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Polymicrobial communities in periodontal disease: their quasi-organismal nature and dialogue with the host

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Abstract

In health, indigenous polymicrobial communities at mucosal surfaces maintain an ecological balance via both inter-microbial and host-microbial interactions that promote their own and the host's fitness, while preventing invasion by exogenous pathogens. However, genetic and acquired destabilizing factors (including immune deficiencies, immunoregulatory defects, smoking, diet, obesity, diabetes and other systemic diseases, aging) may disrupt this homeostatic balance, leading to selective outgrowth of species with potential for destructive inflammation. This process, known as dysbiosis, underlies the development of periodontitis in susceptible hosts. The pathogenic process is not linear but involves a positive-feedback loop between dysbiosis and the host inflammatory response. The dysbiotic community is essentially a quasi-organismal entity, where constituent organisms communicate via sophisticated physical and chemical signals and display functional specialization (*e.g.*, accessory pathogens, keystone pathogens, pathobionts), which enables polymicrobial synergy and dictates the community's pathogenic potential or nososymbiocity. In this review, we discuss early and recent studies in support of the 'polymicrobial synergy and dysbiosis' (PSD) model of periodontal disease pathogenesis. According to this concept, disease is not caused by individual 'causative pathogens' but rather by reciprocally reinforced interactions between physically and metabolically integrated polymicrobial communities and a dysregulated host inflammatory response.

Introduction

It is now well established that, unlike classic infections of single microbial etiology (*e.g.*, diphtheria, tetanus, typhoid fever, leprosy), periodontitis is not caused by a single or even a few select organisms but rather by polymicrobial communities of indigenous microbes acting in concert. In other words, periodontitis is not an infectious condition, but rather a dysbiotic disease, *i.e.*, associated with an alteration in the abundance or influence of individual species within the polymicrobial community, relative to their abundance or influence in health [1,2]. Periodontal dysbiosis is associated with disruption of tissue

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homeostasis, in great part due microbial subversion of the host immune and inflammatory response in the periodontium [3,4] (Figure 1).

Bacteria that have accumulated into polymicrobial biofilms form a defined structure, and attain quasi-organismal status owing to the functional specialization of constituent species and coordination of their activities via sophisticated signaling mechanisms that maintain the structure and function of the biofilm entity [2,5]. In contrast to conventional host-pathogen interactions in the setting of diseases with a single-infective etiology, the simple dichotomous characterization of microbes as either commensals or pathogens is not adequate to describe dysbiotic endogenous communities as drivers of disease. Rather, the commensal or pathogenic properties of bacteria within an organized community are not necessarily intrinsic but rather contextual features. Such properties, therefore, should be considered within the context of the community where the bacteria reside as well as the genetic and immune status of the host. In this regard, nososymbiocity is a newly coined term for a microbial community's collective pathogenic potential that depends upon both the outcome of interbacterial interactions and host susceptibility (Table 1) [6]. Thus, nososymbiocity (*nosos* [Greek for disease] potentially arising from living together with a susceptible host) is a more accurate and context-dependent term than 'pathogenicity', which implies the presence of specific causative pathogens[6]. It becomes apparent that the health- or disease- associated properties of a microbial community represent a continuum from commensalism to pathogenicity that includes many newly recognized categories such as homeostatic commensals, accessory pathogens, keystone pathogens, and pathobionts [2,6] (Figure 1).

Such polymicrobial communities within the sub-gingival dental plaque present unique challenges to the immune system. Whereas communities of mostly eubiotic commensal organisms may contribute to the training and maintenance of homeostatic immune functions (steady-state host defense), dysbiotic communities induce an immune response that is ineffective, uncontrolled and destructive in a setting of disrupted homeostasis (Figure 2). In this review, we describe the establishment and dynamics of subgingival microbial communities and discuss how the interactions among specialized community participants determine an emergent overall function that promotes or destabilizes periodontal tissue homeostasis. Given that periodontitis requires a susceptible host (Table 1), we also examine the host-microbe interplay that shapes both the host immune response and the polymicrobial community in both quantitative and qualitative ways. We conclude that in periodontitis, destructive inflammation and dysbiotic communities co-develop in a reciprocally reinforced way and their interplay spirals out to become the actual driver of this oral disease in susceptible individuals.

Community infrastructure in periodontal health and disease

Within the oral cavity there are distinct microenvironments including the mineralized tissues of the teeth and the epithelial surfaces of the mucosal membranes. The accumulation of heterotypic bacterial communities on these surfaces is a highly orchestrated process, dependent first on a stable association with the substratum, and then on mutual interspecies binding interactions and metabolic compatibility. Local environmental constraints also

determine the nature of the associated microbial communities. For example, the epithelial cells of the gingival epithelium continually turn over, and hence the microbial communities have less time to develop and tend to be less complex than those on the non-shedding surfaces of the teeth. Additionally, to avoid loss following host cell death, many bacterial colonizers of the junctional epithelium invade the tissues and internalize within epithelial cells where they can spread to adjacent cells [7-9]. Polymicrobial communities can also develop intracellularly [10,11], a location that will protect them from the action of many immune effector molecules. On tooth surfaces, a layer, or pellicle, of molecules derived from saliva is rapidly deposited, and it is to these salivary molecules that primary colonizers such as the oral streptococci and actinomyces adhere. Indeed, successful oral colonizers often possess a multiplicity of adhesins with a variety of specificities, a configuration which both increases the range of available substrates and the affinity of binding [12,13]. Streptococcal adhesins can be fimbrial components (*e.g.*, Cell-surface hydrophobicity protein A in *S. gordonii* and Fimbria-associated protein-1 in *S. parasanguinis*) and surface structural proteins, such as the conserved Antigen I/II family or the amylase-binding proteins A and B [14-16]. This latter example highlights a recurring theme in oral bacterial ecology, namely an association between binding and nutrition. Starch degradation by amylase will produce glucose and maltodextrins in close proximity to bacterial cells, which can then be transported into the bacteria as a source of energy [17]. Among the actinomyces, fimbriae are the major adhesive structures and *Actinomyces oris* adheres to salivary acidic proline-rich proteins and statherin through type 1 fimbriae. Adherence may not be mediated by the structural subunit protein, but rather by accessory proteins at the tip of the fimbrial shaft [18,19]. Both the oral streptococci and actinomyces are well adapted to thrive on tooth surfaces and rapidly increase in number. In this developing polymicrobial community a second major theme of oral microbial ecology rapidly emerges, that of interspecies communication. *S. gordonii* and *A. oris* attach, or coadhere, to each other through the streptococcal surface protein A/B binding to a glucose, mannose and galactose-containing polysaccharide on the surface of the actinomyces [20]. An intricate nutritional communication program develops in the coadhered conglomerates of streptococci and actinomyces. *S. gordonii* scavenges arginine from the *A. oris* cell surface through the action of an extracellular protease, and the arginine-containing peptides or free arginine thus released are internalized and then sensed by arginine repressor family of transcriptional regulators. Expression of arginine biosynthesis genes is repressed and *S. gordonii* gains the capacity to grow in the absence of an exogenous arginine source [21-23].

Communities in which early colonizers such as streptococci and actinomyces predominate are generally associated with gingival health. However, a major corollary of the development of this plaque community is the provision of an attachment substratum for later colonizers with the potential to elevate community nososymbiocity. Indeed, image analysis of human dental plaque communities reveals spatially complex polymicrobial structures resembling ‘hedgehogs’, ‘corncobs’ and ‘cauliflowers’ [5]. Porphyromonads, a taxon which includes the keystone pathogen *P. gingivalis,* can be identified in these structures, for example, in the ‘hedgehog’ structures porphyromonads are closely associated with streptococci and surround a central core organism which is often *Corynebacterium matruchotii* [5]. Interestingly, direct observation of ex vivo plaque samples does not support the widely

accepted notion that the presence of *Fusobacterium nucleatum* is required to function as a bridge between early and late colonizers, a concept that arose from in vitro coaggregation studies [24].

The association between *P. gingivalis* and streptococci such as *S. gordonii* has been extensively studied in vitro and in vivo, and what has emerged is a picture of a complex multidimensional interaction. Initial detection of the streptococcal metabolite 4-amino benzoate by *P. gingivalis* increases activity of a tyrosine kinase, designated *P. gingivalis* tyrosine kinase-1, and the ensuing protein phosphorylation-dependent signaling converges on the fimbrial adhesins designated major fimbrial protein A and minor fimbrial antigen-1 [25]. While *P. gingivalis* thus becomes primed for attachment, pathogenic potential at this stage is diminished, and 4-amino benzoate treated *P. gingivalis* cells are less pathogenic in a murine models of alveolar bone loss [25]. As the two species physically associate, engagement of the minor fimbrial antigen-1 adhesin with the streptococcal surface protein A/B initiates community development and also activates the *P. gingivalis* low-molecular-weight tyrosine phosphatase-1 [26]. *P. gingivalis* tyrosine kinase-1 is dephosphorylated by the low-molecular-weight tyrosine phosphatase-1 which reverses information flow through the phosphoprotein-dependent signaling pathways and suppresses adhesin production [27-29] (Figure 3A). Ultimately, community development is constrained; however, communities of *P. gingivalis* and *S. gordonii* cells are more pathogenic in vivo compared to either species alone[30]. The pathophysiology of *P. gingivalis* also depends on the uptake of heme which is an essential source of iron, and *S. gordonii* can contribute haem acquisition[31]. Hydrogen peroxide produced by *S. gordonii* oxidizes oxyhaemoglobin to methaemoglobin, which is followed by haem release and extraction through the actions of the lysine-specific gingipain protease and the haemophore-like protein known as hemin utilization Y [32]

As a well-adapted human oral colonizer, *P. gingivalis* participates in synergistic interactions with a number of other organisms present in oral microbial communities. *P. gingivalis* produces isobutyric acid which stimulates growth of the oral spirochete *Treponema denticola*, and reciprocally *T. denticola* produces succinic acid which enhances growth of *P. gingivalis* [33]. Contact with *T. denticola* also upregulates the expression of *P. gingivalis* adhesins and proteases [34], and the organisms are synergistically pathogenic in murine models of periodontal disease [33,35]. Cross-kingdom interactions with the pleiomorphic yeast *Candida albicans* have also been documented. The *P. gingivalis* internalin family surface protein J binds to the candidal hyphal protein known as *C. albicans* invasin-like protein, resulting in the upregulation of genes encoding components of the *P. gingivalis* Type IX section system [36]. As the gingipain proteases and other potential virulence factors are secreted through the Type IX section system, communities of *P. gingivalis* and *C. albicans* may have increased nososymbiocity. In support of this, studies of subjects with chronic periodontitis have shown that the *C. albicans* carrier status increases dramatically, together with higher isolation frequencies of *P. gingivalis* [37,38]. *C. albicans* also interact with the oral streptococci, and under host-permissive conditions these organisms can form hypervirulent mucosal biofilms [39].

