

The inflammasome and danger molecule signaling: at the crossroads of inflammation and pathogen persistence in the oral cavity

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The innate immune system is critical in the initial defense against pathogenic microorganisms; however, the host faces major challenges from microbial pathogens that have developed mechanisms to evade detection by the host immune system. As the first line of host defense, the innate immune system relies heavily on the presence of evolutionarily conserved pattern-recognition receptors, the best characterized of which include membrane-bound Toll-like receptors, retinoid-inducible gene 1-like receptors, C-type lectin receptors and nucleotide-binding-domain-like receptors, to recognize various pathogens or their components (1, 55). These special receptors, which are expressed by many cell types, including macrophages, neutrophils, monocytes and epithelial cells, can sense 'pathogen-associated molecular patterns' and also respond to 'danger-associated molecular patterns' (also known as 'damage-associated molecular patterns') which may be host (ATP, DNA or cholesterol crystals) or environmentally (asbestos, silica, alum or nanoparticles) derived (73). The activation of pattern recognition receptors by pathogen-associated molecular patterns and their post-receptor signaling via stimulation with danger-associated molecular patterns can ultimately drive the recruitment of 'inflammasome' complexes and play a crucial role in the activation of specific inflammatory cascades (1). The term 'inflammasome' was coined by the late Jürg Tschopp and his research team in 2002 (54). Inflammasomes are nucleotide-binding-domain-like receptors containing multiprotein complexes functioning as

a molecular platform and are activated by exposure to cellular danger or stress signals, which trigger the maturation and secretion of pro-inflammatory cytokines, such as interleukin-1 β and interleukin-18 (54). According to the specific recognition receptors involved and the activated caspase cascades, more than five inflammasome families have been identified to date and new ones are still being discovered (52, 55).

Nucleotide-binding-domain-like receptors are a family of cytosolic pattern recognition receptors that are critical in surveying the cytoplasm for pathogen-associated molecular patterns or danger-associated molecular patterns (55). Several members of the nucleotide-binding-domain-like receptor gene family participate in the assembly of inflammasomes, and the main members demonstrated to form inflammasomes in cells are NLR family, pyrin domain containing 1 (NLRP1), NLR family, pyrin domain containing 3 (NLRP3) and NLR family, CARD domain containing 4 (NLRC4) (1, 37). NLRP3 is the most comprehensively characterized inflammasome and has been shown to be associated with several autoinflammatory and nonautoimmune chronic conditions (73). NLRP3 has lately become an important target molecule in understanding how various pathogens and related danger signals could orchestrate the inflammasome complexes in order to redirect host immune responses.

Inflammasomes can control the mediation of pro-inflammatory responses in a diverse group of chronic diseases, such as gout, cancer and bacterial and viral infections (20, 55). The role of inflammasomes in

mediating host metabolic responses and the dysregulation of inflammasome components are associated with various inherited chronic inflammatory and immune disorders, highlighting their relevance in human disease (73). In particular, the imbalance of interleukin-1beta activity is among the focal points of both microbial-associated and nonmicrobial inflammatory diseases. The progression of periodontitis is inflammatory in nature, with the main triggers of oral inflammation usually residing in the oral microbiome and the balance of its components (27). The advancement of periodontal disease has been proposed to correlate with modulations of innate immunity, in particular, with up-regulated levels and/or unbalanced production of pro-inflammatory cytokines, which can lead to severe tissue damage (6). Therefore, inflammasomes are emerging as chief regulators of the host innate immune defense system in chronic inflammatory diseases, and their role against microbial pathogens is becoming crucially important in controlling and limiting invading microbes. On the other hand, increasing numbers of microorganisms and their virulence factors are found to function by targeting inflammasomes and modulating interleukin-1beta processing, which, all together, could be involved in the development and/or progression of various inflammatory diseases, including periodontal disease (14, 48, 82). In the light of recently accumulated evidence on the role of inflammasomes and danger molecule signaling in the coordination of multiple inflammatory processes in the oral mucosa, this review aims to describe and present the specific interactions and functions of inflammasomes, focusing on the current research on NLRP3, in relation to microbial pathogenesis, and particularly the potential implications for human chronic diseases and periodontal disease. The signaling components of the inflammasome as plausible intervention targets will also be briefly discussed.

ATP and purinergic signaling as core regulators of NLRP3 inflammasome

Considered as one of the key molecules in the innate immune system, NLRP3 is one of the best characterized nucleotide-binding-domain-like receptor family members as a result of its unique involvement in the recognition of microbial and danger components, having an active role in the induction of pro-inflammatory host responses (52). A number of exogenous and endogenous factors have been shown to promote

activation of the NLRP3 inflammasome. These include microbial infection (37, 48), individual microbial components (50) and host-derived small danger molecules, such as extracellular ATP, which are indicative of cellular stress or damage (14, 32, 69, 73). The stimulation of cytosolic NLRP3 receptors leads to its assembly with the adaptor protein, apoptosis-associated speck-like protein containing a carboxyl-terminal caspase recruitment domain, and the effector protein, caspase-1 (57). This complex then results in the activation of caspase-1, which ultimately cleaves pro-interleukin-1beta and pro-interleukin-18 into their biologically active mature forms (54). NLRP3 inflammasome activation generally requires two signals. The first signal is induced when pathogen-associated molecular patterns (i.e. lipopolysaccharide, bacterial DNA and viral RNA) stimulate a pattern-recognition receptor and trigger the production of the interleukin-1 precursor (35). The second signal is induced by the ligation of danger-associated molecular patterns [small danger signal molecules, either host-derived (i.e. extracellular ATP) or environmentally associated] to danger signal receptors. A growing number of research findings are highlighting the crucial role of extracellular ATP in the regulation of the NLRP3 inflammasome through purinergic receptors (P2X), which are important mediators of apoptosis and initiators of inflammatory responses as well as necessary for controlling infections (26) (Fig. 1).

