction

Innate immunity is a phylogenetically ancient system of host defense and represents the inherited resistance to infection (75). Until relatively recently, the innate immune response was viewed as a non-specific and temporary expedient to “buy time” until the activation of adaptive immunity, which comprises the system of B and T lymphocytes, each of which expresses antigen receptors of exquisite specificity (41). Although lacking the ability to make such fine structural distinctions, innate immunity is nevertheless endowed with considerable specificity. Indeed, germ-line encoded receptors (collectively known as pattern-recognition receptors) can detect and respond to conserved and generally distinct microbial structures, which are shared by related groups of microorganisms (*e.g.*, lipopolysaccharide of gram-negative bacteria or lipoteichoic acid of gram-positive bacteria) (106). Most importantly, innate immunity is sophisticated enough to make judgments that instruct the initiation and progression of the adaptive immune response (41,[106]). In this regard, the acquired specificity of the antigen receptors is not the result of co-evolution with microbes but the outcome of randomly generated

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gene recombination. Thus, even though the adaptive immune receptors can bind virtually any structure, they have no clue on the biological context of the encountered antigen (*i.e.*, should they respond or not?). This information, however, is provided by innate immune mechanisms, which act as mediators between detection of infection and induction of the adaptive response. Not surprisingly, therefore, successful pathogens which disarm or subvert host defenses target preferentially innate immunity (42) and particularly central systems such as the complement and the Toll-like receptor (TLR) family of pattern-recognition receptors (97,[141]).

In the oral cavity, innate immunity contributes significantly to antimicrobial defense, although inadequate or overexuberant activation of the innate response may lead to oral disease, such as periodontitis (36,[53]). In this context, periodontal health represents a dynamic state where proinflammatory and antimicrobial activities to control infection are optimally balanced by anti-inflammatory mechanisms to prevent unwarranted inflammation (43). This homeostatic balance may be disrupted, however, either by genetic defects in host immunity or by pathogens that undermine host defense mechanisms (43,[89],[93]). It should be noted that pathogen-instigated immune suppression of specific pathways and destructive inflammatory responses in the periodontium are not necessarily mutually exclusive, since the latter may arise as a consequence of the inability to control infection (52).

This review focuses on two key innate immune components, the complement and the TLRs, and discusses how these systems interact with each other and with periodontal bacteria. The ability of periodontal pathogens to persist and establish chronic infections suggests that they may have evolved ways to disarm these defense mechanisms or subvert them to their advantage. Understanding the mechanisms of periodontal host-pathogen interplay can offer important insights into the disease pathogenesis and facilitate the rational design of therapeutic interventions. A brief background on complement and TLR biology is presented below to facilitate the discussion on their interactions with periodontal bacteria.

Complement, TLRs, and potential for crosstalk

The term “complement” was coined by Paul Ehrlich in the late 1890s to describe a heat-sensitive activity in serum that is *complementary* to that of antibody in causing lysis of bacteria (153). In line with this early view, the complement has been traditionally considered as an antimicrobial enzyme system found in serum and inflammatory exudates like the gingival crevicular fluid (7,[111],[124]). However, it is now well appreciated that complement constitutes a fundamental component of innate immunity, by virtue of its ability to orchestrate critical events during immune and inflammatory responses, including regulation of other innate or adaptive immune pathways (67,[99],[104],[183]).

The triggering of the complement system involves sequential activation and proteolytic cleavage of a series of serum proteins, leading to recruitment and activation of inflammatory cells, microbial opsonization and phagocytosis, and direct lysis of targeted pathogens (104). In addition to the serum components, the integrated complement system also includes membrane-bound regulators and receptors for interactions with various mediators of the immune system. Complement activation can proceed through three distinct mechanisms, namely the classical, lectin, or alternative pathways (104) (Fig. 1). All three pathways converge at a central step, involving activation of the third component of complement (C3) by pathway-specific C3 convertases (96,[104]). The activation of the classical pathway is initiated by antigen-antibody complexes, whereas the lectin pathway is triggered through interaction of a secreted pattern-recognition receptor (the mannose-binding lectin) with specific carbohydrate groups on the surface of a variety of microorganisms. To ensure fast and immediate response to invading pathogens, the complement cascade is maintained at a low level of activity (“tick-over”) by the so-called alternative pathway. This pathway is initiated by spontaneous

hydrolysis of C3 to C3(H_2O), thereby inducing a conformational change that allows binding to complement factor B and formation of the initial alternative pathway C3 convertase. This results in rapid propagation of the alternative pathway, as long as there is no sufficient negative regulation as normally occurs with non-self surfaces (*e.g.*, bacteria). In addition to this mechanism, the alternative pathway can be induced by bacterial lipopolysacharide and lipooligosacharide molecules in a way that strictly requires the participation of the plasma protein properdin (86). The alternative pathway may represent up to 80% of complement activation (104). In all three pathways, proteolytic cleavage of a series of proteins downstream of C3 leads to the generation of effector molecules, including opsonins (C3b, iC3b) and anaphylatoxins (C3a, C5a). The iC3b fragment is generated by further cleavage of microbe-attached C3b and mediates phagocytosis by complement receptor-3 (Fig. 1). The inflammatory anaphylatoxins C3a and C5a activate seven-transmembrane domain G-protein-coupled receptors, known as the C3a receptor and C5a receptor (CD88), respectively. A newly identified but modestly characterized alternative receptor for C5a is the so-called C5a receptor-like 2; this was originally believed to be an anti-inflammatory decoy receptor, but is now thought to play a novel and distinct role in sepsis (173). Another C5 cleavage product, the C5b, initiates the assembly of the C5b-9 membrane attack complex, which induces lysis of complement-targeted bacteria (104) (Fig. 1).

TLRs comprise a family of pattern recognition receptors named after their similarity to the *Drosophila* Toll protein (100,[107]). Their discovery in the late 1990s has sparked a resurgent interest in innate immunity. Indeed, the study of TLRs has helped appreciate the economical specificity of the innate immune system and that adaptive immunity did not evolve to replace innate immunity, but rather evolved around it. TLRs are transmembrane glycoproteins comprising an N-terminal leucine-rich repeat domain, a transmembrane region, and a C-terminal cytoplasmic signaling domain (76,[83]). These receptors are primarily expressed by first-line professional phagocytes (*e.g.*, neutrophils, macrophages, and dendritic cells) and are thus strategically located for early recognition of microbial pathogens (1). To date, 10 human TLRs have been identified which generally sense and respond to distinct types of microbial structures (Fig. 2). For instance, TLR3 responds to double-stranded viral RNA, TLR4 responds to enterobacterial lipopolysaccharide, TLR5 to bacterial flagellin, and TLR9 to microbial CpG DNA. TLR2 is unique in that it heterodimerizes with signaling partners (TLR1 or TLR6) for detecting and responding to microbial cell wall components, such as lipoteichoic acid, lipoproteins, fimbriae, or yeast zymosan (1,[12],[90]). Those TLRs which are mainly responsible for detecting extracellular microbial structures are expressed on the host cell surface (TLRs 1, 2, 4, 5, and 6), whereas those specializing in detecting viral or bacterial nucleic acids are appropriately located intracellularly on endocytic vesicles or organelles (TLRs 3, 7, 8 and 9) (Fig. 2). Following ligand binding, TLR signaling is triggered upon recruitment to the cytoplasmic TLR domains of adaptor proteins, which help propagate the signals to downstream kinases and transcription factors. This ultimately leads to induction of immunoregulatory genes that activate or suppress the innate immune and inflammatory response (for a detailed review on TLR signal transduction see (119,[120]). The presence of both common and selective adaptors, in conjunction with the apparent compartmentalization of the TLRs, allows the induction of individual signaling pathways (for at least some TLRs) in addition to a core TLR response (120). It is thus possible that activation of diverse TLR intracellular pathways, dependent upon different TLR ligand specificities, may allow the host to tailor a response that is appropriate against a given pathogen.

Both complement and TLRs are rapidly activated by most pathogens upon encounter with the host, and common microbial molecules like gram-negative bacterial lipopolysaccharide and yeast zymosan can act both as TLR ligands and complement activators. It is conceivable that the coordination of the early innate response would require a crosstalk between the complement and the TLR systems. In this regard, a systematic analysis of crosstalk in intracellular signaling

pathways has revealed that a great number of microbe-induced stimuli converge on a relatively limited number of effector signaling pathways (116). In principle, a molecular crosstalk between complement and TLRs could result in cross-regulation of the two systems, including potential synergistic or even antagonistic interactions. These interactions may help enhance host defense or regulate it to prevent excessive inflammatory responses. However, it is also plausible that at least some crosstalk interactions may be instigated by the pathogens themselves for deregulating or modifying the host response in a way that favors their survival. Though only recently has this issue started to be addressed, available evidence indicates bidirectional cooperation between the complement and the TLR system, since complement regulates TLR activation (67,[183]), whereas TLR signaling transmodulates the activity of complement receptors (60,[63]).

