Advances in Experimental Medicine and Biology 1197

Georgios N. Belibasakis
George Hajishengallis
Nagihan Bostanci
Michael A. Curtis *Editors*

# Oral Mucosal Immunity and Microbiome

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# Oral Mucosal Immunity and Microbiome

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# Preface

This textbook presents a variety of research topics that were addressed by leaders in the field of oral health and disease during the 1st International Conference on Oral Mucosal Immunity and the Microbiome. The Conference took place at the Avra Imperial Hotel, Kolymbari, Chania, Crete, Greece between 26th and 30th September 2018 under the auspices of Aegean Conferences. This collection of topics and themes highlights the recent progress in mucosal barrier immunity and its associated microbiome from an oral health perspective. The book offers a unique cross-disciplinary perspective for bridging our understanding of oral diseases and beyond.


Stockholm, Sweden — Georgios N. Belibasakis
Philadelphia, PA, USA — George Hajishengallis
Stockholm, Sweden — Nagihan Bostanci
London, UK — Michael A. Curtis

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# Advances in Oral Mucosal Immunity and the Microbiome

Georgios N. Belibasakis and George Hajishengallis

## Concepts in Homeostatic Breakdown and Oral Dysbiosis

Recent advances from microbiome and immunological studies highlighted that periodontitis is not a classical bacterial infection, but the outcome of dysbiosis owing to a homeostatic host-microbe breakdown. Homeostatic mechanisms are essential for the integrity of mucosal barrier sites, including the periodontium. Dysbiosis represents a perturbation of the microbiome composition driven by changes in the local environmental conditions, among which inflammation is key. An altered (dysbiotic) microbial community is adapted to survive in and take advantage of the nutritionally favorable inflammatory environment and may actively contribute to the persistence of a deregulated host immune-inflammatory state. Thus, inflammation and dysbiosis positively reinforce each other in a self-sustained loop and constitute the actual driver of periodontitis.

Understanding these concepts can be critical for the development of novel treatments for microbially driven inflammatory diseases, such as periodontitis. Such strategies may involve microbial community manipulation or targeted modulation of the host response, limiting inflammatory destruction and promoting resolution. Targeting the host inflammatory response may restore environmental conditions associated with health and consequently promote a symbiotic microbial community. Both a host-centric and a microbe-centric perspective were considered in understanding the shifts from oral symbiosis to dysbiosis, and from health to disease, discussed by George Hajishengallis (University of Pennsylvania) with an emphasis on complement-targeted therapeutic approach to periodontitis and Mike Curtis (King's College London).

## T Cells in Oral Mucosal Immunity

Mucosal colonization of *Porphyromonas gingivalis*, an important component of the dysbiotic microbiome, can regulate the plasticity of interstitial Th17 and Treg cells leading to an unfavorable balance that promotes disease (Massimo Costalonga, University of Minnesota). The IL-17A-producing cell populations include Th17 cells, γδ T cells, and a unique population of CD3+ CD4/CD8 double-negative T cells termed mucosa-associated invariant T (MAIT) cells. MAIT cells represent an unconventional type of T lymphocytes with innate-like characteristics,

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can be located also in the oral mucosa, and respond to oral commensals and pathogens. A subset of MAIT cells was shown to exhibit high levels of IL-17 production (Johan Sandberg, Karolinska Institutet).

γδ T cells reside also in the gingiva and are localized preferentially in the epithelium adjacent to the dental biofilm. Vγ6+ cells are a major source of IL-17 in gingiva. Chimeric mice and parabiosis experiments indicated that the main fraction of gingival γδ T cells is radioresistant and tissue resident, persisting locally independent of circulating γδ T cells. Notably, gingival γδ T-cell homeostasis is regulated by the microbiota as the ratio of Vγ6+ and Vγ4+ cells was reversed in germ-free mice, and their activation state was decreased. Ablation of γδ T cells results in elevated gingival inflammation and subsequent alterations to oral microbial diversity, indicating that oral mucosal homeostasis is shaped by the interplay of γδ T cells and the local microbiota (Avi-Hai Hovav, The Hebrew University).

Expansion of Th17 cells and neutrophil accumulation are required for inflammatory tissue destruction in experimental periodontitis in mice as well as in human periodontitis. This is dependent on the local dysbiotic microbiome and necessitates both IL-6 and IL-23, as opposed to homeostatic oral Th17 cells, which accumulate in a commensal-independent and IL-6-dependent manner (Nicolas Dutzan, NIH). The IL-23–IL-17 axis is involved in the early-onset periodontal immunopathology in leukocyte adhesion deficiency 1 (LAD1), which is characterized by deficiencies in adhesion of neutrophils to endothelium and transmigration into tissues. Targeted treatment with an IL23-blocking antibody is shown to yield impressive clinical outcomes in periodontal therapy (Niki Moutsopoulos, NIH).

IL-17 drives dysbiotic changes of the oral microbiome also in diabetes, with implications in the pathogenesis of periodontal disease. Dysbiotic microbiota transferred from diabetic mice to germ-free recipients induced greater expression of osteolytic cytokines, bone loss, and neutrophils, compared to bacteria transferred from normoglycemic animals. Treatment with IL-17 antibody reduced these effects, concluding that regulation of IL-17 in diabetes is central in altering the oral microbiota, rendering it more pathogenic (Dana Graves, University of Pennsylvania). An interplay among B cells, Th17 cells, and diabetes is also revealed in periodontal disease. Diabetes-primed B cells supported Th17 cytokine profiles, whereas B cells had a modest effect on T-cell function in normoglycemic individuals. T2D diabetes also alters B-cell and T-cell mitochondrial function, which perpetuates a Th17 milieu. These findings collectively reveal a novel role of lymphocytes in T2D-potentiated periodontitis (Barbara Nikolajczyk, University of Kentucky).

In other mucosal inflammation murine models, such as colitis, both commensal bacteria and the pathobiont *Helicobacter typhlonius* were required to mediate an exacerbating CD4 T-cell–mediated inflammatory response, leading to colonic tissue destruction. This is accompanied by metabolomic changes in the feces of the affected mice and the functions of monocyte/macrophages in the affected intestine (Christopher Muller, University of Berne).

## Dendritic Cells and Macrophages in Oral Mucosal Immunity

Environmental signals deriving from the indigenous microbiota may prime regulatory cues in dendritic cells (DCs) so that they can promptly respond to pathogen encounter, by eliciting cytokine, chemokine, and T-cell responses. The indigenous microbiome can indeed control type I interferon (IFN-I) production and IFN-I receptor signaling in DC in steady state, poising these cells for pathogen combat. Nevertheless, this basal state may as well prime disadvantageous T-cell responses, which can in turn be counteracted by barriers of peripheral tolerance barriers (Andreas Diefenbach, Charitè—Universitätsmedizin Berlin).

The role of the dysbiotic microbiome in the expansion of inflammatory blood myeloid dendritic cells (mDCs) and conversion of regulatory Treg cells to Th17 cells has also been considered in patients with periodontitis. In response to

periodontal therapy, the levels of certain periodontal pathogens in subgingival biofilms as well as their content in panDCs were reduced. Administration of antibiotics further reduced the frequency of mDCs and the frequency of Treg to Th17 conversion to healthy control levels. Hence, a dysbiotic oral microbiome may have a role on tissue homing DCs and bone-damaging T cells in periodontal disease (Christopher Cutler, Augusta University).

Periodontitis is associated with an imbalanced ratio of proinflammatory M1 and anti-inflammatory M2 macrophages, favoring the proinflammatory state. C-C motif chemokine ligand 2 (CCL2) is known to promote M2 chemotaxis, whereas IL-4 induces M2 polarization. Controlled local release of microparticles delivering CCL2 and IL-4 into the inflamed periodontium in mice showed significant reduction of alveolar bone loss, accompanied by an increase in the M2 subset and a decrease in the M1 subset. Therefore, local M2 immunomodulation could be a novel approach for periodontal treatment (Mostafa Shehabeldin, Charles Sfeir, University of Pittsburg).

## Epithelial Cells in Oral Mucosal Immunity

Oral and gingival epithelial cells are at the host–microbe interface and play important roles in shaping the host immune and inflammatory response. *P. gingivalis* interacts with gingival epithelial cells and can modulate the downstream neutrophil-specific chemokine expression via its unique lipid A deacylase, which has the potential to alter TLR4-mediated homeostasis (Richard Darveau, University of Washington). In oral epithelial cells, *P. gingivalis* can also induce Notch-1 signaling resulting in the production of the antimicrobial and proinflammatory protein phospholipase A2 group IIA ($PLA_2$-IIA) that can affect commensal bacteria in a manner that promotes dysbiosis (Octavio Gonzalez, University of Kentucky). When challenged with individual species in planktonic form, the gene expression changes elicited in oral epithelial cells were significantly different from those elicited in response to a multispecies biofilm composed of the same bacteria (Jeffrey Ebersole, University of Nevada). A three-dimensional implant-mucosa model was developed, defined by a stratified oral mucosa firmly attached to the implant. Culture with commensal *S. oralis* biofilm led to morphological differences of the mucosa at the implant site, accompanied by an altered gene expression and cytokine/chemokine secretion (Carina Mikolai, Hannover Medical School).

## Fungal Pathogenesis

*Candida albicans* is a commensal colonizer of the oral mucosa in humans, yet responsible for causing fungal infections in immune-compromised hosts. The interplay of the resident oral mucosal bacterial microbiota, such as mitis group streptococci, and *C. albicans* can be crucial in oropharyngeal candidiasis associated with cancer chemotherapy. Antibiotic-mediated depletion of oral commensals leads to severe dysbiotic changes in mucosa-associated bacterial communities and oropharyngeal candidiasis and reveals divergent relationships between mucosa-associated bacteria and *C. albicans* in the upper and lower gastrointestinal tract (Anna Dongari-Bagtzoglou, University of Connecticut).

*Candida albicans* can interact with *Streptococcus mutans* in oral biofilms. Understanding the *Candida*–streptococcal inter-kingdom interactions is particularly relevant to severe early-childhood caries. The interaction is characterized by changes in the spatial organization, extracellular polysaccharide matrix, and metabolic activity of bacterial–fungal biofilms, and their impact on clinically relevant outcomes such as drug resistance and virulence (Michel Koo, University of Pennsylvania).

*Candida albicans* itself produces a cytolytic peptide toxin (candidalysin) that is critical for pathogenesis and immune activation, which intercalates into and destabilizes the structural integrity of epithelial cell plasma membranes. This results in the release of alarmins and cell stress, leading to epithelial activation, eliciting an

inflammatory response, which helps clear the fungal infection (Jason Naglik, King's College London).

## Viral Pathogenesis

Microbially mediated oral diseases can signal underlying HIV/AIDS progression in HIV-infected adults. The Adolescent Master Protocol of the Pediatric HIV/AIDS Cohort Study is a longitudinal study of perinatally HIV-infected and HIV-exposed, uninfected youth. Data indicate that species richness and alpha diversity differed little between the two groups. However, significant differences in average counts were observed in two *Corynebacterium* species (associated with oral health), which were lower in the infected group. Caries odds increased with increasing levels of the genera, *Streptococcus*, *Scardovia*, *Bifidobacterium*, and *Lactobacillus* (Bruce Paster, Forsyth Institute).

In another study, involving children receiving care at a tertiary facility in Nigeria, no significant differences in bacterial diversity in saliva were found when comparing HIV-infected and HIV-exposed but not infected to completely uninfected and unexposed children. Younger ages and immune suppression levels were strongly associated with lower microbial diversity indices, whereas the relative abundance of taxa *Bacteroidales, Megasphaera, Prevotella, Tannerella*, and *Treponema* were associated with either HIV infection or exposure. In HIV-infected children, the odds for dental pathologies increased with a relative abundance of genera *Streptococcus, Rothia, Veillonella, Fusobacterium,* and *Lactobacillus* (Modupe Cocker, Dartmouth College).

## Microbiome and Cancer

There is increasing evidence that microbiome is involved in carcinogenesis, either directly through the produced metabolites or indirectly via induction of inflammation. There is also increasing evidence that *P. gingivalis* is strongly linked to the development and severity of aspiration pneumonia, rheumatoid arthritis, esophagus cancer, and Alzheimer's disease. A number of plausible mechanistic models implicate *P. gingivalis* virulence factors, gingipains, and peptidyl arginine deiminase (PPAD) in pathogenesis of some of these conditions (Jan Potempa, Jagiellonian University and University of Louisville).

*Fusobacterium nucleatum*, a common oral anaerobe associated with periodontitis, is also enriched in colorectal adenocarcinoma where it is associated with tumor acceleration, drug resistance, and poor disease outcome. Fusobacteria use the Fap2 lectin to attach to colorectal adenocarcinoma-displayed Gal-GalNAc, and they may translocate from the oral cavity via the hematogenous route during transient bacteremia (Gilad Bachrach, the Hebrew University-Hadassah). *F. nucleatum* has also been associated with intrauterine infection and adverse pregnancy outcomes. Specifically, *F. nucleatum* triggers placental inflammation in maternal endothelial cells via toll-like receptor (TLR)-4 signaling, whereas supplementation of pregnant mice with of omega-3 fatty acids during gestation suppressed placental inflammation, reduced *F. nucleatum* proliferation in the placenta, and increased fetal and neonatal survival (Yiping Han, Columbia University).

Sjögren's syndrome and focal lymphocytic sialadenitis are associated with changes in the oral microbiome. A substantial part of the changes in the oral microbiota of Sjögren's syndrome is shared with that of drug-induced sicca, although *Prevotella melaninogenica* appeared to be more closely associated with Sjögren's syndrome. Many bacterial cells were present within the lymphocytic infiltration and ductal cells and in areas of focal lymphocytic sialadenitis associated with the syndrome (Youngnim Choi, Seoul National University).

Fanconi anemia is a rare genetic disease associated with oral squamous cell carcinoma with the potential involvement of the oral microbiome. Microbial, metabolomic, and inflammatory profiling showed differences between siblings discordant for this disease. Higher abundance *Streptococcus* and *Candida* species were observed

in disease, along with a reduction in bacterial-derived catabolites produced from amino acids, polyamines, amino acids, and dipeptides (Flavia Teles, University of Pennsylvania).

Oral mucositis primarily results from the cytotoxic effect of chemotherapeutics on the rapidly dividing oral epithelium, but these medications also cause shifts in the mucosal microbiome contributing to the development of this condition. The microbial dysbiosis accompanying oral mucositis is characterized by enrichment of gram-negative proinflammatory pathobionts and depletion of abundant health-associated symbionts. The affected oral epithelium responds to this dysbiotic microbiome by up-regulating genes involved in proteolysis of cell junctions and apoptosis, such as kallikrein-5. Exposure to the symbiont *Streptococcus salivarius* decreases kallikrein-5 and reduces desmosomal degradation, suggesting that this depleted symbiont may actually protect against this condition (Patricia Diaz, University of Connecticut).

## Microbiome and Omics

Proteomic technologies have greatly advanced our understanding of host inflammatory pathways, host-microbe cross-talk, as well as the metaproteomic characterization of the oral microbiome. The plethora of molecular information revealed in oral biological fluids such as gingival crevicular fluid and saliva catalogued in open public databases can be fundamental for the identification of diagnostic/prognostic markers of the disease. Yet, the future challenge arising from these vast data gathering lies within its bioinformatic evaluation and biological interpretation (Nagihan Bostanci, Karolinska Institutet).

Human microbiome studies have focused on characterizing changes in the composition of the microbial communities. For instance, comparative genome-sequencing efforts have identified the identities and genomic compositions of oral treponemes in healthy and periodontally diseased oral niches. By focusing on genes that encode for housekeeping functions and surface-exposed virulence factors, it has been possible to decipher genetic lineages of widely distributed oral treponemes within the general population, as well as ones associated with periodontal disease (Rory Watt, University of Hong Kong). Microbiome profiles at active and inactive lesions suggest that changes in periodontal status cannot be explained solely by differences in subgingival microbial composition, but their metabolic activities are essential in driving dysbiotic processes. Hence, metatranscriptomic (gene expression) analysis of the oral microbiome may help identify changes in metabolic activities of the community that could reflect functional dysbiosis and explain the transition from health to disease (Jorge Frias-Lopez, University of Florida). The dynamics of the microbiome can also be demonstrated by its interaction with antibiotics. Administration of antibiotics induces notable changes in the gut and the oral microbiomes, yet, the latter is found to be significantly more resilient toward this exogenous disturbance. Moreover, rather than the use of antibiotics, the baseline levels of microbial diversity, presence of specific taxa, or certain microbial co-occurrences were able to predict better clinical treatment outcomes in patients with periodontitis (Wim Crielaard, Academic Center for Dentistry Amsterdam).

The human microbiome has co-evolved with the host, forming together a "holobiont." The microbiome is able to adapt to genotypic and environmental mandates of the host, dynamically maintaining oral health. Thus, identifiable host factors capable of shaping the oral microbiome could serve as prognosticators of disease state and activity or therapeutic intervention targets (Purnima Kumar, Ohio State University). As the microbiome has evolved together with the host, it is not surprising that bacterial taxa cohabiting the same niche are dependent on one another for growth and nutritional or signaling support. It is, therefore, often impossible to isolate certain microorganisms when their nutritional benefactors are not present. Co-culture and hybridization-directed enrichment approaches have led to the isolation of oral representatives of the phyla *Synergistetes*, *Chloroflexi,* and *Saccharibacteria*, and previously uncultured

members of the family *Lachnospiraceae* and the genera *Tannerella* and *Prevotella* (William Wade, King's College London).

Microbiome research using next-generation sequencing has admittedly reached a peak of productivity, aligning with the advancing technological trends. Yet methodological limitations and quality issues related to study design and sampling procedures influence the outcomes, necessitating hypothesis-driven, rather than technology-driven microbiome research (Egija Zaura, Academic Center for Dentistry Amsterdam). It is now evident that multi-omics data layering and aggregation is required for delineating the biological understanding of oral disease, combining both "deep" and "wide" analyses. A key element in this process is the identification and interrogation of biologically informed versus solely clinically defined disease traits ("endophenotypes" of biologically homogeneous disease signatures that might present with overlapping or heterogeneous clinical signs). Advancements in this direction will require commitment to systems biology and team science models (Kimon Divaris, University of North Carolina).

## Microbial Virulence and Dysbiosis

*Porphyromonas gingivalis* acts synergistically with accessory pathogen *Streptococcus gordonii* in heterotypic microbial communities to induce dysbiotic inflammatory responses. Their interaction involves mechanisms of co-adherence and metabolic cooperation. In *P. gingivalis*, these systems are governed by a signaling pathway dictated by protein tyrosine (de)phosphorylation, which converges on the expression of colonization and virulence factors (Richard Lamont, University of Louisville). *P. gingivalis* employs a number of mechanisms that enable it to avoid intracellular killing and survive within the oral mucosa. It can achieve this in gingival epithelial cells by attenuating the bactericidal effect of myeloperoxidase, inducing an antiapoptotic phenotype by the cells, and forming endoplasmic reticulum-rich vacuoles in which it can replicate and be protected from lysosomal degradation (Ozlem Yilmaz, Medical University of South Carolina). Novel insights into targeting inflammation indicate that A20, an ubiquitin-editing enzyme that terminates NF-κB activation, can restrain periodontal inflammation and cytokine production in responses to *P. gingivalis* via its negative regulatory effect on NF-kB signaling, as shown in in vivo and ex vivo models (Sinem Sahingur, Virginia Commonwealth University).

*Tannerella forsythia* is another example of species that has developed strategies to survive within the oral cavity. It has evolved pathways for the transport and recycling of *N*-acetylmuramic acid (MurNAc) and peptidoglycan fragments released by cohabiting bacteria during their cell wall turnover. It also produces methylglyoxal (MGO) that can modify host proteins to generate advanced glycation end products (AGEs) and kill bacteria that lack MGO-detoxification systems, thus releasing peptidoglycan by them. Hence, *T. forsythia*'s peptidoglycan-scavenging activities and MGO production might drive immune dysregulation and microbial dysbiosis (Ashu Sharma, State University of New York at Buffalo).

*Aggregatibacter actinomycetemcomitans*, a microorganism long associated with aggressive periodontitis, is shown to be necessary, but not sufficient, to cause disease requiring a consortium of bacteria. In a rhesus monkey model, it was shown that *A. actinomycetemcomitans* colonization was associated with lactate-producing species such as *Leptotrichia*, *Abiotrophia*, and *Streptococcus* in line with the capacity of this species to utilize lactate as a carbon source. Highly leukotoxin-producing strains showed a more favorable colonization pattern, associated with up-regulation of colonization-related genes (Daniel Fine, Rutgers University). In a murine abscess model of pairwise co-infections, *A. actinomycetemcomitans* was more abundant when in the presence of non-oral microbes. Approximately 40 of its core genes were essential for its survival in this process, encoding for proteins involved in ATP synthase, pili, regulation, secretion and efflux, and DNA replication. *A. actinomycetemcomitans* required more genes for survival in co-infection than in mono-infection, indicating that the co-infection can alter the host environment by reliev-

ing gene essentialities (Marvin Whiteley, Georgia Institute of Technology).

*Filifactor alocis* and *Peptoanaerobacter stomatis* are two gram-positive anaerobes that have recently emerged as important periodontal pathogens, recognized by human neutrophils primarily through TLR2/6. *F. alocis* survives in neutrophils for longer times, prevents granule recruitment to bacteria-containing phagosome, induces minimal respiratory burst response, and may inhibit neutrophil extracellular trap (NET) formation. In contrast, *P. stomatis* despite its low phagocytic index, induces neutrophil degranulation and a robust respiratory burst response, which promotes NETs formation (Silvia Uriarte, University of Louisville).

## Conclusion

The distilled message from this breakthrough conference (Fig. 1) is that oral mucosal immunity is orchestrated by distinctive immune and epithelial cell populations and functions in a reciprocal relationship with the oral microbiome in establishing health or disease. Efforts to understand jointly the microbial and immune components of the disease by scientists of either discipline will drive the development of predictable preventive, diagnostic, and treatment strategies (Fig. 2). Cross-disciplinary research is the way forward. The conference concluded with Greek dances and a Gala dinner and was renewed for October 11–16, 2020, in Kos, the island of Hippocrates.

**Fig. 1** The poster announcement of the 1st International Conference on Oral Mucosal Immunity and Microbiome

**Fig. 2** Lively discussions during the course of the conference

# Trained Innate Immunity and Its Implications for Mucosal Immunity and Inflammation

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## Introduction

Historically, the adaptive arm of immunity (etymologically derived from *immunis*; Latin for "exempt") has been described in contrasting terms with regard to innate immunity. Features, such as receptor repertoire diversity, specificity, and memory, which underlie the concept of vaccination, were considered to be the exclusive prerogative of adaptive immunity (Roitt et al. 1989).


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For instance, whereas innate immune receptors are encoded in the germline resulting in limited diversity, adaptive immune receptors are generated randomly by gene recombination and somatic hypermutation, thereby leading to an unlimited receptor repertoire. Adaptive immunity is exquisitely specific being able to discriminate even between individual microbes of the same species (strain-dependent antigenic variability). Moreover, immunological memory, the ability of the adaptive immune system to "remember" past encounters with pathogens, enables the immune system to swiftly and specifically recognize a subsequent challenge with the same pathogen and initiate a faster and stronger immune response that is often protective for the host. Lack of immunological memory in innate immunity meant that the innate immune response is invariable and thus not enhanced upon repeated encounters with a pathogen; the perceived significance of the innate response lied in its ability to act fast and "buy" time until the adaptive immunity becomes activated.

However, the defining differences between the adaptive and innate forms of immunity are not as unambiguously defined as previously thought. For instance, innate immunity is endowed with adequate specificity to distinguish between different classes of microorganisms as well as to discriminate between healthy and damaged/stressed host cells (Miyake 2007; Fearon 1997; Medzhitov and Janeway 2000). Moreover, recent


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advances show that, despite lacking the fine antigen specificity, clonality, and longevity of adaptive lymphocytes, innate immune cells can retain "memory" of earlier challenges (e.g., infection or vaccination) and thereby display increased responsiveness upon rechallenge with the same or even unrelated pathogen (Netea et al. 2016; Goodridge et al. 2016).

This enhanced state of immune activation that is based on innate immune memory is known as "trained innate immunity" or more briefly "trained immunity" (Netea and van der Meer 2017). As implied above, innate immune memory lacks specificity to the initial infection (or other inflammatory stimuli); indeed, exposure to a certain pathogen can confer enhanced protection against a future challenge with a different pathogen even from a different kingdom (e.g., the original challenge could be a fungal pathogen and the secondary one could be a bacterial or viral pathogen). Innate immune memory is not necessarily expressed as trained immunity but also as "innate immune tolerance." In this regard, prior exposure of myeloid cells such as monocytes/macrophages to relatively high doses of bacterial lipopolysaccharide (LPS) induces a state of reduced ability to elicit proinflammatory cytokines to subsequent restimulation with LPS (homologous tolerance) or other proinflammatory stimuli (heterologous tolerance) (Jacinto et al. 2002). In this review, we will focus on trained immunity and will discuss its evolutionary origins, its functional consequences at mucosal barrier sites, as well as the mechanisms that underlie the induction of a trained immune phenotype and how this can be exploited therapeutically.