The danger signal molecule, extracellular ATP, and its degradation products (such as adenosine) can play a pivotal immunoregulatory role in the microenvironment during infection (78, 88). Extracellular ATP is released by infected, stressed or dying cells, and is among the most potent pro-inflammatory stimulus involved in both autocrine and paracrine signaling in host tissues (26). Multiple animal models have provided evidence for the role of extracellular ATP in pro-inflammatory induction during the processes of inflammatory and autoimmune chronic diseases (16, 88, 94). This small molecule has lately been documented to elicit a range of specific signaling events critical for inflammasome activation, cell death, organelle function and control of infections upon ligation with the P2X₇ purinergic receptors, a family of cation-permeable ligand-gated ion channels (53). The significance of the P2X₇ receptor for inflammasome signaling has been widely studied in myeloid cells, such as monocytes, macrophages and dendritic cells. However, the role of P2X₇ in epithelial cells which line the mucosal tissues, forming an initial barrier to invading microbes and functioning as an

Periodontal disease, diabetes type 2, gout, rheumatoid arthritis, cancer

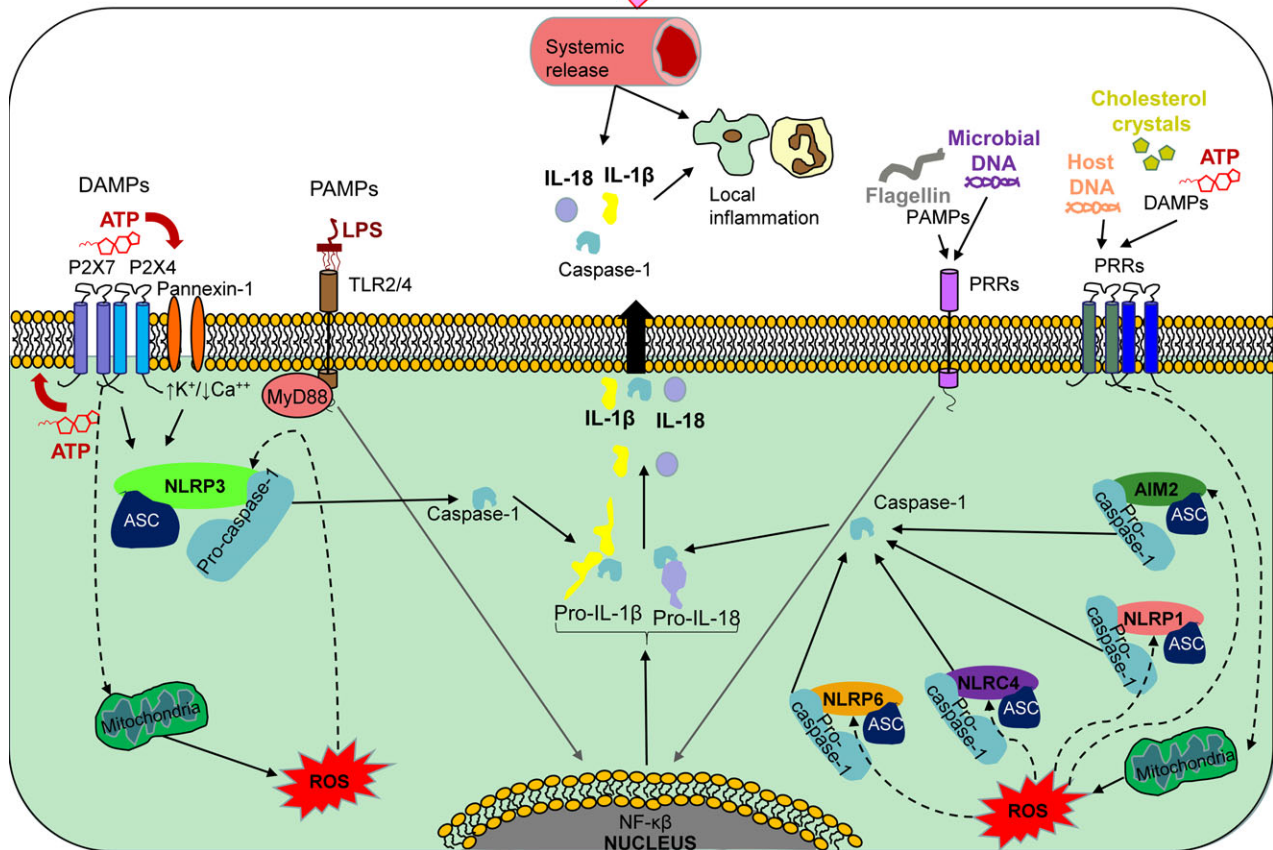


Fig. 1. Schematic representation of the proposed mechanisms of stimulation of different inflammasomes and their relationship with the development of chronic inflammatory diseases. Several members of the nucleotide-binding-domain-like receptor (NLR) family, such as NLR family, pyrin domain containing 1, 3 and 6 (NLRP1, NLRP3 and NLRP6, respectively), NLR family, CARD domain containing 4 (NLRC4) and absent in melanoma 2 (AIM2), are known to participate in the assembly of the inflammasome complex. A first signal is induced with the activation of NLRs by pathogen-associated molecular patterns (PAMPs), such as microbial lipopolysaccharides (LPS), through pattern recognition receptors (PRRs) such as Toll-like receptors 2 and 4 (TLR2/4). Stimulation of PRRs with PAMPs leads to the increased expression of pro-cytokines [pro-interleukin-1beta (pro-IL-1 β) and pro-interleukin-18 (pro-IL-18)] that are not biologically active. A second signal, provided through the activation of danger recognition receptors (DAMPs), such as extracellular ATP, cholesterol crystals or damaged host DNA, is required for inflammasome activation and assembly, leading to the activation of