Periodontitis, associated bacteria, and complement/TLR immunity

Periodontal disease is possibly the most common chronic disorder of infectious origin in humans, resulting in inflammatory destruction of the tooth-supporting tissues (126). The disease is initiated by certain species of subgingival gram-negative anaerobic bacteria co-existing within dynamic communities of highly-organized architecture (31,[158]), originally termed “dental plaque” which predates the more modern term “biofilm” (46,[109]). In fact, seldom would discussions on pathogenic biofilms omit the classical example of dental plaque (25,[88]). In periodontal health, the ordered structure of the dental plaque biofilm consists predominately of gram-positive, facultative anaerobic bacteria, although the onset of the disease is associated with a shift to gram-negative anaerobic bacteria which begin to colonize the subgingival pocket with greater frequencies (157). Using a color-coded system, Socransky and colleagues characterized these microbial communities as red, orange, green, purple and yellow complexes, on the basis of cluster analysis, community ordination, and associated disease severity (158). A high prevalence of red complex members such as Porphyromonas gingivalis, Treponema denticola, and Tanerella forsythia correlates strongly with periodontal tissue destruction (70,[158]). *Prevotella intermedia* and *Fusobacterium nucleatum*, both members of the orange complex, are also associated with various forms of periodontal disease (28,[158],[178]).

While the bacteria constitute an essential etiologic factor, it is the host inflammatory reaction to bacterial challenge that primarily mediates periodontal tissue damage (43). This is not to say, however, that the challenge in periodontitis involves simply the issue of controlling the inflammatory response. In a related context, purely anti-inflammatory therapies in sepsis clinical trials have generally failed even if the initial hyperinflammatory stage was controlled; indeed, many patients would succumb to the infection itself at later stages of the disease (138). Therefore, periodontal and other infection-driven inflammatory diseases should be dealt with in ways that address both infection and inflammation. This in turn requires adequate understanding of both protective and destructive aspects of the host response and how pathogens may evade the former and contribute to the latter.

There is strong evidence that complement and TLRs form an important link between infection and various local or systemic autoimmune or inflammatory conditions, such as septic shock, ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, atherosclerosis, ischemia/reperfusion injury, and asthma (3,[16],[24],[136],[172]). There is also evidence for complement and TLR involvement in periodontitis. In this regard, the complement is abundantly found in the gingival crevicular fluid, where it is present at up to 70% of the serum concentration (131). Activated complement fragments are abundantly found in the gingival crevicular fluid of periodontitis patients, whereas they are absent or present in lower concentrations in the crevicular fluid from healthy individuals (7,[17],[147],[148]). The functionality of the complement components of the gingival crevicular fluid has been confirmed (26),

whereas activated complement fragments have also been detected in the gingival connective tissue (26). Importantly, induction of experimental gingivitis in human volunteers causes progressive elevation of complement cleavage products and correlates with increased microbial plaque accumulation, clinical inflammation, and bleeding on probing (124). These clinical findings suggest a role for complement involvement in periodontal pathogenesis. Moreover, in vitro mechanistic studies have demonstrated complex interactions between periodontal bacteria and the complement system (61,[105],[128],[131],[156]).

In addition to elevated complement activity, the inflamed periodontium is infiltrated by TLR-expressing inflammatory cells, whereas healthy gingivae display significantly lower levels of TLR expression (112,[113],[135]). Besides professional inflammatory cells, gingival epithelial cells and fibroblasts also express TLRs and the level of expression correlates with disease activity (94,[135],[162],[171]). In terms of function, TLRs (particularly TLR2 and to a much lesser extent TLR4) have been shown to regulate important immune and inflammatory responses to periodontal bacteria in vivo and in vitro (6,[15],[23],[30],[40],[57],[62],[122],[182]).

However, the precise roles, whether protective or destructive, played by the complement and the TLRs in periodontal infection and inflammation are poorly understood. This is partly because these issues have not been systematically investigated as yet. Nevertheless, a substantial body of available literature exists, which, if properly synthesized and interpreted, could provide important new insights for future studies. Below, we aim to contribute to this direction by discussing pertinent studies from the oral microbiological and immunological literature. We will focus on major pathogens for which enough literature exists on their interactions with both the complement and the TLR systems, namely, *P. gingivalis*, *T. denticola*, *T. forsythia*, and *P. intermedia*.

P. gingivalis: master of subversion

In principle, a host inflammatory response can become destructive when it is deregulated and its magnitude gets out of proportion to the microbial threat, or when it is undermined by pathogens leading to persisting but ineffective inflammation in terms of infection control (43,[52],[93],[140]). In the context of periodontitis, *P. gingivalis* could be reasonably characterized as a master of subversion, on the basis of sophisticated sabotage tactics presented below. This gram-negative anaerobic organism expresses an elaborate system of adhesins and proteolytic enzymes (*e.g.*, long and short fimbriae, hemagglutinins, and Arg- and Lys-specific cysteine proteinases known as gingipains), which coordinately enable the pathogen to colonize host tissues and secure critical nutrients (98). As important as these virulence features may be, *P. gingivalis* would probably be unable to establish a chronic infection, unless it could have also evolved ways to evade, undermine, or trick the host immune system. This is lucidly exemplified by its capacity to not only subvert both complement and TLR immunity but, moreover, to exploit crosstalk signaling pathways between complement and TLRs.

Neutralization of complement action

P. gingivalis causes significant inhibition of complement activation, regardless of the initiation pathway involved (classical, lectin, or alternative; Fig. 1), through gingipain-dependent degradation of key complement components, such as the C3 (reviewed in refs. [130],[155]). As a consequence, the deposition of opsonins or the membrane attack complex on the pathogen surface is suppressed, unless its gingipain activity is ablated by chemical or genetic means (145,[156]). All three gingipain enzymes participate in complement inactivation, although the Arg-specific enzymes (HRgpA and RgpB) are more potent in this regard than the Lys-specific gingipain (Kgp) (128). As a further safety measure, the pathogen appears to hijack physiological mechanisms of inhibiting the complement cascade. In this regard, *P. gingivalis* uses its HRgpA to capture the circulating C4b-binding protein on the bacterial cell

surface, thereby acquiring the ability to negatively regulate the classical pathway C3 convertase (132).

The above summarized findings are consistent with observations that *P. gingivalis* is exquisitely resistant to the lytic action of complement (128,[156]). Curiously, however, Arg- and Lys-gingipain mutants are as resistant as the wild-type organism upon their exposure to human serum, even though active complement fragments are readily deposited on their bacterial surface (156). These intriguing observations suggest an inherent protective mechanism that is independent of complement inactivation. Indeed, a surface anionic polysaccharide was implicated in this inherent resistance since *P. gingivalis* mutants lacking this structure become readily susceptible to complement-mediated lysis (156). Although this anionic polysaccharide may directly confer resistance, the possibility for an indirect effect may not formally be ruled out. In this context, certain pathogens (*e.g.*, *Helicobacter pylori* and *Escherichia coli*) acquire resistance against complement lysis by expressing molecules that can bind CD59, a host regulatory protein which inhibits the terminal step of the membrane attack complex formation (97).

It, therefore, appears that *P. gingivalis* may be using a number of different reinforcing mechanisms to ensure its survival in the presence of complement. In this regard, since the inhibitory mechanisms of *P. gingivalis* against complement activation are leaky (128), it makes sense that it has also developed inherent resistance against complement-dependent lysis. However, if the surface anionic polysaccharide is sufficient to provide inherent protection, a plausible question is why the pathogen has additionally evolved ways to suppress a system that cannot kill it. An interesting interpretation is that *P. gingivalis* may have evolved complement inactivation capacity not for its own protection, but for the benefit of other organisms occupying the same subgingival niche. This action may not be as altruistic as it seems; it may actually offer a survival advantage for *P. gingivalis*, as it depends on other periodontal bacteria for enhanced colonization and full expression of virulence (81,[88],[127]). Since *P. gingivalis* is resistant to the lytic action of complement (128,[156]), the ability of the complement system to directly offer host protection against this organism is seriously questioned. Nevertheless, it cannot be ruled out that complement activation may indirectly fight this pathogen through the recruitment and activation of phagocytic cells. However, as discussed in a later section (Exploitation of crosstalk interactions between TLRs and complement), *P. gingivalis* may have evolved strategies to diminish or evade its destruction by phagocytes in the presence of complement.

Evasion and subversion of TLRs

Available evidence suggests that *P. gingivalis* may have also evolved ways to evade or subvert the TLR system, which senses this organism primarily through TLR2, as shown in vitro and in vivo (15,[57]). On the other hand, TLR4 appears to play little or no role in cell activation in response to this oral bacterium (15,[57]). These observations appear curious given that *P. gingivalis* is a gram-negative organism which expresses a lipopolysaccharide. However, the organism elegantly utilizes specific lipid A 1- and 4′-phosphatases and a deacylase which in concert generate a tetra-acylated and dephosphorylated lipid A structure (21). This modification renders the lipopolysaccharide molecule biologically inert, thereby allowing *P. gingivalis* evade TLR4 activation (21). At the same time, this modification confers protection against polymyxin B and perhaps other cationic anti-microbial peptides (21). Intriguingly, the presence of high concentrations of hemin (an environmental nutrient found in diseased sites) suppresses lipid A 1-phosphatase activity and leads to the production of a mono-phosphorylated lipid A, which actively antagonizes TLR4 activation (21,[22]). Thus, even though *P. gingivalis* may express other molecules with intrinsic TLR4 agonistic activity, TLR4 activation is likely suppressed in the context of the whole organism (Fig. 3), as seen both in

vitro and in vivo (15,[57]). In this regard, *P. gingivalis* behaves like certain other, non-oral pathogens which have also opted to modify their surface structures so as to escape TLR4 recognition (8,[110],[152]).