Collectively, the study of the spatial orientation and molecular interactions in oral microbial communities shows that organisms co-ordinate their behavior to optimize physical

position and metabolic potential. Microbes can thus become functionally specialized within communities and their concerted activities resemble a quasi-organismal state. Moreover, the degree of nososymbiocity is to a large extent an unintended consequence of synchronization and synergy of constituent organism physiological activities.

Indigenous microbiota and homeostatic immunity

Although the mucosal immune system is faced with a bewildering diversity and load of bacteria, humans– and mammals in general– are normally healthy and free of clinically significant inflammation. In great part, this is due to homeostatic immunity which restricts microbial expansion and colonization to superficial layers of tissue while preventing unwarranted responses to innocuous antigens [40] (Figure 4). However, the indigenous microbiota also contributes to the maintenance of health and provides protection against exogenous pathogens, in part through colonization resistance. The importance of colonization resistance becomes evident from cases where the indigenous intestinal microbiota is disrupted or suppressed (*e.g.*, by broad-spectrum antibiotics) thereby allowing colonization by exogenous pathogens and outgrowth of indigenous pathobionts with potential for systemic dissemination and induction of septic shock [41]. The mechanistic basis for colonization resistance involves both bacteria-bacteria and bacteria-host interactions. Interbacterial interactions include the inhibitory action of toxic metabolites, bacteriocins, antibiotics, and type VI secretion systems, as well as competition for space and nutrients [41,42]. The indigenous microbiota also regulates certain basic developmental features and functions of the immune system in ways that prime it for vigorous defense against overt pathogens while maintaining tolerance to innocuous antigens, such as food proteins [41,43].

The use of germ-free mice and specific-pathogen-free (“conventional”) mice with deletions in genes controlling different bacterial recognition systems, has led to an appreciation that microbial colonization is required for the development of a fully competent immune system at the tissue, cellular, and molecular level [43-45]. For instance, in the absence of the gut microbiota, mice have incomplete development of the gut-associated lymphoid tissues with relatively fewer and smaller Peyer's patches and mesenteric lymph nodes. The commensal microbiota can regulate dendritic and other innate immune cells in a way that promotes the differentiation of effector B and T cells. Particularly with regard to T cell development, the indigenous commensal microbiota regulates the induction of T helper 17 cells, a lineage of $CD4^+$ T helper cells that produce interleukin (IL)-17 and IL-22, the combined action of which induces neutrophil recruitment, enhances the production of mucus and antimicrobial proteins (*e.g.*, Regenerating islet-derived proteins IIIβ and IIIγ), resists pathogen invasion, and promotes epithelial regeneration and tissue repair [46-49]. In the small intestine of mice, colonization with a single organism, a segmented filamentous bacterium, is sufficient to induce T helper 17 cells [50]. The resident microbiota also contributes to the development of $CD4^+$ $Foxp3^+$ regulatory T cells, which produce IL-10 and help control host inflammatory responses, thereby promoting tissue homeostasis [51]. In this regard, metabolites (such as the short-chain fatty acids butyrate and propionate) produced by gut commensals during starch and fiber fermentation were shown to promote the differentiation of colonic regulatory T cells [52,53]. Commensals can stimulate epithelial cells to produce antimicrobial peptides and to reinforce tight junctions [43-45]. Moreover,

exposure to certain commensal organisms or their products induces metabolic and epigenetic changes in myeloid progenitor cells that give rise to monocytes/macrophages in a 'trained' state that enables enhanced immune responses to subsequent encounters with pathogens [54-56]. Overall therefore, symbiotic commensals can contribute to host-microbe homeostasis through niche protection (colonization resistance) and immune education in a manner that promotes homeostatic immunity.

On subgingival tooth surfaces, colonizing bacteria first assemble into physiologically compatible communities, and the organisms within these biofilms communicate through sophisticated signaling mechanisms [2,57]. Overgrowth and overt pathogenicity are controlled by the host immune and inflammatory responses and, in fact, a controlled immuno-inflammatory state is not only normal in healthy gingiva but is required to maintain health. A well-regulated host immune response to the subgingival biofilm can thus maintain a balanced host-microbe interplay and contribute to homeostasis that characterizes the healthy periodontium [58]. Genetic deletion or inhibition of either immune or regulatory cells (*e.g.*, γδ T cells or regulatory T cells, respectively) or genetic deficiency of key immune defense or regulatory proteins (*e.g.*, lysosomal-associated membrane protein-2 or developmental endothelial locus-1, respectively) greatly increase periodontal disease susceptibility in mouse models [59-63]. Under steady state conditions, the periodontium is constantly patrolled by neutrophils, a network of antigen-presenting cells (dendritic cells and macrophages) and a predominantly T cell-rich infiltrate of lymphocytes [58,64]. Thus, even in clinically healthy gingiva, a low-level of inflammation with controlled recruitment of neutrophils is constantly maintained to constrain bacterial outgrowth or other types of insults [65,66]. In contrast to T cells, a clinically healthy periodontium contains minimal numbers of B and plasma cells. Populations of γδ T cells and of innate lymphoid cells can also be seen in healthy gingiva and are thought to contribute to the maintenance of tissue homeostasis [58,59,67,68].

Studies in germ-free mice have shown that the recruitment of neutrophils to the periodontium does not require commensal bacterial colonization, which however further promotes this function. Specifically, the commensal microbiota selectively upregulates the expression of the neutrophil-specific chemokine CXC motif ligand-2 (but not the highly homologous CXC motif ligand-1) leading to increased neutrophil recruitment to the periodontium as compared to the germ-free state [64]. Interestingly, at least in mice, CXC motif ligand-1 is predominantly derived from activated endothelial cells and pericytes, whereas CXC motif ligand-2 is produced mainly by neutrophils [69]. These two homologs, perform distinct functions; whereas CXC motif ligand-1 promotes neutrophil crawling on the endothelium and subendothelium, CXC motif ligand-2 mediates neutrophil transmigration through endothelial junctions [69]. These findings are in line with the recently proposed location-dependent homeostatic principle, according to which compartmentalized expression of the same or related molecules enables optimal performance with cell type-specific actions and spatiotemporal regulation of the immune response [70]. Microbe-induced CXC motif ligand-2 production requires the presence of Myeloid differentiation primary response gene 88 (MyD88) [64], a signaling adaptor of most Toll-like receptors (TLR), *e.g.*, TLR2, TLR4, TLR5, and TLR9 [71]. The oral commensal microbiota is required also for MyD88-dependent induction of epithelial cell production of growth arrest-specific gene 6 [72]. Growth arrest-specific gene 6 is a ligand of a group of receptor tyrosine kinases (Tyro3,

Axl, and Mer) collectively known as the TAM (for Tyro3, Axl and Mer) receptor family which function as homeostatic regulators in adult tissues [73] including the periodontium [72]. Consistent with this, Growth arrest-specific gene 6-deficient mice develop microbial dysbiosis and inflammation in the gingival tissue [72].

In addition to bacteria, other challenges that may contribute to the training of oral mucosal immunity include ongoing damage from mastication and perhaps dietary and airborne allergens/particles [58]. A study investigating the mechanisms of T helper 17 induction in the periodontium under steady-state conditions has surprisingly found that the induction of gingival T helper 17 cells was not dependent on commensal bacterial colonization; indeed, the steady-state T helper 17 cell population was indistinguishable between conventional and germ-free mice [74]. Interestingly, the development of gingival T helper 17 cells was dependent upon signals from epithelial cell-derived IL-6, which was induced by mechanical damage as occurs physiologically through mastication and abrasion [74]. In contrast to homeostatic T helper 17 that physiologically arise in the oral mucosa independently of microbial triggers [74], in the setting of periodontitis, pathologic T helper 17 amplification is triggered by the local dysbiotic microbiota, thus suggesting divergent T helper 17 regulation in health vs. disease [75]. In summary, physiological mechanical damage, and not commensal colonization, sets the immune tone of the gingiva under steady-state/homeostatic conditions. This is in contrast to the mechanisms operating in the gut where the microbiota is responsible for steady-state control of CD4+ T cell effector function, as discussed above [43,50].

Despite host immunity and the potential benefits of the indigenous microbiota, a variety of factors can initiate the transition from a eubiotic to a dysbiotic state (Figure 2). For instance, alterations affecting the immuno-inflammatory status of the host could modulate the composition or the metatranscriptional landscape of a polymicrobial community, or the ability of certain bacteria to translocate to normally sterile sites [40-42]. As a community develops, changes to the microenvironment, such as availability of certain type of nutrients, oxygen or pH can provide physiological support for the outgrowth or over-representation of dysbiotic organisms [76-78]. If the conditions are such that dysbiotic communities can reach a metastable state, the potential for disease development could persist for an extended time. Below we review a triad of protagonists in a dysbiotic community (accessory pathogens, keystone pathogens, and pathobionts) and discuss their roles in inter-bacterial and host-bacterial interactions.

Accessory pathogens: unwitting participants?

In diseases with a single-infective etiology, microbial virulence may be predicted by the capacity of the organism to express virulence factors, such as cytotoxins, capsular polysaccharide, or invasins. In contrast, in diseases associated with dysbiotic microbial communities, ‘virulence’ may refer to any microbial trait that can elevate the collective pathogenicity or nososymbiocity of the community. In other words, whether a constituent organism behaves as a ‘pathogen’ or ‘commensal’ may not necessarily be predicted from its ability, or lack thereof, to express classical ‘virulence factors’. Indeed, a microbial trait or function that on its own may not contribute to disease, may do so in an interactive

polymicrobial community of functionally specialized organisms. This notion is an integral part of the PSD concept that has challenged the commensal-pathogen duality in favor of a more subtle, context-dependent view of microbial pathogenic potential [1,2,57].