pro-caspase-1 into the active caspase-1 form. Activated caspase-1 cleaves the pro-cytokines into their active IL-1 β or IL-18 forms, leading to the extracellular secretion of these inflammatory cytokines. NLRP3 is the most well studied NLR member. Currently, there are three models for activation of the NLRP3 inflammasome – the reactive oxygen species (ROS) model, the lysosomal burst model and the ATP-triggered K⁺-efflux model. For simplicity, only the ROS model is represented here. The proposed mechanisms are depicted by dashed arrows. NLRP3, in particular, has recently been associated with the development of chronic inflammatory diseases, such as periodontal disease, type 2 diabetes, gout, rheumatoid arthritis and cancer. Most of these diseases have been related to overproduction, imbalance and/or systemic release of the inflammasome-dependent cytokines IL-1 β and IL-18, leading to local inflammation, as well as to systemic effects. ASC, apoptosis-associated speck-like protein containing a carboxyl-terminal caspase recruitment domain; NF- κ B, nuclear factor-kappaB.

important arm of the innate immune system, has recently been explored. Interestingly, the presence and function of this receptor in epithelial cells has become largely characterized in human primary gingival epithelial cells, which form the first line of defense against colonizing microbes in periodontal

pockets (11, 79, 90, 92, 93). Gingival epithelial cells were reported to express functional P2X₇ receptors that mediated extracellular ATP-induced cell death, which was inhibited by the periodontal pathogen *Porphyromonas gingivalis* (11, 93). A recent study indicated that in gingival epithelial cells, extracellu-

lar ATP signaling requires the assembly of P2X₇ and P2X₄ receptors with the pannexin-1 hemichannel, leading to the production of intracellular reactive oxygen species, which, in turn, act as intermediate signaling molecules for the subsequent activation of the NLRP3 inflammasome (16, 33). The study also demonstrated that when both P2X₇ and P2X₄ receptors were individually down-regulated after treatment of small interfering RNA, *P. gingivalis*-infected gingival epithelial cells showed a significant reduction in secretion of extracellular ATP-induced interleukin-1beta (33). Overall, the involvement of P2X₇ in the activation of the NLRP3 inflammasome indicates that P2X₇ is likely to function as a master regulator in this inflammatory pathway. This has also been validated by *in vivo* studies, in which P2X₇-knockout mice display impaired inflammasome activation, reduced levels of interleukin-1beta release and decreased disease severity in a murine model of arthritis (49) or a disruption of extracellular ATP-induced processing of pro-interleukin-1beta in macrophages (77). Several change-of-function polymorphisms (causing inactivation or reduced function of the P2X₇ receptor) and their associations with resistance or susceptibility to different human diseases have further highlighted the important mechanistic role of purinergic signaling in chronic diseases (9, 25).

As extracellular ATP–P2X₇ coupled inflammasome activation and subsequent pro-inflammatory cytokine secretion is one of the central self-defense mechanisms that leads to the recruitment of specialized pathogen-fighting cells (e.g. macrophages and neutrophils) and alerts neighboring cells to the danger of infection, it is often targeted by persistent pathogens in their evolution toward adaptation and successful colonization of host tissues (13, 59). What is interesting is that successful opportunistic pathogens, such as *P. gingivalis*, can inhibit the extracellular ATP–P2X₇ pathway by directly preventing inflammasome activation through secreting an effector called ‘nucleoside diphosphate kinase’ (11, 80). This ATP-hydrolyzing enzyme homolog is secreted by *P. gingivalis*, as well as by other successful persistent microbial species, such as *Mycobacterium tuberculosis*, possibly to negate the robust host immune response and modify host-cell biology for successful persistence (70, 80). It appears that extracellular ATP signaling, followed by inflammasome activation, may function to influence the existing oral mucosal microenvironment, which is in constant contact with a large variety of microorganisms, thus leading to a series of signaling events modulating the pathogenicity

of the residing microbiome (79). The consequences of these interactions are perhaps linked not only to local inflammatory diseases but may also have the potential to cause systemic effects.

Inflammasomes and their relevance in chronic inflammatory diseases and other chronic diseases

