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The Inflammasome and Danger Molecule Signaling: At the Crossroads of Inflammation and Pathogen Persistence in the Oral Cavity

Özlem Yilmaz[1,2,*] and **Kyu Lim Lee**[1]

[1]Department of Periodontology, University of Florida, Gainesville, FL 32610, USA

[2]Emerging Pathogens Institute, University of Florida, Gainesville, FL 32610, USA

Abstract

Inflammasomes are an oligomeric assembly of multiprotein complexes that activate the caspase-1-dependent maturation and the subsequent secretion of inflammatory interleukin-1β and interleukin-18 cytokines in response to a ‘danger signal’ in vertebrates. The assessment of their significance continues to grow rapidly as the complex biology of various chronic inflammatory conditions are better dissected. Increasing evidence links inflammasomes and host-derived small ‘danger molecule ATP’-signaling strongly with the modulation of the host immune response by microbial colonizers as well as potential altering of the microbiome structure and inter-microbial interactions in host. All of these factors eventually lead to the destructive chronic inflammatory disease state. In the oral cavity, a highly dynamic and multifaceted interplay takes place between the endogenous danger molecule signaling and colonizing microbes on the mucosal surfaces. This interaction may redirect the local microenvironment to favor the conversion of the resident microbiome towards pathogenicity. This review outlines the major components of the known inflammasome complexes/mechanisms and highlights their regulation, in particular, by oral microorganisms in relation to the periodontal disease pathology. Better characterizations of the cellular and molecular biology of the inflammasome will likely present important potential therapeutic targets in the treatment and prevention of periodontal disease as well as other debilitating chronic diseases.

While the innate immune system is critical for the initial defense against pathogenic microorganisms, the host faces major challenges with microbial pathogens that have developed mechanisms to circumvent host immune detection. As first line of host defense, the innate immune system heavily relies on the presence of evolutionarily conserved pattern-recognition receptors, which include the best characterized membrane-bound Toll-like receptors, Retinoidinducible 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 encompassing macrophages, neutrophils, monocytes, and epithelial cells, can sense ‘pathogen-associated molecular patterns’, and also respond to ‘danger-associated molecular patterns’, also known as

[*]Correspondence: Department of Periodontology, College of Dentistry And Emerging Pathogens Institute, University of Florida, Gainesville, FL, 32610-0434 Tel: (352) 273 8003 oyilmaz@ufl.edu.

'damage-associated molecular patterns', which can be host- (ATP, DNA or cholesterol crystals) or environmentally-derived (asbestos, silica, alum or nanoparticles) (73). The activation of pattern recognition receptors by pathogen-associated molecular patterns and their post-receptor signaling via stimulation by 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 late Jurg Tschopp and his research team in 2002 (54). Inflammasomes are nucleotide-binding-domain-like receptors containing-multi-protein complexes functioning as a molecular platform activated upon cellular danger or stress signals which trigger the maturation and secretion of pro-inflammatory cytokines such as interleukin-1β and -18 (54). Depending on the specific recognition receptors involved and the activated caspase-cascades, over five inflammasome-families have been identified 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 NLRP1, NLRP3 and NLRC4 (1, 37). From these families, the NLRP3 is the most comprehensively characterized inflammasome, shown to be associated with several autoinflammatory and non-autoimmune chronic conditions (73). NLRP3 has lately become an important target molecule to understand 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 ranging from gout to cancer, to bacterial and viral infections (20, 55). The role of the inflammasomes in mediating host metabolic responses and the dysregulation of inflammasome components are associated with various inherited chronic inflammatory and immune disorders, highlighting its relevance in human disease (73). In particular, the imbalance of interleukin-1β activity is among the focal points of both microbial-associated and non-microbial inflammatory diseases. The progression of periodontitis is inflammatory by 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, while their role against microbial pathogens is becoming crucially important in controlling and limiting the invading microbes. On the other hand, there are increasing numbers of microorganisms and their virulence factors found to function by targeting the inflammasome and modulating interleukin-1β processing, which all together could be involved in the development and/or progression of various inflammatory diseases, including periodontal disease (14, 48, 82). In light of the recently accumulated evidence on the role of the inflammasome 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

the inflammasome, focusing on the current research on NLRP3, in relation to microbial pathogenesis, and particularly the potential implications to human chronic diseases and periodontal disease. The signaling components of the inflammasome as plausible intervention targets will also be discussed briefly.