The above considerations may explain why TLR2, rather than TLR4, is the predominant TLR involved in *P. gingivalis* recognition. Induction of TLR2 signaling by *P. gingivalis* requires a signaling partner (TLR1 or TLR6), takes place in membrane lipid rafts where the receptors are recruited ad hoc, and is facilitated by a non-signaling coreceptor (CD14) which constitutively resides in lipid rafts (57). Although the host TLR2 response may be potentially protective, *P. gingivalis* has developed ways to undermine the intended host response. Indeed, the pathogen was shown to manipulate the TLR2 response by instigating a molecular crosstalk between TLR2 and the CXC-chemokine receptor 4 in macrophage lipid rafts (62). Specifically, the binding of *P. gingivalis* fimbriae to CXC-chemokine receptor 4 induces cAMP-dependent protein kinase A signaling, which in turn suppresses TLR2-dependent activation of nuclear factor-κB and induction of nitric oxide (Fig. 3) (62). The inhibition of production of this key antimicrobial molecule promotes the ability of *P. gingivalis* to survive in vitro and in vivo (62).

The impact of TLR2 signaling on the ability of *P. gingivalis* to cause experimental periodontitis was examined by two independent studies, which found that TLR2-deficient mice (but not TLR4-deficient or wild-type controls) are protected against periodontal bone loss (15,[47]). These findings are consistent with the notion that TLR2 signaling is manipulated by *P. gingivalis* in a way that promotes its virulence. However, an alternative or additional interpretation is that the observed enhanced bone loss in normal mice could be attributed to *P. gingivalis* induction of TLR2-mediated inflammatory osteoclastogenesis (169).

Exploitation of crosstalk interactions between TLRs and complement

TLR2 activation by *P. gingivalis* induces two distinct signaling cascades (60). One of the cascades leads to induction of proinflammatory and antimicrobial responses, and represents the pathway that is manipulated by *P. gingivalis* through exploitation of CXC-chemokine receptor 4, as outlined above. The other cascade represents a proadhesive pathway and involves a crosstalk between TLR2 and the complement system (60). Specifically, *P. gingivalis* induces TLR2 inside-out signaling which transactivates the adhesive capacity of complement receptor-3 (64) (Fig. 4). This crosstalk is made possible by the property of complement receptor-3 to cluster with TLRs in lipid rafts of *P. gingivalis*-stimulated cells (57). Once transactivated, however, complement receptor-3 becomes a target of subversive activity by *P. gingivalis*.

Indeed, *P. gingivalis* uses its fimbriae to bind complement receptor-3, which in turn mediates the uptake of this oral pathogen by macrophages (59). Intriguingly, this phagocytic mechanism does not promote the killing of *P. gingivalis* (170), possibly because complement receptor-3 is not linked to vigorous microbicidal mechanisms (140). In contrast, when *P. gingivalis* is phagocytosed by alternative receptors, *i.e.*, when complement receptor-3 is blocked or genetically ablated, the intracellular killing of this pathogen is dramatically enhanced (170).

The interaction of *P. gingivalis* with complement receptor-3 also activates the extracellular signal-regulated kinase 1/2, which in turn selectively inhibits mRNA expression of the p35 and p40 subunits of interleukin-12 (56) (Fig. 4). Interleukin-12 is a key cytokine involved in pathogen clearance through regulatory effects on the production of interferon-γ, which is a potent activator of the macrophage microbicidal capacity (168). Consistent with the above, wild-type mice elicit lower levels of interleukin-12 and interferon-γ and display impaired clearance of *P. gingivalis* systemic infection compared to mice that lack complement receptor-3 (56). Similar results are seen after CR3 blockade with a specific antagonist which, furthermore,

suppresses *P. gingivalis* induction of periodontal bone loss in mice (56). In brief, there is compelling evidence that complement receptor-3 constitutes an Achilles' heel which confers host susceptibility to *P. gingivalis* infection. In this regard, it seems likely that *P. gingivalis* may have actually co-opted a natural anti-inflammatory mechanism to evade innate immunity. Specifically, complement receptor-3 is heavily committed with phagocytosis of iC3b-coated apoptotic cells, which are not normally recognized as danger (84,[108]). This precludes induction of a vigorous host response and, in fact, production of interleukin-12 is inhibited following phagocytosis of apoptotic cells by macrophages (84).

Although *P. gingivalis* inhibits the complement cascade, curiously enough, the pathogen proactively generates one of the active complement fragments. Specifically, all three gingipains (HRgpA, RgpB, and Kgp) act in a C5 convertase-like manner and generate biologically active C5a through limited degradation of C5, whereas the C5b remnant is functionally inert (128, [176]). When C5 is oxidized by hydroxyl radicals (as may occur in the oxidative environment of the inflammatory response) the gingipains generate increased C5a biological activity (35). Furthermore, *P. gingivalis* may indirectly generate functional C5a by exploiting the physiological crosstalk between the coagulation and the complement systems, which activates the so-called extrinsic pathway (71) (Fig. 1). Indeed, HRgpA and RgpB activate prothrombin to form thrombin (73) which, in turn, generates biologically active C5a by acting as a C5 convertase (71) (Fig. 1). Although C5a can potentially play a key role in host defense against infection (50), it seems highly unlikely that *P. gingivalis* uses its enzymes to generate C5a to contribute to its elimination.

An intriguing question, therefore, is whether there is any selective pressure or advantage for *P. gingivalis* to specifically generate C5a, given that this chronically persisting pathogen overall inhibits the complement cascade. A possible scenario is that local generation of excessive levels of C5a could incapacitate the antimicrobial function of gingival crevicular neutrophils rendering them less threatening to *P. gingivalis*. This is because neutrophils become immunologically paralyzed in the presence of high concentrations (10-100 nM) of C5a and thereby fail to carry out functions such as chemotaxis, phagocytosis, and production of antimicrobial and inflammatory mediators (72,[172]). Such immunological dysfunction has been seen both in vitro and in vivo and involves both human and rodent neutrophils (72,[137],[172]). In fact, C5a-mediated inhibition of neutrophil killing of *P. gingivalis* does occur, at least in vitro (J. Krauss and G. Hajishengallis, unpublished data). However, the underlying mechanisms, whether involving immune paralysis or alteration of specific signaling pathways, are currently under investigation. In addition to its potential exploitation by *P. gingivalis*, C5a may amplify periodontal tissue damage through its ability to recruit and activate inflammatory cells. For example, enhanced production of reactive oxygen species by C5a-stimulated neutrophils (50) may contribute to oxidative periodontal tissue destruction (19). On the other hand, this host response would not affect *P. gingivalis*, since it is resistant to killing by reactive oxygen species (58,[114]).

Even if the gingipain activity of *P. gingivalis* is capable of increasing the microenviromental C5a concentrations to paralyzing levels for the neutrophils, this would not impinge on the function of macrophages, which can also be recruited to the gingival crevice or additionally interact with the pathogen in the periodontal connective tissue (33,[166]). Indeed, macrophages are quite resistant to the deleterious effects of high C5a concentrations, because they express relatively modest levels of the C5a receptor relative to the neutrophils (172). For instance, whereas the ability of neutrophils to induce tumor necrosis factor-α (and other innate responses) is inhibited in the presence of C5a at ≥ 10 nM, the macrophages display potentiated tumor necrosis factor-α responses under the same C5a concentrations (72,[137]).

Therefore, even at high levels, C5a does not exert a general immunosuppressive influence on macrophages. Strikingly, however, C5a can specifically downregulate cytokines of the interleukin-12 family. Indeed, C5a-induced signaling in macrophages interferes with TLR-induced expression of mRNA for the interleukin-12 p35, interleukin-12/interleukin-23 p40, and interleukin-23 p19 subunits (67,[95]). These regulatory effects are possibly mediated through C5a-induced phosphatidylinositol-3 kinase and extracellular signal-regulated kinase 1/2 signaling, which in concert suppress critical transcription factors, the interferon regulatory factor-1 and -8 (67) (Fig. 4). At the protein level, the production of interleukin-12 is inhibited both in vitro and in vivo, leading to suppression of T-helper type 1 cell-mediated immunity (67,[183]). Moreover, the ability of C5a to inhibit mRNA expression of both interleukin-23 subunits strongly suggests that C5a can interfere with the capacity of this cytokine to support the development of the T-helper type 17 cell subset (11). The physiological significance of these C5a regulatory effects is likely to attenuate potential tissue damage mediated by T-helper type 1 and T-helper type 17 cells, as seen in various pathological inflammatory conditions (43,[102],[144]). However, undesirable outcomes may arise when C5a is not produced physiologically but through the uncontrolled action of microbial enzymes, such as the *P. gingivalis* gingipains. Since interleukin-12 is important for immune control of *P. gingivalis* (56), it is possible that this pathogen may exploit the C5a-induced crosstalk with TLR2 for inhibiting IL-12-dependent immune clearance. Such evasion mechanism may be complementary, rather than redundant, since the interaction of *P. gingivalis* with complement receptor-3 causes partial inhibition of interleukin-12 production (about 60%) (56). The notion that *P. gingivalis* hijacks C5a for its own benefit is additionally supported by observations that the intracellular survival of this pathogen in macrophages is promoted in the presence of C5a (M. Wang and G. Hajishengallis, unpublished observations).