With the increased knowledge gained over the last decade, inflammasomes have gradually become recognized for their association with hereditary and acquired chronic inflammatory diseases and conditions of humans, such as cancer, gout, type-2 diabetes, rheumatoid arthritis and periodontal disease (14, 20, 73). Gout, which is characterized by joint inflammation progressing to arthropathies, is strongly linked to metabolic dysfunctions leading to elevated blood uric acid levels and monosodium urate crystals in joints, thus stimulating the activation of the NLRP3 inflammasome and subsequently causing chronic interleukin-1beta and interleukin-18 secretion and neutrophil recruitment locally (73). The emerging role for the NLRP3 inflammasome as a sensor of metabolic stress is reinforced by its involvement in the development of type-2 diabetes. Type-2 diabetes has been proposed to be linked to the NLRP3-induced secretion of interleukin-1beta and to this cytokine’s ability to mediate prolonged hyperglycemic toxicity in pancreatic islets, contributing to the destruction of beta cells and dysregulation of glucose-induced insulin secretion (73). The recent clinical trials using interleukin-1 receptor antagonists in the treatment of type-2 diabetes underscore the role of the NLRP3 inflammasome in the disease (51). NLRP3 has also been demonstrated to contribute to the pathogenesis of the autoimmune disorder, rheumatoid arthritis, which is also characterized by chronic inflammation of the joints and surrounding tissues, particularly the synovial membrane (56). Atherosclerosis, a progressive inflammatory disease characterized by arterial wall injury and deposition of atherosclerotic plaques (89), has been associated with *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *P. gingivalis* and other bacteria known for their modulation of interleukin-1beta release through the NLRP3 inflammasome complex (29, 30). The inflammasome-dependent cytokines interleukin-1beta and interleukin-18 have been implicated in the pathogenesis and progression of diverse chronic diseases and, more recently, in various types of cancers, such as gastric and colon cancers, as well as oral and esophageal

squamous cell carcinomas, although the exact mechanisms are, as yet, not well defined (10, 20, 41).

Whilst inflammasome signaling is becoming well established in the progression of various diseases, intriguingly there is also mounting evidence supporting the association of the oral microbiome with the same array of diseases (2, 29). The oral cavity is essentially a diverse ecosystem, harboring vast numbers of oral microorganisms, and can serve as a reservoir for possible systemic dissemination of microorganisms or their components and the release of inflammatory signals, possibly leading to inflammation at distant body sites (2, 29, 68). With advances in technologies for microbial detection, a diverse group of oral species has additionally been directly detected in several systemic chronic diseases (15, 61, 62). Recent clinical studies have shown not only a significant increase in the incidence of periodontal disease in patients with rheumatoid arthritis (15, 61), but also the presence of DNA of periodontal bacteria, such as *P. gingivalis*, in the synovial fluid of patients who have both diseases (62). The implication of oral bacteria, such as *A. actinomycetemcomitans* and *P. gingivalis*, in the initiation and progression of atherosclerotic disease is also well documented (24, 29). Moreover, there is an increasingly recognized association of orodigestive cancers, and particularly oral squamous cell carcinoma, with certain periodontal bacteria, such as *P. gingivalis* (2, 34, 42, 86). The accumulating evidence points to the specific microbe-induced alteration of inflammatory mediators through regulation of inflammasome activation, potentially serving as major factors in the development of diverse chronic diseases. Therefore, the following sections will focus on the importance of inflammasome regulation and activation by different periodontal pathogens, with emphasis on the currently identified mechanisms developed by successful oral colonizers to subvert the host immune response.

Inflammasome signaling and periodontal disease

Is there a specific role for the inflammasome in the etiology of periodontal disease?

Inflammasome complexes appear to assume a pivotal role in periodontal disease and the inflammasome-associated inflammatory mediators involved in the progression of the disease have been high-

lighted through several clinical studies (6, 63, 84). The relationship between the interleukin-1 cytokine family and the NLRP3 inflammasome complex has been revealed in a recent clinical study (6). The findings indicated that higher expression levels of NLRP3 and NLRP2, but not of apoptosis-associated speck-like protein containing a carboxyl-terminal caspase recruitment domain (which mediates the binding of nucleotide-binding-domain-like receptor to caspase-1), were detected in gingival tissue samples from patients with three forms of periodontal disease (gingivitis, chronic periodontitis and generalized aggressive periodontitis) when compared with healthy subjects. Consistent with previous findings (21, 38, 63), the expression levels of both interleukin-1beta and interleukin-18 mRNA were also enhanced in those patients, and thus the findings revealed a positive correlation between NLRP3 and expression of interleukin-1beta and interleukin-18 in periodontal disease (6). Although the enhanced expression of inflammasome complexes may be an indicator of the presence of periodontal disease, the study showed no difference in expression levels amongst the different forms of periodontal diseases, thus suggesting that those complexes may not be used to determine the severity of the disease. The study further incorporated an *in vitro* experiment to investigate the association between inflammasome-component expression and the regulation of this expression by *P. gingivalis*. Interestingly, the *in vitro* findings mirrored a similar pattern of elevated levels of interleukin-1beta, interleukin-18 and NLRP3, as observed in patients with different forms of periodontal disease, emphasizing the potential role of *P. gingivalis* in the molecular mechanisms involved in periodontitis. A more recent study supported the finding of elevated levels of interleukin-1beta and interleukin-18 by measuring these markers in the gingival crevicular fluids of patients with chronic periodontitis (65). Additionally, the study showed up-regulated levels of NLRP3, absent in melanoma 2 and caspase-1 in the gingival tissues of patients with periodontitis, suggesting the participation of at least two separate inflammasomes in the disease-associated increased interleukin-1beta levels. Absent in melanoma 2 is a recently named inflammasome that activates caspase-1 in response to cytosolic double-stranded DNA derived from invading bacteria and viruses (57). Hence, these new findings suggest that multiple inflammasome machineries may be contributing simultaneously to the microbiome-associated induction of inflammation during periodontal disease, such as the interplay between various mi-

crobiome components and specific inflammasome molecules.