The term 'accessory pathogen' defines a subset of microbes that, while generally perceived as symbiotic commensals, under certain conditions can act synergistically to promote the virulence of disease-associated organisms (Figure 1). Although traditionally seen as a prototypical oral commensal, *Streptococcus gordonii* is now considered an accessory pathogen, as it facilitates colonization and virulence of the keystone periodontal pathogen *P. gingivalis* (discussed above) [79-81]. *S. gordonii* also enhances the pathogenicity of another periodontitis-associated organism, *Aggregatibacter actinomycetemcomitans*, through similarly multilayered, spatially-constrained communication mechanisms (Figure 3B). Streptococcal-derived hydrogen peroxide is an environmental cue to which *A. actinomycetemcomitans* responds by activation of the the positive regulator of hydrogen peroxide-inducible genes, known as Oxygen Resistance [82]. The Oxygen Resistance transcriptional regulator controls expression of the gene encoding *Actinobacillus putative invasin A,* and higher levels of this surface protein increase resistance to complement, intracellular invasion and proinflammatory cytokine production [82]. The same transcriptional regulator also controls transcription of the gene encoding catalase A, and increased production of catalase A enhances degradation of hydrogen peroxide produced by both streptococci and neutrophils, thus protecting *A. actinomycetemcomitans* from oxidative damage [83]. Hydrogen peroxide also increases the bioavailability of oxygen, allowing *A. actinomycetemcomitans* to shift from a primarily fermentative to a respiratory metabolism, an interaction termed cross-respiration, which enhances the growth and fitness of *A. actinomycetemcomitans* in vivo [84]. A second level of communication involves transport of streptococcal lactate into *A. actinomycetemcomitans* through the proton-driven lactate permease, and conversion to pyruvate by lactate dehydrogenase [85]. Pyruvate suppresses autophosphorylation of E1 which then decreases uptake of phosphotransferase system carbohydrates such as glucose [86]. This is termed carbon resource portioning, and preferential utilization of lactate, even in the presence of organisms that can metabolize glucose more efficiently, provides a competitive advantage to *A. actinomycetemcomitans*. Communities of *A. actinomycetemcomitans* and *S. gordonii* can become restricted for iron, and this induces expression of Dispersin B which is under the transcriptional control of the Fur transcriptional regulator [87]. Dispersin B is a beta-hexosaminidase which cleaves β (1,6)-linked N-acetylglucosamine polymers in the biofilm matrix, and facilitates release of *A. actinomycetemcomitans* from biofilm communities [88]. The counteracting influences of oxidative stress and metabolic synergy, together with opposing constraints of retention/dispersal elicit a finally balanced spatial configuration whereby *A. actinomycetemcomitans* maintains an optimal distance from streptococci in dual-species communities in vivo [83].

Accessory pathogenic mechanisms operate also in other mucosal sites of the body. For instance, although *Gardnerella vaginalis* is not uropathogenic by itself, it can trigger the emergence of *Escherichia coli* from intracellular reservoirs into the lumen of the bladder, where it can translocate to the kidney and cause inflammation and systemic infection [89]. In a Drosophila model of cholera the action of an otherwise commensal organism, *Acetobacter pasteurianus*, is required for *V. cholerae* pathogenesis [90]. Moreover, in the

intestine, *Bacteroides thetaiotaomicron* produces fucosidases that generate fucose (from host-derived glycans), which activates a fucose sensor of enterohemorrhagic *E. coli*; this sensor comprises a two-component signal transduction system, designated FusKR, where FusK is the histidine sensor kinase and FusR is the response regulator. The activation of FusKR leads to the expression of genes that enhance the metabolism and pathogenicity of enterohemorrhagic *E. coli* [91]. Consistent with the contextual nature of microbial influence on other organisms and on the host, *P. gingivalis* can also function as an accessory pathogen in the setting of respiratory infection. Indeed, *P. gingivalis*, which may be aspirated into the lungs [92], promotes the capacity of *Pseudomonas aeruginosa* to invade respiratory epithelial cells and modulates its apoptosis-inducing activity, thereby potentially enhancing its ability to establish infection [93,94].

Given their widespread distribution and abundance, oral streptococci (and other accessory pathogens such as the gastrointestinal *Bacteroides*) are unlikely that they have specifically evolved as accessory pathogens. Their role is more likely ‘unwitting’, rather than ‘willing’, participants. In this regard, whereas *S. gordonii*-derived 4-amino benzoate acts as signal for *P. gingivalis* to upregulate fimbrial expression and enhance its colonization, it also suppresses extracellular polysaccharide production by *P. gingivalis* and decrease its virulence in the mouse abscess model [95]. It would be expected, however, that as the biomass of *P. gingivalis* aggregates increases, the accessibility of streptococcal 4-amino benzoate will decrease, thereby resulting in increased *P. gingivalis* pathogenicity.

Keystone pathogens: community service at host’s expense

The term ‘keystone’ was introduced in the ecological literature to describe species whose influence on their communities is disproportionately large relative to their abundance, thus serving as the ‘keystone’ of their community’s structure [96]. Accordingly, in the context of microbial pathogenesis, the keystone-pathogen hypothesis holds that certain low-abundance microbes can have a major role in the community structure by promoting the emergence of dysbiotic communities that can precipitate disease [65,97,98] (Figure 1). In remodeling a eubiotic microbiota into a dysbiotic one, keystone pathogens can mediate both quantitative (increased counts) and qualitative (emergence of newly dominant species) alterations the microbiota. The qualitative alterations involve changes in microbial composition as well as the metatranscriptome and the metaproteome of the community. Keystone pathogen-induced changes to the microbiota may involve both indirect and direct effects; the former through subversion of the host immune response and the latter via interspecies interactions with other member species [65,98-101]. Since the proposal that *P. gingivalis* may be a keystone species of the dysbiotic periodontal microbiota [65,97,98,102], a great number of keystone species have been identified in the human, plant, and soil microbiomes [103].

Manipulation of complement and Toll-like receptor function.

In the oral gavage model of periodontitis [104], *P. gingivalis* fails to cause alveolar bone loss in germ-free mice even though it can colonize this host [98]. In conventional (specific-pathogen free) mice, however, this oral bacterium remodels the periodontal commensal microbiota into a dysbiotic community that causes bone loss, as long as the mice have

intact complement and Toll-like receptor (TLR) signaling pathways [98,99]. This is because the keystone-pathogen status of *P. gingivalis* depends heavily on its ability to induce subversive crosstalk between complement C5a receptor-1 (C5aR1) and TLR2 on leukocytes, thereby leading to selective inhibition of antimicrobial responses and promotion of destructive inflammation [98,99,105-107] (Figure 5). In fact, not only does the impairment of host immunity allow uncontrolled bacterial growth, but also the resulting inflammatory environment favors the development of a subset of inflammophilic species that can thrive on nutritional substrates derived from tissue breakdown (*e.g.*, degraded collagen as a source of amino-acids) [76,77,108]. *P. gingivalis* therefore can disrupt host-microbe homeostasis and lead to the emergence of a dysbiotic/inflammophilic microbiota.

The above-discussed influence of *P. gingivalis* on the microbial community is greater than should be expected from its low abundance which was < 0.01% of the total microbiota in the mouse periodontitis model [98]. *P. gingivalis* is a quantitatively minor constituent also in human periodontitis-associated biofilms. Indeed, in contrast to findings from early culture-based microbiological studies, most recent studies using culture-independent molecular methods show that *P. gingivalis* is a quantitatively minor constituent of human periodontitis-associated biofilms [109-112]. However, *P. gingivalis* has also been detected at relatively high abundance in some sites [113]. It is possible that dysbiosis can be initiated at low *P. gingivalis* colonization levels, and subsequently the relative abundance of *P. gingivalis* might increase due to elevated inflammation.

Subversion of neutrophils.

C5aR1 and TLR2 lie at the core of the ability of *P. gingivalis* to subvert innate immunity and this subversion mechanism affects different phagocytic cell types. The relevant mechanisms operating in neutrophils are briefly described here. TLR2 is critical for *P. gingivalis* recognition by neutrophils *in vivo* [114], whereas C5aR1 is under the control of *P. gingivalis* gingipains that can locally generate C5a ligand independently of complement activation [107,115]. In both human and mouse neutrophils, the *P. gingivalis*-induced C5aR1-TLR2 crosstalk triggers ubiquitination and proteasomal degradation of a major TLR2 signaling adaptor, MyD88 (Figure 5), thereby suppressing its antimicrobial effects that can eradicate *P. gingivalis*, presumably through downstream activation of Intereleukin-1 receptor associated kinase 4-dependent neutrophil granule exocytosis [99,116,117]. However, *P. gingivalis*-induced degradation of MyD88 also inhibits downstream proinflammatory signaling, and, in fact, a similar but host-controlled mechanism of ubiquitin-mediated MyD88 degradation regulates TLR-induced inflammation in the periodontal tissue [118].

Given the inhibition of the MyD88 pathway, the question arose as to how *P. gingivalis* rescues the nutritionally favorable (for the bacteria) inflammatory response. The answer came quite unexpectedly. Pharmacological blockade of C5aR1 or TLR2 in MyD88-deficient mice promoted bacterial killing by neutrophils, suggesting that *P. gingivalis* evades killing via an additional, MyD88-independent mechanism involving the two receptors. The dissection of this alternative pathway revealed that another TLR2 adaptor, Mal, induces phosphoinositide 3-kinase signalling, which in turn inhibits the GTPase Ras homolog family member A-dependent actin polymerization and hence suppresses *P. gingivalis*

phagocytosis [99]. Intriguingly, the same phosphoinositide 3-kinase pathway was shown to stimulate a robust inflammatory response, since genetic or pharmacological ablation of Mal or phosphoinositide 3-kinase inhibits the production of pro-inflammatory cytokines by neutrophils *in vitro* or *in vivo* [99]. Thus, *P. gingivalis* substitutes TLR2-phosphoinositide 3-kinase in place of TLR2-MyD88 signaling to preserve the inflammatory response and at the same time uncouples inflammation from the bactericidal activities of neutrophils (Figure 5).

The subversion of neutrophil function by *P. gingivalis* promotes the survival of bystander bacteria species, such as *F. nucleatum,* which are otherwise susceptible to neutrophil killing [99]. Conversely, inhibition of phosphoinositide 3-kinase or any of the two upstream receptors, C5aR1 or TLR2, reverses the capacity of *P. gingivalis* to protect itself or *F. nucleatum* against neutrophil killing. Importantly, local inhibition of phosphoinositide 3-kinase, C5aR, or TLR2 in the periodontium of *P. gingivalis*-colonized mice leads to elimination of *P. gingivalis*, reverses the increase in total microbiota counts induced earlier by *P. gingivalis* colonization, and blocks periodontal inflammation and bone loss [98,99]. In the same study, local inhibition of TLR4 has no effect, consistent with earlier findings that TLR4-deficient neutrophils are phenotypically similar to wild-type neutrophils as both display normal inflammatory responses to *P. gingivalis* while failing to kill the organism [114]. This intriguing observation is likely attributed to the capacity of *P. gingivalis* to express atypical lipid A structures that prevent TLR4 activation [119]. In summary, *P. gingivalis* manipulates neutrophils through distinct mechanisms that together ensure the survival of the microbial community and the perpetuation of inflammation.