## Trained Immunity, an Evolutionary Conserved Function for Broad-Based Protection Against Infections

Trained innate immunity is seemingly an evolutionarily conserved way of remembering past challenges as it is not present only in vertebrates but also in non-vertebrate animals and in plants (Penkov et al. 2019). Although plants lack adaptive immunity, they use sophisticated mechanisms to anticipate and effectively fight infections. Systemic acquired resistance (SAR) of plants is a mechanism of inducible defense that provides long-lasting protection (weeks to months) against a broad range of microbial pathogens (Durrant and Dong 2004). SAR spreads from the site of the infection to the entire tissues of the plant through production of mobile signals, accumulation of salicylic acid (which, in plants, functions as a defense factor), increased expression of pattern-recognition receptors, and secretion of antimicrobial proteins, thereby establishing protection against secondary infections (Fu and Dong 2013; Reimer-Michalski and Conrath 2016). Thus, SAR endows the plant with memory to the initial infection by priming even remote tissues for enhanced defense to subsequent infections. In a manner analogous to immunization in vertebrates, plants inoculated with attenuated microorganisms acquire long-term protection against subsequent infections but in a relatively nonspecific manner (cross-protection against bacterial, fungal, and viral pathogens) (Durrant and Dong 2004; Fu and Dong 2013). Importantly, SAR involves adaptations in the chromatin state and thus can be transmitted to progeny through epigenetic mechanisms (Fu and Dong 2013; Reimer-Michalski and Conrath 2016), suggesting that the enhanced innate resistance of plants to reinfection involves memory mechanisms similar to those of trained immunity in jawed vertebrates (Netea and van der Meer 2017).

Adaptive immunity is thought to have arisen in the first jawed vertebrates (gnathostomes; Greek for jawed mouth), i.e., the placoderm fish (Flajnik and Kasahara 2010). Therefore, the investigation of the presence of innate immune memory can be better studied in invertebrates, such as insect models, that lack adaptive immune memory. A number of studies have demonstrated that the immune system of insects can be primed for long-lasting immune responses that can often afford protection against future infections. A few examples will be given here. Priming of *Drosophila melanogaster* with a sublethal dose of *Streptococcus pneumoniae* protected the insect for life against an otherwise-lethal reinfection

with the same pathogen (Pham et al. 2007). This protective effect required the Toll pathway and phagocytes (Pham et al. 2007). When initially challenged with LPS, larvae of the mealworm beetle, *Tenebrio molitor*, elicited a long-lasting antimicrobial response that protected the larvae against a subsequent exposure to an entomopathogenic fungus (Moret and Siva-Jothy 2003). *Anopheles gambiae* mosquitoes infected with *Plasmodium falciparum* were shown to be primed for a long-lasting enhanced antibacterial response that could efficiently reduce *Plasmodium* survival upon reinfection (Rodrigues et al. 2010). The primed state was induced when plasmodium ookinetes breached the gut barrier and bacteria gained access to injured epithelial cells, leading to induction of quantitative changes and qualitative differentiation of hemocytes (insect equivalent of leukocytes) that lasted for the lifespan of the *Anopheles* mosquito (Rodrigues et al. 2010).

Besides infection itself, other cues that may confer protection against secondary microbial challenge is apoptotic corpse engulfment. Indeed, a recent study has shown that innate immune memory can be induced in *Drosophila* embryonic macrophages (hemocytes) following phagocytosis of apoptotic cells (Weavers et al. 2016). This priming is triggered by calcium-induced JNK signaling, which in turn up-regulates the damage receptor Draper and the cell is thus poised to respond more rapidly to subsequent infection or injury. Unlike primed or "trained" hemocytes, naive hemocytes that have not engulfed apoptotic cells are not efficient in detecting sterile tissue damage in vivo and migrating accordingly. Moreover, untrained hemocytes are also inefficient to recognize infection with *Escherichia coli* and respond by phagocytosing the pathogen (Weavers et al. 2016).

Emerging evidence suggests that the free-living nematode (roundworm) *Caenorhabditis elegans* also can build long-lasting and even transgenerational innate immune memory (Penkov et al. 2019). This memory allows the worm to effectively avoid or resist secondary pathogen encounters. For instance, brief exposure to virulent or avirulent strains of enteropathogenic *E. coli* (EPEC) primes *C. elegans* to survive an otherwise-lethal re-encounter with EPEC (Anyanful et al. 2009). *C. elegans* worms exposed to *Pseudomonas aeruginosa* were "trained" to avoid this pathogenic bacterium; however, in the absence of relevant training, the worms failed to develop aversive learning behavior against pathogenic *P. aeruginosa* (Lee and Mylonakis 2017). Intriguingly, moreover, second-generation *C. elegans* feeding on pathogenic bacteria can avoid bacterial infection by entering diapause, a hibernation-like state that arrests development (Palominos et al. 2017).

These findings have called for a reevaluation of the earlier assumption that invertebrate innate immunity lacks memory qualities and the current burden of proof is consistent with the presence of innate immune memory in lower organisms. Together, the studies discussed in this section indicate that innate immune memory is a conserved function that has persisted in evolution.

## Trained Immunity in Mammals: Protection Against Mucosal and Systemic Infection

*Candida albicans* is a commensal fungus at mucosal surfaces of healthy individuals but behaves as an opportunistic pathogen in immunocompromised patients, where it frequently causes superficial infections in the oral cavity and the vagina (Williams et al. 2013). In immunocompromised individuals, moreover, *C. albicans* (and other *Candida* species) can also cause systemic infections, which constitute the fourth leading cause of nosocomial bloodstream infection in modern hospitals (50,000 new cases of systemic candidiasis per year in the U.S.) with high morbidity and mortality (Lionakis 2014). *C. albicans* is also frequently detected in high numbers in the biofilm that develops on the tooth surfaces ("dental plaque") of toddlers with early childhood caries, a severe form of tooth decay where this fungus contributes to its pathogenesis (Hajishengallis et al. 2017).

*C. albicans* is a prototype organism used to consolidate the concept of trained immunity. In this regard, T- and B-cell–deficient mice that

were previously "trained" with low-dose *C. albicans* or purified β-glucan (major fungal cell wall constituent) displayed increased protection, as compared to untrained controls, against reinfection with *C. albicans* through enhanced production of cytokines and phagocytic killing (Quintin et al. 2012). Thus, trained innate immune cells, such as monocytes/macrophages, acquire enhanced responsiveness after secondary stimulation with the fungus, which can thereby be eliminated more readily by innate immune means, irrespective of adaptive immunity. As alluded to above, innate immune memory is not specific, as adaptive immune memory is, since exposure to one pathogen can afford enhanced protection against a second, unrelated pathogen. Indeed, systemic infection with attenuated *C. albicans* protected mice not only against secondary challenge with virulent *C. albicans* but also against *Staphylococcus aureus* in a macrophage-dependent manner (Bistoni et al. 1986). Innate immune memory is not an exclusive feature of the monocytic lineage but also resides within other innate immune cell lineages, such as natural killer cells, which rapidly degranulate and produce cytokines upon reactivation with cytomegalovirus (Sun et al. 2009).

Epidemiological studies on the effectiveness of vaccines showed that specific immunizations had off-target beneficial effects, i.e., were associated with protection against diseases that were not intended to fight (Netea and van der Meer 2017; Goodridge et al. 2016). For instance, a randomized trial of bacillus Calmette–Guérin (BCG) vaccination suggested that BCG has nonspecific protective affects against neonatal sepsis and infections of the respiratory mucosa (Aaby et al. 2011). Moreover, the off-target effects of BCG include protection against malignant tumors (Falk et al. 1973; Martinez-Pineiro and Muntanola 1977; Simmons and Rios 1971). This "bonus" protection can be explained by vaccine-induced trained immunity. In this regard, besides protein immunogens, vaccines contain adjuvants either intrinsically (whole microbial cells contain inherent adjuvant substances) or extrinsically, such as alum, the most commonly used adjuvant in vaccine formulations. Adjuvants can directly or indirectly (through the release of danger-associated molecular pattern-containing molecules) activate pattern-recognition receptors on innate immune cells (Sanders and Feavers 2011; Mizel and Bates 2010; Marrack et al. 2009). Thus, the off-target effects of vaccines may be mediated, at least in part, by adjuvant-dependent induction of trained innate immunity (Arts et al. 2018; Kleinnijenhuis et al. 2012). Indeed, BCG vaccination of healthy adults resulted in elevated ex vivo induction of monocyte-derived cytokines (TNF and IL-1β) in response to stimulation with unrelated bacterial or fungal pathogens (Kleinnijenhuis et al. 2012). This enhanced responsiveness of monocytes was dependent on the NOD2 receptor and persisted for at least 3 months after vaccination (Kleinnijenhuis et al. 2012). Moreover, a randomized placebo-controlled human challenge study showed that BCG vaccination confers protection against experimental infection with an attenuated vaccine strain of yellow fever virus, as evidenced by reduced viremia that correlated with elevated IL-1β (Arts et al. 2018).

The findings discussed in the previous section that apoptotic cell phagocytosis by hemocytes in *Drosophila* primes these cells for enhanced responses to subsequent infection or injury (Weavers et al. 2016) seems to deviate from the functional consequences of apoptotic cell phagocytosis (efferocytosis) in mammalian macrophages. In this regard, efferocytosis activates liver X receptor (LXR) signaling and reprograms the macrophage toward a pro-resolving phenotype (Kourtzelis et al. 2017, 2019; Ravichandran and Lorenz 2007). However, the effects of LXR on inflammation in mammals appear to be context dependent. For instance, LXR signaling inhibits LPS-induced TLR4 activation in macrophages when LPS and LXR agonists are added together (simulating conditions when efferocytosis occurs at the declining stage of infection and initiation of resolution). However, LXR signaling enhances LPS-induced TLR4 activation if the macrophages are pretreated with LXR agonist 48 h prior to LPS challenge (Fontaine et al. 2007; Rigamonti et al. 2008), a priming scenario analogous to the experimental design in the *Drosophila* study (Weavers et al. 2016). In other words, LXR

signaling primes or trains the macrophage for heightened antimicrobial responses upon subsequent encounters with pathogens; however, if the infection has already occurred, LXR signaling acts to mitigate associated inflammation and promote transition to resolution.

## Mechanisms of Trained Immunity

Recent studies have shown that the acquisition of a trained immunity state by innate immune cells involves metabolic, epigenetic, and transcriptional reprogramming, which is sustained for months (Bekkering et al. 2018; Arts et al. 2016a; Saeed et al. 2014; Cheng et al. 2014; Norata et al. 2015; Penkov et al. 2019). Before discussing this evidence in greater detail, it would be instructive to briefly discuss the importance of epigenetic rewiring in the context of trained immunity.

Many loci encoding inflammatory genes are in a repressed state in myeloid cells. Upon activation with a proinflammatory stimulus, important changes occur in the genomic elements (enhancers and promoters) that regulate gene expression. Specifically, there is increased histone acetylation and chromatin opening and enhanced recruitment of certain transcription factors (e.g., NF-κB, AP-1, and STAT proteins) and RNA polymerase II (Glass and Natoli 2016; Saccani et al. 2001). In other words, chromatin becomes more accessible to the transcriptional machinery. Importantly, such enhanced accessibility persists over time as the acquisition of specific chromatin marks (histone acetylation or methylation) is well maintained (only partially lost after stimulus elimination). For instance, a latent enhancer, which may be unmarked epigenetically in unstimulated cells, may acquire histone modifications typical of active enhancers (e.g., H3K4me1; monomethylation of histone H3 at K4) (Ostuni et al. 2013). This epigenetic adaptation underlies trained immunity as it could be maintained well after the removal of the inductive stimulus and promotes more efficient induction of genes and protection in response to future challenges (Netea et al. 2016). In this regard, BCG vaccination-induced training that protects against experimental infection with attenuated yellow fever virus was correlated with BCG-induced genome-wide epigenetic adaptations in monocytes and increased production of IL-1β (Arts et al. 2018).

Similar to trained innate immunity, innate immune tolerance, the other side of the coin in terms of innate immune memory, is also regulated by chromatin modifications, which, however, silence genes encoding proinflammatory mediators (Foster et al. 2007). Epigenetic modifications also underlie the fact that myeloid cells, such as macrophages, exhibit tissue-specific functions that are largely instructed by microenvironmental stimuli (Stout et al. 2009; Matzinger 2007). Indeed, the local microenvironment shapes the enhancer landscape of macrophages beyond what could be attributed to developmental origin, thus contributing to their plasticity in a tissue-specific context (Lavin et al. 2014).

Training induced by *C. albicans* or β-glucan in monocytes/macrophages is mediated through binding to the C-type lectin receptor dectin-1 that activates a noncanonical Raf-1–dependent pathway, which in turn leads to genome-wide alterations in epigenetic marks, such as H3K4me1, H3K4me3, and H3K27Ac (Netea et al. 2016). The mechanisms by which epigenetic changes are induced and how these in turn regulate trained immunity involve complex cross-talk interactions between metabolism, the epigenome, and the immune response (Fig. 1). Resting monocytes and macrophages primarily use oxidative phosphorylation (OXPHOS). However, during β-glucan–induced trained immunity, macrophages undergo a rapid shift toward aerobic glycolysis (also known as the Warburg effect), a process that can swiftly accommodate the increased metabolic needs associated with cell activation and expansion, specifically in terms of energy and building blocks required for cell membranes (cholesterol and phospholipids) and DNA (purines) (Cheng et al. 2014; Norata et al. 2015). This metabolic switch from OXPHOS to aerobic glycolysis during β-glucan–induced training involves activation of the Akt–mTOR–HIF1α signaling pathway (Cheng et al. 2014).

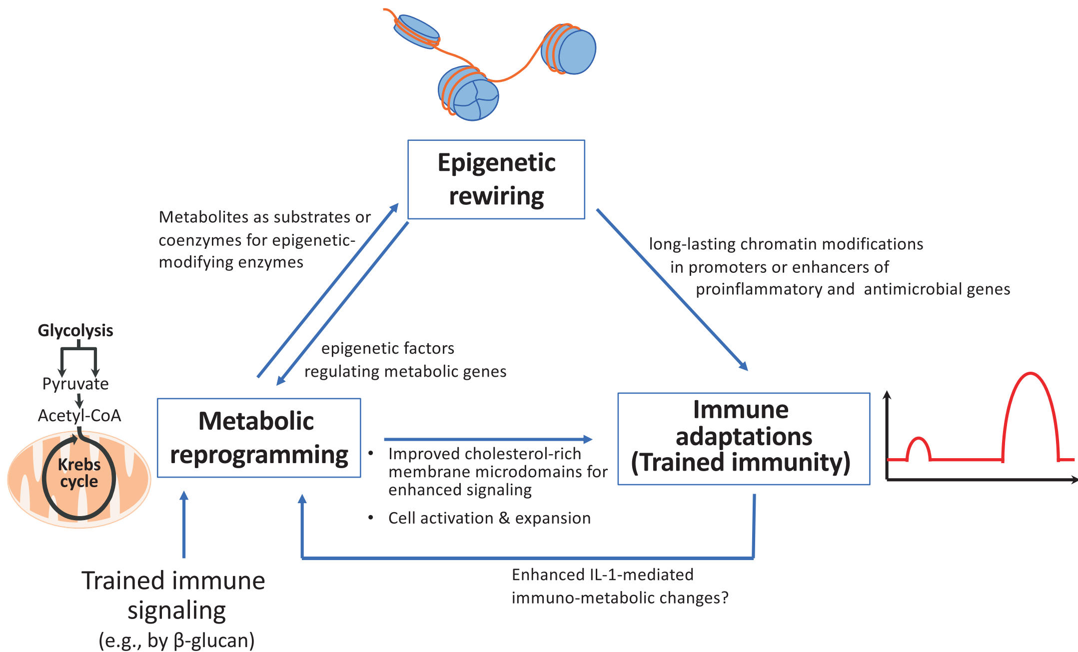


**Fig. 1** Immuno-epigeno-metabolic cross talk in trained innate immunity. Outline of major mechanisms by which innate immune signaling-triggered metabolic pathways induce epigenetic changes and how these prime for heightened secondary immune responses or trained immunity, which also cross talks with metabolism. See text for details

The β-glucan-induced metabolic alterations are functionally connected to the epigenetic rewiring. In this regard, Krebs cycle metabolites have the ability to regulate the function of key epigenetic enzymes. Whereas citrate is exported to the cytoplasm for synthesis of cell membrane building blocks (cholesterol and phospholipids), fumarate accumulates in response to β-glucan stimulation of macrophages (owing to glutamine replenishment of the Krebs cycle and glutaminolysis) (Arts et al. 2016b). Fumarate in turn inhibits KDM5 histone (H3K4) demethylases, thus bringing about epigenetic reprogramming by increasing histone methylation (Arts et al. 2016b). Another example of a link between trained immune signaling, cellular metabolism, and epigenetic reprogramming involves the acetyl coenzyme A (acetyl-CoA), which serves as an acetyl donor to the Krebs cycle for oxidation and energy production. Acetyl-CoA also provides the acetyl group for histone acetylation, a histone mark that promotes gene transcription (Gut and Verdin 2013). Moreover, lactate, the end product of anaerobic glycolysis, can inhibit the activity of histone deacetylases (HDAC) and, hence, promote gene accessibility and transcription (Latham et al. 2012). The cholesterol synthesis pathway also contributes to trained immunity as the latter can be inhibited in myeloid cells by statins. However, the essential effector of the trained phenotype is not cholesterol itself but the intermediate metabolite mevalonate, which via the insulin-like growth factor-1 receptor and downstream mTOR signaling leads to histone modifications in inflammatory pathways (Bekkering et al. 2018). In summary, metabolites exert a significant impact on the epigenetic landscape by serving as signaling molecules or as substrates and/or cofactors for chromatin-modifying enzymes. The reverse may also be true since the modified chromatin structure in turn can regulate the expression of metabolic genes (Hino et al. 2013). Overall, innate immune cells exposed to appropriate stimuli modify their metabolism and remodel their chromatin in a manner that can elicit an “educated” (much faster and stronger)

response to subsequent encounter with the same or different stimuli. However, this concept also generated a paradox.

The long-term effects of trained immunity on circulating monocytes have been puzzling, as these cells have a relatively short lifespan (Yona et al. 2013) in the circulation. This paradox was resolved by two recent reports that appeared in the same issue of *Cell* (Kaufmann et al. 2018; Mitroulis et al. 2018). These studies have shown for the first time that the processes induced by trained immunity involve long-term adaptations (metabolic, epigenetic, and transcriptional) in hematopoietic progenitor cells that give rise to lines of differentiated innate immune cells in the bone marrow (BM) (Kaufmann et al. 2018; Mitroulis et al. 2018). For instance, β-glucan reprograms several pathways associated with cell proliferation, glycolysis, and cholesterol biosynthesis and induces the expansion of hematopoietic stem and progenitor cells (HSPCs) in the BM (Mitroulis et al. 2018) (Fig. 2). Appropriately enough, β-glucan increases the frequencies of myeloid-biased $CD41^+$ long-term hematopoietic stem cells (LT-HSCs) and of multipotent progenitors (MPP) that are biased toward myelopoiesis, namely the MPP3 subset. These effects do not involve direct β-glucan contact with hematopoietic progenitors but are instead mediated by β-glucan–induced innate immune mediators in the BM microenvironment, namely IL-1β and granulocyte-macrophage colony-stimulating factor (GM-CSF). This β-glucan–induced trained phenotype persists for at least several weeks, can be adoptively transferred to untrained recipient mice, and protects against (a) DNA damage in LT-HSCs induced by a secondary challenge with LPS and (b) chemotherapy-induced myelosuppression (Mitroulis et al. 2018) (Fig. 2). Similarly, BCG can also induce expansion of HSCs and promote myelopoiesis leading to the generation of trained monocytes/macrophages that protect against *Mycobacterium tuberculosis* infection (Kaufmann et al. 2018). This BCG-induced training is dependent on IFN-γ signaling. Thus, trained innate immunity is initiated by modulation of the progenitors of myeloid cells in the bone marrow in a manner that enhances the replenishment of innate immune cell populations upon stress associated with infectious, inflammatory, or chemotherapeutic challenges and leads to protective immunity.

## Maladaptive Trained Immunity

Although trained innate immunity can protect against subsequent systemic or mucosal infections or chemotherapy-induced myeloablation (Mitroulis et al. 2018; Kaufmann et al. 2018; Quintin et al. 2012; Arts et al. 2018; Kleinnijenhuis et al. 2012), there may be settings in which trained immunity can do more harm than good. As reasoned above, trained immunity may have evolved as a form of innate immune memory to provide broad cross-protection against reinfections. However, in modern societies, trained immunity may be a potential contributory factor to age-related chronic inflammatory diseases. Such maladaptive trained immunity could be inappropriately induced by microbial or endogenous stimuli (damage-associated molecular patterns; DAMPs) and lead to exaggerated immune responses that drive or exacerbate inflammatory or autoimmune diseases. In other words, if the immune system is epigenetically trained (due to earlier infection, vaccination, or injury) to elicit a heightened immune response, this enhanced responsiveness may aggravate existing inflammatory/autoimmune diseases or prove deleterious in hosts that are genetically susceptible to inflammatory/autoimmune diseases. These notions are consistent with clinical observations or experimental studies in animal models.

As mentioned above, mevalonate has been implicated as an inducer of the trained immunity phenotype. Intriguingly, monocytes of patients with hyper immunoglobulin D syndrome (HIDS), who accumulate mevalonate due to mevalonate kinase deficiency, constitutively exhibit a trained phenotype both epigenetically and immunologically (Bekkering et al. 2018). This finding may explain, at least in part, the susceptibility of these patients to autoinflammatory disorders. Indeed, HIDS patients suffer from recurrent febrile episodes often associated with lymphadenopathy,

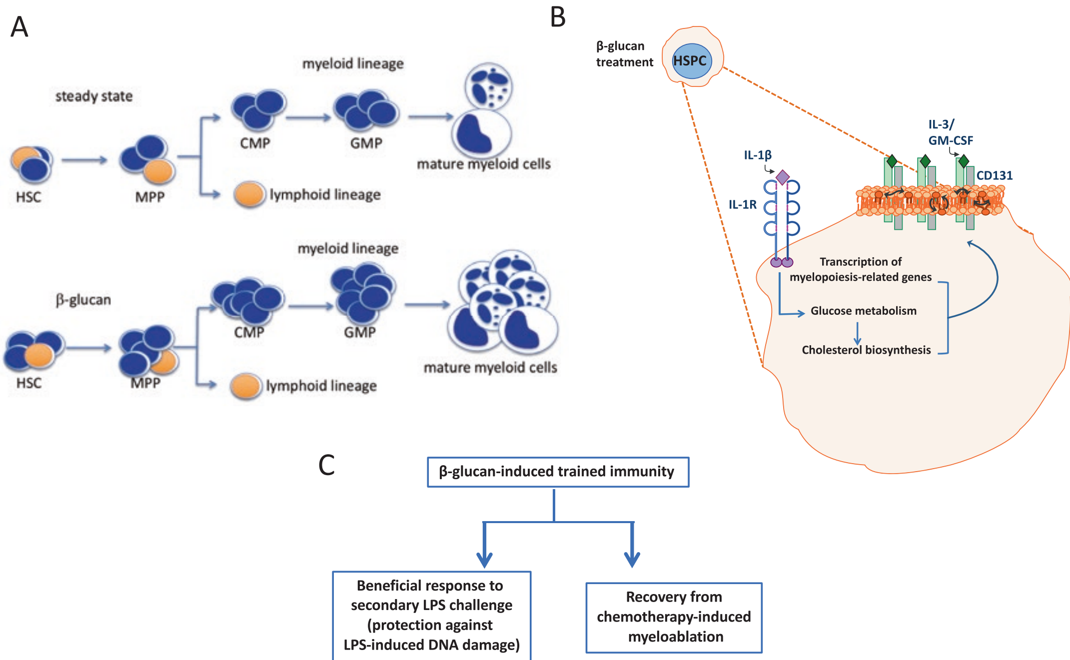


**Fig. 2** Trained innate immunity acts at the level of myeloid progenitors. (**a**) Trained immunity as induced by β-glucan mediates a myelopoiesis bias in the bone marrow. *CMP* common myeloid progenitor, *GMP* granulocyte-monocyte progenitor, *HSC* hematopoietic stem cell, *MPP* multipotent progenitor. (**b**) The β-glucan–dependent myelopoiesis bias is mediated by an early activated IL-1β pathway associated with enhanced glycolysis and lipid metabolism changes in hematopoietic stem and progenitor cells (HSPC) resulting in enhanced cholesterol-rich membrane microdomains and thereby enhanced GM-CSF/CD131 signaling, which drives myelopoiesis. (**c**) Induction of trained immunity in HSPCs protects them from LPS-induced DNA damage and enables beneficial responses of the bone marrow to a secondary LPS challenge as well as to chemotherapy-induced myelosuppression

abdominal and joint pain, hepatomegaly, splenomegaly, and skin rash among other inflammatory symptoms (van der Meer et al. 1984).

Low concentrations of oxidized low-density lipoprotein (oxLDL) were shown to induce a long-lasting proinflammatory phenotype in monocytes associated with increased histone methylation (H3K4me3) in the promoter regions of several pro-atherogenic genes; the proinflammatory IL6, MCP1, IL8, TNF, MMP2, and MMP9; and the scavenger receptors CD36 and scavenger receptor-A (Bekkering et al. 2014). Upon rechallenge with TLR agonists, the oxLDL-trained monocytes showed increased production of pro-atherogenic cytokines, whereas upon rechallenge with oxLDL, the trained monocytes express higher levels of scavenger receptors and show increased foam cell formation (Bekkering et al. 2014). This study suggests that the proinflammatory nature of high-fat western diets (WD) could induce maladaptive trained immunity. Using LDL receptor (Ldlr)-deficient mice on a WD as a model of metabolic syndrome-induced autoinflammation, a recent study showed that WD induces long-lasting inflammasome-mediated trained immunity in myeloid cells (Christ et al. 2018). Mice that were doubly deficient in both Ldlr and the Nlrp3 inflammasome did not exhibit a trained phenotype and developed significantly reduced systemic inflammation and atherosclerotic plaque size in response to WD feeding (Christ et al. 2018).

In a possibly analogous manner involving nonimmune cells and high glucose as an endogenous proinflammatory stimulus with potential for epigenetic alterations (Yun et al. 2011), transient hyperglycemia was associated with persistent up-regulation of NFκB-p65 gene expression

in human vascular endothelial cells attributed to chromatin modifications (enhanced H3K4 and reduced H3K9 methylation) (Brasacchio et al. 2009; El-Osta et al. 2008). This so-called "hyperglycemic memory" (Brasacchio et al. 2009) may represent a form of maladaptive trained immunity that could contribute to the pathogenesis of diabetes. This study also suggests that the trained immunity phenotype may not be restricted to professional immune cells and additional examples will be given in later sections.