Interestingly, unlike C5a, C3a is extensively degraded by *P. gingivalis* gingipains and does not retain biological activity (176). Whether this is beneficial for the pathogen is uncertain, but it should be noted that C3a exerts direct antimicrobial effects and readily kills both gram-negative and gram-positive bacteria such as *E. coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* (117). If C3a can kill *P. gingivalis* as well, then its gingipain-mediated inactivation would serve to protect *P. gingivalis*.

In summary, it appears that *P. gingivalis* does not have a purely defensive agenda in dealing with the complement system. In other words, the pathogen may not restrict its action to simply inhibiting the complement cascade, but rather may proactively employ specific complement components (such as the complement receptor-3 and the C5a) for bidirectional crosstalk interactions with TLR2 that favor the pathogen (Fig. 4). These and other subversive mechanisms (for a review see refs. [52],[130],[181]) justify the characterization of *P. gingivalis* as a keynote periodontal pathogen, in that it can contribute virulence attributes that are essential for the survival of the entire biofilm community (28,[52]).

T. denticola: spiraling out of control

In health, spirochetes (spiral-shaped bacteria with periplasmic flagella) inhabit the periodontal tissues at relatively low frequencies. In sharp contrast, progressive decline in periodontal health positively correlates with a surge in the spirochete population, which constitutes a substantial percentage of the plaque microbiota in clinically diseased sites (38,[149]). Although their role in the initiation of periodontal diseases is uncertain, *Treponemes* are literally at the forefront of established periodontal lesions in dominating numbers (38). In particular, the elevated presence of *Treponema denticola*, a relatively well-characterized oral spirochete, correlates with progressive disease activity and signifies a defining risk factor for advanced pocketing and periodontal attachment loss (149,[158]). Being a member of the red complex, *T. denticola* can engage in specific co-aggregating interactions with the other members (*P. gingivalis* and *T.*

forsythia) and with *F. nucleatum*, mediated mostly by its 53-kDa major outer sheath protein (139,[149]).

The ability of *T. denticola* to colonize and invade the periodontium can be attributed, at least in part, to its remarkable motility, multiple adhesive properties, and high proteolytic activity (18). In this regard, dentilisin, a cell-surface adhesin with chymotyrpsin-like properties, appears to facilitate invasion of connective tissues via degradation of epithelial cell tight junctions (20). Moreover, dentilisin promotes bacterial invasion of the gingivae by activating matrix metalloproteinases to degrade extracellular matrix, a mechanism which at the same time contributes to periodontal tissue destruction (34,[38]). However, the capacity of *T. denticola* to chronically persist in periodontal tissues suggests that the pathogen may have additionally evolved ways to inactivate or escape immune defense mechanisms. In this context, we review below its interactions with the complement and the TLR systems.

Complementary employment of the enemy's arsenal

Due to its potentially destructive nature for host tissues, complement activation is tightly controlled by membrane-bound as well as soluble regulatory host proteins. One such soluble control protein is factor H, a 155-kDa glycoprotein which controls the alternative pathway by inhibiting the formation and accelerating the decay of the alternative pathway C3 convertase (2). Moreover, factor H contributes to cleavage and inactivation of C3b to iC3b, and thus can attenuate the activation of the classical pathway C5 convertase and the downstream proinflammatory events (2).

Although the most direct way whereby pathogens can inhibit complement activation involves the use of virulence proteases which degrade and inactivate complement components, another evasion mechanism depends upon the ability of the pathogen to hijack and employ circulating complement regulators (97). In this regard, *T. denticola* expresses a 11.4-kDa cell surface lipoprotein which can bind factor H (105). This factor H-binding protein has little or no homology to other bacterial virulence proteins with similar function, suggesting convergent evolution for possible evasion of the complement system. Strikingly, however, once full-length factor H becomes associated with *T. denticola*, the organism uses its serine protease dentilisin to generate a 50-kDa factor H fragment that remains attached to the bacterial surface (105). This seems paradoxical since dentilisin appears to negate the action of the factor H-binding protein. However, the dentilisin-mediated proteolysis of factor H is quite slow since this complement protein remains essentially intact after 40 min and complete digestion requires 1h (105). Since complement activation is a remarkably rapid process, there is plenty of useful time during which *T. denticola* can escape complement killing by means of surface-bound, biologically active factor H. An interesting question that remains unanswered is whether the attached fragment retains useful complement regulatory activity or whether it might be employed to serve a different virulence role. What is quite certain, however, is that proactive acquisition of factor H and its proteolytic processing would not be selected if it was detrimental for the pathogen.

The *T. denticola* dentilisin can additionally use another complement component as substrate, to generate a biologically active fragment. Specifically, this bacterial enzyme generates iC3b upon hydrolysis of the α chain of C3 (179). This is consistent with an early study showing that *T. denticola* is readily opsonized with iC3b (146). Since iC3b-opsonized bacteria are taken up by phagocytes via complement receptor-3, it would seem counterproductive for a pathogen to help the host generate even more iC3b. However, as alluded to above, iC3b-mediated phagocytosis is often associated with weak killing mechanisms or even immunosuppressive signaling (10,[84],[108],[177]) which may be exploited by certain pathogens. Indeed, in addition to *P. gingivalis* discussed above, other microbial pathogens such as *Mycobacterium tuberculosis*, *Bordetella pertussis*, and HIV-1 promote their survival by exploiting complement

receptor-3–mediated entry, either by direct interaction with the receptor or upon opsonization with iC3b (39,[68],[97]).

In summary, although *T. denticola* definitely cannot prevent the initiation of the complement cascade (146), it may be protected from its killing potential by hijacking and employing specific downregulatory factors or specific opsonins for safe intracellular entry. However, additional functional studies are warranted to confirm these plausible mechanisms. Intriguingly, even if *T. denticola* was unable to successfully resist complement-dependent killing, its co-existence with *P. gingivalis* in shared niches (158) may provide it with effective protection mechanisms. In vivo observations that mixed infections with both pathogens are more virulent in mouse models than monoinfections (80,[81],[85]) are consistent with this notion, although the precise synergistic mechanisms operating in vivo have not been elucidated.

Interactions with TLRs: Paying a Toll to escape?

Studies in gingival epithelial cells, macrophages, and transfected cell lines have shown that *T. denticola* activates TLR2, rather than TLR4 (5,[13],[118],[142]). When purified molecules of *T. denticola* were examined for cell activation, the major outer sheath protein was confirmed to be a TLR2 agonist, although its lipooligosaccharide induced production of cytokines and nitric oxide in a TLR4-dependent but TLR2-independent manner (118). It is uncertain at the moment whether the lipooligosaccharide is not properly exposed on the bacterial cell surface or the pathogen possesses TLR4-antagonitic activity, like *P. gingivalis* does (22), as a means to evade TLR4 activation. Although macrophages readily elicit cytokine production in response to *T. denticola* (118,[142]), gingival epithelial cells stimulated with the same pathogen fail to induce interleukin-8 (5,[13]). Whereas degradation of this cytokine may in part explain the findings (5), it appears that the inhibitory mechanism in the epithelial cells is mainly exerted at the transcriptional level (13). Since gingival epithelial cell production of interleukin-8 is important for chemoattraction and activation of neutrophils, *T. denticola* appears capable of proactively suppressing or delaying the neutrophil influx. Such mechanism, if indeed operating in vivo, would be most important especially in the initial stages of the infection process, *i.e*., before a relatively recalcitrant pathogenic biofilm has been established. The capacity to suppress IL-8 production, as well as to inhibit induction of the human β-defensin 2 (13), is consistent with observations that the pathogen is frequently seen in close association with the gingival epithelium (38). Moreover, the *T. denticola* mechanism to suppress TLR2-induced production of interleukin-8 is reminiscent of the so-called “local chemokine paralysis” mechanism used by *P. gingivalis* (29).

Interestingly, *T. denticola* may use an additional mechanism to prevent the influx of neutrophils into its niche. Specifically, its major outer sheath protein was shown to impair Rac1-dependent neutrophil chemotaxis (103). Rac1 is a small GTPase involved in various cellular functions, including a role in the activation of the NADPH oxidase during the generation of the neutrophil oxidative burst (37). Interestingly, *T. denticola* was shown to suppress the induction of this oxygen-dependent killing mechanism in neutrophils (150), which is a TLR-dependent function (143). Whether the underlying mechanism involves the ability of *T. denticola* to inhibit Rac1 activity is uncertain, however. In addition to its roles in chemoattractant-induced directional motility (175) and the oxidative burst (37), Rac1 is also involved in TLR2-induced activation of nuclear factor-κB (4). Thus, it would be interesting to determine whether the capacity of *T. denticola* to interfere with this central signaling regulator causes extensive or even global inhibition of the neutrophil antimicrobial function.