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 due to 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 the activation of the NLRP3 inflammasome. Those 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-1β 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, viral RNA) stimulate a pattern-recognition receptor and trigger the production of the interleukin-1 precursor (35). The second signal is brought by danger-associated molecular patterns that are small danger signal molecules, either host-derived (i.e. extracellular ATP) or environmentally-associated, ligating to danger signal receptors. A growing number of research findings are heightening 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 for controlling infections (26) (Figure 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 $P2X_7$ purinergic receptors, which are a family of cation-permeable ligand gated ion channels (53). The significance of the $P2X_7$ receptor for inflammasome signaling has been widely studied in the myeloid cells such as monocytes, macrophages, and dendritic cells. However, the role of $P2X_7$ in epithelial cells which line the mucosal tissues forming an initial barrier for invading microbes and functioning as an important arm of innate immunity has recently been explored. Interestingly, this receptor presence and function in epithelial cells has become largely characterized in human primary gingival epithelial cells that form a first line of

defense to the colonizing microbes in periodontal pockets (11, 79, 90, 92, 93). Gingival epithelial cells were determined to express functional $P2X_7$ receptors mediating 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, extracellular ATP signaling requires the assembly of $P2X_7$ and $P2X_4$ 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 when both $P2X_7$ and $P2X_4$ receptors were individually downregulated through a small interference RNA, *Porphyromonas gingivalis*-infected gingival epithelial cells showed a significant reduction in extracellular ATP-induced interleukin-1β secretion (33). Overall, the involvement of $P2X_7$ in the activation of NLRP3 inflammasome indicates that $P2X_7$ is likely to function as a master regulator in this inflammatory pathway. This has also been validated by *in vivo* studies, where $P2X_7$ -knockout mice display impaired inflammasome activation, reduced levels of interleukin-1β release and decreased disease severity in a murine model of arthritis (49) or a disruption of extracellular ATP-induced processing of pro-interleukin-1β in macrophages (77). Several change-of-function polymorphisms (causing $P2X_7$ receptor inactivation or reduced function) and their associations with resistance or susceptibility to different human diseases have further highlighted the important mechanistic role of the purinergic signaling in chronic diseases (9, 25).

Since extracellular ATP-$P2X_7$ coupled inflammasome activation and subsequent pro-inflammatory cytokine secretion is one of the central self defense mechanisms leading to the recruitment of specialized pathogen-fighting cells (e.g. macrophages, neutrophils) and alerting neighboring cells of danger of infection, it is often targeted by persistent pathogens in their evolution towards adaptation and successful colonization of host tissues (13, 59). What is interesting is that successful opportunistic pathogens, such as *P. gingivalis* have developed mechanisms of inhibiting the extracellular ATP-$P2X_7$ pathway, directly linking with inflammasome activation via secretion of an effector called ‘nucleoside diphosphate kinase’ (11, 80). This ATP hydrolyzing enzyme homolog is secreted by *P. gingivalis* as well as other successful persistent microbial species, such as *Mycobacterium tuberculosis*, to possibly negate the robust host immune response and modify host cell biology for successful persistence (70, 80). It appears that extracellular ATP signaling coupled with the inflammasome 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 with local inflammatory diseases but may also have the potential to cause systemic effects.

Inflammasome and its relevance to chronic inflammatory diseases and other chronic diseases

With the increased knowledge gained over the last decade, the 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 with 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-1β 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-1β, and the cytokine's ability to mediate prolonged hyperglycemic toxicity in pancreatic islets, contributing to the destruction of β 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 also been associated with Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, P. gingivalis, and other bacteria known for their modulation of interleukin-1β release through NRLP3 inflammasome complex (29, 30). The inflammasome-dependent cytokines interleukin-1β 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).

While 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 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 that 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 highlighted 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 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 to healthy subjects. Consistent with previous findings (21, 38, 63), both interleukin-1β and interleukin-18 mRNA expression levels were also enhanced in those patients, and thus, the findings revealed a positive correlation between NLRP3 and interleukin-1β and interleukin-18 expression in periodontal disease (6). While 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 look at 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-1β, 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-1β and interleukin-18 by measuring these markers in the gingival crevicular fluids of patients with chronic periodontitis (65). Additionally, the study showed upregulated 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-1β 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 there may be multiple inflammasome machineries simultaneously contributing to the microbiome-associated induction of inflammation during periodontal disease, such as the interplay between various microbiome 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 also exploring the relations of those biomarkers to the composition of the subgingival microbiome. Recently, a study reported that periodontal species, such as P. gingivalis, Treponema denticola, Tannerella forsythia, and Eubacterium nodaum were shown to be