Subversion of macrophages.

Of course, besides neutrophils, other cell types in the periodontal environment express the implicated molecules (C5aR1, TLR2, phosphoinositide 3-kinase). Thus, the protective effects seen after local pharmacological inhibition of these molecules could additionally involve counteraction of *P. gingivalis* effects on additional cell types. For instance, *P. gingivalis* was shown to induce and exploit phosphoinositide 3-kinase signaling also in gingival epithelial cells, where this bacterium inhibits apoptosis in a phosphoinositide 3-kinase-dependent manner in order to promote its intracellular persistence [120]. Moreover, *P. gingivalis* instigates a subversive C5aR1-TLR2 signaling also in macrophages although the underlying mechanism is different. Here, *P. gingivalis*-activated C5aR1 initiates intracellular Ca^{2+} signaling that synergistically augments the otherwise weak cAMP responses caused by TLR2 activation alone [106]. The ensuing activation of cAMP-dependent protein kinase A inhibits NF-κB and glycogen synthase kinase-3β, in turn suppressing inducible nitric oxide synthase-dependent killing of *P. gingivalis*. Nevertheless, the induction of pro-inflammatory cytokines (such as interleukin (IL)-1β, IL-6 and tumor necrosis factor) by macrophages is upregulated by the *P. gingivalis*-induced C5aR1-TLR2 crosstalk and is also independent of MyD88 [105,107].

The ability of *P. gingivalis* to induce TLR2-mediated inflammation by circumventing MyD88 in both neutrophils and macrophages is unconventional given that typical TLR2 agonists, such as the lipopeptide Pam3CSK4, activate TLR2 in a strictly MyD88-dependent

way [99,105]. In line with the redundant role of MyD88 in *P. gingivalis*-induced inflammation, this organism causes alveolar bone loss in a MyD88-independent manner, whereas the presence of intact TLR2 signaling is required [105]. Equally unconventional is the ability of *P. gingivalis* to exploit TLR2-phosphoinositide 3-kinase signaling to prevent phagocytosis, as it stands in stark contrast to findings with other organisms whose phagocytosis is promoted by TLR2 and/or phosphoinositide 3-kinase signaling [121-123]. Intriguingly, moreover, within those cells that do phagocytose *P. gingivalis* bacteria, TLR2-phosphoinositide 3-kinase signaling suppresses phago-lysosomal maturation, thereby revealing another mechanistic layer whereby this keystone pathogen can evade killing [105] (Figure 5).

Interactions with dendritic cells.

Oddly, whereas *P. gingivalis* readily exploits C5aR1 to evade neutrophils and macrophages, it fails to do so in dendritic cells, which utilize C5aR1 to kill *P. gingivalis* [124]. These differential effects of C5aR1 in dendritic cells and macrophages might be attributed to differential regulation of the cAMP response in these two cell types. In macrophages, activation of C5aR1 leads to increased levels of intracellular cAMP and hence protein kinase A activation, which is critical for suppressing the nitric oxide-dependent killing of *P. gingivalis* [106]. In contrast, in dendritic cells, C5aR1 activation suppresses cAMP production and thus the activation of protein kinase A [125]. The findings that C5aR1 promotes the intracellular killing of *P. gingivalis* in dendritic cells while enhancing the intracellular survival of this organism in neutrophils and macrophages may not be as surprising if one considers that the major threat to *P. gingivalis* in its predominant niche (periodontal pocket) is primarily represented by neutrophils and (secondarily) macrophages, rather than dendritic cells.

Alternatively, *P. gingivalis* may manipulate other innate immune mechanisms to subvert dendritic cells. In this regard, *P. gingivalis* uses its minor fimbrial antigen-1 to interact with a C-type lectin, the dendritic cell-specific ICAM-3 grabbing nonintegrin, and thereby enter dendritic cells in a manner that promotes its survival while suppressing the maturation of the dendritic cells [126,127]. Although dendritic cell-specific ICAM-3 grabbing nonintegrin directs *P. gingivalis* into intracellular vesicles that escape early autophagosomal recognition and degradation, TLR2 antagonizes autophagy evasion and thus inhibits the intracellular persistence of this oral bacterium [128]. Consistent with this finding, *P. gingivalis* displays increased viable counts in TLR2-deficient dendritic cells as compared to wild-type controls, thus further supporting that TLR2 contributes to the intracellular killing of this pathogen [124]. Taken together, these studies suggest that dendritic cell-specific ICAM-3 grabbing nonintegrin promotes the intracellular survival of *P. gingivalis* in dendritic cells, whereas C5aR1 and TLR2 mediate the opposite effect, i.e., promote the clearance of *P. gingivalis.*

Subversion of epithelial and endothelial cells.

P. gingivalis may use additional mechanisms to protect itself and bystander bacteria in the community. In fact, prior to the eventual infiltration of neutrophils in diseased periodontal pockets heavily colonized by subgingival communities, *i.e.*, at an early stage in the colonization process, *P. gingivalis* may be able to inhibit neutrophil recruitment. Indeed, this bacterium was shown to suppress the capacity of gingival epithelial cells to induce IL-8

for chemoattraction of neutrophils [129]. This subversion effect is known as “local chemokine paralysis” and depends on the capacity of *P. gingivalis* to invade the epithelial cells [129] and secrete the serine phosphatase B [130]. *P. gingivalis*-invaded epithelial cells are restrained from eliciting normal IL-8 responses even in the presence of bacteria like *F. nucleatum* that are otherwise potent inducers of IL-8 production [129,131]. Moreover, *P. gingivalis* acts on endothelial cells and prevents the upregulation of E-selectin by other periodontal bacteria [132]. The combined inhibition of E-selectin and IL-8 expression could suppress the extravasation and directed migration of neutrophils to the gingival crevice. Although the subversive effects of *P. gingivalis* on E-selectin and IL-8 expression are transient in vivo [98], at least in principle, they could allow adequate time for *P. gingivalis* and bystander bacteria to establish colonization while delaying the influx of neutrophils. This might be a critical step for periodontal disease pathogenesis. In this regard, a serine phosphatase B-deficient isogenic mutant of *P. gingivalis* (thus unable to cause local chemokine paralysis) induces higher levels of neutrophil recruitment to the periodontium and causes reduced bone loss in rats compared to wild-type *P. gingivalis* [133].

P. gingivalis also suppresses the expression of T-helper-1 cell-biasing chemokines (CXC motif ligands −9, −10, and −11), even in the presence *F. nucleatum* which by itself is a potent inducer of these chemokines [134]. The inhibitory mechanism of T helper 1-associated chemokine expression by *P. gingivalis* is mediated through suppression of the Signal Transducer And Activator Of Transcription 1-Interferon regulatory factor 1 pathway in epithelial cells but also in myeloid cells (neutrophils and monocytes) [134]. The resulting disruption of T helper 1-biasing chemokines may disrupt the balance of protective and destructive cytokines in the periodontium, given that interferon-γ can promote anti-microbial immunity [135] as well as can mitigate osteoclast activation [136,137].

Although many putative mechanisms for *P. gingivalis* immune subversion have been proposed, most have not been tested *in vivo* in the context of experimental periodontitis. For instance, the *in vitro* capacity of *P. gingivalis* to degrade or inactivate antimicrobial peptides or the lytic action of complement could in principle confer *in vivo* protection to bystander bacteria that are otherwise susceptible to these host defense mechanisms [115,138,139].

Interbacterial interactions.

Besides exerting community-wide influence via host modulation, *P. gingivalis* may additionally modulate the commensal microbiota through host-independent, direct effects on bacteria. Indeed, it has been demonstrated that the introduction of *P. gingivalis* into a health-compatible multispecies biofilm changes the pattern of microbial community gene expression [100] and can even induce DNA fragmentation and cell death to certain commensals [101]. The ability of *P. gingivalis* to alter the composition of polymicrobial biofilms *in vitro* depends in part on the expression of the gingipain proteases [140].

P. gingivalis role in human periodontitis.

It is currently uncertain whether *P. gingivalis* can act as a keystone pathogen also in human periodontitis. However, a recent investigation involving metagenomics sequencing and phylogenetic profiling of the microbial communities associated with human periodontitis

lent support, albeit indirect, to the keystone pathogen-induced polymicrobial synergy and dysbiosis model [141]. Direct evidence can be obtained through an interventional study in human periodontitis patients. If an interventional treatment that selectively targets *P. gingivalis* (*e.g.*, a specific vaccine or antimicrobial) leads to significant reduction in *P. gingivalis* counts and at the same time suppresses the entire dysbiotic biofilm and disease development, then *P. gingivalis* can be considered a human keystone pathogen (at least in a subset of patients, as *P. gingivalis* is not an obligatory mechanism of periodontitis; see below). In support of this hypothesis, non-human primates (which naturally harbor *P. gingivalis*) administered a gingipain-based vaccine displayed a decrease in both the counts of *P. gingivalis* and the total subgingival bacterial load [142], suggesting that the presence of *P. gingivalis* benefits the entire disease-associated microbial community.

P. gingivalis may also be detected, although less frequently, in the healthy human periodontium [143-147]. This begs the question of whether there are certain triggers that can enable *P. gingivalis* to initiate disease. Alternatively, or additionally, these findings could be explained by strain and virulence diversity within the population structure of *P. gingivalis* (i.e., different strains are associated with health vs. disease). Regarding the former possibility, major *P. gingivalis* virulence factors, including the gingipains which are required for complement subversion, are regulated by local environmental conditions that likely differ among different individuals [102]. Other environmental factors that can modulate the pathogenicity of *P. gingivalis* are antagonistic bacteria within the microbial community. For instance, *Streptococcus cristatus* inhibits the expression of fimbrial proteins and gingipains in *P. gingivalis* through the signaling action of arginine deiminase [148,149]. This inhibitory action requires a direct contact between *S. cristatus* arginine deiminase and specific receptors on the surface of *P. gingivalis* [149] (Figure 3C). In line with this antagonistic mechanism, the distribution of *P. gingivalis* and *S. cristatus* is negatively correlated in the human subgingival dental plaque biofilm [150]. Furthermore, *S. cristatus* suppresses *P. gingivalis*-induced alveolar bone loss in mice [150].

Host genetic factors may also dictate the virulence potential of *P. gingivalis*. Thus, it is possible that there might be individuals who can resist the capacity of *P. gingivalis* to convert a eubiotic microbiota into a dysbiotic one. Such individuals might have alterations in signaling pathways required for immune subversion by *P. gingivalis* and induction of dysbiosis. Interestingly, rare immune deficiencies leading to dysregulated and destructive periodontal inflammation are associated with a compositionally unique dysbiotic microbiome that lacks *P. gingivalis* [151,152]. Therefore, since the presence of *P. gingivalis* is not necessarily associated with disease, it seems more accurate to consider this organism as an important risk factor rather than a causal agent in periodontitis.