"Microglial priming" in neurodegenerative disorders refers to the property of microglia (the resident macrophages of the central nervous system) to elicit an exaggerated inflammatory response to a secondary inflammatory stimulus, even if that would be a normal subthreshold challenge (Norden et al. 2015; Perry, Holmes 2014). Interestingly, the initial priming stimulus and the secondary challenge may be separated temporally. For instance, an inflammatory challenge in utero may affect microglial reactivity later in life (Knuesel et al. 2014). The microglia can develop a primed state with aging or after stress, traumatic brain injury, or neurodegenerative disease, and the functional consequences of subsequent exaggerated inflammatory responses include development of cognitive dysfunction, compromised synaptic plasticity, and accelerated neurodegeneration (Norden et al. 2015; Perry and Holmes 2014).

A recent review of relevant literature suggests that the priming of microglia, which are unusually long-lived tissue-resident cells (Reu et al. 2017), is mediated by epigenetic mechanisms similar to those of trained immunity involving other types of myeloid cells (histone modifications such as H3K4me1 deposition at latent enhancer sites) (Haley et al. 2017). In other words, exposure to inflammatory stimuli might lead to accumulation of epigenetic alterations in the microglia that confer a trained/hyperactive phenotype that could contribute to destructive immune responses associated with neurodegenerative conditions. As alluded to above, aging is a risk factor contributing to microglia priming. Indeed, activation of the peripheral innate immune system by intraperitoneal injection of LPS leads to increased microglia-dependent neuroinflammation in old as compared with young adult mice (Godbout et al. 2005). This form of maladaptive trained immunity might contribute to the behavioral deficits that frequently follow systemic infections in the elderly.

In a recent study, peripherally administered inflammatory stimuli induced either immune training or tolerance in the brain depending on the frequency of stimulus application. Specifically, a single intraperitoneal injection of LPS-induced tolerance, whereas four consecutive daily intraperitoneal injections of LPS-induced training; these outcomes were associated with differential epigenetic reprogramming of the microglia (Wendeln et al. 2018). This imprinted memory persisted for at least 6 months and, in the form of immune training, exacerbated cerebral inflammation and β-amyloidosis in a mouse model of Alzheimer's pathology. In contrast, pathology in the same model was alleviated when tolerance was induced (Wendeln et al. 2018). Thus immune memory in the brain, induced by peripheral stimuli, represents an important modifier of neuroinflammatory pathology. Although it has long been known that the frequency or dosing of LPS may differentially induce priming or tolerance, this cannot entirely explain the manner by which LPS regulates host immune responses to secondary challenges. Indeed, it was earlier established that tolerogenic LPS-induced chromatin modifications silence genes encoding proinflammatory mediators but not genes encoding antimicrobial effectors, which are instead primed by the same LPS stimulus (Foster and Medzhitov 2009).

## Trained Immunity, β-glucan, and Mucosal Dysbiotic Diseases

*As* mentioned above, *C. albicans* (and other *Candida* species) are commensal yeasts in mucosal surfaces of healthy individuals but may cause systemic infections in immunocompromised patients (Lionakis 2014). At least in principle, at mucosal sites colonized by *C. albicans* or other fungal species, locally produced β-glucans could

induce regional trained immunity in mature myeloid cells and the continued presence of β-glucans could ensure a sustained trained innate immune status. Periodontitis and colitis are mucosal inflammatory diseases with a dysbiotic component (Saleh and Trinchieri 2011; Stecher et al. 2013; Lamont and Hajishengallis 2015; Hajishengallis 2015). Thus although tissue damage is primarily inflicted by an exaggerated or dysregulated host immune response, suboptimal host immunity is also an unfavorable situation as it may facilitate transition from a symbiotic to a dysbiotic, hence, disease-provoking microbiota. It is therefore not easy to predict the effects of trained immunity, e.g., as induced by β-glucan, on mucosal dysbiosis-driven inflammatory disease.

β-Glucans produced by fungi at mucosal sites, such as the lung, can enter the circulation where they can persist and reach remote sites, such as the joints where β-glucans were shown to activate synovial cells (Obayashi et al. 1995; Yasuoka et al. 1996; Yoshitomi et al. 2005). Moreover, β-glucan from *C. albicans* could substitute for Freund's complete adjuvant to cause collagen-induced arthritis in DBA/1 mice (Hida et al. 2005). Of course, rheumatoid arthritis primarily represents "sterile" inflammation (Chen and Nunez 2010) and if disseminated β-glucans indeed induce trained immunity at remote sites, the trained phenotype would likely exacerbate existing rheumatoid arthritis (or perhaps trigger arthritis in susceptible individuals). However, the above-discussed findings (Obayashi et al. 1995; Yasuoka et al. 1996; Yoshitomi et al. 2005) suggest a hypothetical, if not plausible, scenario by which β-glucans released locally at mucosal barrier sites may reach the bone marrow at concentrations sufficient enough to train myeloid progenitors. Alternatively, *C. albicans*-derived β-glucan may act on local macrophages and induce IL-1β (and/or other cytokines), which in turn may reach the bone marrow to mediate trained immunity.

Although fungal pathogenesis has primarily been associated with immunocompromised states, even in systemically healthy individuals, *C. albicans* appears to contribute to the pathogenesis of periodontal disease. In this regard, although the main reservoir of *C. albicans* is the buccal mucosa, tongue, and palate, these fungal organisms can co-aggregate with bacteria in subgingival biofilms and also adhere to and infect gingival epithelial cells (Sardi et al. 2010; Sztukowska et al. 2018; Dongari-Bagtzoglou et al. 2005). *C. albicans* was shown to colonize the periodontal pockets of approximately 15–20% chronic periodontitis patients, and, in fact, hyphae were found within the underlying gingival connective tissue (Reynaud et al. 2001; Urzua et al. 2008; Jarvensivu et al. 2004). Importantly, the presence of *C. albicans* in the periodontal pockets was associated with the severity of chronic periodontitis (Canabarro et al. 2013). However, the underlying mechanisms for this association, whether causal or not, are uncertain. Equally uncertain is whether the presence of *C. albicans* in periodontal pockets is linked to induction of trained innate immunity and how this affects periodontal disease.

Gut colonization by *C. albicans* was shown to increase the incidence of allergic diarrhea in sensitized BALB/c mice, promote limb joint inflammation in collagen-induced arthritis in DBA/1J mice, and exacerbate contact hypersensitivity in NC/Nga mice (Sonoyama et al. 2011). This model involves persistent *C. albicans* gut colonization by a single intragastric inoculation in immunocompetent adult mice in the absence of antibiotics or immunosuppressants, and thus preventing systemic disseminated infections by *C. albicans*; this model therefore mimics immunocompetent humans with chronic, latent intestinal colonization by *C. albicans* (Yamaguchi et al. 2005; Sonoyama et al. 2011). The aggravation of allergic diarrhea by *C. albicans* gut colonization was attributed to increased infiltration of the colon with eosinophils and mast cells (Sonoyama et al. 2011). Moreover, the intestinal *C. albicans*-associated aggravation of hapten-induced contact hypersensitivity and collagen-induced arthritis mice was associated with increased myeloperoxidase activity, a marker of neutrophil recruitment in the inflamed tissues. Whether these enhanced host immune responses derive, at least in part, through *C. albicans*-induced trained immunity is

not known but is a plausible possibility. Even more challenging is to explain the mechanisms connecting *C. albicans* gut colonization with exacerbation of inflammatory pathology in tissues distant from the gut.

In models of colitis, β-glucan was shown to have variable effects. Mice orally pretreated with β-glucans for 14 days before initiation of dextran sulphate sodium (DSS)-induced colitis exhibited worsened colitis with increased colonic levels of inflammatory cytokines and chemokines, compared to vehicle-treated mouse controls (Heinsbroek et al. 2015). The authors speculated that this might be due to training of the immune system by β-glucan treatment (Heinsbroek et al. 2015). In contrast, another study found that β-glucan given to C57BL/6J mice as part of their diet for 26 days attenuated DSS-induced colitis, which was initiated 5 days before the end of the experimental period (Zhou et al. 2014). In another investigation, oral administration of β-glucan also mitigated DSS-induced colitis associated with improved structural integrity of tight junctions and intestinal permeability and with decreased levels of myeloperoxidase, eosinophil peroxidase, and *N*-acetyl-β-D-glucosaminidase (Han et al. 2017). Interestingly, group 2 innate lymphoid cells (ILC2s) were shown to protect against DSS-induced colitis through production of amphiregulin, a molecule that promotes restoration of tissue integrity after acute or chronic inflammation-induced damage (Monticelli et al. 2015; Zaiss et al. 2015). Whether β-glucan can also prime the immune system for enhanced homeostatic/tissue repair responses in the gut is not known. However, at least in some settings, training has been shown to promote tissue repair (below).

## Trained Tissue Repair

Tissue repair during inflammation resolution is a complex process that depends on the regenerative capacity of the tissue and the participation at the site of injury of inflammatory cells, e.g., macrophages, which contribute to wound debridement and produce growth factors, chemokines, and other metabolites that stimulate fibroblasts and other cells involved in wound healing (White and Mantovani 2013; Eming et al. 2017). Epithelial cells are not only involved in repairing the epithelial barrier but also contribute to the general tissue repair process by regulating the proliferation of fibroblasts and their production of collagen required for wound healing (Zhang et al. 2017).

A recent study has shown that the healing capacity of skin epithelial cells is enhanced by previous exposure to inflammatory stimuli (chemical [imiquimod], mechanical, or microbial [*C. albicans*]) (Naik et al. 2017). These findings strongly indicate that pre-conditioning due to previous inflammatory events is not an exclusive property of professional immune cells. Specifically, after acute inflammation, epithelial stem cells can accelerate barrier restoration following future tissue damage. Importantly, resident skin macrophages and T cells are not required for the enhanced wound repair following secondary inflammation in this model. This epithelial cell "memory" was associated with alterations at the chromatin level as determined by Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq). Following the initial inflammatory stimulus, the trained epithelial stem cells maintained chromatin accessibility at key stress response genes, which were thus transcribed faster upon a secondary challenge (Naik et al. 2017). To identify downstream effectors of the epithelial stem cell memory that confer tissue repair advantage, the authors performed pathway analysis of the rapid-response transcripts and identified "inflammasome signaling" among the top featured terms. Gain- and loss-of-function experiments with AIM2, CASP1, IL1β, and IL1R1 confirmed the involvement of the inflammasome pathway in mediating enhanced wound repair to inflammation-trained epithelial stem cells (Naik et al. 2017). In this context, gut epithelial cells can also detect tissue damage by expressing AIM2 and activating the inflammasome (Hu et al. 2016). Therefore, it would be interesting to know if epithelial stem cells in tissues other than the skin share a similar AIM2 inflammasome-associated memory of earlier inflammatory

assaults. It should be noted, however, that such memory may not always be beneficial as, at least in principle, it could amplify tissue damage upon a secondary assault (e.g., in the setting of inflammatory or autoimmune disorders) rather than promoting tissue repair.

## Summary and Perspective

Trained immunity has recently emerged as a new concept that endows innate immunity with immunological memory of past inflammatory events so that the host is poised to respond rapidly and robustly to subsequent challenges. As innate immune memory is not as specific as adaptive immune memory, it can provide cross-protection against different infections. However, by the same mechanism, maladaptive trained immunity can prime the host to become more susceptible to a range of distinct inflammatory or autoimmune disorders. Cellular metabolic pathways and chromatin modifications involved in the induction of trained immunity could be therapeutically modulated to better treat genetic or acquired immunodeficiencies (through restoration or enhancement of immune function), counteract the adverse effects of chemotherapy-induced myelosuppression, or to alleviate autoimmune and inflammatory disorders. Among the primary targets of therapeutic trained immunity are likely infants and the elderly, who are particularly vulnerable to infectious diseases although the elderly are also susceptible to inflammatory and degenerative conditions. Here, it would be important to understand which chronic inflammatory disorders are strongly linked to a trained immune phenotype and how this could be therapeutically manipulated to better treat the disease.

Although the demonstration that trained innate immunity can act at the level of the myeloid progenitor cells has resolved the paradox of long-lasting innate immune memory despite the short life of mature myeloid cells in circulation, there are still many unanswered questions. For instance, although epigenetic adaptations are strongly correlated with trained immunity, formal and specific cause-and-effect connection is currently lacking. Moreover, we are far from an in-depth understanding of the molecular mechanisms of trained immunity and we do not understand the precise mechanisms that allow trained cells to maintain their chromatin in an open state at select loci for a long time (e.g., months). During DNA replication and cell division, chromatin is disassembled and reassembled, which begs the question as to how chromatin landmarks associated with trained immunity are retained or even transmitted in the context of transgenerational innate immune memory. Of course, a low rate of cell division would facilitate maintenance of chromatin memory as compared to high-rate cell division. Another great challenge ahead is to develop ways for targeted pharmacologic manipulation of the metabolic pathways or epigenetic landscapes involved in regulation of trained immunity in order to promote optimal gene expression patterns.

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# Signaling Systems in Oral Bacteria

Daniel P. Miller and Richard J. Lamont

## Introduction

The human mouth supports several hundred species of bacteria, viruses, archaea, and fungi that reside in complex, polymicrobial communities on every hard and soft surface. Typically, this oral microbiome exists in homeostasis with the host. The close proximity of oral bacteria in supra- and subgingival plaque allows for constant communication both at an intracellular level as the bacteria are able to sense and respond to their environment, but also at the interspecies level with a wide variety of complex synergistic and antagonistic relationships (Table 1). The environment oral bacteria find themselves in is constantly changing, with local fluctuations in pH, oxygen availability, and temperature gradients, a constant flow of saliva and gingival crevicular fluid, as well as changes in immune effectors and nutrient availability. Indeed, the composition of the oral community dynamically responds to environmental changes, and through the acquisition of new species can mold the environment to its preferences rather than adapting to its surroundings. These microbiomes can remain in mutualistic balance with the host or can become dysbiotic, or destructive to the host, resulting in increased risk of dental caries and gingivitis along with other more severe periodontal diseases (Lamont et al. 2018). Our goal in this chapter is to provide a broad understanding of the strategies employed by oral bacteria to sense and respond to their environment with special emphasis on those signaling systems participating in cell–cell communication that may coordinate the polymicrobial community and promote dysbiosis.

## Two-Component Signaling in Oral Bacteria

Oral bacteria located in both the supra- and subgingival environments must be able to respond to changes in local pH, osmotic shifts, nutrient availability, as well as chemical and immunological stresses. Bacteria have widely conserved signal transduction systems that sense and respond to these environmental conditions in order to survive and thrive in the dynamic oral environment. There are several broadly conserved classes of signal transduction systems of various complexities that operate through phosphorelay signaling. The best characterized of the signaling mechanisms in bacteria are the two-component systems (TCSs), which are primarily involved in adaptation to external stimuli, but are often involved in interspecies interactions (Stock et al. 2000).

Archetypically, each TCS is composed of two proteins, a transmembrane stimulus-sensing

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**Table 1** Interactions within the oral biofilm

| Synergistic or mutualistic | Antagonistic |
|---|---|
| Co-aggregation<br>Biofilm development | Bacteriocins<br>Acidification |
| Cell–cell communication | Peroxide production |
| Metabolic syntrophy and cross-feeding | Metabolic competition |
| Cross-respiration<br>Immune subversion | Physical interactions impacting expression of adhesins and proteases |
| Transfer of genetic material | |

The oral biofilm is characterized by largely beneficial interactions that enhance the community as a whole (synergistic or mutualistic). However, antagonistic interactions allow for individual species to be more competitive within a microenvironment also occur

protein and a cytosolic response regulator (RR), which carries out some cellular change in response to the stimulus (Gao and Stock 2009). The sensor protein (normally a histidine kinase; HK) functions as a periplasmic sensing protein composed of a sensing domain and a cytoplasmic autophosphorylation domain. The N-terminal sensing domain typically comprises a signal receiver flanked by one or more transmembrane domains. While this architecture is generally conserved among HKs, the sensing domain is highly variable, allowing for broad specificity of signals to be detected. The highly conserved C-terminus is composed of the autophosphorylation domain followed by the ATPase-like catalytic domain. Almost all HKs function as homodimers with dimerization occurring within the autophosphorylation domain. Autophosphorylation of HKs was previously thought to only occur *in trans*, but some recent reports indicate HKs can autophosphorylate *in cis* (Sankhe et al. 2018). The RR is also a modular protein composed of a receiver domain and an output domain. Recognition of the extracellular signal by the HK stimulates ATP-dependent phosphorylation of a histidine residue followed by subsequent transfer of the phosphate to an aspartate residue in the receiver domain of the RR. Phosphorylation of the RR promotes conformation changes and, in some cases dimerization of the RR, that activate the RR output domain to carry-out various functions (discussed further below). Most of the TCSs are encoded as an operon with the HK, RR, and some accessory genes being co-transcribed. In some cases, termed “orphan” systems, the HK and RR are not associated and the genes encoded the two components are spatially separated.

## TCSs of *P. gingivalis*

The *P. gingivalis* genome contains relatively few complete TCSs with some strain variability on the number of complete and orphan TCS encoding genes (Table 2). The best characterized TCS in *P. gingivalis* is FimSR (33277: PGN_0904-0903; W83: PG_1432-1431). The FimSR system regulates the production of the long FimA-component fimbriae that are associated with binding to gingival epithelial cells, host matrix proteins, other bacteria, and with biofilm development. Functional analysis demonstrated that FimR positively regulates the expression of FimA and that a *fimS*-deficient strain does not express *fimA* or produce the FimA fimbriae (Nishikawa et al. 2004). Most of the work to characterize the regulation of FimSR has been done in the 33277 strain as the W83 strain contains a mutation in the HK kinase domain, preventing FimS from binding ATP and autophosphorylation. As expected, W83 lacks production of the FimA fimbriae (Nishikawa and Duncan 2010). The regulation of *fimA* gene expression is an indirect function of FimR as it does not directly bind the *fimA* promoter. The exact mechanism by which FimR regulates FimA expression remains unclear, with conflicting studies involving the genes immediately upstream of the *fimA* operon. Deletion of the HK FimS also abolishes FimA fimbrial production, confirming that a fully functional FimSR TCS is required for fimbrial gene transcription (Nishikawa et al. 2004). There is also evidence that FimR can regulate the Mfa1-component minor fimbriae, and that this regulation is mediated by direct binding of FimR to the *mfa1* promoter (Wu et al. 2007). The FimSR system appears to be constitutively

**Table 2** TCSs in select oral bacteria

| Organism | Complete TCS | Orphan components (HK/RR/hybrid) |
|---|---|---|
| *P. gingivalis* 33277 | 3 | 1/3/1 |
| *P. gingivalis* W83 | 4 | 1/2/1 |
| *T. denticola* 35405 | 5 | 4/4/1 |
| *F. nucleatum* 35586 | 6 | 1/2/0 |
| *A. actinomycetemcomitans* D11S | 3 | 2/3/1 |
| *T. forsythia* 43037 | 9 | 1/3/4 |
| *P. intermedia* 17 | 4 | 1/2/2 |
| *F. alocis* 35896 | 9 | 0/0/0 |
| *S. constellatus* C1050 | 10 | 0/2/0 |
| *S. mutans* UA159 | 14 | 0/1/0 |
| *S. gordonii* Challis | 14 | 1/2/0 |

Data were obtained using the prokaryotic two-component system (p2cs) database (Ortet et al. 2015)

active during in vitro growth. The FimS sensor domain contains multiple tetratricopeptide (TPR) domains involved in protein-binding interactions, suggesting that FimSR may sense and respond to protein or peptide levels, regulating costly fimbrial production in nutrient-deplete conditions for the asaccharolytic *P. gingivalis*.

*P. gingivalis* possesses multiple systems to acquire and transport heme as an essential source of iron (Smalley and Olczak 2017). The HaeSR system (33277: PGN_0752-0753; W83: PG_0719-0720) was identified and named for its regulation of genes involved in heme acquisition by *P. gingivalis* (Scott et al. 2013). HaeR is a transcriptional regulator, and based on chromatin immunoprecipitation and high-throughput sequencing (ChIP-seq) data, can recognize two promoter architectures allowing it to function as either an activator or a repressor. Utilizing ChIP-seq and electromobility shift assays (EMSA), Scott et al. showed that HaeR can bind to the 5′ UTR directly upstream of genes encoding known iron transport system proteins *ihtA* (PGN_0704; PG_0668), *htrA* (PGN_0687; PG_0648), and *hmuS* (PGN_0556; PG_1553) and, moreover, positively regulates their expression in response to heme (Scott et al. 2013). The same studies also identified a number of ABC transporters and TonB-dependent transporters negatively regulated by HaeR in response to heme concentrations. In addition to heme transport, HaeR is also known to directly control the expression of the Kgp and RgpA gingipains in response to heme-deplete conditions. The gingipains play a role in iron acquisition through the binding and degradation of hemoglobin, and other heme-containing proteins, to release heme. Gingipains work in concert with HmuY to capture the released heme for transport into the cytoplasm (Smalley et al. 2011). Additionally, gingipains are instrumental in the conversion of heme into μ-oxo-bisheme, which *P. gingivalis* accumulates on the surface, causing the normal black-pigmented appearance of colonies on solid media and providing protection against hydrogen peroxide (Smalley and Olczak 2017).

The PorXY TCSs are orphan proteins due to the fact they are not located in a genetic locus but they have been shown to function as a bona fide TCS. PorY (33277: PGN_2001; W83: PG_0052) is a HK that autophosphorylates and is able to transfer the phosphate to PorX (33277: PGN_1019; W83: PG_0928) (Kadowaki et al. 2016). PorXY have been shown to regulate expression of genes associated with the type IX secretion system (T9SS) of *P. gingivalis* that is responsible for transporting the gingipains and other important effector proteins across the outer membrane of the cell (Lasica et al. 2017). Mutations of either *porX* or *porY* have shown reduced secretion of T9SS proteins, reduced extracellular gingipain activity, and non-pigmentation of colonies (Sato et al. 2010). However, PorX is an atypical RR in that it does not contain a DNA-binding domain but rather is annotated to contain an alkaline phosphatase

domain. Indeed, direct examination of PorX function using EMSAs found that PorX did not directly bind to the promoter regions associated with T9SS expression (Vincent et al. 2016). Rather, PorX acts indirectly by binding to the extracytoplasmic sigma factor, SigP, which has been shown to directly interact with the promoter of T9SS-encoding genes (Kadowaki et al. 2016). The exact role PorX plays in regulating SigP activity remains unclear, whether PorX can dephosphorylate SigP or potentially displace its anti-sigma factor to promote activity remains an open question for future study. Another study found that the PorX was able to bind to the T9SS component protein PorL, but again the biological significance of this interaction remains unstudied (Vincent et al. 2016).

*P. gingivalis* also possess a hybrid TCS, GppX (PGN_1768; PG_1797), in which the sensor and response regulator domains are contained in the same protein (Hasegawa et al. 2003). GppX regulates the post-translational maturation and localization of gingipains (Hasegawa et al. 2003) and also negatively regulates expression of the *luxS* gene encoding quorum sensing molecules (see below) (James et al. 2006). RNA-Seq of a *gppX* mutant strain revealed that in addition to *luxS*, GppX controls expression of proteins important for both monospecies and heterotypic biofilm formation (Hirano et al. 2013).

An orphan RR, RprY (33277: PGN_1186; W83: PG_1089), has also been well characterized in *P. gingivalis*. There is no protein encoded by *P. gingivalis* with homology to the cognate HK, RprX. Activation of RprY responds to sodium depletion as a RprY-LacZ fusion protein in *E. coli* was specifically induced in sodium-deplete media, and an *rprY*-deficient strain of *P. gingivalis* was unable to grow under sodium depletion (Krishnan and Duncan 2013). EMSA and ChIP-on-chip assays determined the RprY regulon to include oxidative stress response genes such as *clpB*, *dnaK*, *groES*, and alkyl hydroperoxide reductase C (Duran-Pinedo et al. 2007). In the absence of RprX, *P. gingivalis* activation of RprY may involve more complex regulation of transcriptional regulatory activities. Where most TCS proteins are co-transcribed, RprY is co-transcribed with a protein acetyltransferase (*pat*; PGN_1185), which has been shown to acetylate RprY in vitro (Li et al. 2018). Acetylation of RprY is reversed by the deacetylase CobB (PGN_0004). Acetylation of RprY was shown to reduce binding to target promoters while phosphorylation increased binding to promoter DNA, suggesting multiple layers of RprY regulation. RprY was determined to be acetylated in vivo; however, the exact role of in vivo acetylation of RprY and what protein(s) are responsible for phosphorylation of RprY remain uncharacterized (Li et al. 2018).

## TCS of *T. denticola*

*T. denticola* ATCC 35405 encodes 5 complete TCSs along with 9 orphan TCS proteins (4 HK, 4 RR and 1 HK-RR hybrid protein) (Table 2). Only two of the TCSs of *T. denticola* have been characterized and will be discussed here. The first system is AtcSR (TDE0032-0033) and the second is Hpk2/Rrp2 (TDE1969-1970). Both of these TCSs are expressed throughout growth but are up-regulated during late-log and stationary phases (Frederick et al. 2011). Hpk2/Rrp2 is common in spirochetes and other oral bacteria, whereas AtcSR is unique to *T. denticola*, suggesting a possible role in niche-specific signaling. AtcS is a typical HK although it lacks a consensus sensing domain. It does, however, possess a conserved autoinducer (AI)-2 transport domain and may sense and respond to the presence of AI-2 (AI-2–dependent signaling will be discussed later in the chapter). AtcS has been shown to transfer phosphate to its cognate RR, AtcR (Frederick et al. 2008). AtcR contains a LytRT domain, the only RR in oral spirochetes to possess this DNA-binding domain, and indeed of the over 81,000 predicted bacterial RRs, only ~4% possess a LytTR domain (according to the prokaryotic two-component system database as of January 2019; http://www.p2cs.org/) (Ortet et al. 2015). A recent analysis in *T. denticola* identified 25 LytTR-binding sites composed of 3 distinct promoter architectures that regulate a possible 50 genes (Miller et al. 2014). These studies used

bioinformatics approaches to identify the LytTR-binding sites in the ATCC 35405 genome and confirmed function by EMSA studies. AtcR was shown to directly bind to promoters upstream of TDE2770 (the first gene in the flagellar assembly operon), TDE1514 (an operon encoding an ABC transport system), TDE2496 (a methyl-accepting chemotaxis protein), and TDE1971 (first gene in the operon encoding Hpk2/Rrp2). Binding to TDE2770 and TDE2496 strongly suggests that AtcSR plays a role in regulating the motility and chemotaxis of *T. denticola*. Both motility and chemotaxis of *T. denticola* are required for biofilm development in mono- and dual-species biofilms (Yamada et al. 2005). Additionally, the ability to regulate the expression of another TCS (Hpk2/Rrp2) suggests that AtcSR sits atop a complex signaling cascade with global regulatory potential.