present at significantly higher levels in the gingival crevicular fluids of chronic periodontitis patients, which were also showing high levels of interleukin-1β and interleukin-18 (84). The study also compared clinically healthy sites from both periodontitis patients and periodontally healthy controls. Interestingly, the subgingival microbiome composition also differed between these two groups. Periodontally healthy subjects had higher proportions of certain species, some of which include *Actinomyces odontolyticus*, *Streptococcus gordonii*, and *A. actinomycetemcomitans* (belonging to the Purple, Yellow and Green complex, respectively), while the individuals with periodontitis had significantly higher levels of species comprising *F. nucleatum* and *P. gingivalis*, *T. denticola*, *T. forsythia* (belonging to the Orange and 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-1β and interleukin-18). On the other hand, Purple, Yellow and Green complex species displayed a negative association with interleukin-1β (84). The observed increased expression levels of interleukin-1β and interleukin-18 in clinically healthy sites of periodontitis patients suggested that inflammatory responses possibly occur early on before they can be seen clinically and are perhaps due to the higher colonization of the Red and Orange Complex species rather than resulting from genetic predisposition.

While the contribution of microbes in the etiology of periodontal disease is largely recognized, 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 in the oral cavity has also lately been associated with a variety of related systemic diseases (2, 27). Being recognized for its ability to modulate the composition of the oral microbiome highly effectively, *P. gingivalis* has developed number of distinct mechanisms for manipulating host inflammatory responses, such as reducing the innate immune response for its own benefit and at the same time, offering an advantageous environment to 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 to 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-1β in co-infections

compared to single-infections of mouse periodontal tissues (67). On the other hand, a recent work performed in an *in vitro* mouse macrophage model depicted that *P. gingivalis* can synergistically regulate *F. nucleatum* invasion into the host cells through inhibiting both *F. nucleatum*-induced interleukin-1β 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 where *P. gingivalis* was found to downregulate NLRP3 expression, induce pro-interleukin-1β production but only promote mature interleukin-1β secretion upon danger signal extracellular ATP stimulation (92).

Thus, a compelling amount of research pinpoints *P. gingivalis* is able to modulate specific known inflammasome components and successfully colonize and persist in host cells. Along the same lines, a recent work further underscored this ability of the microorganism. It showed that NLRP3 inflammasome and interleukin-1β expression was significantly downregulated in gingival fibroblast cultures when *P. gingivalis* was introduced in a subgingival biofilm comprised of 9 species (Campylobacter rectus, F. nucleatum, Prevotalla intermedia, T. forsythia, T. denticola, Veillonella dispar, Actinomyces oris, Streptococcus anginosus, and *Streptococcus oralis*) compared to the same biofilm without *P. gingivalis*, which showed NLRP3 and interleukin-1β expression close to control levels (4). Interestingly, 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 expression levels were not affected in either subgingival biofilm models. Altogether, it appears that *P. gingivalis* can selectively target host immune responses and orchestrate other microbial responses to its own advantage, especially since 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-1β secretion in a human acute monocytic leukemia cell type (THP-1) differentiated to macrophages and discovered that both NLRP3 and absent in melanoma 2 activation are necessary for *P. gingivalis*-induced caspase-1 dependent interleukin-1β secretion via Toll-like receptor 2 and 4 (65). Apoptosis-associated speck-like protein containing a carboxyl-terminal caspase recruitment domain, NLRP3, absent in melanoma 2 and caspase-1, which are components of the NLRP3 and absent in melanoma 2 inflammasomes, were increased in the presence of *P. gingivalis* and were required for the secretion of interleukin-1β. Additionally, in macrophages *P. gingivalis*-induced interleukin-1β secretion resulted in a highly inflammatory cell death known as ‘pyroptosis’, whereas in primary gingival epithelial cells, *P. gingivalis* induces a pro-survival phenotype even upon treatment with high levels of ATP (91). Another *in vitro* study where the human myelomonocytic cell line Mono-Mac-6 was challenged with *P. gingivalis* observed that while the untreated cells showed low NLRP3 expression levels, the infected cells showed highly elevated expression levels of NLRP3, interleukin-1β and interleukin-18 (6). Within the same cell type, a different *in vitro* study reported that *P. gingivalis* differentially regulated interleukin-1β and interleukin-18, where mRNA levels of both cytokines were upregulated by whole *P. gingivalis* infection, as well as by the heat-inactivated organism or stimulation by the chemically inhibited gingipains or , with purified *P. gingivalis*lipopolysaccharide (28). Interestingly, only interleukin-1β and not interleukin-18 was secreted in these cells under the examined conditions. These differences in the outcomes may be due to the nature of the different cell types, the microenvironment,