T. forsythia and *P. intermedia* vs. innate immunity: Uncharted territories under exploration

T. forsythia was described as a new species associated with periodontitis in 1986 and was originally named *Bacteroides forsythus* (164). Now, 23 years later numerous clinical studies in humans and animal model studies firmly confirmed the prominent role of this bacterium in the initiation and progression of periodontal disease (165). As a member of the red complex (together with *P. gingivalis* and *T. denticola*), *T. forsythia* is consider a major periodontal pathogen. *P. intermedia* (formerly *Bacteroides melaninogenicus* ss. *intermedius* then *Bacteroides intermedius*) an obligatory anaerobic, black-pigmented, gram-negative rod is frequently associated with adult periodontitis, acute necrotizing ulcerative gingivitis (Trench Mouth), and pregnancy gingivitis (27,[51]). This organism is also involved in extraoral infections including aspiration pneumonia and intra-abdominal infection (9,[14]). *P. intermedia* is a member of the orange complex (158) but can also co-aggregate with *P. gingivalis* and *T. forsythia* (79,[160]). Relatively little is known about how *T. forsythia* and *P. intermedia* interact with innate immunity, and only recently have studies been conducted to elucidate the way their interplay with complement and Toll-like receptors. The two bacteria will be examined together in the following sections.

T. forsythia and *P. intermedia* interactions with complement

Given the importance of *T. forsythia* in periodontitis, it is perplexing that literally nothing is known how this pathogen interacts with complement. Extensive search of PubMed, Medline, Web of Science and Scopus data basis using combination of key words *Bacteroides forsythus*/complement and *Tannerella*/complement revealed not even a single publication. Since *T. forsythia* is exposed to complement in gingival crevicular fluid it can only be speculated that, like other major periodontopathic bacteria, the bacterium is either resistant to complement, or it is protected from the microbicidal activity of complement by other inhabitants of dental plaque (see below). Indeed, preliminary data strongly suggest that akin to other periodontopathogens, *T. forsythia* proteases may also play an essential role in neutralizing complement (M. Bojko, J. Potempa, K. Abdulkarim, and A. Blom – unpublished results).

Several lines of evidence from early studies suggest that complement is a potentially important host defense mechanism against *P. intermedi*a. First, heat inactivation of complement in normal human serum significantly reduces opsonic activity for *P. intermedi*a (167). Second, in the absence of the classical pathway, *P. intermedia* is opsonized by the alternative pathway probably in response to its lipopolysacchride (69,[121]). Nevertheless, the classical pathway is also important since kinetic studies have revealed that opsonization proceeds at significantly faster rates when the classical pathway is intact (167). Third, different strains of *P. intermedia* show variable sensitivity to the complement-dependent bactericidal activity of serum (161). Finally, the alternative pathway was shown to contribute to the killing of serum-sensitive strains, while the classical pathway was primarily responsible for killing strains with intermediate sensitivity (161).

Interestingly, a number of *Prevotella* subspecies and strains, including *P. intermedia*, which have been associated with gingivitis and periodontitis display higher proteolytic activity than that recorded for commensal strains isolated from healthy mouths (180). This suggests that, akin to the gingipains of *P. gingivalis*, proteases produced by *P. intermedia* may also contribute to resistance against the antibacterial activity of complement. Indeed, recent and detailed investigations clearly implicate interpain A (InpA), a streptopain-like (SpeB-like) cysteine protease secreted by some *Prevotella intermedia* strains, in conferring 100% resistance to killing by 20% normal human serum (131). In contrast an isogenic InpA-deficient strain

showed only 78% survival rate (131). This protective effect is exerted by efficient degradation of complement factor C3, which is the central component of the entire complement system (Fig. 1). Significantly, proteolytic inactivation of C3 by InpA occurs in whole human serum, which explains the observation that opsonization and/or opsonisation-dependent phagocytosis of *E. coli* and *Proteus mirabilis* are impaired when the serum is pretreated with certain *Bacteroides* species, including *P. intermedia* (77,[115],[167]).

Moreover, it appears that *P. intermedia* has developed additional, protease-independent strategies for evading killing by complement. This can be inferred from its significant survival rate, even when the bacterium is incubated with serum containing the broad-spectrum cysteine protease inhibitor, E-64 (129). This inhibitor should ameliorate the proteolytic activity not only of InpA, but also of two other streptopain-like proteases expressed by *P. intermedia* (129). Although it cannot be excluded that serine proteinase activity, insensitive to E-64 inhibition (151), may be employed by *P. intermedia* to neutralize complement, it seems more likely that other mechanisms, such as binding of host-derived complement inhibitors, or the molecular composition of the bacterial surface, may confer exceptional resistance against complement-dependent lysis.

Oral bacterial co-aggregation is important for dental plaque formation and *P. intermedia* has been shown to co-aggregate with *P. gingivalis* (78,[79]). In the context of this specific interaction and the general pathogenicity of biofilms, it becomes important to note that the interpain of *P. intermedia* synergizes with the gingipains of *P. gingivalis* in complement inactivation (131). It is plausible that secreted interpain and gingipains aid in the survival of bystander bacterial species in dental plaque, which could otherwise be eliminated by the complement bactericidal activity. This bacterial synergy may be one of the mechanisms that contribute to a favorable biofilm environment that could foster the survival of the entire microbial community.

In interacting with complement, *P. intermedia* shares one important feature with *P. gingivalis*. At low concentrations, the interpain, similarly to gingipains, is able to activate the C1 complex in serum causing deposition of C1q on bacterial surfaces (131). Furthermore, the interpain can directly release anaphylatoxin C3a while gingipains additionally release C5a (128,[131]). Significantly, although high concentrations of these proteases inhibit the complement cascade, the anaphylatoxins are still released by direct protease action. Taken together, the following scenario can be envisioned (Fig. 5). *P. gingivalis* and *P. intermedia* activate complement when present at low numbers, resulting in a local inflammatory reaction which does not eliminate them since these bacteria are resistant to complement-mediated lysis. However, complement activation can suppress complement-sensitive bacteria, which could otherwise compete for space and nutrients. The inflammatory serum exudate, moreover, provides *P. gingivalis* and *P. intermedia* with precious nutrients (such as hemin, a source of essential iron). At later stages of infection, the concentration of proteases is high enough to destroy C3 and inhibit complement activation, thus promoting the survival of the entire biofilm community by helping bystander bacteria evade complement killing. Even under these conditions that suppress the physiological activation of the complement cascade, the proactive release of biologically active C5a can stimulate inflammatory serum exudate for acquisition of hemin and other nutrients. Furthermore, since anaphylatoxins are potent mediators of inflammatory reactions (91), their local generation at sites heavily populated with bacteria can contribute to the pathological processes associated with periodontal tissue destruction. Moreover, C5a could be additionally exploited for immune evasion (see above, Exploitation of crosstalk interactions between TLRs and complement), thus further promoting pathogen survival and ensuing inflammation. It is thus becoming apparent that periodontal pathogens have evolved in ways that allow them to not only endure inflammation but exploit it for promoting their adaptive fitness, as long as they can shut down critical host responses (such as induction of nitric oxide production (62)) that could eliminate them.

P. intermedia and *T. forsythia* interactions with TLRs

TLR-mediated recognition of *P. intermedia* and *T. forsythia* and subsequent activation of signal transduction pathways culminating in the generation of inflammatory cytokines can have direct bearing periodontal pathogenesis. Unfortunately, our knowledge about TLR interactions with these two pathogens is limited. Similar to *P. gingivalis* and *T. denticola* which predominantly activate TLR2, *P. intermedia* and *T. forsythia* sonicates exclusively stimulate TLR2 in human cell lines transfected with various combinations of TLRs and co-receptors (82). Consistent with these results, purified lipoproteins of *T. forsythia* and a nonendotoxic glycoprotein of *P. intermedia* activate TLR2 signaling in human monocytic THP-1 cells and/or human gingival fibroblasts (65,[159]). On the other hand, highly purified lipid A derived from the lipopolysaccharide of *P. intermedia* activates murine peritoneal macrophages to secrete interleukin-6 through a TLR4-dependent pathway (66). However, the fact that whole cells of *P. intermedia* fail to activate TLR4 (82) suggests the possible presence of TLR4 antagonistic activity associated with the bacterial cell surface, although this possibility has not been addressed.

In addition to lipoproteins, another cell surface-associated and secreted protein, the BspA of *T. forsythia* also activates TLR2, resulting in the production of interleukin-1, interleukin-8, and tumor necrosis factor-α upon interactions with human gingival epithelial cells or human monocytic THP-1 cells (55,[122]). In the former interaction, TLR1 serves as a signaling partner (122), whereas the latter interaction is additionally dependent on the CD14 co-receptor (55). From a pathological point of view, it is important to note that *T. forsythia*, *T. denticola*, and *P. gingivalis* can induce strongly synergistic interleukin-6 production (163). Since interleukin-6 activates osteoclasts (74), this synergistic action may significantly reduce the number of bacteria required to stimulate alveolar bone resorption. This concept becomes biologically relevant in the light of recent evidence that the three pathogens synergize in inducing periodontal bone resorption in vivo (81).