The other characterized treponemal TCS is Hpk2/Rrp2, so named for its homology with a TCS well characterized in the Lyme disease spirochete *Borrelia burgdorferi* (Sarkar et al. 2010). Hpk2 contains an N-terminal Per-Arnt-Sim (PAS) domain, which is predicted to sense redox potential, oxygen, and light. Preliminary characterization of Hpk2 revealed increased autophosphorylation under anoxic conditions compared to an aerobic environment. Deletion of the PAS domain from Hpk2 abolished the difference in phosphorylation under different environmental conditions, suggesting that the PAS domain of Hpk2 acts as an oxygen sensor (Sarkar et al. 2010). More recent structural and functional studies of Hpk2 reveal that binding of heme to the PAS domain promotes homodimerization of Hpk2 and enhances autophosphorylation (Sarkar et al. 2018). As *T. denticola* generally resides at the leading edge of the subgingival oral biofilm, it may be exposed to constant changes in oxygen tension, and Hpk2 through the PAS domain may sense and respond to these environmental cues. Rrp2 is a σ-54–dependent transcriptional regulator, named on the basis of homology with the borrelial Rrp2 RR. Yet, the Rrp2 in *Borrelia* activates the RpoN-RpoS regulatory pathway that controls the tick-to-host cycle, whereas *T. denticola* lacks both the enzootic lifecycle and any homolog for RpoN (Frederick et al. 2011). So while the regulon of Rrp2 in *T. denticola* remains unclear, it is sure to regulate genes in response to environmental cues as Hpk2 activity is oxygen dependent.

## Serine/Threonine and Tyrosine Phosphorylation Systems in Oral Bacteria

Nearly 40 years ago, the first bacterial serine/threonine (Ser/Thr) phosphorylation systems were identified, and after decades of research, we now know that Ser/Thr phosphosystems play an integral role in bacterial pathogenicity regulating important cellular processes including metabolism, stress responses, and cell division. While our understanding of Ser/Thr phosphorylation in bacteria has developed over many years, only recently have we understood that phosphorylation of tyrosine residues also occurs in bacteria, previously thought to be an eukaryotic-exclusive post-translational modification (PTM). Emerging research into this relatively new class of PTM in bacteria also shows that Tyr phosphorylation can influence protein function and cellular localization to regulate key systems including capsule production, stress responses, motility, secondary metabolism, and virulence (Whitmore and Lamont 2012).

Ser/Thr phosphorylation in bacteria is primarily carried out by Hanks-type kinases, and these kinases are historically referred to as "eukaryotic-like" due to their homology with previously well-defined systems, although there is no evidence to suggest that these genes were horizontally transferred to bacteria from eukaryotic sources (Cozzone 2005). Recent phylostratigraphic analysis suggests that bacterial, archaeal, and eukaryotic Hanks-type Ser/Thr kinases share an evolutionary ancestor and did not arise from convergent evolution or a horizontal transfer event (Stancik et al. 2018). Due to this shared lineage, the structural domains and function of all Hanks-type kinases from all organisms are very similar. This is in contrast to the bacterial tyrosine kinases (BY-kinase), which are structurally and

mechanistically distinct from those found in eukaryotic systems.

Ser/Thr/Tyr phosphorylation networks do not have specific DNA-binding elements that directly regulate transcription; they rather can phosphorylate multiple transcription factors to activate or repress gene transcription. In this way, Hanks-type kinases and BY-kinases are distinct from TCS in that they phosphorylate many substrates and often influence diverse cellular processes, while HK are highly restricted to a single RR substrate. An ever expanding number of microbiome studies and detailed molecular work in *Bacillus subtilis* and *Mycobacteria tuberculosis* clearly demonstrate that Ser/Thr/Tyr phosphorylation can all occur on the same substrates and often involves phosphorylation of other kinases (Shi et al. 2014; Prisic et al. 2010). There is mounting evidence that both BY-kinases and Hanks-type kinases serve as complex signaling hubs, and their roles in regulating the physiology of oral bacteria are an emerging topic.

*Streptococcus mutans,* the major etiological agent in dental caries, resides in the supragingival oral biofilm. *S. mutans* can efficiently metabolize carbohydrates to produce lactic acid, resulting in a pH reduction that causes demineralization of the enamel and dentin. Major virulence determinants of *S. mutans,* including adherence to hydroxyapatite surfaces and development of biofilms with acidic microenvironments, are all associated with the Hanks-type kinase PknB and a Ser/Thr protein phosphatase PppL. *S. mutans* contains only a single Ser/Thr protein kinase and phosphatase, which makes it an ideal model organism for studying the pleiotropic effects of Ser/Thr phosphorylation networks (Hussain et al. 2006). PppL is located immediately upstream of PknB, and the two genes are co-expressed. Genetic deletion of PknB attenuated biofilm development by reducing biofilm thickness and maturation of nutrient channels compared to the parental strain but without a significant change in long-term growth of *S. mutans*, suggesting a specific role in biofilm development (Hussain et al. 2006). Additional biofilm studies using the *pknB*-deficient strain as well as strains with deletion of *pppL* and a *pknB/pppL* double mutation revealed that the kinase- and phosphatase-deficient strains had comparable reduced biofilm thickness, while the double mutant had an increased reduction in biofilm thickness (Banu et al. 2010). Further study of the *S. mutans* deletion strains showed reduced resistance to acidic growth conditions and diminished ability to survive oxidative and osmotic stresses. The impact of PknB and PppL on *S. mutans* was also demonstrated in vivo using a rat dental caries model (Banu et al. 2010). All three mutants showed reduced advanced caries compared to the parental strain. These data suggest that Ser/Thr phosphorylation plays an important role in the pathogenicity of *S. mutans* and the development of dental caries.

*S. mutans* competes with other oral streptococci in the oral cavity through the production of bacteriocins, antimicrobial peptides that target closely related bacteria. One particular antagonistic relationship is with *S. sanguinis*, with which *S. mutans* primarily competes for nutrients, and which is reciprocally antagonistic to *S. mutans* through the production of $H_2O_2$ (Kreth et al. 2008). Ser/Thr signaling is instrumental to the fitness of *S. mutans* in this competitive oral ecosystem. In one study, examination of a *pknB*-deficient strain demonstrated that PknB is involved in the resistance to oxidative stress from $H_2O_2$, and this strain was thus more sensitive to competition with *S. sanguinis* than the wild type (Zhu and Kreth 2010). The exact role of PknB in oxidative stress remains unclear; however, this study confirms the results previously discussed. Transcriptomic analysis of *S. mutans* suggests that there is signaling cross talk between PknB/PppL and the VicKR and ComDE TCSs (Banu et al. 2010). Deletion of PknB reduced the expression of two bacteriocins and genetic competence genes, which were also down-regulated in a *vicK* mutant. The overlap of the PknB/PppL regulon and that of VicKR may also include resistance to oxidative stress. While no direct evidence has been shown that PknB can either activate the HK or phosphorylate the RR of either system, the phosphorylation of the RR CovR by the *Streptococcus agalactiae* Ser/Thr kinase was found to occur within the signal receiver domain and this prevented binding of CovR to DNA (Lin et al. 2009).

*P. gingivalis* produces a haloacid dehydrogenase family phosphoserine phosphatase, SerB, which is located on the outer membrane of the cell and is secreted upon contact with the gingival epithelium. SerB is transported into epithelial cells and regulates cytoskeletal architecture involving tubulin microtubules and actin microfilaments (Hasegawa et al. 2008; Tribble et al. 2006; Zhang et al. 2005). Most importantly for the oral community as a whole, SerB is able to down-regulate IL-8 secretion from epithelial cells, hence, reducing PMN recruitment (Hasegawa et al. 2008; Takeuchi et al. 2013). The localized dysregulation of IL-8 will induce a transient and localized state of chemokine paralysis and disrupt the local mucosal defense. In this function, *P. gingivalis* may exert immune regulatory functions that can impact the whole subgingival community, consistent with its role as a keystone pathogen (Darveau et al. 2012; Hajishengallis et al. 2012). While SerB has extensively been characterized, a Ser/Thr kinase has yet to be characterized for this species. There is one gene annotated as a Ser/Thr kinase in the *P. gingivalis* genome (33277: PGN_0762; W83: PG_0731) due to a conserved PASTA (penicillin binding and Ser/Thr kinase associated) domain; however, this protein is yet to be tested for kinase activity.

*P. gingivalis* is the only oral species with a characterized Tyr phosphorelay system, consisting of a single BY-kinase (33277: PGN_1524; W83: PG_0436) named Ptk1 and a low-molecular weight protein tyrosine phosphatase named Ltp1 (33277: PGN_0491; PG_1641). The first set of studies sought to identify *P. gingivalis* genes that are differentially regulated in dual-species communities with *S. gordonii* and determined that Ltp1 was significantly induced upon incubation with *S. gordonii* (Simionato et al. 2006). An *ltp1* mutant strain showed increased homotypic and heterotypic biofilm formation with *S. gordonii*, suggesting that it functions to constrain community development (Maeda et al. 2008; Simionato et al. 2006). The regulation of *P. gingivalis* biofilm development may be twofold (at least) as Ltp1 negatively regulates the expression of *luxS* (enzyme responsible for AI-2 synthesis and quorum sensing) and EPS production. Additional studies suggested that Ltp1 operates through the transcriptional regulator CdhR (Chawla et al. 2010). CdhR was determined to negatively regulate expression of *mfa1* and *luxS* while positively regulating the expression of *ltp1* and the *hmu* hemin acquisition operon (Chawla et al. 2010; Wu et al. 2009). Molecular and functional characterization of the BY-kinase Ptk1 demonstrated that it autophosphorylates up to seven Tyr residues within a C-terminal cluster and that kinase activity is essential for EPS production and biofilm development with *S. gordonii* (Liu et al. 2017; Wright et al. 2014). Substrate phosphorylation studies also determined that Ptk1 can phosphorylate EPS-associated genes PGN_0224 and PGN_0613 as well as CdhR (Liu et al. 2017; Wright et al. 2014). The pleiotropic regulation of the Tyr phosphorelay system suggests that it may act as a global regulator of community development and mediate other polymicrobial interactions. While *P. gingivalis* is the only oral bacteria with a characterized Tyr phosphorylation system, there are several other oral bacteria including *Prevotella* spp., oral streptococci, and *Neisseria*, as well as lactobacilli with proteins homologous to known BY-kinases.

## Secondary Messengers and Metabolic Signaling

### Quorum Sensing in Oral Bacteria

The oral biofilm is composed of complex community derived from multiple species that transition from assemblages of individual organisms to stable communities. These stable communities in fact undergo fluctuations in bacterial composition in response to environmental and microbial factors. Community development and maturation is a complex process that is the sum of various synergistic and antagonistic interactions all occurring in oral community but the net effect is a stable polymicrobial environment that is more beneficial to the bacteria for nutrient acquisition and processing, protection from immune responses, and resistance to environmental

stresses. The bacteria within the oral community are able to sense and respond to each other by the production and detection of small chemical signals, secondary messengers, and metabolites. One method oral bacteria utilize to coordinate activities in the subgingival biofilm is quorum sensing. Bacteria can indirectly sense bacterial cell density through detection of increasing concentrations of quorum sensing molecules, termed autoinducers (Waters and Bassler 2005; Whiteley et al. 2017). There are two, chemically distinct autoinducers recognized in bacteria. The first, autoinducer-1 (AI-1), is an acyl-homoserine lactone, and this molecule functions in intraspecies cell–cell signaling. Quorum sensing mediated by AI-1 has not been found in periodontal pathogens and has been suggested to be absent in oral bacteria (Frias et al. 2001). The second cell–cell signaling system is composed of several interconvertible molecules that derive from 4,5-dihydroxy-2,3-pentanedione (DPD), which is a product of the LuxS enzyme. DPD spontaneously rearranges into a series of interconvertible molecules, collectively referred to as autoinducer-2 (AI-2). Interestingly, environmental conditions can influence the forms of AI-2 present at equilibrium, including pH or whether AI-2 can be boronated (Semmelhack et al. 2005). Additionally, two structurally distinct AI-2 receptors have been identified that allow specific detection of the multiple forms of AI-2. A number of *luxS* homologs have been identified in oral bacteria, suggesting that the ability for cell–cell communication via AI-2 is common.

Studies involving AI-2–based signaling among oral bacteria have predominantly focused on interactions with oral streptococci. Using an in vitro biofilm model, *S. gordonii* and *P. gingivalis* form AI-2–dependent dual-species biofilms (McNab et al. 2003). Deletion of *luxS*, and thus reduced AI-2, from both species attenuated biofilm development. These were some of the first studies to link AI-2 signaling to bacterial communication in dental plaque communities. AI-2 has also been shown to be essential for interactions between *S. gordonii* and *Veillonella atypica*, *P. gingivalis* and *Filifactor alocis*, and monospecies biofilm development in *Eikenella corrodens* and *S. anginosus* (Azakami et al. 2006; Wang et al. 2013; Mashima and Nakazawa 2014). Interesting work by Rickard et al. found that *S. oralis* and *Actinomyces naeslundii* dual-species biofilm formation is dependent upon AI-2 (Rickard et al. 2006). Unlike other studies, the interaction between *S. oralis* and *A. naeslundii* is extremely sensitive to the concentration of AI-2. Genetic deletion of *luxS* from *S. oralis* abolished biofilm development and the mutualistic interaction could only be restored using picromolar concentrations of AI-2. If the concentration was below or exceeded picomolar levels, biofilm development was delayed. Context for the significance of these findings is provided by studies showing that supernatant from late-colonizing bacteria (*P. gingivalis*, *Fusobacterium nucleatum,* and *Prevotella intermedia)* stimulated AI-2–dependent responses in a *Veillonella harveyi* reporter strain up to 100-fold more than early colonizers such as *S. mutans*, *S. oralis*, or *S. mitis* (Frias et al. 2001). The ability of pathogenic oral bacteria to induce significantly more quorum sensing responses suggests that they may have a disproportionate role in shaping oral community development and activities. This is most strongly supported by studies involving *P. gingivalis*, which has been shown to form dual-species biofilms with *Aggregatibacter actinomycetemcomitans*, *T. denticola*, and *S. gordonii*, which are all aborted in a *P. gingivalis luxS* mutant strain (McNab et al. 2003; Yamada et al. 2005).

In gram-positive bacteria, quorum sensing is mediated by competence stimulating peptides (CSPs), also termed autoinducing peptides (AIPs), that bind to a membrane HK and the response is transduced through a TCS. In most of these cases, the CSP is produced from the *comC* gene, which is proteolytically cleaved at a Gly-Gly site and the mature CSP is transported through the ComAB ABC transport system. These genes are also commonly found in an operon with the CSP-binding HK (ComD) and the cognate RR (ComE) (Havarstein et al. 1997). CSP-mediated signaling is typically species specific and has been shown to regulate bacteriocin production, genetic competence, and biofilm development. CSPs have been identified in

several oral streptococci including *S. mitis*, *S. gordonii*, *S. constellatus*, *S. mutans*, and *S. intermedius* (Havarstein et al. 1997). In *S. mutans*, the ComC-dependent CSP produces a 18 amino acid peptide that is recognized by ComDE, and ComE has been shown to directly regulate genes involved in CipB bacteriocin biosynthesis (Hossain and Biswas 2012; Perry et al. 2009). With regard to competence, transcription of roughly 30 competence-associated genes is regulated by the sigma factor ComX (also known as SigX) (Khan et al. 2016). ComX is induced by both the ComC-CSP as well as a 7-amino acid CSP derived from the *comS* gene (also known as comX-inducing peptide or XIP) (Khan et al. 2012). While CSP signaling is species specific, a number of oral bacteria have been shown to antagonize *S. mutans* CSP-mediated signaling. Wang et al. reported that *P. gingivalis* and *T. denticola* reduced *S. mutans* transformation efficiency and bacteriocin production by degrading CSP, while *S. gordonii*, *S. mitis*, *S. oralis*, and *S. sanguinis* also degraded CSP leading to reduced *S. mutans* biofilm development (Wang et al. 2011a, b). Oral streptococci can also interact and form biofilms with the pathogenic fungus *Candida albicans*. Interestingly, CSP production by *S. gordonii* was shown to constrain extracellular DNA and dual-species biofilm development with *C. albicans* (Jack et al. 2015). While the complete roles of CSP signaling in the mouth remains unclear, these studies demonstrate the existence of complex, cross-species, and cross-kingdom signaling in the oral biofilm.

## Cyclic Nucleotides as Secondary Messaging Molecules

Another important mechanism for oral bacteria to respond to changes in their environment is through secondary messaging molecules. One of the best characterized is cyclic dimeric guanosine 3′,5′-monophosphate (c-di-GMP), a molecule that has been extensively studied for its role in regulating biofilm development (Hengge et al. 2016). Levels of c-di-GMP in bacterial cells are regulated by the activities of diguanylate cyclases for synthesis, and phosphodiesterases (PDE) that degrade c-di-GMP. Diguanylate cyclases possess a conserved GG(D/E)EF domain that catalyzes the synthesis of c-di-GMP and two molecules of pyrophosphate from two molecules of GTP. The degradation of c-di-GMP is carried out by PDE enzymes that contain either an EAL or HD-GYP domain. Intracellular c-di-GMP is sensed by proteins that carry a PilZ-domain and by c-di-GMP-dependent riboswitches that regulate transcription of genes responsive to the concentrations of the cyclic nucleotide. A number of cellular processes including motility and chemotaxis, cell–cell communication, and exopolysaccharide production have all been shown to be controlled in response to c-di-GMP. Another very recently identified, similar messenger is c-di-AMP, which has also been shown to regulate bacterial biofilm development, fatty acid synthesis, and maintenance of the cell wall (Opoku-Temeng et al. 2016). C-di-AMP is synthesized by diadenylate cyclases and again degraded by the actions of PDEs. Signaling in oral bacteria by cyclic nucleotides has been severely understudied considering the significant impact it may play in promoting synergistic interactions and polymicrobial lifestyles.

*T. denticola* encodes seven proteins with a GGDEF domain, two proteins that contain both a GGDEF and an EAL domain, which are both inner membrane and cytosolic, and two PilZ-containing c-di-GMP binding proteins (Frederick et al. 2011). These systems were determined to be functional, as intracellular c-di-GMP was detected using high-performance liquid chromatography (Kostick et al. 2011). Genetic deletion of TDE0214, a PilZ-domain containing protein, reduced *T. denticola* motility and chemotaxis, biofilm development, and virulence in a murine infection model (Bian et al. 2013).

*P. gingivalis* was originally described as lacking a c-di-GMP signaling network; however, recent work identified intracellular c-di-GMP, thus suggesting that it can be synthesized by *P. gingivalis*. Chaudhuri et al. provided additional insight through the deletion of PGN_1932, revealing this protein to function as a diguanylate cyclase with a 60% reduction in intracellular

c-di-GMP compared the parental strain (Chaudhuri et al. 2014). Deletion of PGN_1932 significantly altered FimA fimbriae production with a subsequent reduction in attachment to solid surfaces and HeLa cells. Bioinformatics analysis of the ATCC 33277 genome suggests that there are two genes (PGN_0239 and PGN_0282) annotated as PDE proteins, but no c-di-GMP binding proteins were identified. While clearly a large amount of remaining research into cyclic nucleotide signaling in *P. gingivalis* is required, these preliminary reports suggest that it's a topic worthy of investigation.

In *S. mutans*, there is evidence that both c-di-GMP and c-di-AMP regulate EPS and biofilm development. Prior genome analysis of *S. mutans* strain UA159 did not identify any diguanylate cyclases genes; however, a study by Yan et al. described a UA159 protein (AAN59731) that when cloned into *E. coli* acted as a diguanylate cyclase (Yan et al. 2010). While AAN59731 does not contain a GG(D/E)EF domain and biochemical or structural characterization of its activity has not been described, an AAN59731 knockout does have a defective biofilm phenotype. Collectively, there is circumstantial evidence that *S. mutans* utilizes c-di-GMP, but there are several questions that remain as to whether the system is truly active and what the biological implication of such a system would be. There is, however, very strong evidence that c-di-AMP plays an important role in *S. mutans* signaling. *S. mutans*, like most gram-positive bacteria, encodes only a single diadenylate cyclase, CdaA. There are also two PDEs that function in *S. mutans*. The first is PdeA (also reported as GdpP for its homology to the first reported PDE in *Bacillus subtilis*), which degrades c-di-AMP to pApA the substrate for the second PDE, DhhP, finally producing two molecules of AMP. DhhP activity is specific for pApA, and c-di-AMP cannot be utilized as a direct substrate (Konno et al. 2018). There are also two c-di-AMP binding proteins, CabPA and CabPB. In initial studies, deletion of the *cdaA* gene resulted in reduced intracellular c-di-AMP levels, decreased resistance to $H_2O_2$, and increased EPS production (Cheng et al. 2016). However, a second study released in the same year found reduced EPS production in the *cdaA*-deficient strain compared to the parental strain (Peng et al. 2016a). More clarity on the role of c-di-AMP in *S. mutans* was gained from deletion of *pdeA* (Peng et al. 2016b). In these studies, c-di-AMP levels were elevated and biofilm development was enhanced. Increased biofilm development was dependent upon the up-regulation of a major glucan-producing protein, GtfB, as deletion of *gtfB* in the PdeA-knockout abolished the enhanced biofilm development. Further investigation convincingly showed that CabPA, but not CabPB, binds to the response regulator VicR, a known transcriptional regulator of *gtfB* (Peng et al. 2016b). Deletion of the second PDE, DhhP, and a double PDE mutant also showed increased biofilm development compared to wild type. Collectively, these results nicely show that c-di-AMP homeostasis in *S. mutans* is critical to the regulation of biofilm development (Konno et al. 2018).

Hence, although initial bioinformatic analysis of oral bacterial genomes determined that many bacteria did not have the essential enzymatic machinery for functional cyclic nucleotide signaling pathways (Romling et al. 2013), recent studies in *P. gingivalis*, *F. nucleatum,* and *S. mutans* dispute those initial annotations and suggest that there may be plenty more to be learned from studying these signaling systems in the future (Chaudhuri et al. 2014; Gursoy et al. 2017; Yan et al. 2010).

## Metabolic Signaling

Within the subgingival biofilm, many bacteria are intimately associated with one or more physiologically compatible species. This close proximity facilitates the action of many of the signaling systems described in this chapter but also allows cooperative metabolism (Fig. 1). Metabolic communication can result from one of two processes; either metabolic cross-feeding where one species secretes small, simple metabolites, which may be utilized to benefit neighboring species or secondly metabolic syntrophy, the sequential metabolism of complex nutrients for community benefit.

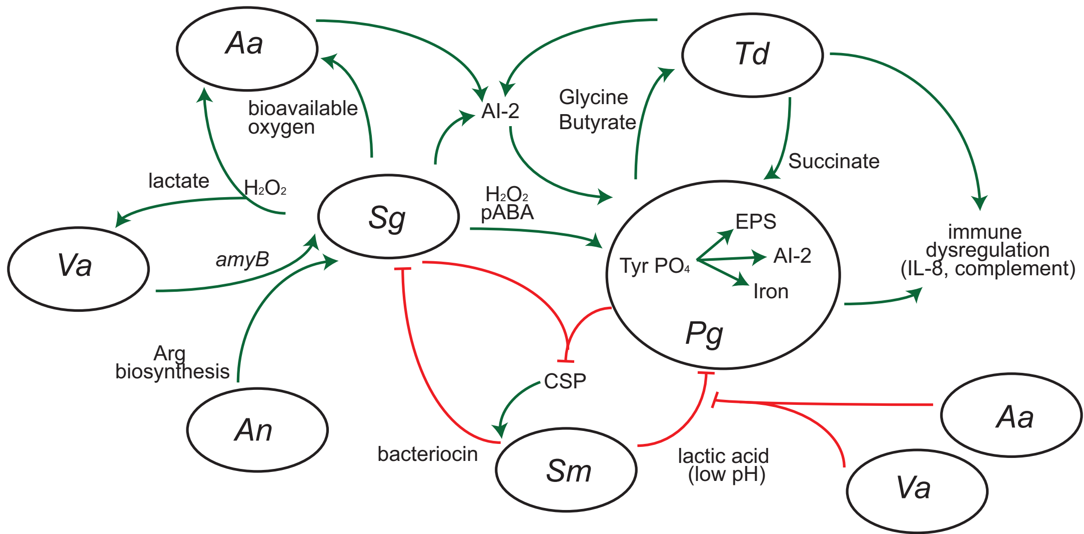


**Fig. 1** Overview of interactions in the oral biofilm. *Abbreviations*: *A. actinomycetemcomitans* (*Aa*), *Veillonella atypica* (*Va*), *Actinomyces naeslundii* (*An*), *Streptococcus gordonii* (*Sg*), *Streptococcus mutans* (*Sm*), *Porphyromonas gingivalis* (*Pg*), and *Treponema denticola* (*Td*). Synergistic interactions are shown with green arrows, and antagonistic interactions are shown with red arrows, with the direction of the arrows indicating the signaling information flow

Multiple studies have demonstrated metabolic communication in dual-species experiments. In one case of synergistic growth, *F. nucleatum* enabled the growth of *P. gingivalis* in oxygenated environments, suggesting that the more aerotolerant *F. nucleatum* can consume oxygen, providing a microenvironment with differential redox potential in oral biofilms to benefit the growth of more oxygen-sensitive organisms (Diaz et al. 2002). In another study, *A. naeslundii* was incubated in a medium that did not support its growth, and then with the addition of *S. oralis*, both species displayed robust growth and co-aggregation, suggesting that close association promotes metabolic cross-feeding and enhanced growth (Palmer et al. 2001). Similarly, *A. naeslundii* enhances the growth of *S. gordonii* by increasing expression of arginine biosynthesis genes and detoxifying hydrogen peroxide (Jakubovics et al. 2008a, b). Metabolism may also have indirect benefits to other community members; the lactic acid utilizing *Veillonella atypica* and *A. actinomycetemcomitans* could prevent the acidification of the oral biofilm, thus protecting the acid-sensitive organisms such as *P. gingivalis*. A study by Egland et al. demonstrated that *Veillonella atypica* induces the expression of the *amyB* gene encoding an α-amylase of *S. gordonii* (Egland et al. 2004). Increased utilization of carbohydrates by *S. gordonii* results in increased production of lactic acid, the preferred energy source for *V. atypica,* and hence, this is a mutually synergistic nutritional interaction.