and the associated inflammasome components. While gingival epithelial cells function both as an initial defense barrier and a primary reservoir for the colonizing bacteria, it is not surprising to see that *P. gingivalis* downregulates NLRP3 in the gingival epithelial cells to successfully facilitate its colonization and possibly dissemination 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 the stimulation of the *P. gingivalis*-infected cells with extracellular ATP leads to moderate interleukin-1β secretion in primary gingival epithelial cells, thus providing evidence that *P. gingivalis*-modulation of the NLRP3 inflammasome activation is possibly achieved through inhibition of the 'second signal'—ATP. The recent further characterization of the mechanisms of *P. gingivalis*-regulation of inflammasome activation revealed that extracellular ATP-induced reactive oxygen species production in gingival epithelial cells involves the formation of a heterocomplex between purinergic $P2X_7$, $P2X_4$ receptors and 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 the inflammasome activation and interleukin-1β secretion (11). In the same *in vitro* study *P. gingivalis* was shown to effectively 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 for their properties as major host anti-microbial response against intracellular invaders (79). Additionally, since 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-1β production and caspase-1 activation through extracellular ATP release, leading to the secretion of active interleukin-1β in monocytic THP-1 cells *in vitro* (40). The components involved were further analyzed where a surface protein of *T. denticola* (Td92) was shown to directly interact with integrin α5β1 to activate the NLRP3 inflammasome, upregulate pro-interleukin-1β synthesis and caspase-1 activation to increase interleukin-1β secretion (40). Although *T. denticola* is a tenacious spirochete known for its ability to cause periodontal tissue damage, it has often been studied for its inter-bacterial relationship in combination 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 herpes viruses 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 involved in elevated levels of a number 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 to the single infections was also demonstrated in an experimental mouse study (81). Moreover, gingival specimens from cytomegalovirus-positive periodontitis lesions showed an upregulation of mRNAs for interleukin-1β along with some other pro-inflammatory cytokines (12). Nevertheless, the specific molecular actions underlying the shared infections by the herpes viruses and *P. gingivalis* (and the other periodontal bacteria) as well as their combined potential effects in the regulation of inflammasome are still waiting to be fully researched.

The aforementioned mechanisms of *P. gingivalis* are likely to be critical factors for the 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 an important role in altering host inflammatory responses as well as 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-1β quantification in gingival crevicular fluids and the study detected not only a high presence of *A. actinomycetemcomitans*, but also elevated levels of interleukin-1β in the diseased sites (44). Moreover, the same study suggested that leukotoxin is the main factor responsible of inducing elevated interleukin-1β by *in vitro* testing in macrophages treated with a highly leukotoxic strain (HK 1519) when compared to the minimally leukotoxic strain (D7SS WT) (44). A separate study also reported that the bacterial leukotoxin induced an excessive pro-inflammatory response by interleukin-1β and interleukin-18 secretion in macrophages through involvement of purinergic receptor $P2X_7$ in the process (43). Contrastingly, another *in vitro* study showed upregulation of NLRP3 and reduction of NLRP6 gene expression but no other inflammasome components, such as the apoptosis-associated speck-like protein containing a carboxyl-terminal caspase recruitment domain, and elevated interleukin-1β and interleukin-18 in human mononuclear leukocytes using leukotoxin and cytolethal distending toxin gene knock out mutant strains of *A. actinomycetemcomitans* (5). Based on this study, there is a possible regulation of inflammasome complexes by additional molecules other than the two best studied virulence factors of the microorganism. A potential candidate may be the putative membrane protein of *A. actinomycetemcomitans* 'bacterial interleukin-1β receptor I', which was found to bind interleukin-1β (64). While the exact details involved in