Keystone pathogens in other settings.

A study investigating the intestinal innate immune response of gnotobiotic zebrafish showed that combinations of distinct bacterial species elicit host responses that do not reflect the numerically dominant species [153]. Instead, it was found that different species exert different ‘per capita’ immunostimulatory effects. The finding that individual members of a community disproportionately regulate the host innate immune response is consistent with

the keystone-pathogen concept and, moreover, suggests that knowledge of specific keystone-like properties of individual species might provide predictive insights into the function of polymicrobial communities [153]. On the other hand, the evidence that minor members in a community can exert dominant effects challenges the notion inherent in compositional analyses that the relative abundances of different taxa or species can have predictive value for disease pathology. In this regard, a study has used a time-series algorithm called Learning Interactions from MIcrobial Time Series to deduce ecological interaction networks in the gut microbiome and identified keystone species with disproportionate influence on the gut microbiome structure. The identified species were under-represented according to metagenomics abundance data [154]. Perturbations applied to these species have been computationally shown to exert a large impact on microbial community structure. Interestingly, one of two such species identified by the Learning Interactions from MIcrobial Time Series algorithm, *Bacteroides fragilis*, is experimentally implicated as a keystone pathogen ("alpha-bug") in colon cancer [155-157]. The other putative keystone species identified is *Bacteroides stercosis* [154], which has also been associated with increased risk of colon cancer [158]. Overall, a great number of microbial keystone species have been recently identified, primarily through computational approaches, in different ecosystems [103]. Regardless of the specific ecosystem, keystone species appear to act as drivers of microbiome structure and function irrespective of their relative abundance [103].

Homeostatic commensals: stabilization of health-compatible communities

In contrast to keystone pathogens, certain commensals that can stabilize a microbiota against perturbations that increase its nososymbiocity were referred to as 'keystone stabilizers' [159]. As such bacteria are not always in low abundance, perhaps a more encompassing term is 'homeostatic commensals' [2]. In this regard, *Bacteroides thetaiotaomicron* induces the antimicrobial peptide angiogenin, which kills opportunistic or pathogenic organisms but not *B. thetaiotaomicron* itself or other indigenous bacteria [160]. Moreover, *B. thetaiotaomicron* inhibits nuclear factor-κB-mediated inflammation in a manner dependent upon the nuclear metabolic receptor peroxisome proliferator-activated receptor γ [161]. Whereas the organism does not block nuclear factor-κB activation in the cytoplasm, it somehow triggers the formation of complex between peroxisome proliferator-activated receptor γ and the p65 subunit of the nuclear factor-κB, which is subsequently exported from the nucleus, thus preventing induction of NF-κB-regulated pro-inflammatory genes [161]. However, as mentioned earlier, *B. thetaiotaomicron* can also upregulate virulence gene expression in enterohemorrhagic *E. coli* [91], highlighting the contextual nature of classifying bacteria in functional categories of disease potential. *Faecalibacterium prausnitzii*, whose counts are massively diminished in inflammatory bowel disease but normally comprises about 5% of total bacteria in feces, was shown to oppose dysbiosis and suppress colitis in mice by secreting a metabolite with anti-inflammatory action [162,163]. In a gnotobiotic zebrafish model, low-abundance *Shewanella* (a probiotic species that is used in aquaculture) secretes an anti-inflammatory factor that overrides the pro-inflammatory effect (intestinal neutrophil influx) of high-abundance species of the community [153]. As numerically minor constituents of microbial communities can either enhance or reduce nososymbiocity, it can be argued

that functional cataloging of community members is a more relevant prognostic factor than abundance determinations.

In a manner similar to that mentioned above for *B. thetaiotaomicron*, certain strains of *F. nucleatum* use a specific lipoprotein to induce oral epithelial cell production of human beta defensins to which they are resistant but the same molecules kill *P. gingivalis* at low micromolar concentrations [164]. Whether these *in vitro* observations are relevant *in vivo* (*i.e.*, to suppress *P. gingivalis*-induced dysbiosis) is currently uncertain. Assuming that this is the case, then those strains of *F. nucleatum* may act as homeostatic commensals locally in the periodontal tissue whereas their dissemination to extra-oral sites may be associated with pathology given their resistance to human beta defensins. In this regard, *F. nucleatum* has been associated with colorectal carcinogenesis and adverse pregnancy outcomes [165-167]. Although homeostatic commensals can induce antimicrobial proteins that preferentially target periodontitis-associated bacteria [164], the reverse is also true. Indeed, *P. gingivalis* activates Notch-1 signaling in oral epithelial cells leading to production of phospholipase A_2-IIA, an antimicrobial protein that differentially affects oral bacterial species in a manner consistent with promotion of dysbiosis [168]. Moreover, in oral epithelial cells, *P. gingivalis* induces CXC motif ligand-14, a chemokine with bactericidal activity against oral streptococci but not against *P. gingivalis*, which is relatively resistant by degrading it [169,170].

In *in vitro* multi-species biofilms, H_2O_2 production by commensal species (e.g., *S. sanguinis, S. oralis, S. gordonii, S. cristatus, S. parasanguinis,* and *S. mitis*) decreases the growth of periodontitis-associated bacteria (*P. gingivalis, A. actinomycetemcomitans and P. intermedia*). However, this growth inhibition is counteracted by enzymes that use H_2O_2 as subsrate, such as myeloperoxidase and catalase, both of which are elevated in the gingival crevicular fluid during periodontitis (myeloperoxidase can be released by neutrophils and catalase from erythrocytes through the action of bacterial hemolysins) [171,172]. Peroxidases, therefore, can potentially contribute to dysbiosis (Figure 4).

As alluded to earlier, another oral homeostatic commensal is likely *S. cristatus*, which uses its arginine deiminase to antagonize the virulence of *P. gingivalis* and suppress its ability to induce periodontitis in mice [148-150]. Moreover, in epithelial cells, *S. cristatus* was shown to inhibit the expression of several epithelial cell pro-inflammatory cytokines in response to the pathobiont *F. nucleatum* while upregulating anti-inflammatory mediators[173]. Thus, it is possible that certain oral streptococci can act as homeostatic commensals and help maintain host-community equilibrium although direct in vivo confirmation is currently lacking.

Inflammophilic pathobionts: commensal-turned pathogens

Pathobionts are generally benign commensal organisms within an indigenous microbial community but their numbers can considerably increase and the organisms can become pathogenic when host-microbe homeostasis is disrupted due to certain conditions, such as, inflammation, antibiotic treatment, tissue damage, dietary shifts, and especially immune deficiencies[2,41,159,174-176] (Figure 1). These conditions can favor the outgrowth of pathobionts and thus further destabilize the microbiota, thereby contributing to or exacerbating immune-mediated or inflammatory disorders. Pathobionts therefore are

resident organisms that are conditionally pathogenic as opposed to exogenously acquired infectious agents. A typical pathobiont is *Clostridium difficile*, which can be found at low levels in the healthy human gut. However, after treatment with broad-spectrum antibiotics that disrupt the indigenous microbiota, the abundance of *C. difficile* is massively increased and correlates with severe intestinal inflammation [41]. Consistently, in a mouse model of *C. difficile* infection, antibiotic treatment promotes the ability of this organism to colonize the gut at high levels and induce inflammation [41].

In the periodontium, inflammation not only mediates tissue destruction but also appears to be a major mechanism for the emergence of pathobionts (Figure 4). As a consequence of inflammatory tissue destruction and bleeding in periodontitis, degraded collagen and heme-containing compounds (haptoglobin, hemopexin, and hemoglobin) can be released into the gingival crevicular fluid and thus be utilized by subgingival proteolytic and asaccharolytic bacteria to obtain essential amino-acids and iron [31,76,77,108]. Thus, a subset of species, termed 'inflammo-*philic*' (loving or attracted to inflammation) [76], can selectively expand at the expense of others that cannot exploit the new environmental conditions, in essence promoting a dysbiotic imbalance in the microbial community [76,108]. In this context, the addition of serum, hemoglobin, or hemin to an *in vitro* generated oral multispecies community selectively induces the outgrowth of pathobionts that upregulate the expression of genes encoding proteases, hemolysins, and molecules mediating acquisition of hemin [177]. The altered community also displays increased proinflammatory potential [177].

Consistent with the concept that potential pathobionts should not only withstand the harsh inflammatory environment of the periodontal pockets but also take advantage of it, a γ-proteobacteria species in the mouse oral cavity, designated NI1060, was shown to selectively accumulate at damaged periodontal tissue, ostensibly to procure nutrients from inflammatory tissue breakdown components [178]. Moreover, NI1060 proactively aggravates destructive periodontal inflammation by activating the cytosolic receptor Nod1. In contrast, other commensals, such as NI440 and NI968, dominate healthy sites and do not thrive under destructive inflammation, thus do not behave as pathobionts [178].

Potassium ion was recently proposed as an additional inflammation-related environmental signal that can remodel the oral microbiome from a eubiotic community to a dysbiotic one [179]. It was previously shown that the concentrations of potassium in gingival crevicular fluid samples correlate positively with mean pocket depths in periodontitis patients [180]. The study showed that an increase in the concentration of potassium ion in an *ex vivo* model altered the composition and virulence of the microbial community. These alterations modified the microbiota interactions with epithelial cells resulting in increased production of proinflammatory cytokines and reduced production of human beta defensin-3 [179].

Another factor contributing to the emergence of dysbiotic communities of pathobionts is the redox potential. During the development of subgingival biofilms, the metabolic activity of facultative anaerobes causes depletion of oxygen and generation of carbon dioxide and hydrogen. This activity gradually lowers the redox potential in the crevice, generating an environment that prohibits the growth of aerobic bacteria but promotes the growth of anaerobic species including strict anaerobes associated with periodontal dysbiosis

[77,181]. In stark contrast, oxygen limitation in the colon (colonic epithelial hypoxia; <1% O_2) promotes host-microbe homeostasis (e.g., through the generation of short-chain fatty acids produced by the anaerobic fermentation of fiber), whereas increased oxygenation drives selective expansion of facultative anaerobic Proteobacteria which are associated with dysbiosis [182]. However, the unifying theme in both periodontal and colon dysbiosis is inflammation. Indeed, inflammatory byproducts generated in the gut, such as tetrathionate and nitrate, can be used as electron acceptors by Proteobacteria which bloom at the expense of anaerobic fermenting microbes [182,183]. Overall, the altered conditions in the oral environment discussed above support a microbial community with higher proportions of anaerobic and inflammophilic bacteria that thrive at the expense of those species that cannot endure inflammation or fail to capitalize on the developing inflammatory environment.