*S. gordonii* is a participant in several instances of mutualistic growth. *S. gordonii* catabolizes carbohydrates and secretes $H_2O_2$ and L-lactate, a major carbon source for *A. actinomycetemcomitans*, and can promote *A. actinomycetemcomitans* growth by cross-feeding (Brown and Whiteley 2007). Interestingly, the relationship between *A. actinomycetemcomitans* and *S. gordonii* is a balance of synergistic cross-feeding and antagonistic interactions. Stacy et al. nicely demonstrated that *A. actinomycetemcomitans* spatially positions itself close enough to *S. gordonii* to benefit from the secretion of L-lactate, but far enough away for it to detoxify $H_2O_2$ through the production of catalase (Stacy et al. 2014). *A. actinomycetemcomitans* up-regulates *dspB*, a gene encoding the enzyme dispersin B that hydrolyzes polysaccharides and promotes *A. actinomycetemcomitans* biofilm dispersal, in response to oxygen

and $H_2O_2$ (Kaplan et al. 2004). Many other oral streptococci produce $H_2O_2$ in the oral environment and similar homeostatic mechanisms to maximize energy gains from metabolic cross-feeding while minimizing oxidative stress may be present in shaping the spatial organization of dental plaque. An additional nuance in the interaction between *S. gordonii* and *A. actinomycetemcomitans* was revealed using an *A. actinomycetemcomitans* transposon-insertion pool followed by high-throughput sequencing (TN-Seq) (Stacy et al. 2016). *S. gordonii* increased the availability of oxygen to *A. actinomycetemcomitans* to use as a terminal electron acceptor, shifting the metabolism of *A. actinomycetemcomitans* from fermentation to oxidative respiration. This was termed cross-respiration and enhanced the growth and fitness of *A. actinomycetemcomitans*.

The presence of *A. actinomycetemcomitans* in an in vitro, multispecies biofilm is able to regulate the proteomic profile of the entire complex community, suggesting *A. actinomycetemcomitans* possesses the capability of global regulation of community development (Bao et al. 2015). Additional studies to understand this community regulator identified the histone-like family of nucleoid structuring (H-NS) protein of *A. actinomycetemcomitans.* H-NS proteins act as translational silencers in many gram-negative bacteria with global regulatory potential. In *E. coli*, H-NS regulates roughly 5% of the transcriptome and deletion of the *hns* gene attenuates biofilm development (Hommais et al. 2001). Characterization of an *hns* deletion strain of *A. actinomycetemcomitans* determined that H-NS promotes monospecies biofilm development as well as adhesin production (Bao et al. 2018). Analysis of the protein expression of the in vitro polymicrobial communities with the *hns* deletion and parental strains of *A. actinomycetemcomitans* demonstrated that H-NS regulates the community metabolic capability, specifically those processes involved in peptide, carbohydrate, and malate metabolism (Bao et al. 2018).

*S. gordonii* is also an essential partner with *P. gingivalis* promoting increased biofilm development and synergistic pathogenicity in rodent models of periodontitis (Daep et al. 2011; Periasamy and Kolenbrander 2009). In addition to the *P. gingivalis* tyrosine phosphorylation system discussed previously, *S. gordonii* metabolism can also regulate dual-species biofilm and virulence of *P. gingivalis*. In one study to examine the role of *S. gordonii*-associated genes that control biofilm development with *P. gingivalis*, *spxB* and *cbe* were determined to be instrumental in promoting biofilm development (Kuboniwa et al. 2006). SpxB is responsible for the production of $H_2O_2$ in aerobic conditions, but its role in promoting biofilm development with *P. gingivalis* remains unclear. Cbe (chorismate-binding enzyme) enzymes synthesize para-aminobenzoic acid (pABA) for secretion and folate biosynthesis. Interestingly, pABA was found to promote survival and colonization of *P. gingivalis* in vivo using a mouse oral model (Kuboniwa et al. 2017). Proteomic and metabolic analyses of *P. gingivalis* utilization of pABA showed that *P. gingivalis* can scavenge streptococcal-derived pABA for folate biosynthesis, and, moreover, pABA reduced the overall stress of *P. gingivalis* while promoting production of both FimA and Mfa1 fimbriae. Most surprisingly, pABA reduced the production of EPS and dampened the virulence of *P. gingivalis* in both mouse oral and abscess models. The regulation of EPS was determined to be through the pABA-induced regulation of Ltp1 previously discussed. These interactions will depend on density and spatial configuration, again supporting the developing narrative that metabolic communication promotes structure and polymicrobial synergy within the oral biofilm.

Both the interactions of *S. gordonii* with *A. actinomycetemcomitans* and *P. gingivalis* highlight the synergistic interactions between early colonizing commensal bacteria and more pathogenic late colonizers. One example of synergism involving two periodontal pathogens is the metabolic cross talk between *T. denticola* and *P. gingivalis*. When cultured together, *P. gingivalis* and *T. denticola* co-aggregate and display enhanced growth (Grenier 1992; Tan et al. 2014). Gas–liquid chromatography of co-grown culture supernatants demonstrated that *P. gingivalis* produces

isobutyric acid that stimulates the growth of *T. denticola*, while *T. denticola* secretes succinic acid that is utilized by *P. gingivalis* (Grenier 1992). Additional transcriptome and metabolic analyses demonstrated that *T. denticola* induces the production and secretion of glycine by *P. gingivalis* to promote its own growth in mixed culture (Tan et al. 2014). Transcriptional evidence also suggests that *P. gingivalis* produces thiamine pyrophosphate, an essential nutrient for *T. denticola*; however, direct cross-feeding of this metabolite has not been established. Recent in vivo metatranscriptomic studies suggest that metabolic cross-feeding takes place during periodontal disease and may be a significant contributor to synergistic pathogenicity (Deng et al. 2018; Nowicki et al. 2018).

There are decades of studies demonstrating enhanced growth among oral bacteria in addition to the few examples detailed here. Mutualistic metabolism and communication promote stability of the oral biofilm and reduce direct competition for nutrients among biofilm constituents. Spatial and temporal arrangement of organisms in the complex polymicrobial community may also be a function of shared metabolism. A bioinformatics analysis of metabolic pathways of 11 oral bacteria found a large metabolic redundancy in the community, and metabolic capabilities varied among organisms associated with specific layers of the biofilm (Mazumdar et al. 2013). A recent metatranscriptomic analysis of periodontally diseased sites compared to patient-matched healthy sites revealed that metabolic capability was more stable during periodontitis than was species level diversity, suggesting the overall metabolic potential of the community may be more correlative with pathogenicity than species composition (Jorth et al. 2014). This overall metabolic stability is likely due to shared environmental conditions and stresses; however, true biofilm homeostasis is a balance between this metabolic redundancy and metabolic cross-feeding. If the metabolic capabilities and necessities of the community were too similar, individual species would be in constant competition for nutrients. However, the conservation of core metabolic functions and mutualistic metabolism between integral species reduces the antagonistic interactions and promotes community homeostasis.

Future studies will provide more insight into the complex web of cell–cell communication and signaling cascades utilized by oral bacteria to promote synergistic interactions. The oral biofilm is substantially more than the sum of its parts, with a community-dependent metabolic potential and a structure stabilized by interactions that promote survival of the community as a whole. Current and future technologies will allow for a better understanding of these community-level interactions. Demystifying the languages of bacterial communication may ultimately lead to more fruitful development of preventative and therapeutic interventions for periodontal diseases.

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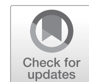

# Origin of Th17 Cells in Type 2 Diabetes-Potentiated Periodontal Disease

Barbara S. Nikolajczyk and Dolphus R. Dawson III

## Introduction

Type 2 diabetes (T2D) is a heterogeneous group of disorders with the common characteristics of insulin resistance, impaired lipid/carbohydrate metabolism, chronic inflammation, and low bone quality (Zhou et al. 2013; Farr et al. 2014; Zhu and Nikolajczyk 2014). Over 30 million U.S. residents have T2D. An additional 34% of the population has prediabetes and are therefore at high risk for T2D (CDC National Diabetes Statistics Report 2017). T2D doubles the risk for periodontitis (PD); therefore, people with T2D disproportionately contribute to the ~50% of the U.S. population that have at least some periodontal disease (Eke et al. 2012, 2015). People with T2D have higher risk of PD and present with fewer teeth and more periodontal attachment loss compared to people who are not hyperglycemic (Botero et al. 2012). The relationship between T2D and PD is reciprocal as evidenced by the impact of periodontal infections on diabetes pathogenesis (Loe 1993; Lamster et al. 2008; Iacopino 2001; Mealey and Rethman 2003). Although the ability of PD to specifically support hyperglycemia in T2D remains controversial (Botero et al. 2012; Engebretson et al. 2013; Borgnakke et al. 2014), modest/transient efficacy of PD treatments in normalizing glycemic control plus the negative impact of T2D on oral tissue regeneration highlights the critical need for new approaches to alleviate periodontal complications that plague people with T2D (Engebretson et al. 2013; Lumelsky 2007; Pacios et al. 2012).

Many studies show that physiological changes in T2D such as increased blood glucose, advanced glycation end product formation, and endotoxemia increase responses of various immune cells ex vivo (Koya and King 1998; Lalla et al. 2000, 2001; Cani et al. 2007; Jagannathan et al. 2010; Defuria et al. 2013; Gyurko et al. 2006; Shanmugam et al. 2003). These studies support the possibility that similar changes occur in vivo to promote PD in T2D, but definitive work is needed to solidify circumstantial evidence that T2D "primes" immune system cells to foster PD. Our recent work significantly narrows the gap between in vitro and in vivo results by showing that (1) B cells are altered to become proinflammatory in T2D; (2) B cells support Th17 T-cell inflammation in T2D; (3) T2D-potentiated changes in B-cell function promote chronic PD; (4) B cells *do not* promote chronic PD in non-T2D hosts; and (5) T cells and monocytes/macrophages are insufficient to promote PD in

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B-cell–null hosts with T2D (Jagannathan et al. 2010; Defuria et al. 2013; Zhu et al. 2014). These findings must be interpreted in the context of data suggesting that T-cell–mediated mechanisms promote chronic PD in lean hosts (Ernst et al. 2007; Vernal et al. 2006) and demonstrations that B-cell–mediated mechanisms promote acute PD in lean hosts (Abe et al. 2015).

This chapter summarizes evidence from our lab and others that B-cell intrinsic changes combine with B-cell–regulated T-cell function to increase cytokine secretion by Th17 cells, a $CD4^+$ T cell subset that has recently become appreciated as a prominent player in human PD (Abusleme and Moutsopoulos 2017). We propose that a T2D-associated increase in Th17 function, supported by B cells, underlies the increased risk of PD in metabolic disease (Fig. 1).

**Fig. 1** Model of lymphocyte function in T2D-potentitated PD. B cells are "primed" by type 2 diabetes (T2D) as indicated by "*". Although the exact nature of priming is unknown, primed B cells support cytokine production by Th17 cells. Given that Th17 cells promote human periodontitis (PD), B-cell priming is one mechanism that may drive increased risk and pathogenesis of PD in T2D

## Th17 T Cells Play Roles in Human PD and T2D

### $CD4^+$ T Cell Subsets in Periodontal Disease

$CD4^+$ T cells are subcategorized into multiple subsets, which include Th1s, Th2s, and Th17s among others (Hirahara and Nakayama 2016). Older studies with the technical limitations of the time concluded that a stable Th1 response characterizes stable periodontal lesions, while Th2 cells dominate progressive periodontitis (Fujihashi et al. 1996; Yamamoto et al. 1997a). These conclusions were based mainly on presence/absence of mRNA for the non-Th1 cytokine IL-10. A more comprehensive analysis indicates that a simple Th1/Th2 model does not adequately describe the immunological drivers of PD (Gaffen and Hajishengallis 2008). A third $CD4^+$ T-cell subset, anti-inflammatory regulatory T cells (Tregs), are also present in the periodontal tissue (Cardoso et al. 2008) and could theoretically attenuate the severity of experimental periodontitis, regardless of Th1 vs. Th2 origins. Diseased periodontal tissues are also populated by abundant Th17 cells (Moutsopoulos et al. 2012), which express the osteoclastogenic cytokine IL-17. *Porphyromonas gingivalis* (*P. gingivalis*) preferentially triggers Th17 differentiation not only by prompting antigen presenting cells to produce Th17-supporting cytokines, but also by differentially degrading cytokines that promote Th1 (over Th17) differentiation (Moutsopoulos et al. 2012). The conclusion that *P. gingivalis* instigates a pathogenic Th17 milieu over a developmentally related Th1 environment counters the conclusion that Th1s characterize nonprogressive lesions that might be beneficial to pathogen survival (Yamamoto et al. 1997b).

Indicators of Th17 cells (cytokine mRNAs/receptors, surface markers) are more frequent in human PD lesions compared to healthy gingiva (Honda et al. 2008; Cardoso et al. 2009). People with natural Th17 defects have less PD (Dutzan et al. 2018), consistent with importance of Th17s in periodontal inflammation and bone loss. IL-17A, the signature cytokine of Th17s, is higher in the gingival crevicular fluid from generalized aggressive compared to chronic periodontitis subjects, is less abundant in gingivitis compared to PD lesions, and is only marginally detectible in healthy gingival tissue (Moutsopoulos et al. 2012; Honda et al. 2008; Shaker and Ghallab 2012; Okui et al. 2012). Taken together with numerous studies cited in a recent review (Abusleme and Moutsopoulos 2017), it is clear that Th17s play important roles in PD pathogenesis in people.

### $CD4^+$ T-Cell Subsets in T2D

Work in mouse models of T2D has variously implicated the Th1/Th2 balance, Tregs, or $CD8^+$ T cells in T2D-promoting inflammation

(Winer et al. 2009; Feuerer et al. 2009; Nishimura et al. 2009). However, work from our lab and others showed that Th17 cells are disproportionately present/functional in adipose tissue and blood from people with insulin resistance/T2D (Jagannathan-Bogdan et al. 2011; Dai et al. 2015; Dalmas et al. 2014; Zeng et al. 2012). Non-biased bioinformatic analysis showed that Th17s are more important than other T-cell subsets in human T2D, as indicated by combinatorial cytokine profiles, for predicting who was non-T2D versus T2D (Ip et al. 2016). Proinflammatory CD4$^+$ Th17s also accumulate in obese adipose tissue in mice (Defuria et al. 2013; Winer et al. 2009), although their relative dominance over other T-cell subsets in murine inflammation is questionable. Together with demonstrations that T cells are at least as abundant in obese human adipose tissue as myeloid cells (Grant et al. 2013), the literature supports the interpretation that Th17s are among the most important source of inflammation in T2D. Higher Th17 cytokine production in T2D, combined with the importance of Th17 cells in PD of non-obese/non-T2D individuals as described above, suggests that Th17-associated mechanisms heighten the risk of PD in people with T2D. Our lab has begun to query roles that regulators of Th17s play in T2D-potentiated PD starting with our understanding of B cells as critical controllers of T-cell function.

## B Cells Predispose PD in T2D

Proinflammatory changes in multiple immune system cells in T2D promote devastating complications, which include periodontal diseases and cardiovascular disease among others (Loe 1993; Mealey and Oates 2006; Van Gaal et al. 2006; Janket et al. 2008). Pro-osteoclastogenic cytokines made by multiple immune cell types, like receptor activator of nuclear factor kappa-B ligand (RANKL), TNFα, IL-1β, and IL-6, are produced in excess in T2D and are top candidates, along with Th17 cytokines (mainly from T cells), for molecules that predispose periodontal complications (Halade et al. 2011; Yasuda et al. 1998; Cao et al. 2009; Khosravi et al. 2013; Jagannathan et al. 2009; Hotamisligil et al. 1993; Park et al. 2010; Fain 2006). The impact of more abundant osteoclastogenic cytokines is further exacerbated by T2D-associated decreases in anti-inflammatory IL-10 and osteoprotegerin (OPG), the bone-protective RANKL decoy receptor (Jagannathan et al. 2010; Halade et al. 2011; Ashley et al. 2011).

B cells produce many pro-osteoclastogenic cytokines including TNFα, IL-6, and RANKL, although we found no evidence that human B cells produce IL-17 (unpublished). B-cell IL-10 is significantly produced by B cells from lean/healthy individuals, but B cells from T2D subjects produce little to no IL-10 (Jagannathan et al. 2010; Harris et al. 2000; Kanematsu et al. 2000; Kawai et al. 2006). These data suggest that a higher pro- to anti-inflammatory cytokine ratio may promote periodontal bone loss in T2D. To test this possibility, we treated two strains of 5-week-old male C57BL/6 mice, WT or B-cell–null μMTs (Kitamura et al. 1991) with low-fat or high-fat diet for 10 weeks, then maintained mice on their respective diets for 6 weeks following PD induction using a standard *P. gingivalis* oral inoculation method (Baker et al. 1994) (Fig. 2a). B cells play an insignificant role in PD in low-fat diet fed (lean) mice as evidenced by similar oral bone loss in both WT and B-cell–null strains (Fig. 2b). This finding was consistent with demonstrations that B cells have minor actions in some other bone loss models (Lee et al. 2006a, b; Li et al. 2007). The presence of B cells also failed to affect *P. gingivalis*-induced periodontal bone loss in young/lean female mice (Zhu et al. 2014). However, serial induction of a T2D-like disease with high-fat diet (Zhu et al. 2014) followed by *P. gingivalis* oral inoculation to induce PD demonstrated that T2D potentiated pathogen-induced oral bone loss in WT but not B-cell–null mice (Fig. 2c), in part due to increase osteoclastogenesis along the periodontal bone (Fig. 2d) and more cytokine production in WT animals (Zhu et al. 2014). These data indicate that fundamentally different mechanisms control PD in lean and T2D mice: PD development is B-cell independent in lean mice, yet requires B cells in mice with pre-existing T2D. This work did not pinpoint cytokine

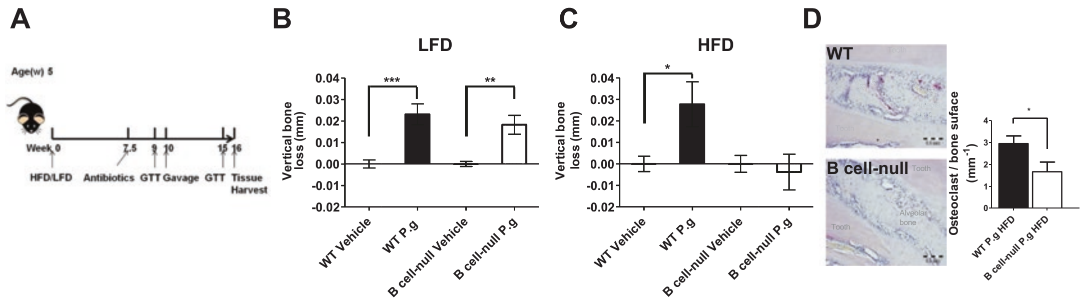


**Fig. 2** B cells promote *P. gingivalis*-induced periodontal bone loss in obese/glucose intolerant but not lean/normoglycemic mice. (**a**) Schematic representation for the combined diet-induced obesity and *P. gingivalis* (P.g) oral inoculation model. Wild-type (WT) or μMT (B-cell–null) mice were fed high-fat diet (HFD) or low-fat diet (LFD) for a total of 16 weeks, starting at 5 weeks of age. Mice were treated with oral antibiotics at 7.5 weeks of diet, and carboxymethylcellulose (vehicle) or *P. gingivalis* (P.g) gavage at 10 weeks of diet. Glucose tolerance tests (GTTs) assessed metabolic health at 9 and 15 weeks of diet with outcomes published (Zhu et al. 2014). P.g vehicle was carboxymethylcellulose. (**b**) Vertical alveolar bone loss of LFD-fed mice after oral inoculation was determined by measuring the distance between the cementoenamel junction and the alveolar bone crest. (**c**) Vertical alveolar bone loss of HFD-fed mice after oral inoculation was determined by measuring the distance between the cementoenamel junction and the alveolar bone crest. (**d**) B cells promote osteoclastogenesis and periodontal inflammation in obesity-associated periodontitis. Left panels: representative images of TRAP staining for periodontal tissue of obese *P. gingivalis*-inoculated mice with genotypes as labeled; arrows highlight TRAP-positive osteoclasts; scale bar represents 0.1 mm. Right panel: quantification of alveolar lining osteoclasts as number of osteoclasts per linear distance of alveolar surface. All data are presented as mean ± SE, $N = 8–9$ per group. $*P < 0.05$, $**P < 0.01$; $***P < 0.001$. (Reprinted from Zhu, M, DeFuria, J, Carr, JD, Belkina, AC, Van Dyke, TE, Gyurko, R, Nikolajczyk, BS. 2014. B cells promote obesity-associated periodontitis and oral pathogen-associated inflammation. *J. Leuk. Biol.* 96:349–357, with permission from John Wiley and Sons (License 4504380151867))

production to B cells, and the exclusive T2D specificity of the outcome contrasted with relatively modest differences in only one PD-associated cytokine, TNFα. The requirement for B cells in T2D-potentiated PD supports the hypothesis that T2D "primes" B cells to potentiate periodontal complications. This work was the first definitive in vivo demonstration that B cells play a minor (if any) role in chronic PD development in lean hosts, and cautions that demonstrations of B cells promoting acute (ligature induced) PD in lean hosts suboptimally query the role B cells play in the chronic PD characteristic of T2D (Abe et al. 2015; Zhu and Nikolajczyk 2015). Although roles for lymph node defects and high BAFF in B-cell–null mice (Golovkina et al. 1999) cannot be completely discounted, these data indicate that the neutrophils, macrophages, T cells, and innate lymphoid cells that remain in B-cell–null mice are not sufficient to promote periodontal complications of T2D, nor are RANKL-producing osteoblasts and osteocytes (Yasuda et al. 1998; Kartsogiannis et al. 1999).

## B-Cell Functions in T2D-Potentiated PD

### Roles for B-Cell Antibody and Cytokine Production in T2D-Potentiated PD

B cells have numerous functions, but the three best understood functions in T2D and/or PD are antibody production, cytokine production, and T-cell regulation. The role of B-cell antibodies in obesity and obesity-associated T2D remains controversial (Defuria et al. 2013; Winer et al. 2011; Nikolajczyk et al. 2012) and will require more investigation, especially in humans. Similarly, antibodies from B cells and plasma cells, the terminally differentiated form of B cells, have been proposed as protective (Hall et al. 2012), destructive (De-Gennaro et al. 2006; Baker et al. 2009), or insignificant (Tew et al. 1989) for PD. Similarly, the role of B-cell cytokines, which have local effects critical for T-cell differentiation/function (Harris et al. 2000), is unclear in

both obesity/T2D and PD. However, the relatively modest amounts of cytokines produced by B cells in these diseases, compared to myeloid and T cells, combined with our data showing partial decreases of only one PD-associated cytokine (TNFα) in B-cell–null mice, suggest indirect effects (Jagannathan et al. 2010; Defuria et al. 2013; Zhu et al. 2014; Ip et al. 2016; Jagannathan et al. 2009). Exceptions include the ability of B cells to produce IL-10, which is severely compromised in T2D (Jagannathan et al. 2010), and B-cell RANKL, which may also play uniquely destructive roles in PD (Kawai et al. 2006). The relevance of general obesity/T2D-associated increases in RANKL (Cao et al. 2009; Jin et al. 2007; Cao et al. 2010) to B-cell RANKL production/function remains untested.

## B Cells Regulate Th17 Cells in Immune-Mediated Disease

Numerous studies are consistent with the conclusion that B cells regulate T-cell function to promote a variety of medical maladies. B cells promote Th17 function in multiple types of immune-related diseases, including classically defined autoimmune diseases. Evidence of this include studies on the B-cell–depleting drug rituxan (anti-CD20), which reduces Th17s in synovial fluid of joints affected with rheumatoid arthritis. In vitro follow-ups confirmed specificity by showing that rituxan-mediated Th17 amelioration required the presence of B cells (van de Veerdonk et al. 2011). Similar lines of evidence from both in vitro and in vivo studies showed that B cells support Th17s/IL-17 production in people with multiple sclerosis, in part through the ability of B cells to produce IL-6 (Ireland et al. 2016; Bar-Or et al. 2010). Broader analyses have shown that people with a variety of generally non-autoimmune primary B-cell deficiencies have lower frequency of circulating Th17 cells (Barbosa et al. 2011). Taken together, these data imply that obesity/T2D-associated changes in B cells, whether it be through local cytokine production or the unique ability of B cells to concentrate antigens for presentation to T cells, support Th17 not only in T2D but also in T2D-potentiated PD.