this interaction, whether bacterial interleukin-1β receptor I only binds interleukin-1β or interacts with other host cytokines, has not been identified, there seems to be a role for interleukin-1β 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). Whether it is a primary cause or a secondary consequence of an already reduced immune response, is not clear 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 there has been no study showing a specific protein of the microorganism inducing inflammasome activation, a recent *in vitro* discovery demonstrated that secretion of aspartic proteases-2 and -6 specifically, were shown to induce interleukin-1β and interleukin-18 production in human monocytes as a result of NLRP3 inflammasome and caspase-1 activation (66). Additionally, a previous study also demonstrated a role for another nucleotide-binding-domain-like receptor molecule, NLRC4, which was shown to be involved in controlling *C. albicans* mucosal infection and preventing the dissemination of the pathogen (87). The study showed that NLRC4 and NLRP3 inflammasomes were both important in the induction of interleukin-1β secretion. These mechanisms display the ability of *C. albicans* to induce excessive inflammatory responses. While *C. albicans* has been shown to be present on the mucosal surfaces of the oral cavity as well as periodontal pockets, the association between its presence in the subgingival microbiome in patients with severe chronic periodontitis was only recently highlighted through a clinical study (8). These special features of *C. albicans* may be necessary to further study 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. The accumulating studies emphasize the centrality of the inflammasome and its constituents, particularly secreted interleukin-1β in the initiation and progression of periodontal disease as well as other chronic inflammatory diseases (21, 50, 63) (Figs. 1 and 2). Whether the inflammasome complexes can serve as diagnostic markers to identify susceptible populations remain to be determined, but recent advances in the understanding of the specific molecular circuitries

involved in inflammasome activation have resulted in multiple clinical trials targeting interleukin-1β as a therapeutic agent (18, 19, 51). The growing involvement of purinergic signaling in particular $P2X_7$-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 the oral pathologies (2).

The ability of opportunistic colonizers such as *P. gingivalis* to successfully alter this vital primary defense mechanism seems to strongly suggest an ancient adaptation between subsets of highly adapted microbiome populations and the host inflammasome, hence the development of specific microbial virulence mechanisms ensuring persistence. However, the gathered knowledge with several oral bacteria illustrates only the highly complex nature of particular interactions that have thus far limited the ability of interventions to prevent inflammation and tissue damage that the bacteria cause. The identification of specific virulence factors and their molecular actions by which successful colonizers modulate host inflammasome networks will help expand current understanding of inflammasome regulation, contributing to the development of well-targeted interventions to control infection and inflammatory disease progression. Unlocking of the existing complex inter-microbial interactions present in the oral cavity with the microbial factors modulating inflammasome activation could be central for the advancement of both the prevention and treatment of periodontal disease and the associated systemic chronic conditions.

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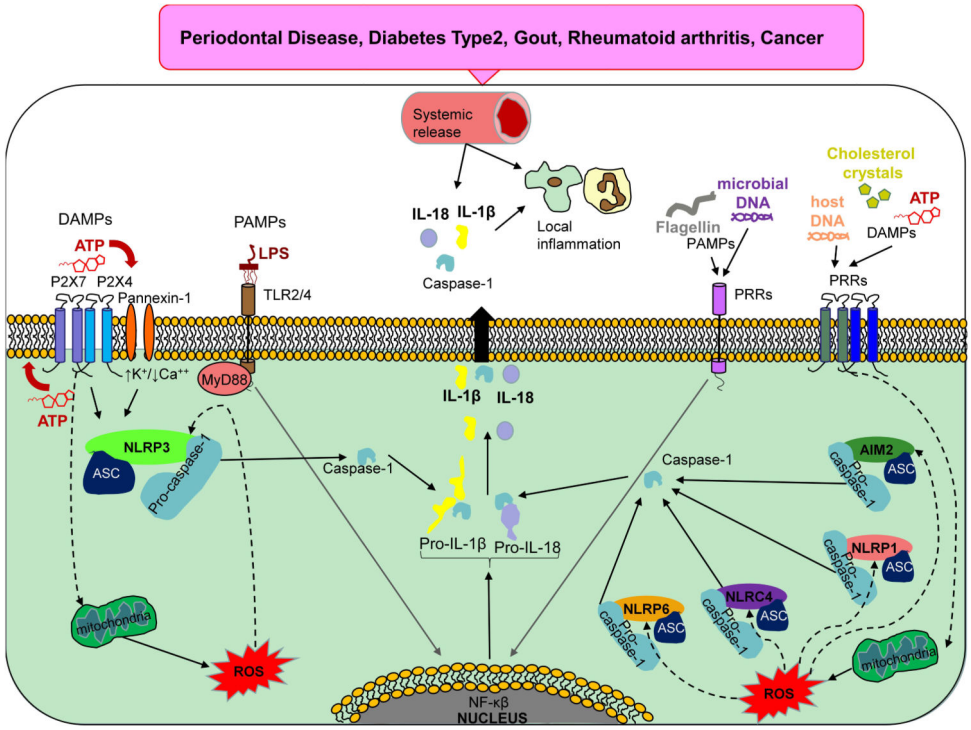