Cumulatively, a large body of evidence indicates that periodontal bacteria preferentially activate immune and non-immune cells using the TLR2 pathway (57,[82],[118],[142],[182]). TLR2 stimulation in the absence of simultaneous signaling through TLR4 favors the development of the T helper 2-type immune response (48,[133],[134]). Interestingly, the immune response in chronic periodontitis is strongly T helper 2-type biased and is strongly associated with the progressive lesion of periodontitis (44,[45]). Therefore, it is reasonable to hypothesize that preferential stimulation of TLR2 by periodontal pathogens contributes to the T helper 2-type skewing of the immune response, which may perpetuate chronic inflammation and periodontal disease progression, as opposed to the T helper 1-type which is more effective in bacterial clearance by professional phagocytes (43). Additional reasons for this TLR2 “preference” may be related to observations that a number of pathogens, including *P. gingivalis* (see above), have evolved mechanisms for successfully dealing with the outcome of TLR2-dependent cell activation (62,[123],[125],[154],[174]). Although there is no compelling evidence that the TLR4 response is necessarily more protective against infection relative to TLR2, it is interesting to note that TLR4, but not TLR2 (as either a TLR2/1 or TLR2/6 heterodimer), activates autophagy in a way that eliminates phagocytosed pathogens by macrophages (32). A notable known exception among oral bacteria that preferentially activate TLR2, is *Aggregatibacter actinomycetemcomitans* which readily activates TLR4 (82,[182]). However, unlike bacteria such as *P. gingivalis*, *T. denticola*, and *P. intermedia* which may survive by suppressing selective aspects of the host response, *A. actinomycetemcomitans* appears to promote its persistence by elaborating powerful toxins (*e.g.*, leukotoxin and cytolethal distending toxin) capable of killing both innate and adaptive immune effector cells (reviewed in ref. [87]).

Conclusions and future directions

As discussed above, complement and TLRs function as major defense systems against microbial infection, whether acting separately or in concert (104,[106],[183]). However, in the course of evolution successful pathogens have "learned" to breach these systems and exploit their communication hubs (97,[141]). Table 1 summarizes putative virulence mechanisms whereby periodontal pathogens may resist or escape complement- and TLR-mediated immunity. These microbial strategies may explain, at least in part, the ability of periodontal bacteria, such as *P. gingivalis*, *T. denticola*, *T. forsythia*, and *P. intermedia*, to persist and establish chronic infections in the otherwise hostile environment of their host. Since these bacteria reside in mixed-species biofilms, it is reasonable to assume a synergy of shared, communal mechanisms of immune subversion to maximize the survival potential of the whole community, a concept that is supported by available initial evidence. This also suggests that even bacteria lacking protective mechanisms on their own, may thrive in such environment, as long as their presence is beneficial to the immune-subversive bacteria. For example, although no immune evasion mechanisms have been reported for *F. nucleatum*, this bacterium is a useful member of the tooth-associated biofilm by forming a co-aggregating bridge between early and late colonizers (92), and can chronically persist in deep periodontal pockets (158).

The analysis of pertinent literature in this review also reveals gaps, especially at the mechanistic level, in our understanding of periodontal bacterial immune evasion. In addition to molecular mechanistic studies which may help identify novel virulence factors, in vivo studies are also warranted to substantiate some of the in vitro findings. Although this review has focused on the host defense potential of the complement and the TLRs and how this is undermined by periodontal bacteria, it should also be noted that both systems contribute to immunopathology in several inflammatory diseases (101,[136]). However, relatively little is known regarding the precise roles of the complement and the TLRs in periodontal pathogenesis. Consequently, it is currently uncertain which specific signaling pathways need to be blocked to attenuate pathology or, conversely, be enhanced to promote host defense. At a first stage, these gaps in our knowledge necessitate mechanistic and interventional studies using appropriate animal models, since causal mechanistic relationships cannot normally be addressed in human studies due to important ethical considerations (49). Successful elucidation of the roles of complement and TLRs in immune and inflammatory responses in the periodontium, and how these are proactively modified by periodontal pathogens, will both offer insights into periodontal pathogenesis and facilitate the rational design of therapeutic interventions.

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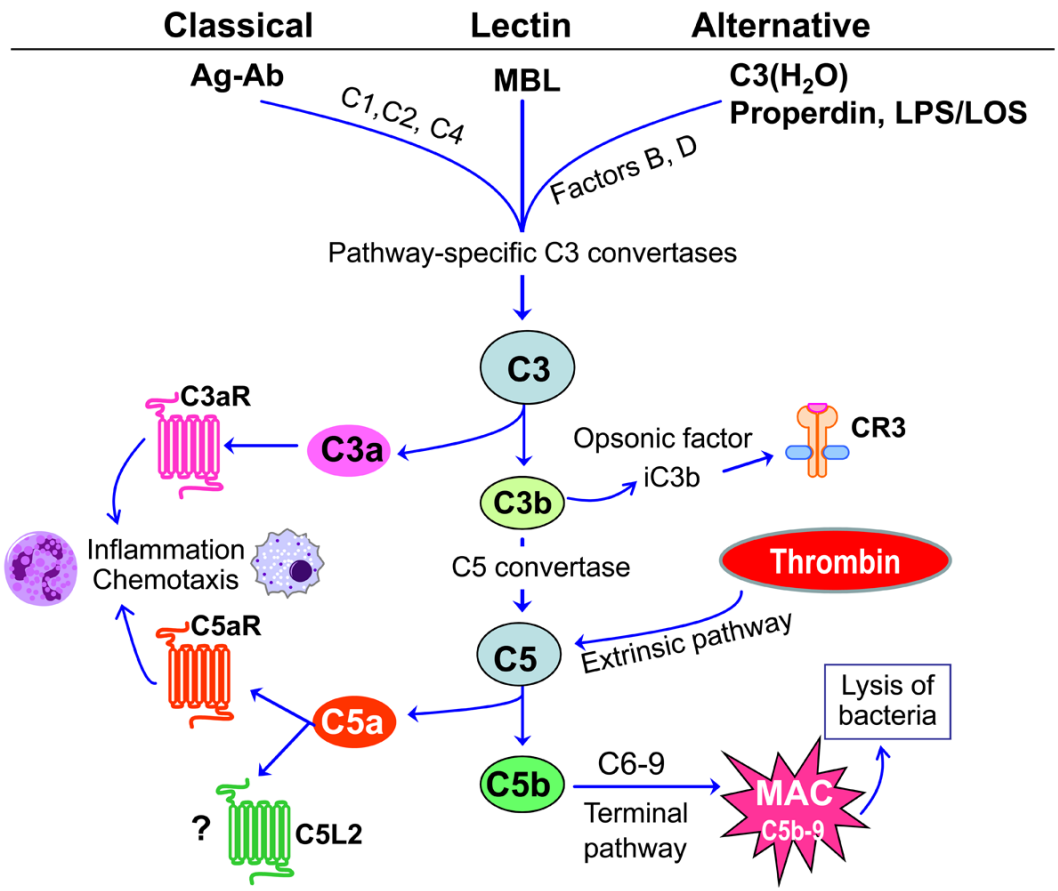

Fig.1. Activation pathways of the complement system

All three pathways converge at a central step, involving activation of the third component of complement (C3) by pathway-specific C3 convertases. The classical pathway is initiated by antigen-antibody (Ag-Ab) complexes and requires the participation of C1, C2, and C4. The lectin pathway is triggered through interaction of the mannose-binding lectin (MBL) with specific carbohydrate groups on the surface of microorganisms. The alternative pathway is initiated by spontaneously hydrolyzed C3 [C3(H_2O)] which can thereby form a complex with factor B, followed by factor B cleavage by factor D and formation of the initial alternative pathway C3 convertase (104). Morerover, the alternative pathway can be induced by bacterial lipopolysacharide (LPS) and lipooligosacharide (LOS) in a properdin-dependent way (86). Proteolytic cleavage of a series of proteins downstream of C3 leads to the generation of potent effector molecules. These include the anaphylatoxins C3a and C5a, which activate specific receptors (C3aR and C5aR, respectively), although C5a also interacts with the so-called C5a receptor-like 2 (C5L2), which is only modestly characterized (91). Additional effectors generated downstream of C3 are the opsonins C3b and iC3b, the latter of which coats microbes and promotes their phagocytosis by complement receptor-3 (CR3). In the terminal pathway, C5b initiates the assembly of the C5b-9 membrane attack complex (MAC), which in turn induces microbial cell lysis (104). Complement activation can also occur through cross-talk with other physiological pathways, such as the coagulation system, in which thrombin acts as a C5 convertase (71).