## Roles of B-Cell/T-Cell Cross Talk in T2D and T2D-Potentiated PD

B cells function at least in part through an ability to regulate T cell and macrophage-mediated inflammation with both the soluble mediators described above and cell contact-dependent mechanisms (Defuria et al. 2013; Harris et al. 2000; Winer et al. 2011). Although myeloid cells can either support or ameliorate T-cell cytokine production depending on the details of the study, and regardless of metabolic status of the PBMC donor for some reports, B cells support Th17 function through a T2D-dependent mechanism (Defuria et al. 2013; Jagannathan-Bogdan et al. 2011; Ip et al. 2016; Tyagi et al. 2017) (Fig. 3a–d). B cells require close approximation with T cells to cause relatively high amounts of Th17 cytokine production characteristic of T2D (Fig. 3e). The obvious candidates for this regulation are the immunological co-receptors like B7, ICOS, and PD1, although detailed molecular mechanisms remain undefined. Given that a combinatorial Th17 profile predicts metabolic status of T2D subjects in bioinformatic analyses (Fig. 4) and activates more conventional T2D-promoting cytokines like TNFα (Fig. 5), B cells play important support roles in defining the diffuse term of "inflammation" in T2D. These data justify future analyses to test the proposal that B-cell support of T-cell inflammation may also potentiate PD pathogenesis.

## Conclusions and Future Directions

Our data lead to the hypothesis that B-cell/T-cell cross talk, which increases Th17 inflammation in T2D, also augments Th17 function in periodontal tissues and thereby T2D-potentiated PD. This possibility does not introduce fundamentally different mechanisms underlying PD etiology in metabolically healthy and T2D individuals strongly supported by our mouse work (Zhu and

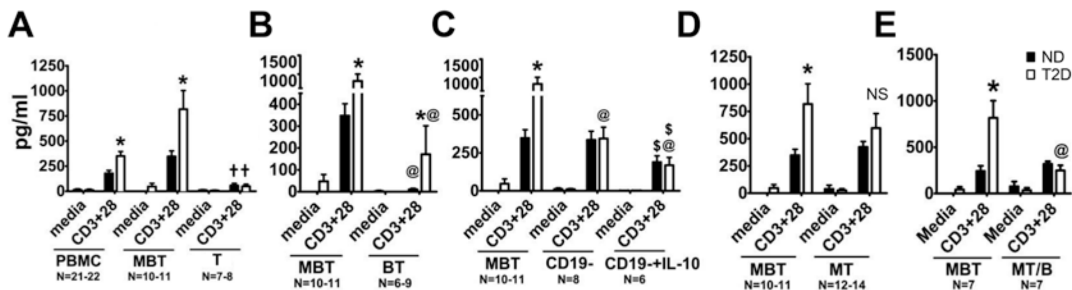


**Fig. 3** Human B cells, but not monocytes, support Th17 function and thus inflammation through T2D-associated mechanisms. (**a**) Supernatants from peripheral blood mononuclear cells (PBMC, left), co-cultured monocytes, B cells, and T cells (M, B, T, respectively, middle), or purified CD4⁺ T cells (T, right) stimulated as indicated and assayed for IL-17A by ELISA. (**b**) IL-17A in supernatants from MBT (left; re-graphed from panel **a**) or BT co-cultures of cells purified from non-T2D or T2D subjects, then stimulated as indicated. (**c**) IL-17A in supernatants from MBT co-cultures (left; re-graphed from panel **a**), B-cell–depleted PBMCs (middle; CD19⁻), or B-cell–depleted PBMCs with recombinant IL-10 supplementation (right; CD19⁻ + IL-10). (**d**) IL-17A in supernatants from MBT (left; re-graphed from Fig. 4a) or MT co-cultures of cells from non-T2D or T2D subjects. (**e**) IL-17A production by MT cultures in the presence of B cells (MBT) or in the presence of B cells physically separated from MT cells by a cytokine-permeable, cell-impermeable transwell membrane (MT/B). MBT and MT/B cultures in panel **e** used a subset of the samples analyzed in panels **a**–**c**. For all panels, ∗ indicates a difference ($P < 0.05$) between T2D (white bars) and non-T2D (black bars) under the same treatment condition; † indicates difference between purified T cells and both MBT and PBMC results (panel **a**); @ indicates difference between MBT and BT (panel **b**) or CD19⁻ ± IL-10 PBMCs (panel **c**) or MT/B (panel **e**) within non-T2D or T2D cohort; $ indicates that CD19⁻ ± IL-10 PBMCs results differ within non-T2D or T2D cohort (panel **c**). All differences were calculated by 3-way ANOVA. IL-17A in all stimulated cultures was higher than relevant unstimulated (media) controls. N for each culture type is indicated below *X* axis. Differences in MT co-cultures from non-T2D and T2D subjects were insignificant (NS, panel **d**). (Originally published in DeFuria, J, Belkina, AC, Jagannathan-Bogdan, M, Snyder-Cappione, J, Carr, JD, Nersesova, Y, Markham, D, Strissel, KJ, Zhu, M, Watkins, A, Allen, J, Bouchard, J, Toraldo, G, Jasuja, R, Obin, MS, McDonnell, ME, Apovian, C, Denis, GV and Nikolajczyk, BS. 2013. B cells promote inflammation in obesity and type 2 diabetes through regulation of T cell function and an inflammatory cytokine profile. *Proc. Nat. Acad. Sci. USA* 110:5133–8. (Copyright ©2013, National Academy of Sciences))

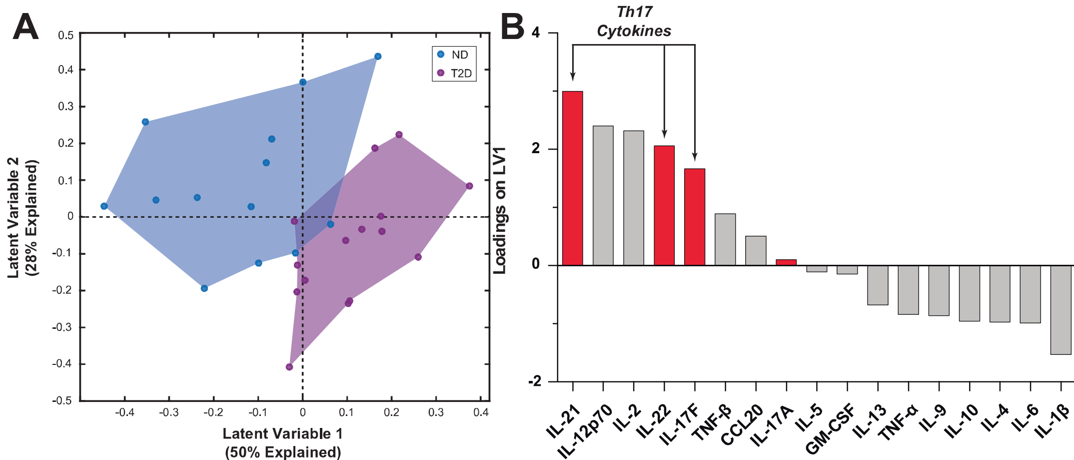


**Fig. 4** An inflammatory T-cell cytokine signature differentiates T2D from ND. (**a**) Partial least squares discriminant analysis (PLSDA) shows mathematically combined cytokines (i.e., cytokine "profiles") from αCD3/αCD28-stimulated PBMCs analyzed for fit into clinical group. Blue: ND; purple: T2D. (**b**) PLSDA loading analysis of latent variable 1 (i.e., separation of ND and T2D cytokine profiles along *X* axis in panel **a**). Leftmost cytokines have highest influence on classifying subjects at T2D. Th17 cytokines (red) are disproportionately important for differentiating cytokine profiles based on disease status. (Reprinted from Ip, B, Cilfone, N, Belkina, AC, DeFuria, J, Jagannathan-Bogdan, M, Zhu, M, Kuchibhatla, R, McDonnell, ME, Xiao, Q, Kepler, TB, Apovian, CM, Lauffenburger, DA and Nikolajczyk, BS. 2016. Th17 cytokines differentiate obesity from obesity-associated type 2 diabetes and promote TNFα production. *Obesity.* 24:102–112 with permission from John Wiley and Sons (License 4504371308870))

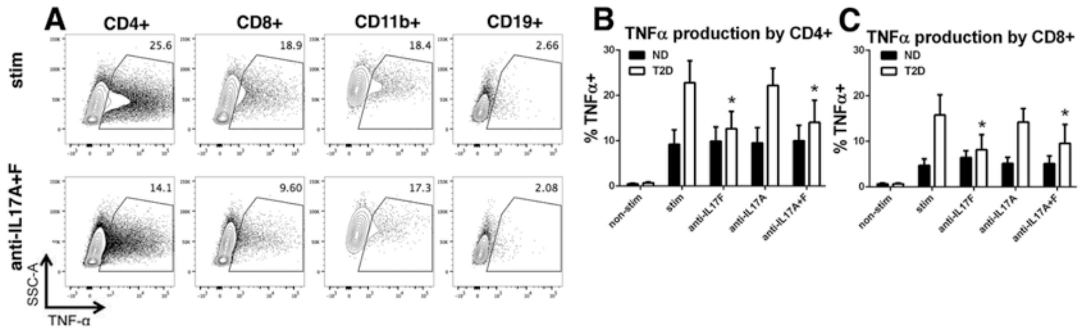


**Fig. 5** IL-17 blockade decreases T-cell TNFα production in T2D. (**a**) Percent intracellular TNFα-positive cells with surface marker expression as indicated in the absence (top panels) or presence (bottom panels) of IL-17 blocking antibody, following stimulation of PBMCs with αCD3/αCD28 (stim). Average percentage and SE of TNF-positive (**b**) CD4+ T cells or (**c**) CD8+ T cells. "Non-stim" indicates PBMCs cultured in the absence of stimuli. "*" indicates significant differences ($P < 0.05$) between αCD3/αCD28 stimulation alone and in combination with antibodies listed on the *X* axis. $N = 4$. (Reprinted from Ip, B, Cilfone, N, Belkina, AC, DeFuria, J, Jagannathan-Bogdan, M, Zhu, M, Kuchibhatla, R, McDonnell, ME, Xiao, Q, Kepler, TB, Apovian, CM, Lauffenburger, DA and Nikolajczyk, BS. 2016. Th17 cytokines differentiate obesity from obesity-associated type 2 diabetes and promote TNFα production. *Obesity.* 24:102–112 with permission from John Wiley and Sons (License 4504371308870))

Nikolajczyk 2014), perhaps due to the paucity of Th17s in the murine high-fat diet model of T2D relative to the dominance of Th17s in human obesity-associated T2D. This proposition also assumes that a higher proportion of Th17 cells or Th17 cytokines than those in lean/healthy individuals increase risk/pathogenesis of PD, although this "rheostat" model has not been systematically tested. Overall, the role of lymphocytes and lymphocyte cross talk in T2D-potentiated periodontal pathogenesis is worthy of more comprehensive investigation in both animal models and humans. The latter studies will absolutely require logistically challenging collaborations between Endocrinology and Periodontology clinics to ensure thorough characterization of both metabolic and periodontal status of study subjects but are likely to be well worth the significant effort.

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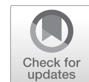


# Gingival Epithelial Cell Recognition of Lipopolysaccharide

Nutthapong Kantrong, Thao T. To, and Richard P. Darveau

## Introduction

Oral, or gingival, epithelium is a specialized lining that protects underlying connective tissues and alveolar bone from potentially harmful burdens derived from oral microbial communities that colonize periodontal pockets. Therefore, the contribution of the gingival epithelium to innate immune defense cannot be underestimated since it is the cell layer that first encounters periodontal bacteria and forms a physical barrier to resist bacterial intrusion.

Simple exclusion of bacterial components as a mechanism to prevent inflammation is not a mechanism employed by gingival tissue. As demonstrated in germ-free mice by us (Zenobia et al. 2013; Greer et al. 2016) and others (Tsukamoto et al. 2012), the healthy innate defense status in fact employs selective bacterial activation to selectively facilitate a low-level inflammation, commonly referred to as inflammatory surveillance (Darveau 2010). A major component of periodontal healthy homeostasis is the regulated transit of neutrophils from the vasculature through gingival tissue and into the gingival crevice (Tonetti 1997). This process is highly regulated, either too few or too many neutrophils can result in destruction of healthy periodontal tissue (Attstrom and Schroeder 1979; Eskan et al. 2012; Moutsopoulos et al. 2014) However, the mechanisms by which neutrophil migration is regulated remain largely unknown. One well-known neutrophil chemoattractant that has been shown to form a concentration gradient in healthy junctional epithelial tissue is IL-8 (Tonetti 1997; Tonetti et al. 1998; Gemmell et al. 1994). In contrast, in chronically inflamed gingival tissues, the IL-8 concentration gradient is disrupted and local areas of both high and low potent expression of IL-8 transcripts IL-8 are observed (Fitzgerald and Kreutzer 1995). These data strongly implicate IL-8 as a key neutrophil chemoattractant in both healthy tissue and diseased tissue, and several studies have demonstrated that oral bacteria induce its expression in gingival epithelial cells (Schueller et al. 2017; Darveau et al. 1998). Moreover, it is not known if lipopolysaccharide, a potent inflammatory stimulator found in both clinically healthy and diseased plaque (To et al. 2016), contributes to the expression of this key gingival inflammatory mediator.

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Furthermore, it is unclear how human oral/gingival epithelial cells employ TLR4 for bacterial recognition. It has been reported that similar to intestinal (Abreu et al. 2001) and airway epithelial cells (Nakamura et al. 1991), gingival epithelial cells do not respond to isolated LPS preparations obtained from either *Escherichia coli* or *Salmonella typhimurium* (Schueller et al. 2017; To et al. 2016) consistent with the notion that TLR4 activation does not occur in this cell type. However, although examination of the IL-8 response to *Prevotella intermedia*, a naturally occurring oral gram-negative bacteria, also revealed no IL-8 response, the contribution of TLR4 was not examined (Sugiyama et al. 2002). This is especially relevant since several reports describe gingival epithelial cell IL-8 responses to *Porphyromonas gingivalis* LPS, although most *P. gingivalis* LPS preparations contain both TLR2 and TLR4 activity (Darveau et al. 2004). The TLR2 activity is most likely due to covalently attached lipoproteins (Glew et al. 2017) and the activity can be removed with extensive lipase treatment (Jain et al. 2013). Therefore, in this report, *F. nucleatum* was chosen as a representative gram-negative oral bacterial species to determine the ability of gingival epithelial cells to utilize TLR4 for recognition of bacterial lipopolysaccharide. This bacterium was selected since it is commonly found in both healthy and diseased dental plaque and its LPS, similar to *E. coli* LPS, selectively activates TLR4 and not TLR2 (To et al. 2016). Considering the ability of *F. nucleatum* to strongly activate TLR4, this study aimed at elucidating the mechanisms of TLR4-mediated IL-8 response in gingival epithelial cells by using *F. nucleatum* LPS.

## Materials and Methods

### Human Gingival Epithelial Cell Culture

Normal human gingival epithelial cells were obtained from the gingival tissue cell collections at the department of Oral Health Sciences, School of Dentistry, the University of Washington. Briefly, normal human gingival epithelial cells were isolated from gingival tissues obtained from healthy adults who underwent the third molar surgical removal procedures. Gingival tissue explant cultures were performed to isolate primary gingival epithelial cells as previously described (Krisanaprakornkit et al. 1998) and then stored in our tissue collection. Primary gingival epithelial cells were grown in keratinocyte serum-free medium (Ker-SFM) (Gibco by Life Technologies, Carlsbad, CA) supplemented with 25-µg/mL bovine pituitary extracts, 0.2-ng/mL human recombinant epidermal growth factor, 5-µg/mL insulin (Sigma-Aldrich, St. Louis, MO), 0.5-µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 0.15-mM calcium chloride, and 10% penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO). Cultured gingival epithelial cells were seeded in 12- or 96-well plates and maintained under a 37 °C humidified atmosphere with 5% $CO_2$. Monolayer of OECs reached confluence prior to the day of bacterial stimulation.

Two telomerase-immortalized cell lines including human oral epithelial cells OKF6/TERT2 (Dickson et al. 2000) and human gingival keratinocytes hTIGK (Moffatt-Jauregui et al. 2013), in addition to primary normal gingival epithelial cells, were utilized in this study. OKF6/TERT2 line was generously provided by the Division of Dermatology, Harvard Medical School. hTIGK line was kindly gifted by Dr. Richard J. Lamont, School of Dentistry, University of Louisville. Both OKF6/TERT2 and hTIGK were grown and maintained in Ker-SFM (Gibco by Life Technologies, Carlsbad, CA) supplemented with 25-µg/mL bovine pituitary extracts, 0.2-ng/mL human recombinant epidermal growth factor, 0.4-mM calcium chloride, and 10% penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO).

### Bacterial Culture and Crude Cell Wall Preparation

*F. nucleatum* ATCC 25586 was grown overnight at 37 °C under an anaerobic gas condition (80% $N_2$, 10% $CO_2$, 10% $H_2$) in trypticase soy yeast

broth (TYK) supplemented with 10-μg/mL hemin (H) and 1-mg/mL menadione. Crude cell wall was prepared as previously described (Krisanaprakornkit et al. 1998) by using French Pressure Cell Press at 15,000 lb/in.$^2$ with the cocktails of protease inhibitors including Pefabloc® SC (Boehringer Mannheim GmbH, Mannheim, Germany), benzamidine (Sigma-Aldrich, St. Louis, MO), and Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (Sigma-Aldrich, St. Louis, MO) at a concentration of 20 μM each and DNase (Sigma-Aldrich, St. Louis, MO). Cell wall fractions were harvested by several centrifugations and suspended in sterile phosphate-buffered saline (PBS) prior to fluorometric protein quantitation using Qubit® protein quantitation kit (Life technologies by Thermo Fisher Scientific, Waltham, MA). Crude *F. nucleatum* cell wall (FnCW) in PBS suspension was kept frozen prior to experimental setup. LPS from *E. coli* and *F. nucleatum* was isolated and purified as previously described (Darveau and Hancock 1983).

## Preparation of Bacterial Culture Supernatant

Bacterial culture supernatants were prepared from *F. nucleatum* ATCC 25586 and *Escherichia coli* JM83 overnight cultures. *E. coli* JM83 was grown in Luria-Bertani (LB) broth under aerobic conditions in a vigorous 37 °C shaker. Bacterial whole cells in an overnight 20-mL culture of each strain were removed by centrifugation at 6500 × *g* for 25 min. Clear culture supernatants were then filtered with Steriflip® filter units with 0.22 μM pore size (Millipore Corporation, Billerica, MA, USA) to ensure a complete removal of bacterial cells and debris from the resulting supernatants. An approximate 20-mL bacterial supernatant was then concentrated into a smaller volume (roughly 500 μL) by using Centricon Plus-20 Centrifugal Filter Devices (Millipore Corporation, Billerica, MA, USA). A concentration of total proteins from each preparation was determined by using Qubit® protein quantitation kit (Life Technologies by Thermo Fisher Scientific, Waltham, MA). Hundred micrograms per milliliter and 10-μg/mL working stocks of supernatants were prepared from original stocks using 1× PBS (Gibco by Life Technologies, Carlsbad, CA) and stored at −20 °C until needed.

## Detection of Endotoxin (LPS) Level in Bacterial Supernatants

In some experiments in which concentrated *F. nucleatum* or *E. coli* culture supernatants were used, the presence of LPS in the supernatants was quantified using ToxinSensor™ Endotoxin Detection System (GenScript, Piscataway, NJ). Briefly, aliquots of either *F. nucleatum* or *E. coli* culture supernatants were incubated with Limulus Ameobocyte Lysate (LAL) reagents for 12 min at 37 °C in 96-well plate. Chromogenic substrate solution was then added to each reaction and allowed 6 min for incubation. Stop solution and color stabilizer were finally added to each well. Optical density at 545 nm wavelength was determined by using a plate reader (VMax microplate reader from Molecular Devices, Sunnyvale, CA). A wide-range dose of EcLPS and FnLPS (100 pg/mL to 1 μg/mL) was used as standards for the readout and PBS served as a negative control.

## HEK293 TLR Activation Assays

Analysis of TLR2- and TLR4-mediated NF-κB activities of bacterial samples and purified agonists was performed using human embryonic kidney 293 cells (HEK293) transfected with plasmid DNAs bearing Firefly luciferase-labeled NF-κB sequence, Renilla luciferase-labeled β-actin sequence, in combination with plasmids encoding human TLR2/1 or TLR4 as described elsewhere (Coats et al. 2007).

Briefly, HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum and seeded in 96-well plates 1 day prior to

transfection. Transfection with plasmids encoding human TLR2/1 or TLR4 was performed by a standard calcium phosphate precipitation method. Next pTLR2 and pTLR1 (0.002 μg each) were co-transfected with pCD14 (0.002 μg) for transient TLR2/1 expression. Similarly, pTLR4 (0.002 μg) and pMD-2 (0.002 μg) were co-transfected with pCD14 (0.002 μg) to induce TLR4 expression. Reporter plasmid pBArenLuc (0.0004 μg) and pNFκBLuc (0.02 μg) were transfected in all wells. Stimulation of transfected HEK293 cells was performed on the following day in triplicate under normal physiological conditions for 4 h, after which the HEK293 cells were rinsed with PBS and lysed with passive lysis buffer (Promega, Madison, WI). Luciferase activity was then assayed by using the Dual Luciferase Assay Reporter System (Promega, Madison, WI). Luminescent signals generated from Firefly luciferase activity were normalized to signals from Renilla luciferase, thereby resulting in the fold activity of NF-κB relative to β-actin internal control.

## TLR4 Gene Silencing

Gingival epithelial cells were seeded into 12-well plates using supplemented Ker-SFM medium containing 1.2-mM calcium chloride. Shortly after seeding gingival epithelial cells, small-interfering RNA was transfected using the fast-forward protocol according to manufacturer's instructions. Specific small-interfering RNA (siRNA) sequences of *TLR4* or non-silencing (NS) scramble RNA sequences (Qiagen, Valencia, CA) were allowed to equilibrate at room temperature for 10 min in the Ker-SFM medium in the presence of HiPerfect transfection reagents (Qiagen, Valencia, CA). Complex of siRNA was subsequently added to the cultured gingival epithelial cells and incubated for 48 h prior to bacterial stimulation. Final concentration of each siRNA used in this study was 50 nM. Efficiency of siRNA gene silencing was determined using quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis to ensure that optimal reduction of *TLR4* mRNA was achieved.

## Gingival Epithelial Cell Stimulation and Intracellular Delivery of LPS

An overnight culture of gingival epithelial cell monolayer was stimulated with live *F. nucleatum*, FnCW, FnLPS, EcLPS, or $Pam_3CSK_4$ with the dose range indicated in the figure for 24 h, prior to IL-8 protein quantification. Supplemented Ker-SFM without antibiotics was used as stimulation medium. For intracellular LPS delivery experiments, LPS–liposome complexes were prepared according to the methodology developed for LPS transfection (Hagar et al. 2013) with a slight modification. Briefly, 500-ng isolated purified EcLPS or FnLPS was pre-mixed with 2-μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA), spun down, and incubated at room temperature for 10 min. Ker-SMF without antibiotics was added and brought up to a final concentration of 1-μg/mL LPS. Cultured gingival epithelial cells were then stimulated with 100-μL LPS–liposome complexes for 24 h, while mock transfection (LPS + medium) and Lipofectamine alone served as negative controls. Positive control wells were infected with 100-ng/mL FnCW. Culture supernatants were assayed for IL-8 protein at 24 h after stimulation. For elucidating the role of endosomal maturation in TLR4 activation, bafilomycin A1 (Sigma-Aldrich, St. Louis, MO) was pre-incubated with gingival epithelial cells for 1 h prior to LPS or bacterial challenging. The use of 400-nM bafilomycin A1 rendered non-toxic to cultured gingival epithelial cells as pre-determined by using CellTiter-Glo® luminescent cell viability assays (Promega, Madison, WI).

Similar to an in vitro delivery of purified LPS, parallel experiments were also performed by using concentrated supernatants harvested from *E. coli* or *F. nucleatum* cultures. In brief, 5 μL from 10-μg/mL culture supernatant was mixed with 2-μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA), spun down, and sat at room tem-

perature for 10 min. Thirteen microliters Ker-SFM without antibiotics was then added to the LPS-Liposome mixture and incubated statically at room temperature for 30 min. Ker-SFM without antibiotics was added and brought up with 480 μL to a final concentration of 100-ng/mL total protein in the supernatant. Hundred microliters prepared samples were added to cultured gingival epithelial cells and incubated for 24 h prior to IL-8 protein assays.

### Measurement of IL-8 Secreted Protein

Culture supernatants were collected for measuring IL-8 levels after 24 h of incubation, unless otherwise indicated. IL-8 monoclonal capture antibody (M801) and detection antibody (M802B) (Thermo Fisher Scientific, Rockford, IL) were used. Avidin-horseradish peroxidase enzyme (HRP) (eBioscience, San Diego, CA) and tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO) were used in the study. Standard sandwich ELISA protocol was performed according to manufacturer's recommendations. Reported concentrations were calculated from the standard curve derived from correlating the OD reading of each sample at 450/570 nm wavelengths (VMax microplate reader from Molecular Devices, Sunnyvale, CA) to the known concentrations of serially diluted recombinant protein standards.

### Data Analysis

All experiments were performed at least three times in triplicate using three different epithelial cell lines, unless otherwise indicated in the figures. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software Inc., La Jolla, CA). One-way Analysis of Variances (ANOVA) with Tukey's post hoc test, unless otherwise stated in the figure text, was used to determine the significance level where $p$-value $<0.05$ was considered statistically different.