A growing number of studies are not only indicating the activation of inflammasome components as potential clinical biomarkers of inflammation in periodontal disease but are also exploring the relationships of those biomarkers with the composition of the subgingival microbiome. Recently, a study reported that certain species of periodontal bacteria, such as *P. gingivalis*, *Treponema denticola*, *Tannerella forsythia* and *Eubacterium nodatum*, were present at significantly higher levels in the gingival crevicular fluid of patients with chronic periodontitis, which also showed high levels of interleukin-1beta and interleukin-18 (84). The study also compared clinically healthy sites of both patients with periodontitis and periodontally healthy controls. Interestingly, the subgingival microbiome composition also differed between these two groups. Periodontally healthy subjects had higher proportions of certain species, including *Actinomyces odontolyticus*, *Streptococcus gordonii* and *A. actinomycetemcomitans* (belonging to the purple, yellow and green complexes, respectively), whilst the individuals with periodontitis had significantly higher levels of species comprising *F. nucleatum* (belonging to the orange complex) and *P. gingivalis*, *T. denticola* and *T. forsythia* (belonging to the red complex) (53). The red complex species, which are established to be strongly associated with periodontal disease etiology and the clinical parameters of increased pocket depth and bleeding upon probing (47), showed a significant, positive correlation with the expression of the pro-inflammatory cytokines (interleukin-1beta and interleukin-18). On the other hand, purple, yellow and green complex species displayed a negative association with interleukin-1beta expression (84). The increased expression levels of interleukin-1beta and interleukin-18 observed in clinically healthy sites of periodontitis patients suggest that inflammatory responses possibly occur early, before they can be seen clinically, and are perhaps caused by the higher colonization of red and orange complex species rather than by genetic predisposition.

The contribution of microbes in the etiology of periodontal disease is largely recognized, and there is a growing interest in determining the potential interaction between periodontal microbes and their ability to modulate the inflammasome response (17, 82, 92) (Fig. 2). The confounding questions are, 'which are the likely major players participating in the regulation of the inflammasome-associated inflammatory responses in the oral cavity?' and 'what are the mechanisms involved in the shift from homeostasis to disease in the host?'

Key players in periodontal disease and inflammasome signaling

Porphyromonas gingivalis

Porphyromonas gingivalis is a gram-negative host-adapted anaerobe and a prominent bacterium present in the tissues of patients with chronic severe periodontitis (3, 74, 90). Its colonization of the oral cavity has also lately been associated with a variety of related systemic diseases (2, 27). Being recognized for its highly effective ability to modulate the composition of the oral microbiome, *P. gingivalis* has developed a number of distinct mechanisms for manipulating host inflammatory responses, such as reducing the innate immune response for its own benefit and, simultaneously, providing a favorable environment for co-habitants, such as *F. nucleatum* and *T. denticola* (36). The pathogenic altruism between *P. gingivalis* and other oral species, such as *F. nucleatum*, has been shown in a mouse subcutaneous chamber model (58). The study reported that when the two bacteria were introduced together (co-infection), there was an increase in the colonization of both species in the chamber model when compared with single infections (54). Another mouse model study illustrated that *P. gingivalis* and *F. nucleatum* exhibited an enhanced virulence phenotype with accelerated bone loss and higher levels of interleukin-1beta in co-infections compared with single infections of mouse periodontal tissues (67). On the other hand, recent work performed in an *in vitro* mouse macrophage model depicted that *P. gingivalis* can synergistically regulate invasion of host cells by *F. nucleatum* through inhibiting both *F. nucleatum*-induced interleukin-1beta and interleukin-18 processing and *F. nucleatum*-promoted cell death (83). This host-signaling-modifying ability of *P. gingivalis* was previously established in the primary gingival epithelial cell model, in which *P. gingivalis* was found to down-regulate NLRP3 expression and induce production of pro-interleukin-1beta but only promote secretion of mature interleukin-1beta upon stimulation with danger signal extracellular ATP (92).

Thus, a compelling amount of research pinpoints that *P. gingivalis* is able to modulate specific known inflammasome components and successfully colonize and persist in host cells. Along the same lines, recent work further underscored this ability of the microorganism. It showed significant down-regulation of expression of the NLRP3 inflammasome and interleukin-1beta in gingival fibroblast cultures when *P. gingivalis* was introduced in a subgingival biofilm