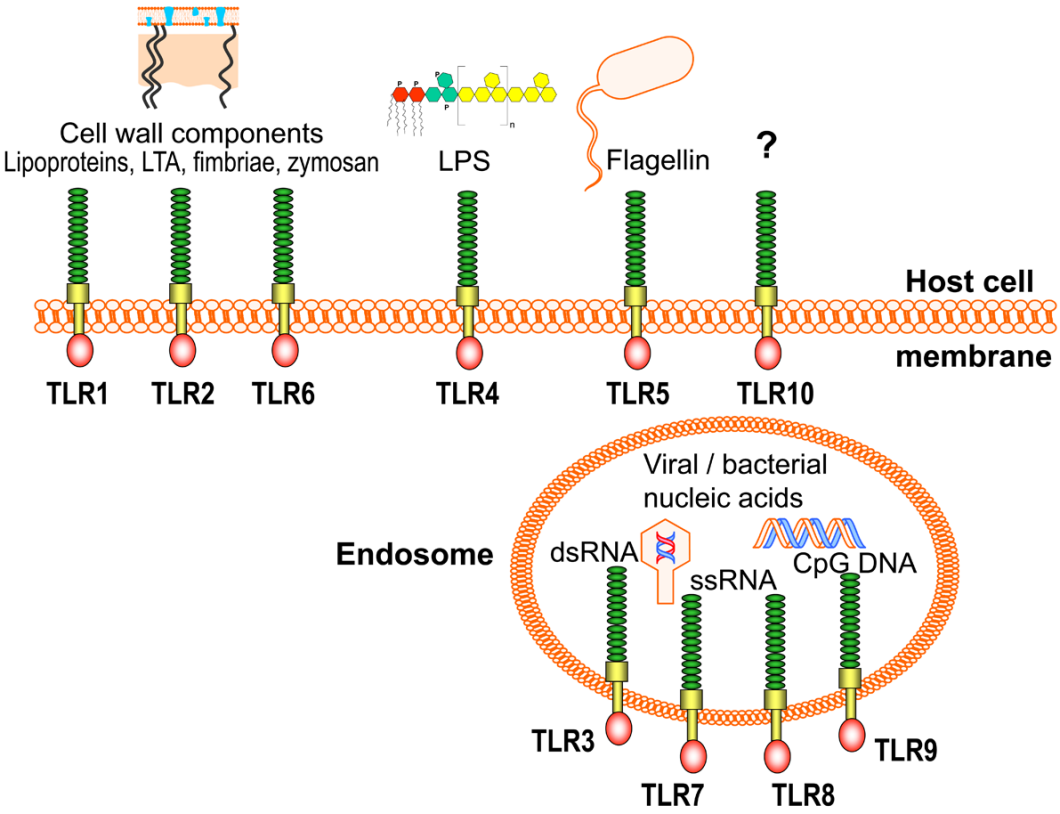

Fig. 2. Microbial ligand specificities of human TLRs

Those TLRs which recognize extracellular microbial structures (*i.e.*, TLRs 1, 2, 4, 5, and 6) are expressed on the host cell surface. TLR2 in cooperation with its signaling partners, TLR1 or TLR6, detect mostly microbial cell wall components, such as lipoproteins, lipoteichoic acid (LTA), firmbriae, or yeast zymosan (1,[12]). TLR4 and TLR5 recognize lipopolysaccharide (LPS) and bacterial flagellin, respectively, whereas no ligand has been identified for TLR10. Those TLRs specializing in detecting viral or bacterial nucleic acids (i.e., TLRs 3, 7, 8 and 9) are expressed intracellularly on endocytic vesicles. TLR3 recognizes double-stranded viral RNA, TLR7 and TLR8 single-stranded viral RNA, and TLR9 detects microbial CpG DNA.

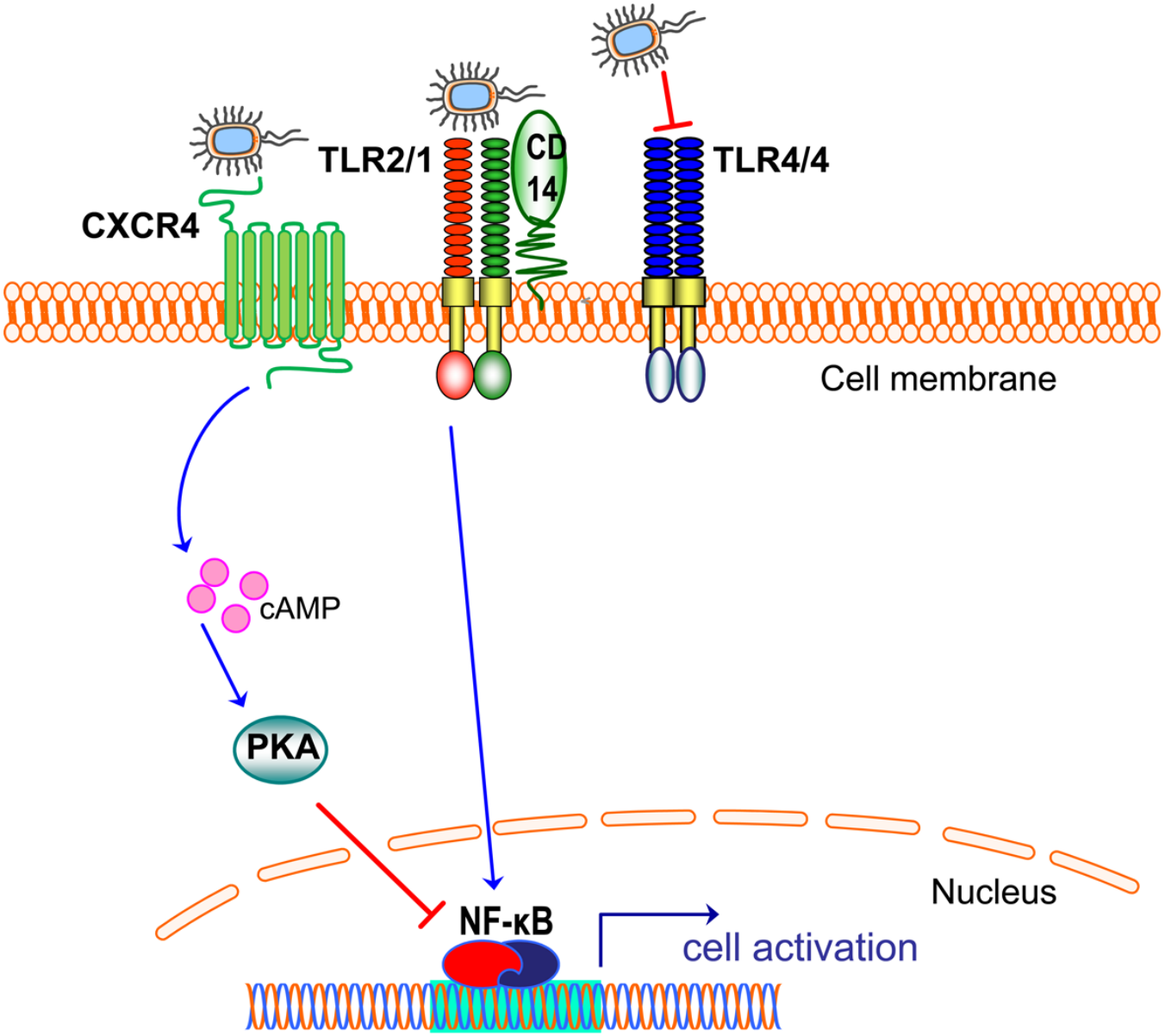

Fig. 3. Evasion or subversion of TLR activation by *P. gingivalis*

P. gingivalis uses an elaborate system of lipid A phosphatase and deacylase activities that modify the lipid A structure of its lipopolysaccharide (21,[22]). These modifications result in lipopolysaccharide molecules that can either evade or actively antagonize TLR4 activation (depicted as a homodimer; TLR4/4) (21,[22]). Although the activation of the TLR2/TLR1 heterodimer (TLR2/1) is not antagonized at the TLR receptor level, *P. gingivalis* instigates a molecular cross-talk between the CXC-chemokine receptor 4 and TLR2/1. Unlike CD14 which facilitates TLR2/1 activation by the pathogen (57), CXCR4 suppresses TLR2 signaling (62). Mechanistically, *P. gingivalis* uses its fimbriae to bind CXCR4 and induce cAMP-dependent PKA signaling, which in turn inhibits the activation of nuclear factor-κB (NF-κB) activation (62).