## Results

### *F. nucleatum* LPS Selectively Activates TLR4

*F. nucleatum* was selected to determine the TLR4 utilization of gingival epithelial cells in their response to LPS due to its ubiquitous presence in dental plaque and its LPS, similar to *E. coli* LPS selectively activates TLR4 and not TLR2 (To et al. 2016). In this study, we repeated those results and additionally examined activation of TLR2 and TLR4 in response to viable *F. nucleatum* and its cell wall fraction (Fig. 1). As observed previously (To et al. 2016), isolated *F. nucleatum* LPS selectively activated TLR4 while both TLR2 and TLR4 activity was observed with both whole cells and isolated cell walls. These data are consistent with the observation that gram-negative bacteria and their isolated cell walls contain both LPS (TLR4 agonist), lipoprotein and peptidoglycan (TLR2 ligands) (Takeuchi et al. 1999).

### Gingival Epithelial Cells Secrete IL-8 in Response to Bacteria and Cell Walls But Not LPS

Next, the ability of gingival epithelial cells to secrete IL-8 in response to *F. nucleatum* and its isolated cell wall and LPS components was examined. In order to account for potential variability in different gingival epithelial cell lines, two immortalized, OKF6 (Dickson et al. 2000) and hTIGK (Moffatt-Jauregui et al. 2013), as well as two primary gingival epithelial cell lines, nGEC1 and nGEC2, obtained from Dr. Whasun Oh Chung's tissue bank collection were employed.

*F. nucleatum* and *E. coli* LPS failed to elicit IL-8 secretion in any of the four gingival epithelial cell lines examined (Fig. 2) consistent with the notion that epithelial cells do not "see" bacterial LPS (Abreu et al. 2001; Nakamura et al. 1991). In contrast, potent, dose-dependent IL-8 secretion was observed in all cell lines examined with both *F. nucleatum* whole cells as well as isolated cell walls (Fig. 2). Furthermore, in con-

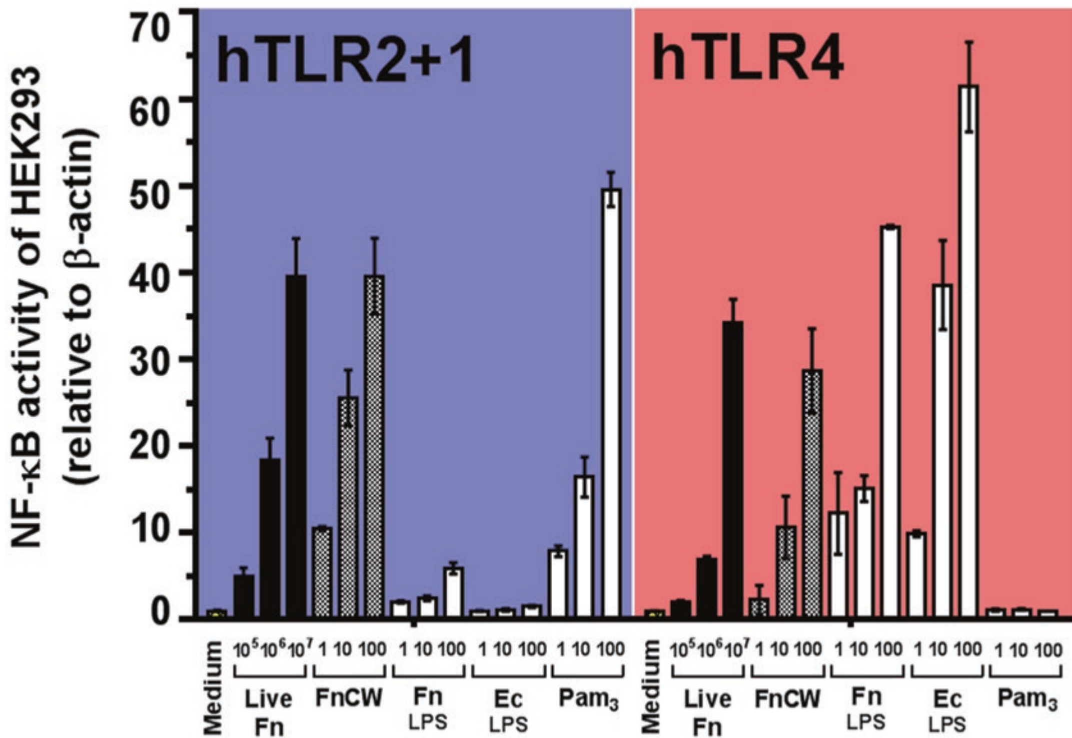


**Fig. 1** TLR2 and TLR4 activation by live *F. nucleatum* or its cell walls. Titration of live *Fn* ($10^5$–$10^7$ bacteria/mL) and FnCW (1–100 ng/mL) were tested for their abilities to activate NF-κB specifically via TLR2 or TLR4, relative to β-actin internal control. Synthetic lipoprotein $Pam_3CSK_4$ (1–100 ng/mL) served as a positive control for TLR2 assays. *E. coli* LPS (1–100 ng/mL) and *F. nucleatum* LPS (1–100 ng/mL) were used as positive controls of TLR4 stimulation assays. Experiments were performed in triplicate wells in two independent setups ($n = 2$) that yielded similar results. Data are shown as average fold TLR activity ± SEM, relative to unstimulated controls, from one independent experiment

trast to the uniform lack of response to isolated LPS, heterogeneity in the IL-8 response to the TLR2 ligand $Pam_3CSK_4$ in that one immortalized cell line (hTIGK) and one primary cell line (nGEC1) secreted significant IL-8 was observed (Fig. 2). These results confirm that gingival epithelial cells do not secrete IL-8 in response to isolated LPS and that these cells display heterogeneity in their response to a TLR2 ligand.

## Gingival Epithelial Cells Do Not Respond to Naturally Shed LPS

LPS is naturally shed from gram-negative bacteria both as a mechanism of normal outer membrane turnover and as a specifically incorporated component into released membrane vesicles (Ellis and Kuehn 2010). Therefore, to more accurately reflect the in vivo environment where dental plaque sheds LPS to the external environment, concentrated culture supernatants of *E. coli* and *F. nucleatum* were tested for the abilities to induce IL-8 expression in gingival epithelial cells. Initially, the presence of *F. nucleatum* and *E. coli* LPS in their respective bacterial culture supernatants was quantified with a limulus amebocyte assay (Fig. 3). These assays confirmed the abundance of LPS in the culture supernatants harvested from both *E. coli* (Fig. 3a) and *F. nucleatum* (Fig. 3b). Notably, a substantial amount of *F. nucleatum* LPS was detected at a lower concentration than *F. nucleatum* supernatant proteins (0.1 and 1 ng/mL), relative

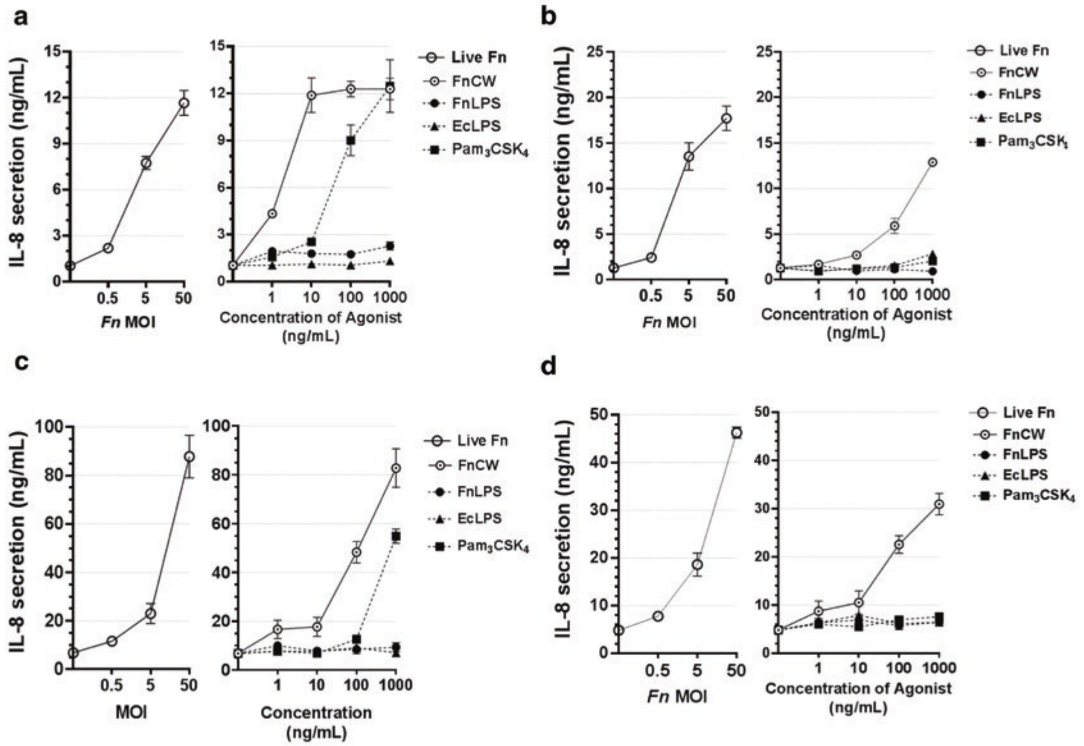


**Fig. 2** IL-8 response to *F. nucleatum* infection by different lines of oral/gingival epithelial cells. Cultured hTIGK (**a**), OKF6 (**b**), normal GEC from donor 1 (nGEC1) (**c**), and from donor 2 (nGEC2) (**d**) were challenged with wide doses of live *Fn*, FnCW, EcLPS, FnLPS, or $Pam_3CSK_4$ for 24 h prior to IL-8 protein determination by ELISA. IL-8 quantification was performed in triplicate samples derived from four different donors. Actual concentrations of secreted IL-8 are shown as mean ± SEM and represent similar results from one of two independent experiments performed in triplicate ($n = 2$). MOI indicates multiplicity of infection used for live *F. nucleatum* stock preparation

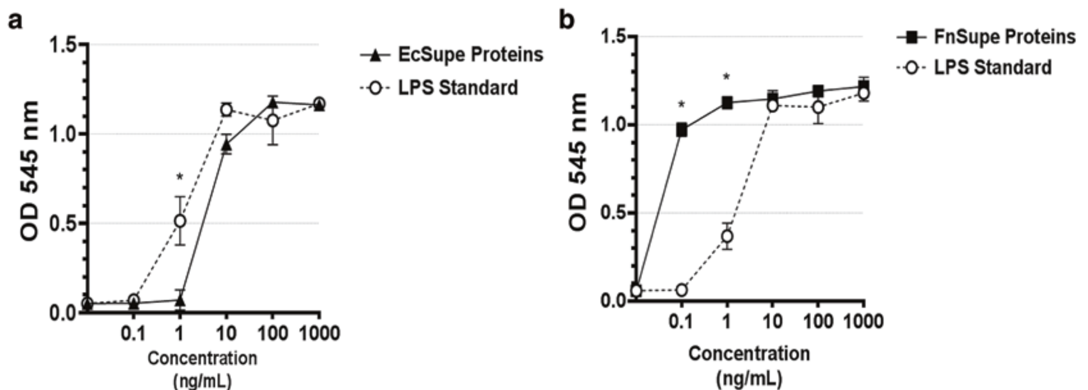


**Fig. 3** Relative quantity of LPS in bacterial culture supernatants. Supernatants from *E. coli* (**a**) and *F. nucleatum* (**b**) cultures were quantified for the amount of shed LPS by using LAL assays. Data represent mean OD545 ± SEM, obtained from one of two independent experiments in triplicate that yielded identical results ($n = 2$). EcLPS and FnLPS served as positive controls and showed similar OD values. Statistical difference was tested using two-way ANOVA with Bonferroni's multiple comparison test. Asterisks indicate statistical significance ($*p$-value < 0.05), compared to LPS standard

to LPS standard controls (Fig. 3b), demonstrating that this bacterium sheds a significant amount of LPS into the culture supernatant. It was found that three of the four gingival epithelial cell lines examined failed to secrete IL-8 in response to either *F. nucleatum* or *E. coli* culture supernatants, whereas the OKF6 cell line secreted IL-8 at the highest concentration of *F. nucleatum* culture supernatant employed (Fig. 4). These data further support the notion that gingival epithelial cells fail to respond to either isolated or naturally shed LPS.

## Gingival Epithelial Cell IL-8 Secretion in Response to LPS Internalization

Similar to oral epithelial cells, both intestinal (Abreu et al. 2001) and airway epithelial cells (Nakamura et al. 1991) do not secrete IL-8 in response to LPS. However, it has been reported that intestinal epithelial cells contain TLR4 associated internally with the Golgi apparatus respond to LPS after it has been internalized into the cell (Hornef et al. 2002, 2003). However, it is not known if oral epithelial cells respond to LPS after internalization. Therefore, the ability of the four oral epithelial cell lines we previously employed to respond to internalized LPS by the secretion of IL-8 was examined (Fig. 5). A liposome–LPS formation technique (Hagar et al. 2013) was employed to deliver either *E. coli* or *F. nucleatum* LPS into the gingival epithelial cells. After 24-h incubation of gingival epithelial cells with liposome–LPS complexes, a significant IL-8 was induced (Fig. 5a) in all test lines, indicating that LPS needs to be internalized to elicit an IL-8 response. Furthermore, an increase of IL-8 secretion was observed when *F. nucleatum* culture supernatants were internalized consistent with the notion that released LPS also requires epithelial cell internalization to elicit an IL-8 response (Fig. 5b). In contrast, IL-8 secretion was not significantly increased when *E. coli* cell culture supernatants were employed.

## TLR4 Significantly Contributes to Internally Delivered LPS-Mediated IL-8 Secretion from Gingival Epithelial Cells

Next, it was determined if IL-8 secretion in response to LPS is mediated by TLR4 after internalization into gingival epithelial cells. TLR4 was silenced with specific siRNA sequence for 48 h prior to live *F. nucleatum* or *E. coli* LPS

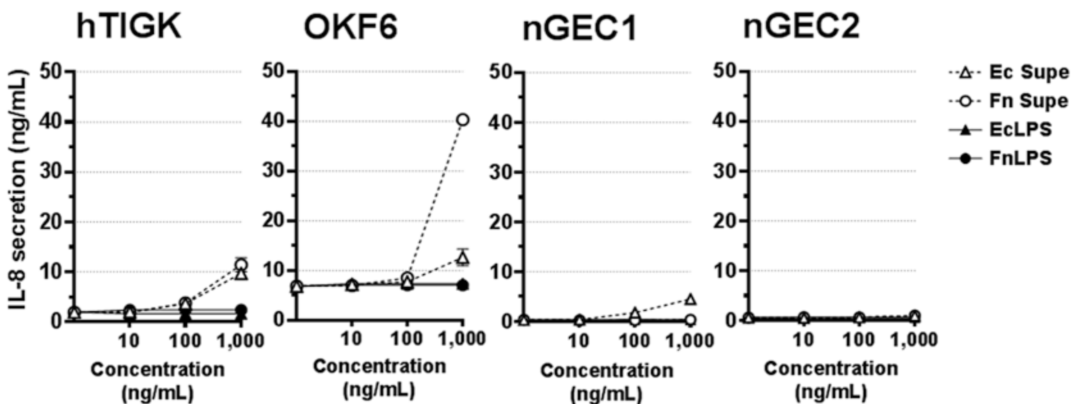


**Fig. 4** Induction of IL-8 expression by bacterial culture supernatants in oral/gingival epithelial cells. LPS-containing bacterial culture supernatants were used for OEC stimulation assays. IL-8 protein levels were determined by ELISA assays. Data are representatives of similar results derived from one of two independent experiments ($n = 2$) in triplicate in all four test lines and shown as mean actual concentration ± SEM

stimulation. The efficiency of siRNA knockdown was determined by qRT-PCR showing that approximately 50% reduction of mRNA expression levels was achieved in each experimental setup (data not shown). After 24 h of stimulation with *F. nucleatum* or *E. coli* LPS, a significant reduction of the amount of secreted IL-8 levels in silenced TLR4 cells was observed (Fig. 6). These data demonstrate that after LPS internalization, TLR4 is required for maximal IL-8 secretion.

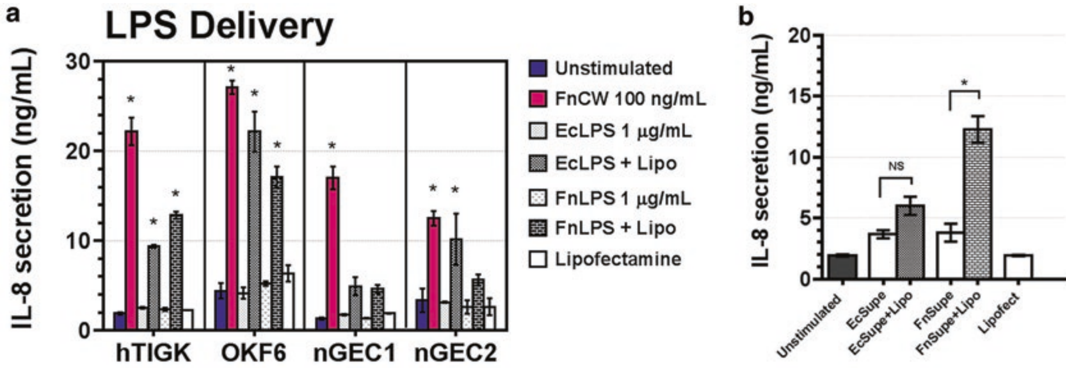


**Fig. 5** Liposome-coupled LPS induction of IL-8 expression in oral/gingival epithelial cells. In (**a**), IL-8 secretion from cultured OECs incubated for 24 h with LPS (as indicated in the Figure) pre-mixed with Lipofectamine: In (**b**), IL-8 secretion by hTIGK in response to Lipofectamine-mediated internalization of shed LPS. Data are shown as mean concentration ± SEM from one of two independent experimental setups ($n = 2$) performed in all four test lines, in triplicate. Statistical significance was determined by using two-way ANOVA with Tukey's post hoc test. Asterisks indicate significant difference (∗*p-value* < 0.05), compared to unstimulated controls (for FnCW) or species matched LPS-stimulated controls (for LPS + Lipofectamine)

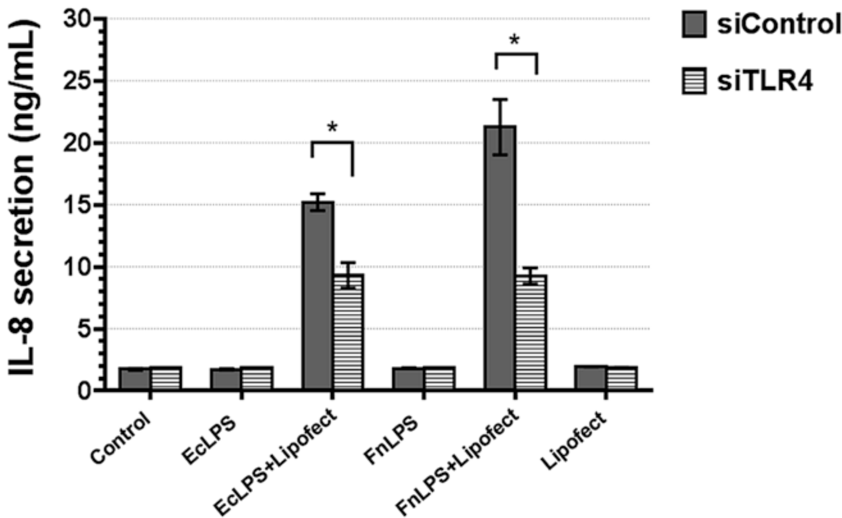


**Fig. 6** Silencing of TLR4 attenuated the IL-8 response to internalized LPS. Cultured oral/gingival epithelial cells were knocked-down for TLR4 by siRNA for 48 h before 24-h stimulation with either *E. coli* or *F. nucleatum* LPS. *F. nucleatum* LPS with and without Lipofectamine treatment. IL-8 protein was quantified by ELISA. Data are shown as means of actual IL-8 concentration ± SEM from four independent experiments ($n = 4$). IL-8 quantification was performed in duplicate samples derived from two donors (hTIGK and OKF6). Differences were tested using two-way ANOVA and Tukey's multiple comparison test. Asterisks indicate significant differences (∗*p-value* < 0.05), compared to corresponding non-silenced control cells

## Endosome Activation Is Required for IL-8 Secretion in Response to LPS

It has been reported that intracellular TLR4 present in the Golgi apparatus of murine intestinal epithelial cells results in NF-κB–dependent CXCL-2 secretion (Hornef et al. 2002). Furthermore, several lines of evidence have also suggested the role of endosomal TLR4 in LPS-dependent cellular signaling in myeloid cells (Uronen-Hansson et al. 2004) and epithelial cells from other body sites (Ueta et al. 2004). Therefore, the contribution of the endosomal processing to IL-8 secretion in response to LPS in gingival epithelial cells was determined (Fig. 7). For these experiments, gingival epithelial cells were pre-incubated with 400-nM bafilomycin A, a known inhibitor of endosomal acidification used in numerous in vitro studies investigating intracellular TLR signaling (Johnson et al. 1993). Bafilomycin completely abrogated IL-8 secretion in response to LPS in both hTIGK and OKF6 epithelial cell lines (Fig. 7). Our data indicate that TLR4 is expressed on the endosomes in gingival epithelial cells, and its activation requires cytosolic engulfment of bacterial LPS in order to deliver LPS to TLR4 found in endosomes, consistent with a previous proposed mechanism for LPS sensing (Husebye et al. 2006).

## Discussion

In addition to being a natural physical barrier that prevents the penetration of microbial components into deeper connective tissues, gingival epithelial cells also act as a surveillance system to mount a protective inflammatory surveillance response to polymicrobial colonization, i.e., an induction of antimicrobial peptides, inflammatory cytokines, and chemokines (Darveau 2010). In this study, the role of human TLR2 and TLR4 in gingival epithelial cells was elucidated by employing *F. nucleatum* as an example of a ubiquitous oral bacterium present in both healthy and diseased sites to reveal the underlying mechanisms of TLR2 and TLR4 sensing associated with IL-8 induction.

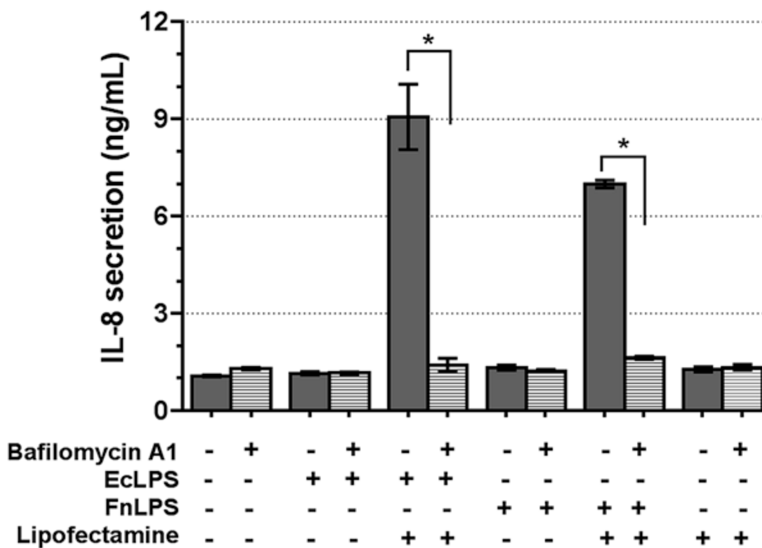


**Fig. 7** Inhibitory effects of bafilomycin A1 on IL-8 induction in oral/gingival epithelial cells in response to internalized LPS. Cultured oral/gingival epithelial cells were pre-incubated with bafilomycin A1 for 1 h prior to LPS delivery. IL-8 secretion was assayed using ELISA. Data are shown as mean actual concentration ± SEM, derived from four independent experiments ($n = 4$). IL-8 quantification was performed in triplicate samples derived from two different donors (hTIGK and OKF6). Asterisks indicate statistical significance ($*p$-value $< 0.05$, NS = not significant), compared to corresponding controls

Previous studies have demonstrated that intestinal epithelial cells do not normally secrete IL-8 in response to isolated LPS preparations unless TLR4 and MD2 were overexpressed (Abreu et al. 2001). However, it was reported that LPS when taken up in epithelial cells will induce a CXCL-2 response requiring TLR4 (Hornef et al. 2002, 2003). Collectively, the data presented here were in agreement that LPS when taken into the cell will elicit an IL-8 response. The inability of gingival epithelial cells to respond to LPS is consistent with a microbial editing function necessary in this tissue in order to maintain a controlled innate defense response associated with the maintenance of periodontal health. Importantly, it was determined that even shed LPS was unable to induce an IL-8 response without internalization revealing the strict control gingival epithelial cells have over excessive IL-8 secretion.

Canonical LPS signaling in eukaryotic cells usually exploits two distinct pathways depending on the location of TLR4, on either the cell surface or endosomal compartments (Kagan et al. 2008). Our data clearly showed that only internalized LPS was detected by gingival epithelial cells for the IL-8 response that required acidification of endosomal compartments. It appears therefore that gingival epithelial cells, similar to intestinal epithelial cells, are programmed to employ only an intracellular route to respond to LPS (Hornef et al. 2003), possibly as a means to prevent overstimulation of the gingival epithelium.

In contrast to LPS-mediated signaling in gingival epithelial cells, TLR2 ligand $Pam_3CSK_4$ induced an IL-8 response in two of epithelial cell lines examined. Although not determined, the unusually high amount of $Pam_3CSK_4$ required to induce an IL-8 response in the primary cell line is consistent with the notion that naturally TLR2 responses are restricted from the lack of functional TLR2 expression (Melmed et al. 2003) and that in some immortalized cell lines this restriction is relaxed. However, it is unclear what may have caused the heterogeneity in the IL-8 response to the TLR2 ligand.

Maintaining periodontal health is associated with the disruption of microbial community and an alteration of host local immune response (Darveau 2010). In this study, it has been shown that one component of maintaining an orchestrated healthy protective innate host response is the inability of gingival epithelial cells to respond to either isolated or naturally shed LPS. Considering the abundance of gram-negative bacteria found in both supra- and subgingival plaque, this protective mechanism may represent a key component of a healthy periodontal state. It has been reported that the expression of TLR4 is significantly different in healthy and diseased gingival tissues (Ren et al. 2005). Therefore, it is tempting to speculate that an increase in gingival epithelial cell responsiveness to LPS contributes to the dysregulated inflammation during episodes of periodontitis. However, further investigations are needed to define the clinical roles of this LPS protection mechanism and its impact on the health of periodontium.