Consistent with the above-discussed concepts, the ecological succession from periodontal health to disease has demonstrated the emergence of newly-dominant community members rather than of novel species [111]. Thus, genera or species that dominate microbial communities in periodontitis are also present albeit at severely reduced relative abundance in health, as predicted by the ecologic plaque hypothesis. This holds that ‘periodontal pathogens’ can be found in the normal microbiota but at levels too low to initiate disease; however, changes in ecologic conditions may favor the outgrowth of such organisms (now known as pathobionts) beyond a threshold level that is sufficient to induce periodontitis [181]. In keeping with the notion that inflammation is exploited by pathobionts to serve their nutritional needs, *in situ* community-wide transcriptomic analysis of subgingival biofilms from periodontitis patients revealed increased expression of proteolysis-related genes and genes for iron acquisition and lipopolysaccharide synthesis [184]. Moreover, the bacterial numbers of periodontitis-associated subgingival biofilms increase with increasing clinical inflammatory markers [111]. Conversely, but consistently, intervention studies in preclinical models have shown that different anti-inflammatory treatments not only inhibit experimental periodontitis in mice, rats, or rabbits but also reduce the periodontal bacterial load and reverse dysbiosis [63,98,185-188].

Besides fostering the growth of inflammophilic bacteria, inflammation may have direct effects on shaping the microbial virulence phenotype. In this regard, certain bacteria can sense proinflammatory cytokines and, in response, upregulate the expression of virulence genes. For example, IL-1β and tumor necrosis factor can promote, respectively, the growth and virulence potential of certain pathogens (*e.g.*, *Shigella flexneri* and virulent strains of *E. coli*) that use specific receptors to bind these cytokines [189,190]. Similarly, *P. aeruginosa* uses the outer-membrane porin F to bind interferon-γ, thereby upregulating the expression of quorum-sensing–dependent virulence determinants (pyocyanin and a lectin known as PA-I), which disrupt epithelial cell function [191]. Whether periodontal pathobionts use similar inflammation-dependent mechanisms to enhance their virulence is uncertain. In this context, combined metagenome/metatranscriptome longitudinal analysis of the subgingival dental plaque microbiome in progressing and non-progressing sites has shown that progressing sites (those showing an increase in pocket depth and clinical attachment level) contain communities that express genes associated with periodontal disease pathogenesis (related to oxidative stress response, ferrous iron transport, amino-acid transport, and lipopolysaccharide synthesis) [192]. On the other hand, the relatively stable

environment of non-progressing sites displays almost the same metagenomic composition and gene expression profile at baseline and two months later [192]. These data imply that the inflammatory environment of the pocket regulates the gene expression pattern of dysbiotic communities to perpetuate periodontal disease activity. Alternatively, however, it could be argued that if the community is sufficiently pathogenic to upregulate virulence genes, this propensity can eventually promote inflammatory tissue destruction. Consistent with the above, an independent study showed that the core microbiomes of clinically healthy sites in individuals with periodontitis are functionally more aligned (in terms of virulence gene expression) with diseased sites than healthy sites from periodontitis-free individuals [193], implying a potential for disease initiation or the presence of environmental conditions conducive for dysbiosis. Interestingly, investigation of changes in gene expression associated with the initial stages of dysbiosis showed that, among those bacteria traditionally associated with the red complex, only *P. gingivalis* was actively expressing virulence factors at baseline; in contrast, *T. denticola* and *T. forsythia* upregulated expression of virulence genes later when tissue breakdown was clinically detected [192]. These findings are consistent with a keystone pathogen role for *P. gingivalis* (thus contributing to dysbiosis initiation) and with a role as pathobionts for *T. denticola* and *T. forsythia*, which may aggravate periodontitis when tissue homeostasis breaks down.

Summary and concluding remarks

Periodontitis is not caused by individual bacteria perceived as ‘causative agents’, but rather by a multi-species community under the influence of organisms with specific functions and environmental conditions that can tip the balance from homeostasis to dysbiosis and destructive inflammation. In an inflammatory environment, the dysbiotic community continues to develop and further stimulates inflammatory responses (Figure 2). In susceptible hosts, the inflammatory response is poorly controlled and ineffective at constraining the dysbiotic microbiota. Even worse, frustrated and misdirected responses contribute to tissue damage and thus perpetuate the inflammophilic community of pathobionts [76].

From the discussion in the earlier sections, it becomes evident that polymicrobial communities exhibit a sophisticated level of structural and functional integration among its constituent species that confers to them a quasi-organismal status. The functional interdependence among constituent members of a polymicrobial community is consistent with the ‘Black Queen Hypothesis’ [194]. According to this concept, those functions that are energetically costly can be discarded as dispensable by ‘cheaters’, as long as they are not lost completely from the community, in other words retained by a subset of community members (‘helpers’) thus benefiting the entire community. This provides a theoretical foundation for the emergence of keystone pathogens (and perhaps other specialized roles) that provide an indispensable public benefit to the community. Whereas exogenous pathogens responsible for classic infectious diseases employ strategies to overcome colonization resistance, endogenous bacteria with pathogenic potential, such as keystone pathogens, exploit the inherent metabolic and/or colonization properties of their microbial neighbors (accessory pathogens) to increase the nososymbiocity of the community.

The functional categories associated with the PSD model (‘accessory pathogens’, ‘keystone pathogens’, and ‘pathobionts’) are not invariable intrinsic properties of specific bacterial species or strains but rather refer to contextual properties of constituent members in a nososymbiotic community. As discussed above, the same bacteria may act as homeostatic commensals in one context and as accessory pathogens in another. Moreover, bacteria can function as keystone or accessory pathogens in different settings.

The PSD model bypasses the ‘chicken-or-the-egg’ question whether dysbiosis initiates inflammation or vice-versa, a dilemma that, at least in the context of periodontal disease pathogenesis, may not be relevant. In other words, periodontal disease pathogenesis does not involve a linear process with a distinct first cause, dysbiosis or inflammation, as neither of the two can become sufficiently strong to drive periodontitis without interaction with the other. Thus, the PSD model places an emphasis on the continuous cyclic process where dysbiosis and inflammation are reciprocally reinforced ultimately becoming the driver of periodontal disease pathogenesis. Indeed, whereas dysbiosis promotes destructive inflammation, inflammation creates a nutritionally favorable environment for further expansion of inflammophilic pathobionts, thereby creating a feed-forward loop that can ultimately cause overt periodontitis in susceptible individuals (Figure 2). In this regard, the PSD model provides mechanistic underpinnings for the ecological plaque hypothesis [181]. The notion that certain commensals can conditionally exacerbate destructive inflammation is consistent with the emerging association with periodontitis of previously underappreciated bacteria, including the Gram-positive *Filifactor alocis* and *Peptoanaerobacter stomatis* and other species from the genera *Prevotella*, *Megasphaera*, *Selenomonas*, and *Desulfobulbus* [111,112,195]. Although most of these species are as yet uncultivated, evidence derived from more tractable organisms has revealed virulence credentials consistent with a pathobiotic status. For example, *F. alocis* was shown to resist immune and inflammatory host responses and thrive in a host-destructive inflammatory environment to which it contributes[196-199]. Pilot evidence also supports a pathobiotic status for *Desulfobulbus oralis* [200].

In a state of disrupted homeostasis, the expansion of pathobionts marks a potential tipping point in the development of nososymbiocity as host-microbe homeostasis is unlikely to be restored without intervention to control a robust, tissue-destructive inflammatory response. Approaches to inhibit inflammation and promote its resolution, restore immune function in case of immunodeficient patients, and control environmental variables that promote dysbiosis (*e.g.*, antibiotics and diet) in patients regardless of immune status, should contribute to immune homeostasis in periodontitis and other mucosal inflammatory disorders [77,201-205]. Moreover, strategies to interfere with the synergistic mechanisms that drive nososymbiocity (*e.g.*, targeting key interspecies interactions or host signaling pathways exploited by the microbes to subvert the host response) should also be useful for the treatment of periodontitis and other polymicrobial diseases [80,99,108,149,206-210].

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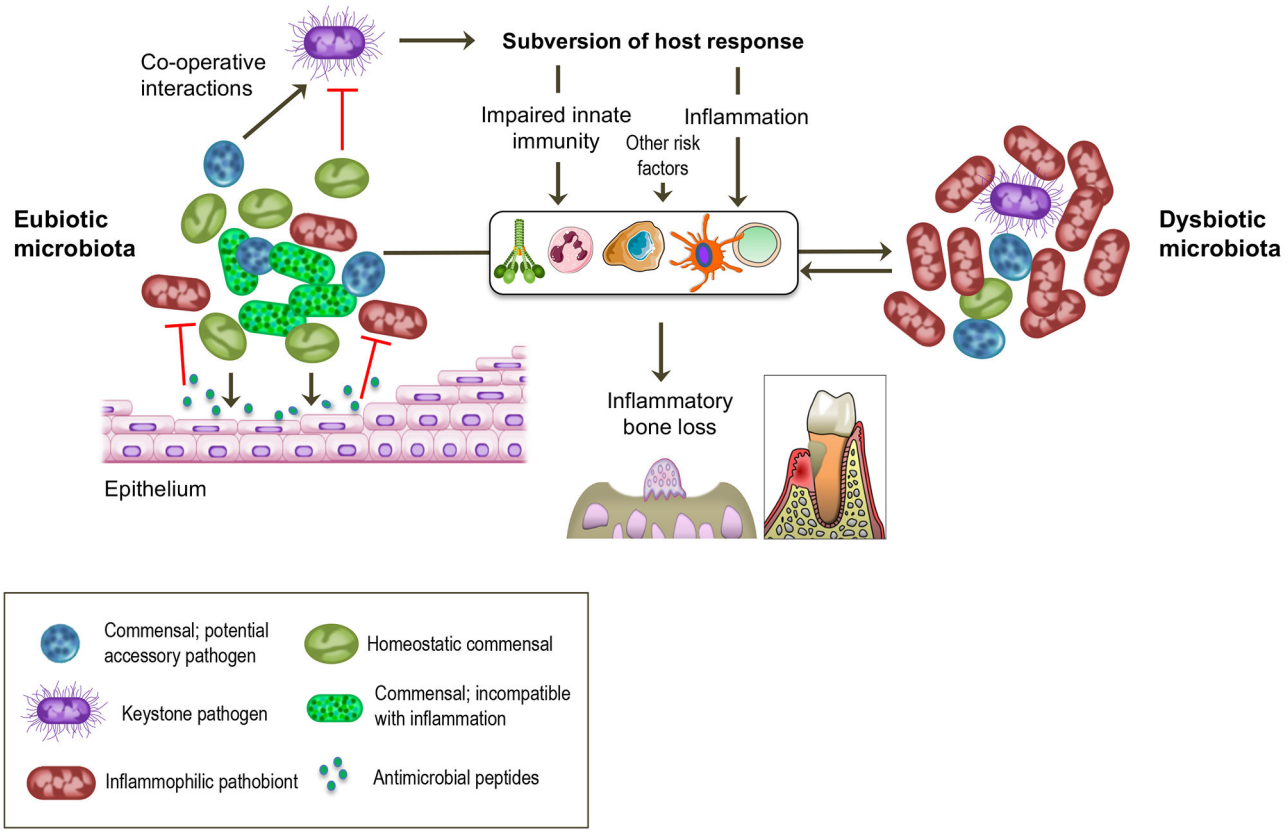

Figure 1. Functional categories among bacteria in polymicrobial communities.