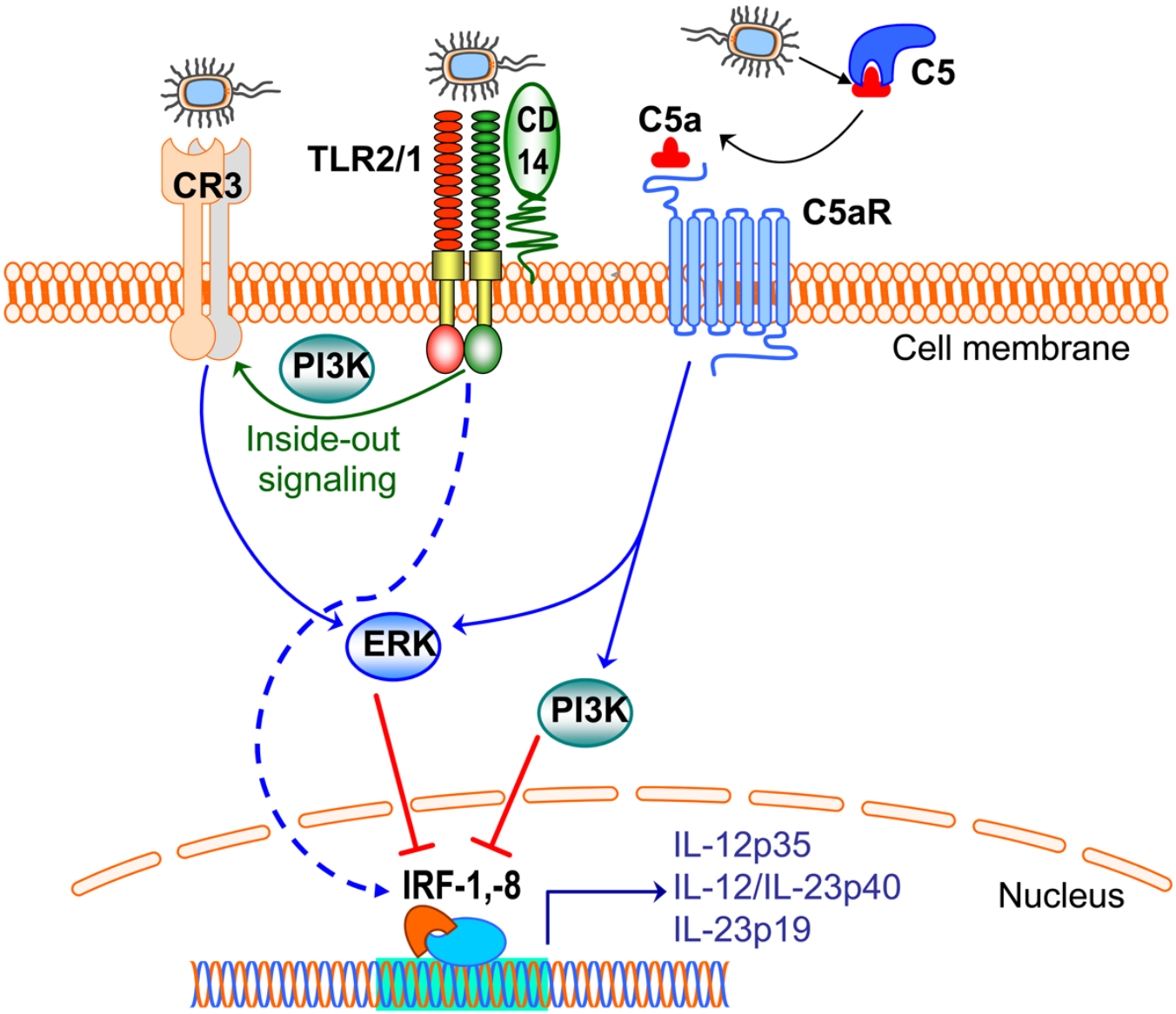

Fig. 4. Cross-talk pathways between TLRs and complement in *P. gingivalis*-activated macrophages

TLR recognition of *P. gingivalis* is predominantly mediated by the TLR2/TLR1 heterodimer (TLR2/1), aided by the CD14 co-receptor (57). This interaction induces phosphatidylinositol 3-kinase (PI3K)-dependent inside-out signaling, which transactivates the high-affinity state of complement receptor-3 (CR3) (54,[64]) (CR3). Interestingly, *P. gingivalis* interacts with activated CR3 and induces extracellular signal-related kinase 1/2 (ERK1/2) signaling, which in turn downregulates mRNA expression of cytokines of the interleukin-12 family (56). Moreover, *P. gingivalis* uses its gingipains to attack C5 and release biologically C5a (128, [176]). Through its receptor (C5aR), C5a can activate PI3K and ERK1/2, which in turn suppress critical transcription factors (the interferon regulatory factors 1 and 8; IRF-1, -8), required for expression of cytokines of the interleukin-12 family (67). Intriguingly, inhibition of bioactive interleukin-12 though these mechanisms results in impaired immune clearance of *P. gingivalis* in vivo (56), suggesting that the pathogen exploits TLR/complement cross-talk signaling to promote its virulence.

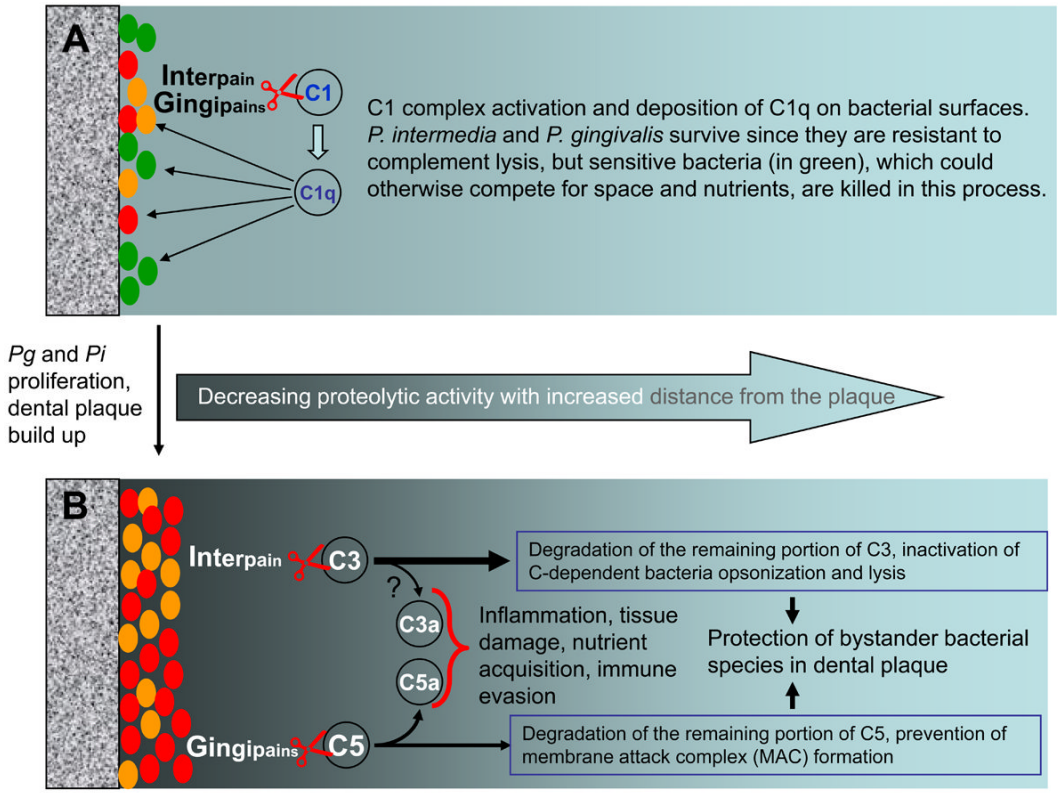

Fig. 5. Biphasic virulence effects of *P. gingivalis* and *P. intermedia* cysteine proteases

(A) Effects of low concentrations of the proteases, *i.e.*, early in the clonization process, which activate the classical pathway. (B) Effects of high concentrations of the proteases, *i.e.*, in a developed biofilm, which suppress the physiological activation of the complement cascade, but selectively generate anaphylatoxins via limited degradation of C3 and C5. *P. gingivalis and P. intermedia* are exceptionally resistant to the bactericidal activity of complement and released proteases, gingipains and interpain, respectively, contribute to the resistance. At low concentrations of the proteases, likely to occur at the early stages of infection or at a long distance from the dental plaque, the released proteases activate the C1 complex leading to deposition of C1q on the bacterial surface (128,[131]). Complement activation may eliminate complement-sensitive commensal bacteria which could otherwise compete with pathogens for space and nutrients. At high concentrations, the proteases synergistically inhibit the bactericidal activity of complement by degrading C3 and C5, thus protecting complement-sensitive bacteria in their proximity and promoting biofilm development. At the same time, released anaphylatoxins (C3a and C5a; although only the latter was confirmed to retain its biological activity) fuel inflammation resulting in tissue damage and nutrient generation, and are exploited for immune evasion (see also Fig. 4).

Table 1

Exploitation of complement, TLRs, or their crosstalk by oral pathogens

	Mechanism	Pathogen	Effector molecule	Refs.
1	a. Inhibition of complement activation through digestion of the central complement component (C3)	*P. gingivalis*	Gingipains, especially HRgpA and RgpB	(129)
		P. intermedia	Interpain (InpA)	(132)
	b. Synergy in complement inactivation	*P. gingivalis* & *P. intermedia*	Gingipains and interpain	(132)
2	Inherent resistance to complement-mediated lysis	*P. gingivalis*	Surface anionic polysaccharide	(157)
3	Hijacking complement regulatory proteins			
	a. C4b-binding protein	*P. gingivalis*	HrgpA	(133)
	b. Factor H	*T. denticola*	11.4-kDa factor H-binding lipoprotein	(106)
4	Generation of specific complement fragments			
	a. iC3b (promotes phagocytosis linked to poor microbicidal activity)	*T. denticola*	Dentilisin	(98, 180)
	b. C5a (inhibits TLR-induced interleukin-12)	*P. gingivalis*	HRgpA, RgpB, and Kgp	(68, 129, 177, 184)
5	Promotion of intracellular survival via complement receptor 3-mediated entry	*P. gingivalis*	Fimbriae	(171)
6	TLR4 evasion by expressing dephosphorylated and tetra-acylated lipid A	*P. gingivalis*	Lipid A 1- and 4′-phosphatases and deacylase	(21)
7	TLR4 antagonism by expressing monophosphorylated tetra-acylated lipid A	*P. gingivalis*	Lipid A 4′-phosphatase and deacylase (lipid A 1-phosphatase suppressed by hemin)	(21,22)
8	Suppression of TLR2-induced IL-12 via complement receptor-3 binding	*P. gingivalis*	Fimbriae	(56)
9	Suppression of TLR2 activation through instigated crosstalk with the CXC-chemokine receptor 4	*P. gingivalis*	Fimbriae	(62)

	Mechanism	Pathogen	Effector molecule	Refs.
10	Inhibition of TLR2-induced interleukin-8 in gingival epithelial cells	*P. gingivalis* *T. denticola*	Unspecified; requires the use of whole cells	(13,29)
11	Suppression of the neutrophil oxidative burst (TLR-dependent)	*T. denticola*	Unspecified; inhibitory factor released into the culture medium	(144, 151).