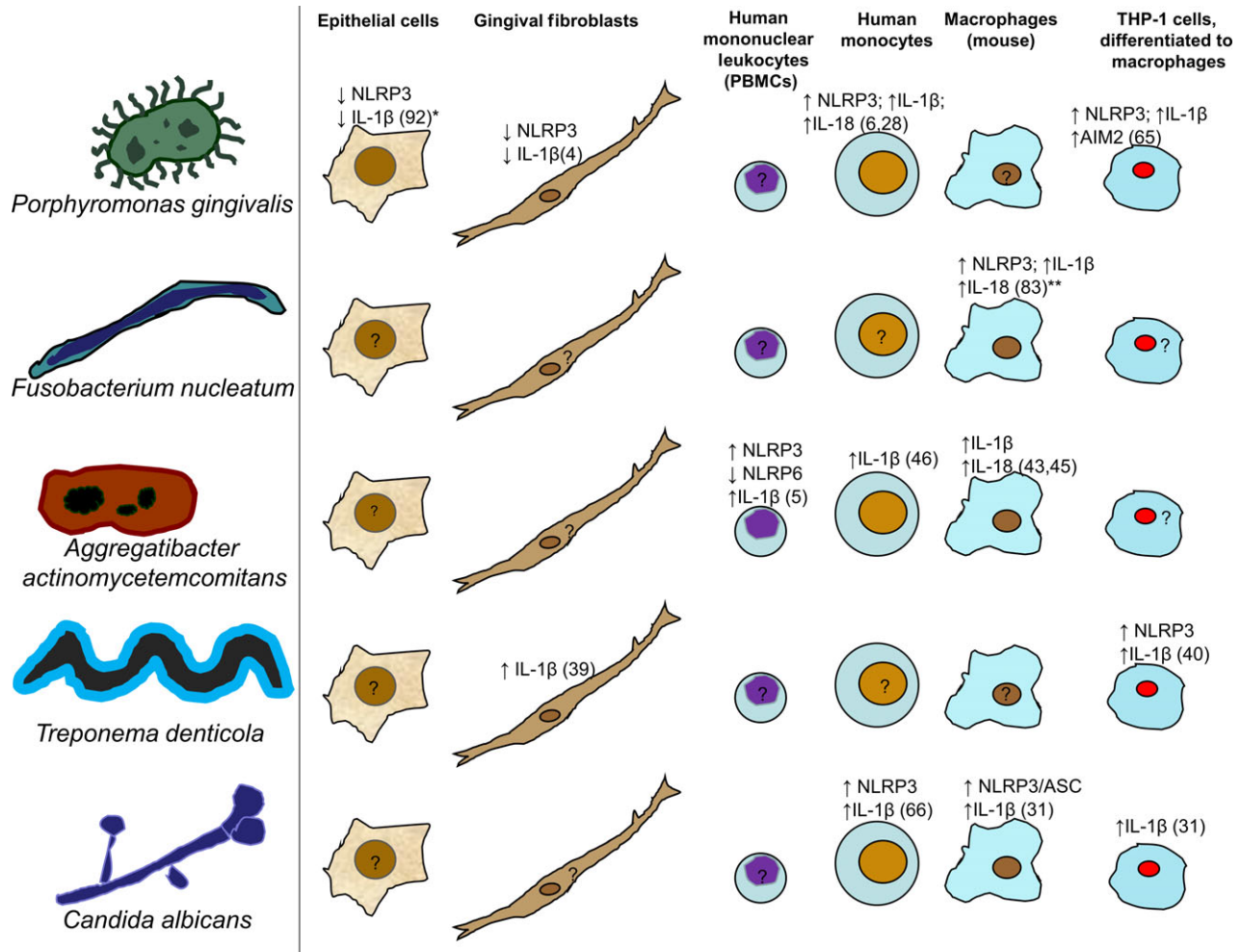


Fig. 2. Modulation of NLR family, pyrin domain containing 3 (NLRP3) inflammasome and interleukin-1beta (IL-1 β) and/or interleukin-18 (IL-18) expression/secretion in different host-cell types by specific periodontal bacteria during infection. *Extracellular ATP treatment induces up-regulation of both NLRP3 and IL-1 β secretion through the purinergic signaling system. **The indicated *Fusobacterium nucleatum*-induced inflammasome/cytokine levels were shown to be down-regulated during co-infection with *Porphyromonas gingivalis*. ?Currently there are no

reported data observing the inflammasome-associated host inflammatory responses in the specific cell types. Arrows represent up-regulation or down-regulation of the studied cytokine or inflammasome components. AIM2, absent in melanoma 2; ASC, apoptosis-associated speck-like protein containing a carboxyl-terminal caspase recruitment domain; PMBCs, peripheral blood mononuclear cells. (References 4–6, 28, 31, 39, 40, 43, 45, 46, 65, 66, 83, 92 are cited in the figure.)

containing nine species of bacteria (*Campylobacter rectus*, *F. nucleatum*, *Prevotella intermedia*, *T. forsythia*, *T. denticola*, *Veillonella dispar*, *Actinomyces oris*, *Streptococcus anginosus* and *Streptococcus oralis*), compared with the same biofilm without *P. gingivalis*, which showed that the levels of NLRP3 and interleukin-1beta were similar to those of the control (4). Interestingly, the expression levels of the adaptor molecule, apoptosis-associated speck-like protein containing a carboxyl-terminal caspase recruitment domain, the effector molecule, caspase-1, and absent in melanoma 2 were not affected in either subgingival biofilm models. Altogether, it appears that *P. gingiva-*

lis can selectively target host immune responses and orchestrate other microbial responses to its own advantage, especially as NLRP3 activation by *P. gingivalis* varies in different cell types (Fig. 2). For example, a recent *in vitro* study examined the mechanisms of activation of NLRP3 and interleukin-1beta secretion in a human acute monocytic leukemia cell type (THP-1) differentiated to macrophages and discovered that activation of both NLRP3 and absent in melanoma 2 are necessary for secretion of *P. gingivalis*-induced caspase-1 dependent interleukin-1beta via Toll-like receptors 2 and 4 (65). Apoptosis-associated speck-like protein containing a carboxyl-termi-

nal caspase recruitment domain, NLRP3, absent in melanoma 2 and caspase-1, all of which are components of both the NLRP3 inflammasomes and the absent in melanoma 2 inflammasomes, were increased in the presence of *P. gingivalis* and were required for the secretion of interleukin-1beta. Additionally, in macrophages, *P. gingivalis*-induced secretion of interleukin-1beta resulted in a highly inflammatory cell death known as 'pyroptosis', whereas in primary gingival epithelial cells, *P. gingivalis* induces a prosurvival phenotype, even upon treatment with high levels of ATP (91). Another *in vitro* study, in which the human myelomonocytic cell line, Mono-Mac-6, was challenged with *P. gingivalis*, observed that while the untreated cells showed low levels of expression of NLRP3, the infected cells showed highly elevated levels of expression of NLRP3, interleukin-1beta and interleukin-18 (6). Within the same cell type, a different *in vitro* study reported that *P. gingivalis* differentially regulated interleukin-1beta and interleukin-18, where mRNA levels of both cytokines were up-regulated by infection with whole *P. gingivalis*, as well as with the heat-inactivated organism, or stimulation with the chemically inhibited gingipains or with purified *P. gingivalis*-lipopolysaccharide (28). Interestingly, only interleukin-1beta (and not interleukin-18) was secreted in these cells under the test conditions. These differences in outcome may be a result of the nature of the different cell types, the microenvironment and the associated inflammasome components. Whilst gingival epithelial cells function both as an initial defense barrier and a primary reservoir for the colonizing bacteria, it is not surprising that *P. gingivalis* down-regulates NLRP3 in the gingival epithelial cells to facilitate its colonization, and possibly disseminate to underlying tissues in the oral mucosa. Macrophages, on the other hand, are professional immune cells designed to fight microbial invaders, which may explain the observed opposing pro-inflammatory behavior of *P. gingivalis*.