Inflammatory bone loss in periodontitis is induced by a polymicrobial community, where different members have distinct and synergistic roles that promote destructive inflammation. Keystone pathogens — which are aided by accessory pathogens in terms of nutritional and/or colonization support — initially subvert the host immune response and contribute (along with other risk factors; see Table 1) to the emergence of a dysbiotic microbiota. Within this altered microbiota, commensal-turned pathobionts overactivate the host response and thrive within the resulting inflammatory environment. In contrast to an accessory pathogen, a homeostatic commensal tends to stabilize a eubiotic community either by directly antagonizing potentially pathogenic microbes or by inducing antimicrobial peptides that preferentially target potential pathogens.

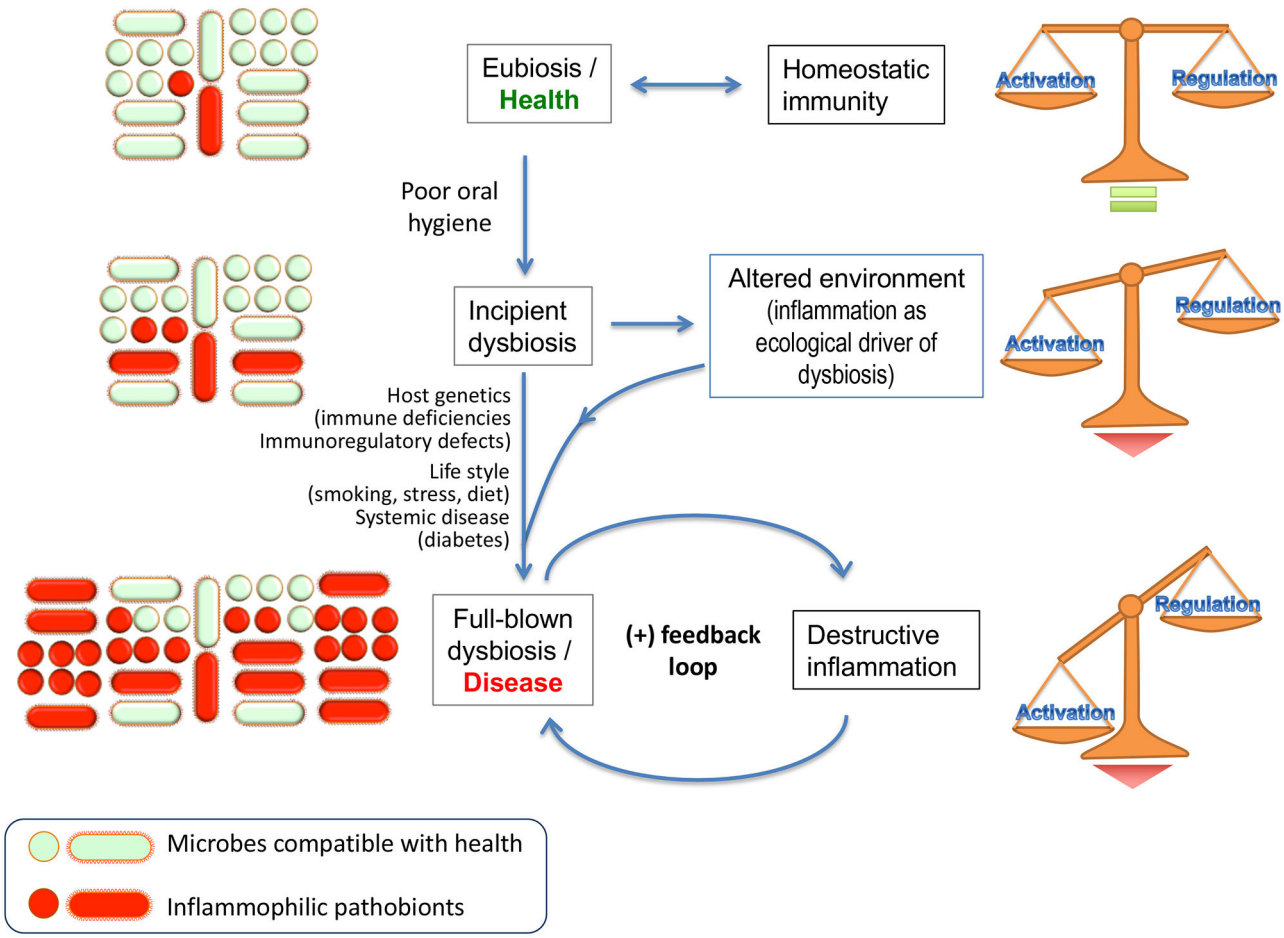

Figure 2. Interplay between inflammation and dysbiosis in periodontitis.

A eubiotic microbial community contributes to the induction and maintenance of homeostatic immunity, where immune activation is optimally regulated to control the health-associated microbiota without collateral tissue damage. Inflammatory responses to a growing biofilm due to poor oral hygiene (as it occurs in experimental gingivitis studies) may cause incipient dysbiosis which will further increase inflammation. Inflammation, in turn, may selectively favor the expansion of pathobionts which can capitalize on the altered environmental conditions (*e.g.*, use inflammatory byproducts to increase their metabolism and growth). The blooming pathobionts further exacerbate inflammation, eventually causing overt periodontitis in susceptible individuals (*e.g.*, owing to genetic or acquired alterations; see Table 1). In susceptible hosts, inflammation is ineffective, uncontrolled, and destructive and engages in a positive-feedback loop with dysbiosis, each reinforcing the other.

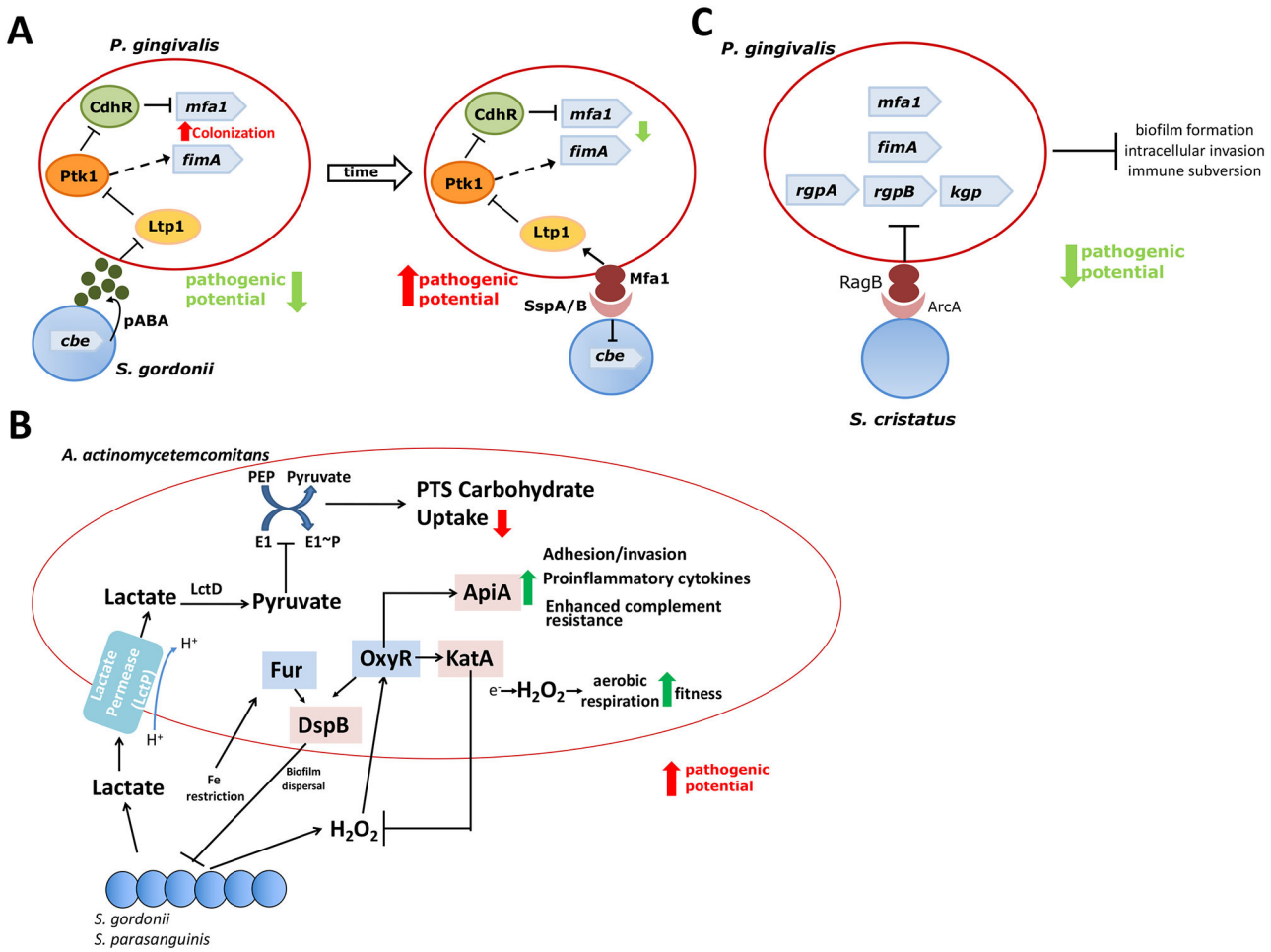

Figure 3: Interactions among bacterial species which impact nososymbiocity.