Fig. 1. Schematic representation of the proposed mechanisms of stimulation of different inflammasomes and their relation to the development of chronic inflammatory diseases

Several members of the nucleotide-binding-domain-like receptor (NLR) family such as NLRP1, NLRP3, NLRC4, NLRP6, and AIM2, are known to participate in the assembly of the inflammasome complex. NLRs are activated by pathogen-associated molecular patterns (PAMPs) such as microbial lipopolysaccharides (LPS) through pattern recognition receptors (PRRs) like the Toll-like receptors 2 and 4 (TLR2/4). The stimulation of the PRRs by PAMPs leads to the increased expression of pro-cytokines (pro-interleukin-1β (pro-IL-1β) 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-1β or IL-18 form leading to the extra-cellular secretion of these inflammatory cytokines. NLRP3 is the most well studied NLR-member. Currently there are three models for the activation of the NLRP3 inflammasome – the reactive oxygen species (ROS) model, the lysosomal burst model, and the ATP-triggered K^+ -efflux model. For simplicity here only the ROS model is represented. 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, diabetes type 2, 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-1β and IL-18, leading to local inflammation, as well as systemic effects.

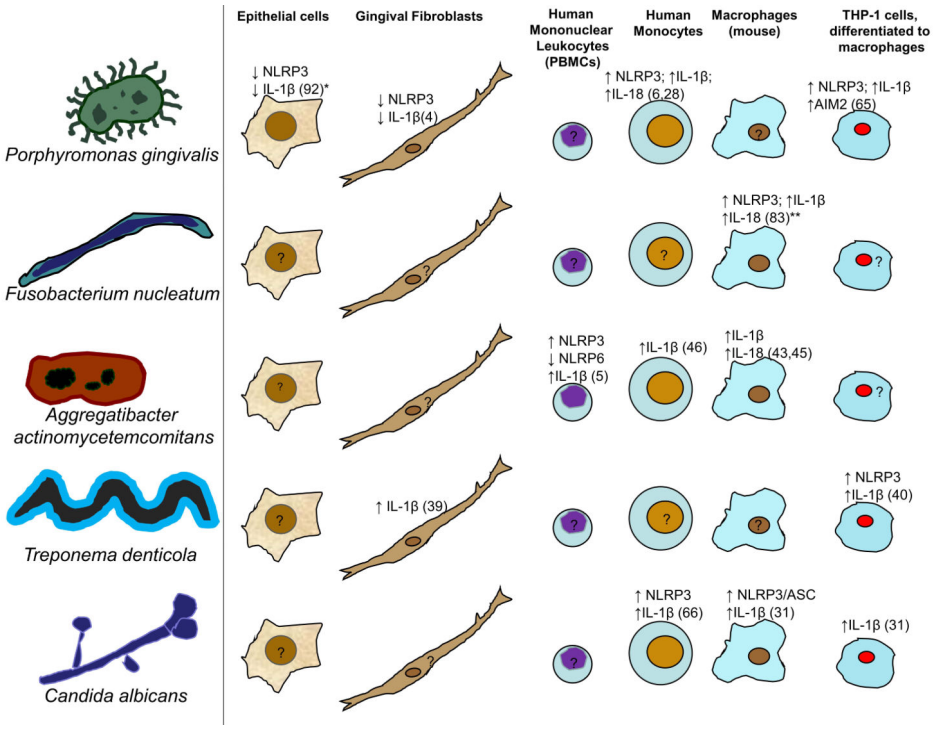

Fig. 2. NLRP3 inflammasome modulation and IL-1β and/or IL-18 expression/secretion in different host-cell types by specific periodontal bacteria during infection

"*"extracellular ATP treatment induces upregulation of both NLRP3 and interleukin-1β (IL-1β) secretion through the purinergic signaling system

"**"Indicated *Fusobacterium nucleatum*-induced inflammasome/cytokine levels were shown to be downregulated during co-infection with *Porphyromonas gingivalis*

"?" Currently there is no reported data observing the inflammasome associated host inflammatory responses in the specific cell types

Arrows represent upregulation or downregulation of the studied cytokine or inflammasome components.

References cited in the figure; (4-6, 28, 31, 39, 40, 43, 45, 46, 65, 66, 83, 92)