As mentioned previously, only stimulation of the *P. gingivalis*-infected cells with extracellular ATP leads to moderate interleukin-1beta secretion in primary gingival epithelial cells, thus providing evidence that modulation of activation of the NLRP3 inflammasome by *P. gingivalis* is possibly achieved through inhibition of the 'second signal' – ATP. The recent further characterization of the mechanisms of activation of *P. gingivalis* regulation of inflammasomes revealed that extracellular ATP-induced production of reactive oxygen species in gingival epithelial cells involves the formation of a heterocomplex among purinergic P2X₇ and P2X₄ receptors and the pannexin-1 hemichannel

(33). Silencing of pannexin-1 expression in gingival epithelial cells resulted in the inhibition of extracellular ATP release during *P. gingivalis* infection, highlighting the importance of pannexin-1 in activation of the inflammasome and secretion of interleukin-1beta (11). In the same *in vitro* study, *P. gingivalis* was shown to modulate reactive oxygen species levels in infected primary gingival epithelial cells through its effector molecule, nucleoside-diphosphate-kinase, to establish successful survival in the host cells (11). Reactive oxygen species are well known as a major host antimicrobial response to intracellular invaders (79). Additionally, as the role of reactive oxygen species as key upstream mediator molecules for NLRP3 activation is emerging, reactive oxygen species signaling represents an attractive target for highly adapted facultative intracellular pathogens, such as *P. gingivalis*, to evade immune recognition and secure persistence (73). Conversely, other intracellular invaders associated with severe periodontitis, such as *T. denticola*, do not inhibit extracellular ATP signaling and have been shown to induce both pro-interleukin-1beta production and caspase-1 activation through extracellular ATP release, leading to the secretion of active interleukin-1beta in monocytic THP-1 cells *in vitro* (40). The components involved were further analyzed and a surface protein of *T. denticola* (Td92) was shown to interact directly with integrin $\alpha 5\beta 1$ to activate the NLRP3 inflammasome and up-regulate pro-interleukin-1beta synthesis and caspase-1 activation to increase secretion of interleukin-1beta (40). Although *T. denticola* is a tenacious spirochete known for its ability to cause periodontal tissue damage, it has often been studied for its interbacterial relationship with other periodontal pathogens, including *P. gingivalis*. It is tempting to speculate that the ability of *P. gingivalis* to modulate inflammasome signaling may contribute to increased colonization and persistence of *T. denticola*, and possibly other periodontal bacteria.

While the contributions of particular bacterial species in the etiology and/or progression of periodontal disease are widely accepted, a few specific herpesviruses have also been suggested to be intensely implicated in the disease process (12, 76, 85). The presence of subgingival cytomegalovirus or Epstein-Barr virus has been described to be particularly linked with increased levels of a variety of major periodontal pathogens including *P. gingivalis*, *T. forsythia*, *Dialister pneumosintes*, *P. intermedia*, *Prevotella nigrescens*, *C. rectus* and *T. denticola* (71, 72, 75). A critically important interaction between cytomegalovirus and *P. gingivalis* co-infection, leading to the damage of liver and spleen when compared with

single infections, was also demonstrated in an experimental mouse study (81). Moreover, gingival specimens from cytomegalovirus-positive periodontitis lesions showed up-regulation of mRNA for interleukin-1beta along with mRNAs for some other pro-inflammatory cytokines (12). The specific molecular actions underlying the shared infections with herpesviruses and *P. gingivalis* (and the other periodontal bacteria), as well as their combined potential effects in the regulation of inflammasomes, need to be fully researched.

The aforementioned mechanisms of *P. gingivalis* are likely to be critical factors for development of the dysbiotic stage in the resident microbiome and disruption of the host homeostatic defenses. Further studies of the mechanisms of action for modulating inflammasome signaling by *P. gingivalis*, and the microorganism's interaction with other emerging infectious agents, may serve as important targets for future prevention and/or control of periodontal disease.