Oral bacteria interact through multiple pathways which can be demarcated both spatially and temporally. Shown are major threads of communication between (A) *P. gingivalis* and *S. gordonii*; (B) *A. actinomycetemcomitans* and *S. gordonii*; and (C) *P. gingivalis* and *S. cristatus*, which either increase or decrease pathogenic potential, as indicated (see text for detailed description). Adapted with permission from reference [2]. ApiA, *Actinobacillus* putative invasin A; ArcA, arginine deiminase; Cbe, chorismate binding enzyme; CdhR, Community Development and Hemin (transcriptional) Regulator; DspB, Dispersin B; fimA, fimbrial protein A; Fur, ferric uptake regulatory (protein); KatA, catalase D; kgp, Lysine-specific proteinase; LctD, lactase D; Ltp1, low-molecular-weight tyrosine phosphatase-1; Mfa, minor fimbrial antigen; RagB, Receptor antigen gene B; OxyR, Oxygen Resistance (positive regulator of hydrogen peroxide-inducible genes); pABA, 4-amino benzoate; Pdk1, *P. gingivalis* tyrosine kinase-1; PTS, phosphotransferase system; rgpA, Arginine-specific cysteine proteinase A; rgpB, Arginine-specific cysteine proteinase B; SspA/B, streptococcal surface protein A/B.

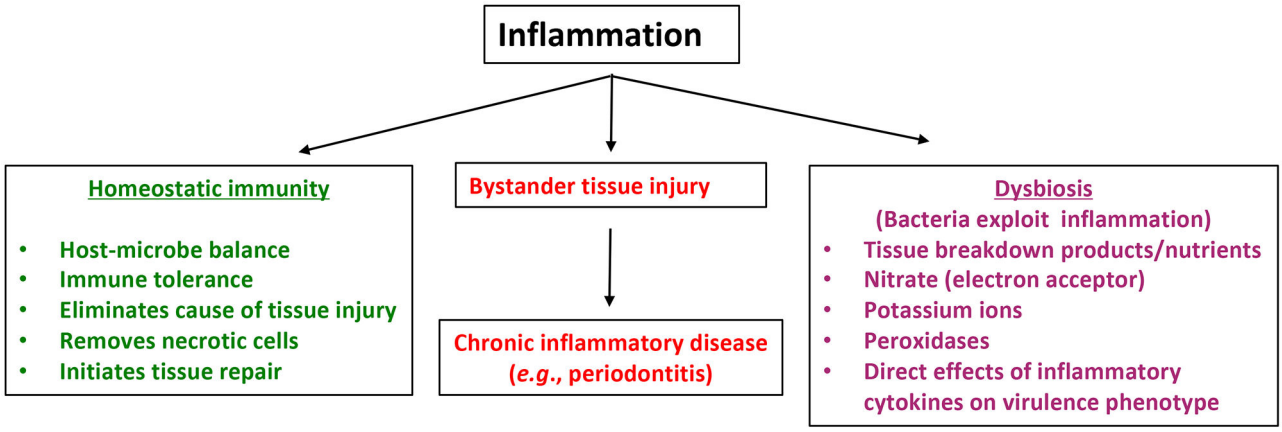

Figure 4: Significance of inflammation in host-microbe interactions.

Ideally, inflammation is an integrated component of a homeostatic process aiming to maintain host-microbe balance and immune tolerance to innocuous antigens and, if necessary, to isolate and destroy causes of tissue injury (*e.g.*, microbial pathogens), remove necrotic cells and cellular debris, and repair tissue damage, thereby restoring normal function. However, excessive inflammation can cause bystander tissue damage and, if not resolved, may become chronic and cause an inflammatory disease, such as periodontitis. Inflammation is also exploited by inflammophilic pathobionts to promote their metabolism, virulence, and adaptive fitness (see text for details on individual molecules exploited by pathobionts).

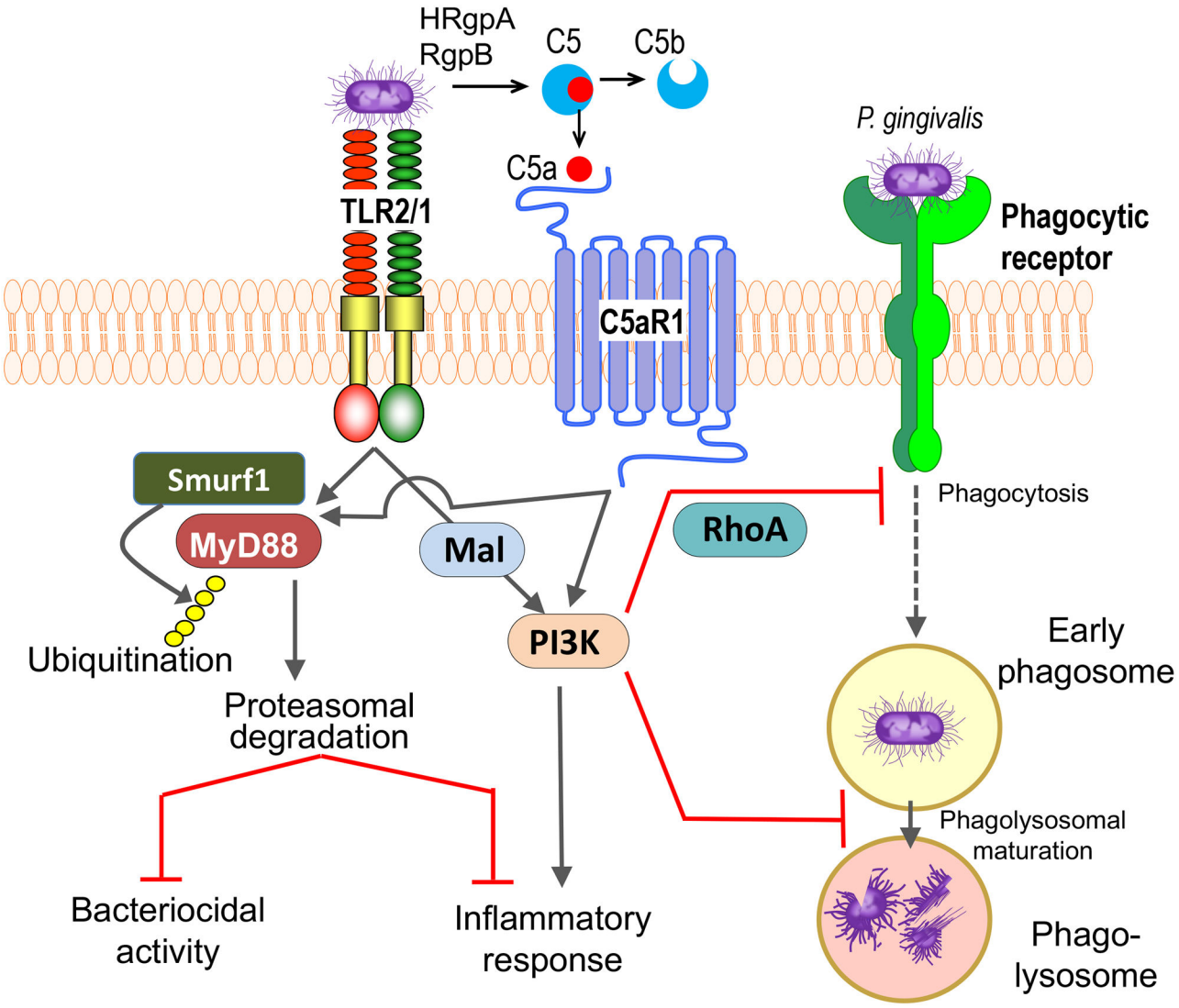

Figure 5. *P. gingivalis* impairs innate host defenses while promoting inflammatory responses in phagocytic cells.

P. gingivalis expresses cell-surface molecules that activate the TLR2–TLR1 complex (TLR2/1) and secretes enzymes (the gingipains designated HRgpA and RgpB) that act on the complement component C5 to generate high local concentrations of C5a, a ligand of C5aR1. The bacterium can thus co-activate C5aR1 and TLR2 in phagocytic cells such as neutrophils and macrophages. In both of these myeloid cell types, *P. gingivalis* can bypass MyD88 and thus prevent the associated bactericidal activity [99,116], which in neutrophils is possibly mediated by downstream activation of IRAK4-dependent neutrophil granule exocytosis [117]. In neutrophils, the inactivation of MyD88 involves its ubiquitination via the E3 ubiquitin ligase Smurf1 and its subsequent proteasomal degradation. Although MyD88-dependent inflammation is blocked by *P. gingivalis*, this bacterium induces PI3K-dependent inflammatory cytokine in both neutrophils and macrophages[105,107]. Similarly, in both cell types, *P. gingivalis*-induced activation of PI3K leads to inhibition of phagocytosis [99,105]. In neutrophils, this activity is mediated by the ability of PI3K to suppress RhoA

GTPase and actin polymerization [99]. Intriguingly, even within those macrophages that do manage to phagocytose *P. gingivalis* bacteria, PI3K signaling suppresses phago-lysosomal maturation, thereby preventing pathogen destruction [105]. These tactics compromise innate immunity while promoting inflammation that leads to the selective expansion of inflammophilic pathobionts. Conversely, inhibition of C5aR1, TLR2, or PI3K reverses dysbiotic inflammation and periodontitis in mice [98,99]. Adapted with permission from ref [2]. C5aR1, Complement C5a receptor-1; HRgpA; hemagglutinin arginine-specific cysteine proteinase A; IRAK4, Interleukin-1 receptor-associated kinase-1; Mal, MyD88-adaptor-like; PI3K, phosphoinositide 3-kinase; RgpB; arginine-specific cysteine proteinase B; RhoA, ras homolog family member A; Smurf-1, Smad ubiquitin regulatory factor-1; TLR2/1, Toll-like receptor 2/Toll-like recptor 1 complex.

Table 1.

Factors that modify the host response and/or the microbiome and promote susceptibility to periodontal disease.

Factors	References
Genetics *	211-214
Sex and gender **	215-219
Aging	220-225
Obesity	226-228
Diabetes	226,229-231
Stress / Depression	232-236
Tobacco smoking	30,237-239
Diet ***	227,231,240-242
Alcohol consumption	243,244
Viral infection	245,246

* Although a genetic basis for periodontitis is strongly supported by adult twin studies, it is uncertain whether specific individual genes determine susceptibility, as periodontitis is a polygenic disease in which multiple genes may contribute cumulatively to the overall disease risk (or protection). In contrast, single genes play a causative role in monogenic forms of aggressive periodontitis (*e.g.*, in patients with leukocyte adhesion deficiency due to mutations in the *ITGB2* gene).

** Despite sexual dimorphisms in immune function, greater risk for destructive periodontitis in men may predominantly be due to gender-based behavioral differences.

*** Can modify the host response in either protective or destructive manner.