Aggregatibacter actinomycetemcomitans

Aggregatibacter actinomycetemcomitans in subgingival biofilms has been closely associated with the loss of periodontal tissue attachment in affected sites of both adults and juveniles. The presence of *A. actinomycetemcomitans* is a well-identified indicator of the initiation of localized aggressive periodontitis (23). The pathogenicity of the microorganism is also underlined by its well-characterized virulence factors, such as leukotoxin and cytolethal distending toxin, which are suggested to play important roles in altering host inflammatory responses as well as in contributing to periodontal disease progression (22). Accordingly, in an earlier clinical study, two periodontitis sites from a patient with localized aggressive periodontitis were sampled for microbial analysis by swabbing and for interleukin-1beta quantification in gingival crevicular fluids; the study detected not only a high level of *A. actinomycetemcomitans*, but also elevated levels of interleukin-1beta in the diseased sites (44). Moreover, following *in vitro* testing of macrophages treated with a highly leukotoxic strain (HK 1519) vs. a minimally leukotoxic strain (D7SS WT), the same study suggested that leukotoxin is the main factor responsible for inducing elevated levels of interleukin-1beta (44). A separate study also reported that the bacterial leukotoxin induced an excessive pro-inflammatory response in macrophages, through the secretion of interleukin-1beta and interleukin-18 and the involvement of purinergic receptor P2X₇ in the process (43). In contrast, in another *in vitro* study where leukotoxin and cytolethal distending toxin

gene knockout mutant strains of *A. actinomycetemcomitans* were used to infect human mononuclear leukocytes, only up-regulation of NLRP3, interleukin-1beta, interleukin-18 and reduction of NLRP6 were observed (but no other inflammasome components, such as apoptosis-associated speck-like protein containing a carboxyl-terminal caspase recruitment domain, were affected) (5). Based on the results of this study, there is possible regulation of inflammasome complexes by additional molecules other than the two best-studied virulence factors of the microorganism. A potential candidate may be 'bacterial interleukin-1beta receptor I', a putative membrane protein of *A. actinomycetemcomitans*, which was found to bind interleukin-1beta (64). Although the exact details of this interaction (i.e. whether bacterial interleukin-1beta receptor I binds interleukin-1beta only or also interacts with other host cytokines) have not been identified, there seems to be a role for interleukin-1beta uptake in the virulence of *A. actinomycetemcomitans*. There is a need for future studies addressing the role of host immune signaling cascades involved in the virulence of this pathogen. Further research on the identified inflammasome components in relation to *A. actinomycetemcomitans* infection may significantly contribute to the characterization of the microorganism and its association with aggressive severe forms of periodontitis.

Candida albicans

Candida albicans is an opportunistic fungal pathogen that commonly resides on human mucosal surfaces and, when overgrown under immunocompromised conditions, causes inflammation and other systemic infections (87). It is not clear whether *C. albicans* is a primary cause or a secondary consequence of an already reduced immune response; however, it has been hypothesized that disruption of epithelium and immunosuppression during periodontal disease may facilitate colonization of *C. albicans* in subgingival pockets (7, 8). One of the most characterized virulence-associated factors belongs to the family of the 'secretion of aspartic proteases', which has been shown to induce secretion of pro-inflammatory cytokines in human monocytes (60). While no study has shown a specific protein of *C. albicans* to be responsible for inducing inflammasome activation, a recent *in vitro* discovery demonstrated that secretion of aspartic proteases-2 and -6 specifically were responsible for inducing interleukin-1beta and interleukin-18 production in human monocytes as a result of activation of NLRP3 inflammasome and caspase-1 (66). Additionally, a previous study also demonstrated a

role for another nucleotide-binding-domain-like receptor molecule, NLR4, which was shown to be involved in controlling *C. albicans* mucosal infection and preventing dissemination of this pathogen (87). The study showed that both NLR4 and NLRP3 inflammasomes were important in the induction of interleukin-1beta secretion. These studies demonstrate the ability of *C. albicans* to induce excessive inflammatory responses. *Candida albicans* has been shown to be present on the mucosal surfaces of the oral cavity as well as in periodontal pockets; however, its presence in the subgingival microbiome in patients with severe chronic periodontitis was only recently highlighted during a clinical study (8). It may be necessary to study these special features of *C. albicans* in further detail, especially in the context of the multifactorial nature of periodontal disease and the possible effects of *C. albicans* upon the composition of the subgingival biofilm.

Outlook on future inflammasome studies, challenges and what can be expected

It is becoming evident that a highly elaborate relationship exists between the components of the oral microbiome and the host's innate immune response. Accumulating evidence from studies on inflammasomes emphasize the centrality of the inflammasome and its constituents (particularly secreted interleukin-1beta) in the initiation and progression of periodontal disease, as well as in other chronic inflammatory diseases (21, 50, 63) (Figs 1 and 2). It remains to be determined whether the inflammasome complexes can serve as diagnostic markers to identify susceptible populations, but recent advances in the understanding of the specific molecular circuitries involved in inflammasome activation have resulted in multiple clinical trials targeting interleukin-1beta as a therapeutic agent (18, 19, 51). The growing involvement of purinergic signaling, in particular P2X₇-ATP coupling, in the modulation of specific inflammasome-associated processes and their recent pharmacological targeting in chronic diseases, also presents great potential for these receptors as target molecules for controlling inflammation and the treatment of oral pathologies (2).

The ability of opportunistic colonizers, such as *P. gingivalis*, to alter this vital primary defense mechanism seems to suggest an ancient adaptation between subsets of highly adapted microbiome populations and the host inflammasome; hence, the devel-

opment of specific microbial virulence mechanisms ensuring persistence. However, the knowledge gathered on several species of oral bacteria illustrates only the highly complex nature of particular interactions that have thus far limited the ability of interventions to prevent the inflammation and tissue damage caused by the bacteria. The identification of specific virulence factors and their molecular actions, by which successful colonizers modulate host inflammasome networks, will help to expand our current understanding of inflammasome regulation, contributing to the development of well-targeted interventions to control infection and inflammatory disease progression. Unlocking the existing complex intermicrobial interactions present in the oral cavity with the microbial factors modulating inflammasome activation could be central for advancement of both the prevention and treatment of periodontal disease and the associated systemic chronic conditions.

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