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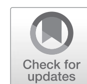

# The Relationship of *Candida albicans* with the Oral Bacterial Microbiome in Health and Disease

Martinna Bertolini and Anna Dongari-Bagtzoglou

## Introduction

Fungi comprise an important constituent of the human oral microbiome, known as the oral mycobiome. Each healthy individual carries around 9–23 fungal genera, with *Candida* species being the most frequently detected oral fungi, followed by *Cladosporium, Aureobasidium, Saccharomyces, Aspergillus, Fusarium,* and *Cryptococcus*. In total, more than 70 cultivable and 11 uncultivable fungal genera were identified by pyrosequencing of ITS sequences in oral rinse samples (Ghannoum et al. 2010). Interestingly, the frequency, intensity, species, and strains of oral *Candida* spp. varied with age (Kleinegger et al. 1996), and elders with high *Candida* load had a lower diversity in their salivary microbiome and a distinct bacterial composition dominated by streptococci (Kraneveld et al. 2012). Gathering additional information on the diversity and composition of the healthy state of the human core mycobiota is important for subsequent studies of fungal community shifts in oral diseases.

The impact of mucosal fungal infections attributed to *Candida* species on public health and healthcare expenditures continues to rise. Candidiasis is the most prevalent mucosal fungal infection in patients with weakened or immature immune systems, such as HIV+ children, neonates, and patients with malignancies (Nagy et al. 1998; Brent 2001; Nicolatou-Galitis et al. 2001). These infections are associated with high morbidity and can lead to systemic disease. The main portal of entry of *C. albicans* into the blood vessels is through the esophageal and gastrointestinal (GI) epithelium, while the oral mucosa is thought to be a fungal reservoir seeding the lower GI tract and alveolar mucosa (Cole et al. 1996). In intensive care or pharmacologically immunosuppressed patients (e.g., transplant, hematologic malignancies, or other cancers), *C. albicans* causes systemic infections with mortality ranging 25–30% (Jarvis 1995). Candidemia, the most common form of systemic candidiasis, is the third most prevalent hospital-acquired bloodstream infection in the US (CDC statistics, 2017) and is considered 50% more deadly than bacterial infections including gram (−) septicemias (Perlroth et al. 2007). This alarmingly high mortality for systemic infections is due to ineffective antifungal therapies, lack of accurate diagnostic tests for early detection, and lack of an effective vaccine. Persistent oroesophageal thrush is refractory to most antifungals and a significant clinical problem (Lucatorto et al. 1991; Redding et al. 2002). Economically, the cost of *Candida* infections is considerable. Annual US healthcare costs for *Candida* infections is approximately $2 billion, and antifungal sales account for ~10% of

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all anti-infectives globally (Moran et al. 2010; Wilson et al. 2002). Therefore, *Candida* species and *C. albicans* in particular (which accounts for ~75% of *Candida* infections) carry an immense health burden, representing a major public health challenge for communities worldwide. Given that the majority of systemic *Candida* infections are acquired across the alimentary-tract mucosae (Gow et al. 2011; Cole et al. 1996), it is of paramount importance to discover new approaches to treat or prevent these infections.

Several studies have shown pathogenic synergy between *C. albicans* and certain mucosal bacteria that leads to more severe oral infection and systemic dissemination. However, very few studies have focused on the interplay between *C. albicans* and resident mucosal bacteriomes in health and disease. Recent experimental evidence has shown that *C. albicans* synergizes with commensal gram-positive cocci to promote invasive mucosal infection (Xu et al. 2014, 2016, 2017; Bertolini et al. 2019; Kong et al. 2015). Based on our work and work by others discussed in this chapter, we posit that future therapeutic and prevention approaches for *Candida*-induced infections should target both the mucosal indigenous microbiota and host mechanisms that restrict fungal breach of mucosal barriers.

## Relationship Between Bacterial Microbiota and *C. albicans* in Mucosal Homeostasis: Lessons Learned from Murine Models

*C. albicans* colonizes the oropharyngeal mucosa of 30–70% healthy humans within the first few weeks of life and persists as a commensal (Russell and Lay 1973). The alimentary tract mucosa also harbors diverse bacterial communities, which are distinct in the oropharynx compared to the lower GI tract (Cho and Blaser 2012). Most bacteria and fungi exist in polymicrobial communities at mucosal sites and their ability to form well-structured biofilms forms the basis for numerous interactions with the host that affect human health. The oral and gastrointestinal mucosa allow polymicrobial community assembly and sessile growth, under both physiologic and pathologic conditions (Pei et al. 2004; Swidsinski et al. 2005). Mice are not colonized by *C. albicans* in a healthy state, although they harbor other indigenous *Candida* species (Iliev et al. 2012). Recent combined genomics and culture approaches have identified 76 culturable bacterial species in the murine lower GI tract (Lagkouvardos et al. 2016). A similar large-scale cultivation study in the murine oral cavity, which would allow more precise taxonomic and functional classification of bacterial sequences, is not yet available. However, Operational Taxonomic Unit (OTU)-level analyses of 16S rRNA gene reads have revealed lower abundance OTUs detected in the mouse oral mucosa that are closely related to culturable members of the human oral microbiome (Dutzan et al. 2017). Low-level colonization of the murine cecum of healthy C57B/L6J mice by *C. albicans* leads to shifts in the community structure such that bacterial communities in the colonized mice are distinct from and more diverse than naive mice (Mason et al. 2012b). *C. albicans* non-pathogenic colonization of the lower GI tract in mice may promote the growth of Bacteroidetes (Mason et al. 2012a). Our studies showed that daily oral inoculation with *C. albicans* induced a decrease in bacterial diversity in the oral mucosa, whereas diversity in the jejunum increased (Bertolini et al. 2019), consistent with other reports in the lower GI tract (Mason et al. 2012b). Although oral community structure changes were not striking, there was an increase in the relative abundance of the genus *Enterococcus* (mostly *E. faecalis*) and a decrease in *Lactobacillus* with oral exposure of mice to different *C. albicans* strains. The positive effect of *C. albicans* inoculation on *Enterococcus* species was evident even with pseudohyphal strains associated with low virulence. Ours was the first study that examined the influence of *C. albicans* colonization on the resident bacteria of mice with unperturbed indigenous microbiota (Bertolini et al. 2019).

The role of indigenous bacteria in alimentary tract colonization of healthy mice has been studied mostly using combinations of broad-spectrum

antibiotics and monitoring the growth of both bacteria and *Candida* in the post-antibiotic period. Enhanced oropharyngeal and intestinal colonization of *C. albicans* was noted with most broad-spectrum antibiotics in mice (Fan et al. 2015; Shankar et al. 2015; Mason et al. 2012a, b). It is well-established that commensal anaerobic bacteria are critical in limiting *Candida* intestinal colonization in mice, and colonization levels are generally proportional to the level of antibiotic depletion of anaerobic bacteria (Koh 2013). However, such an antagonistic relationship between oral anaerobic bacteria and *C. albicans* colonization has not been established in the murine oral mucosa. An increase in *Enterococcus* species in the stomach and small and large intestine (Shankar et al. 2015, Mason et al. 2012a, b) and *Streptococcus* species in the colon (Shankar et al. 2015) in the post-antibiotic period were associated with increased *C. albicans* colonization. On the other hand, abundance of *Lactobacilli* was inversely associated with *C. albicans* colonization in lower GI tract mucosa (Shankar et al. 2015; Mason et al. 2012b). Using innovative predictive statistical models, Shankar and colleagues (Shankar et al. 2015) showed greater dependence of *Candida* colonization in the murine ileum and colon on certain bacterial genera than on the host cytokine environment in the two sites. This study utilized improved regression models to more accurately assess strength of associations between fungal colonization, and bacterial and host parameters in the mouse gut showing a synergy between *C. albicans* and streptococci in mouse gut colonization. These experiments revealed that certain bacterial genera (e.g., *Veillonella*) may influence *C. albicans* colonization by modulating the GI mucosal response, whereas others (e.g., *Streptococcus*) may have a direct impact on *Candida* physiology that does not involve the intestinal immune response (Shankar et al. 2015). Along these lines, a different group showed that *Bacteroides thetaiotaomicron*, a commensal of the murine intestinal mucosa, prevents *C. albicans* colonization by stimulating production of the epithelial antimicrobial proteins cathelicidins (Fan et al. 2015).

## Relationship Between the Resident Bacterial Microbiota and *C. albicans* in Mucosal Disease

### Murine Models of Immunosuppression

In a healthy host, certain members of the resident bacterial microbiota may play an important role in limiting *Candida* infection. This may be accomplished by direct fungal–bacterial cell interactions involving secreted bacterial products. For example, certain *Enterococcus faecalis* strains secrete a bacteriocin that promotes a non-filamentous avirulent phenotype of *C. albicans* in nematodes and orally inoculated mice (Cruz et al. 2013; Graham et al. 2017). In a similar function, a secreted protein of *Salmonella enterica* attenuates the virulence of *C. albicans* in *C. elegans* and promotes a symbiotic state in the nematode gut (Tampakakis et al. 2009; Kim and Mylonakis 2011). Alternatively, resident bacteria may act indirectly to inhibit infection by enhancing the mucosal immune response to *Candida*. An example of this is *Corynebacterium mastitidis*, a member of the resident bacterial microbiota in the murine ocular mucosa, which prevents *C. albicans* infection by driving IL-17 production from mucosal T cells (St Leger et al. 2017). However, in certain non-physiologic host backgrounds, resident bacteria may enhance a tissue-invasive phenotype of *C. albicans*. For example, in germ-free mice, *C. albicans* exists predominantly in the yeast form in the lower GI tract, suggesting that in the absence of resident bacteria, the ability of fungal cells to form tissue-invasive hyphae is limited (Bohm et al. 2017).

All aspects of *C. albicans* virulence and pathogenesis must be examined in the context of the specific host immune status, since this organism does not trigger infection in an immunocompetent host. Two well-recognized risk factors for candidiasis in humans are chronic use of corticosteroids and cancer chemotherapy (Nicolatou-Galitis et al. 2001; Passalacqua et al. 2000; Teoh and Pavelka 2016). Cortisone immunosuppression is the most common oral/esophageal candidiasis mouse model, also characterized by

dissemination in livers and kidneys of orally infected mice (Clancy et al. 2009; Solis and Filler 2012). Using this immunosuppression model, we revealed mutualistic relationships between *C. albicans* and mitis group streptococci. We showed that when *C. albicans* is orally co-inoculated with *Streptococcus oralis*, there is an increase in mucosal biofilms and *Candida* virulence (Xu et al. 2014, 2017; Sobue et al. 2016). Our work further showed that *S. oralis* synergizes with *C. albicans* to augment mucosal invasion both directly by transcriptional activation of fungal filamentation pathways and indirectly by activating epithelial calpain enzymatic pathways that compromise barrier function (Xu et al. 2014, 2016, 2017). Others have shown pathogenic synergy between *C. albicans* and *Staphylococcus aureus* in a similar oral infection model (Kong et al. 2015). In a peritonitis model, rates of disease progression and microbial load in mice infected with both *S. aureus* and *C. albicans* were significantly higher compared to those with monomicrobial infections (Peters and Noverr 2013). In our cortisone oropharyngeal model and the peritonitis model developed by the Noverr lab, pathogenic synergy between gram-positive cocci and *C. albicans* was shown to be host response mediated via induction of a significantly higher proinflammatory response (Xu et al. 2014; Peters and Noverr 2013). In the peritonitis model, pathogenic synergy was primarily eicosanoid mediated (Peters and Noverr 2013), whereas in our oropharyngeal model, an exaggerated TLR-2–dependent chemokine and neutrophil response was involved (Xu et al. 2014).

Under inflammatory or immunocompromised host conditions, ecological resources such as space and nutrients may be diverted to favor indigenous bacterial species that form mutualistic relationships with *C. albicans*, leading to a well-coordinated dysbiosis, which amplifies mucosal damage. For example, in a mouse gastritis model, indigenous enterococci and inoculation with *C. albicans* were both implicated in pathogenesis (Mason et al. 2012a, b). The recent development of a cytotoxic cancer chemotherapy model offered to us the opportunity to study the role of oral bacteria in fungal pathogenesis, since in this model, indigenous bacterial burdens rise in parallel with *C. albicans* infectious burdens (Bertolini et al. 2017, 2019). In patients colonized with *C. albicans* along the GI tract, immunosuppressive effects of cancer chemotherapy combined with damage to the oropharyngeal and gastrointestinal mucosa are thought to result in invasion of the mucosal tissue by *Candida* hyphae and ultimately dissemination via the hepatic portal vein to the liver (Teoh and Pavelka 2016; Meunier et al. 1989). Older models of gastrointestinal candidiasis associated with cytotoxic chemotherapy were based on a single high dose of 5-FU or cyclophosphamide to induce acute neutropenia, a well-known risk factor for disseminated disease of gastrointestinal origin (Clemons et al. 2006; Hata et al. 2011). In contrast, our mouse model of 5-FU chemotherapy is based on low dose, repeated intravenous administration of 5-FU that recapitulates both the oral and gastrointestinal toxicity (mucositis), and neutropenic effects of chemotherapy in cancer patients (Bertolini et al. 2017, 2019). In this model, mice orally inoculated with *C. albicans* gradually develop severe concurrent oroesophageal and intestinal candidiasis over the course of 8 days (Sobue et al. 2018; Bertolini et al. 2019). Thus, this model gave us the opportunity to longitudinally examine site-specific indigenous bacterial changes associated with infection. Mice receiving chemotherapy had an endogenous bacterial overgrowth on the oral but not the small intestinal mucosa. Time-dependent analysis of beta diversity changes in the microbiomes of these mice showed that *C. albicans* caused a profound disruption of the tongue and small intestinal community structure after 6 days of chemotherapy. *C. albicans* infection was associated with loss of mucosal bacterial diversity in both sites, with indigenous *Stenotrophomonas, Alphaproteobacteria,* and *Enterococcus* species dominating the small intestinal, and *Enterococcus faecalis* representing >90% of the oral mucosal communities. *E. faecalis* isolated from mice with oropharyngeal candidiasis was implicated in degrading the epithelial junction protein *E-cadherin* and increasing the permeability of the oral epithelial barrier in vitro. Importantly,

depletion of these organisms with antibiotics in vivo attenuated oral mucosal E-cadherin degradation and *C. albicans* invasion without affecting fungal burdens, indicating that bacterial community changes represent overt dysbiosis (Bertolini et al. 2019).

Although clinically similar oropharyngeal infections are present with either cortisone or cytotoxic chemotherapy, different immune and local mucosal response factors may be involved in pathogenesis under these two host conditions. For example, in pulmonary and oral infection models, mice given cortisone have an increased number of functionally competent infiltrating neutrophils, whereas mice given cytotoxic chemotherapy are severely neutropenic (Bertolini et al. 2017, 2019; Sawyer and Harmsen 1989). That different aspects of the host response are involved in these two models are also illustrated by the fact that prophylactic treatment with granulocyte colony stimulating factor affords significant protection against systemic infections caused by *C. albicans* in chemotherapy-treated but not cortisone-treated mice (Polak-Wyss 1991). We recently showed that cortisone treatment alone causes a small but statistically significant increase in the total bacterial biomass on the mouse oral mucosa (Bertolini et al. 2019). However, although the total bacterial biomass increased, the enterococcal biomass decreased up to 40% compared to untreated control mice, suggesting that the total bacterial biomass increase was due to overgrowth of other bacterial species. This was in contrast to 5-FU treatment where enterococci increased in abundance together with other species. Importantly, infection with *C. albicans* in cortisone-immunosuppressed mice caused a further increase in total bacterial burdens, while enterococci rebounded at or slightly above untreated levels. Taken together, our studies show that while both types of immunosuppression increase oral bacterial burdens, they influence bacterial biodiversity in different ways. We also conclude that *C. albicans* infection favors the growth of endogenous oral enterococci under different immunosuppressive states.

Disease promoting interactions between *Candida* and resident mucosal bacteria may be mucosal site specific, and as fungal genomic technologies are evolving in the recent years, studies of the mycobiome in different organs have started to shed some light on the complexity and heterogeneity of fungal communities at different environments and different sites. For example, mice pretreated with antibiotics and then inoculated with *C. albicans* experience significant inflammatory, ulcerative gastric lesions, but do not develop signs of inflammation in the cecum, despite having similar fungal burdens at this site (Mason et al. 2012a, b). Similarly, in the lower GI tract, gram-negative anaerobes and enteric bacilli limit the growth and dissemination of *Candida* (Sjovall et al. 1986), and after treatment with broad-spectrum antibiotics, *C. albicans* can invade the gut mucosa (Ponnuvel et al. 1996; Krause et al. 2001). In contrast, reports of oral candidiasis or oral mucosal invasion after antibiotic treatment are rare and associated with severely compromised host backgrounds (Gligorov et al. 2011). Thus, the effect that indigenous bacteria have on *Candida* invasion is likely to differ at different mucosal sites due in part to anatomically distinct innate immune epithelial response to infection (Yano et al. 2010).

## Emerging Evidence from Human Oral Microbiome Studies in Oropharyngeal Candidiasis

Studies exploring shifts in the mucosal microbiome during pharmacologic immunosuppression and oral fungal infection in humans are scarce and more studies are sorely needed. Apart from experimental evidence reviewed above, there is clinical evidence that implicates streptococci and *C. albicans* in the pathogenesis of pulmonary and oral infections in immunocompromised patients (Maeda et al. 2011; Abusleme et al. 2018), supporting our earlier work in a mouse model of oropharyngeal candidiasis (Xu et al. 2014, 2016, 2017). Importantly in a recent study, *S. oralis* was identified as the top bacterial species implicated in oral dysbiosis in humans with hyper-IgE inflammatory syndrome-associated oropharyngeal candidiasis (Abusleme et al. 2018).

Moreover, a recent systematic review focusing on evaluation of the impact of chemotherapeutic treatment on the oral microbiota in patients with cancer showed that during chemotherapy, there is an increase in bacteria of the *Enterobacteriaceae* family and in *Streptococcus* species, potentially contributing to local oral manifestations, such as oral mucositis, or even systemic infections such as septicemia (Villafuerte et al. 2018). The type of immunosuppression can have a detrimental role in modifying the local microbial environment where *C. albicans* infections occur. For example, a study of the colon microbiome in transplant patients receiving immunosuppressants showed major shifts with a predominant increase in the proportion of enterococci and a decrease in other Firmicutes, evident as early as the immediate post-transplant period when these patients are extremely susceptible to gastrointestinal candidiasis (Holler et al. 2014). Interestingly, in patients who developed graft-versus-host disease, this shift was even more pronounced, raising the possibility of enterococci acting as pathobionts in this clinical setting (Holler et al. 2014).

## Conclusions and Future Directions

Polymicrobial infections with *C. albicans* and bacteria are not only more prevalent but also have higher morbidity and mortality in immunosuppressed patients (Boktour et al. 2004; Puig-Asensio et al. 2015). We propose a pathogenesis model whereby immunosuppression coupled with *C. albicans* colonization results in a bacterial dysbiosis with dominant species that have the ability to promote fungal virulence, thus acting as synergistic pathobionts (Fig. 1). We recently demonstrated that oral *C. albicans* infection leads to a profound taxonomic imbalance with a striking dominance of *Enterococcus* that contributes to pathology (Bertolini et al. 2019). Like *C. albicans, Enterococcus* species are a major concern in critical care areas due to resistance to multiple antibiotics (Sparo et al. 2018). In the human oral cavity, enterococci are traditionally considered transient commensals and carriage rates in healthy adults are below 20% (Gonçalves et al. 2009; Komiyama et al. 2016). However, the oral carriage rate of *Enterococcus* species (predominantly *E. faecalis*) in patients on chemotherapy treatment (Yang et al. 2013) and patients with underlying systemic disease such as HIV rises up to 82% (Gonçalves et al. 2007, 2009). These are also some of the most high-risk populations for oropharyngeal candidiasis; thus, our findings may have serious clinical implications in these patient populations (reviewed by Lalla et al. 2010 and Olczak-Kowalczyk et al. 2012).

Our studies provide compelling evidence and impetus for a novel oropharyngeal candidiasis pathogenesis framework, which includes the fungus, the resident bacterial microbiota, and a host-permissive environment (Fig. 1). A comparison of the 5-FU and cortisone models showed that both types of immunosuppression allow overgrowth of endogenous bacterial organisms on the oral mucosa. However, the two treatments had different effects on enterococci, with 5-FU promoting and cortisone curtailing their growth, supporting qualitatively different effects on biodiversity. This is not surprising since mice given cortisone have an increased number of functionally competent oral mucosa infiltrating neutrophils (Xu et al. 2014), whereas mice given cytotoxic chemotherapy are severely neutropenic (Bertolini et al. 2017, 2019). Given the central role of neutrophils in the control of endogenous enterococcal overgrowth in mice (Leendertse et al. 2009), the finding that a neutropenic state induced by 5-FU led to overgrowth of these organisms was anticipated. Alternatively, mucosal injury or associated type of inflammatory responses could explain the differences observed between the two types of immunosuppression. Based on the different effect of cortisone and 5-FU–induced immunosuppression on oral enterococci (Bertolini et al. 2019), we propose that the type of immunosuppression influences the type of bacterial dysbiosis associated with mucosal candidiasis.

In conclusion, it is both timely and important to consider the pathogenesis of mucosal candidiasis as being integrally related to the physiology of the resident microbial communities within

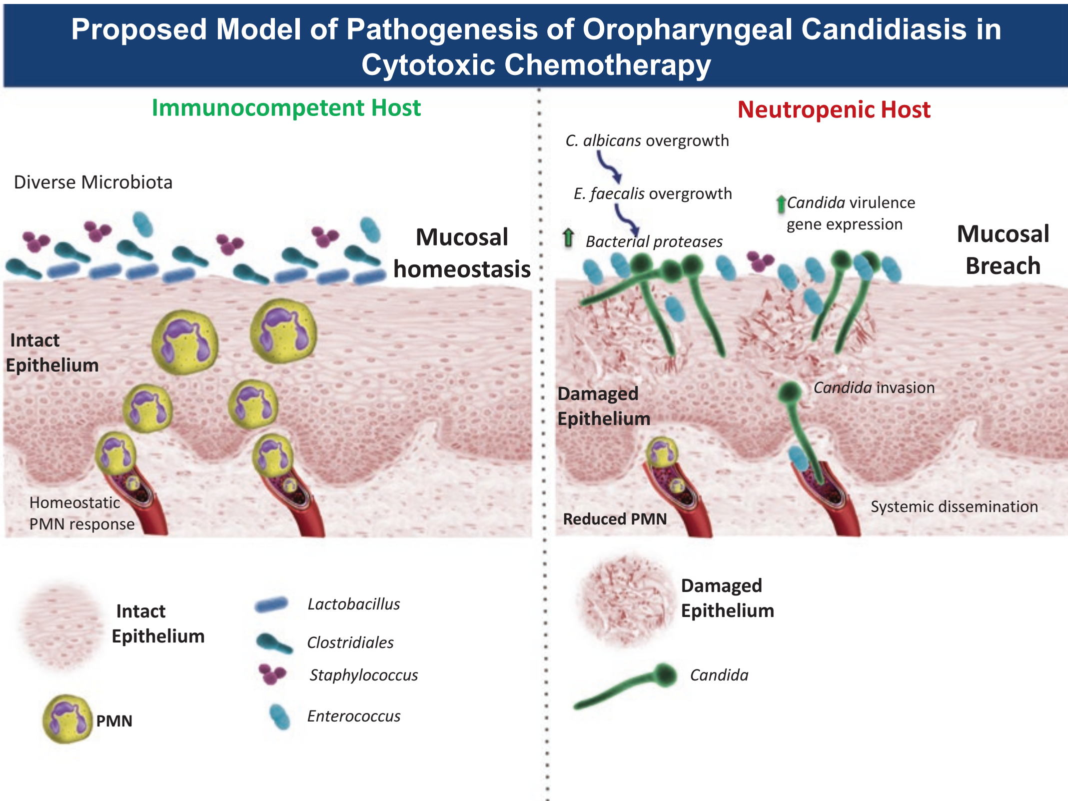


**Fig. 1** Model of oral mucosal candidiasis in cytotoxic cancer chemotherapy. Cytotoxic chemotherapy promotes a dysbiotic state characterized by overgrowth of *C. albicans* and certain resident bacterial species, such as *Enterococcus faecalis*, which have mutualistic relationships with the fungus. Bacteria act as accessory pathogens partly via release of proteolytic enzymes, which augment mucosal barrier breach and hematogenous dissemination by *C. albicans*

which *C. albicans* resides as a commensal or causes disease. Resident bacteria can directly modulate the virulence of *C. albicans* by altering virulence gene expression or indirectly by modulating the host response (Fig. 1). Therefore, site- or niche-specific fungal responses to the local microbiota and reciprocal effects on mucosal bacteria are an important component of fungal pathogenesis. Going forward, mucosal infection models should take advantage of the knowledge gained from the metagenomics field to identify resident commensals with the potential to become pathobionts and define mechanisms of pathogenic synergy with *C. albicans* in different host immunosuppression backgrounds. Such studies will pave the way for better targeted preventative and therapeutic strategies in mucosal candidiasis.

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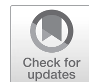

# A Potential Role of Phospholipase 2 Group IIA ($PLA_2$-IIA) in *P. gingivalis*-Induced Oral Dysbiosis

Octavio A. Gonzalez, Vanessa Euzebio-Alves, Yelena Alimova, Ahmad Al-Attar, and J. L. Ebersole

## Introduction

Variation in the abundance of bacterial species that normally colonize epithelial surfaces and favors overgrowth of pathobionts (i.e., microorganisms that are part of the indigenous oral flora but transition to pathogenic after disruption of homeostasis) with a concomitant decrease in bacterial species normally associated with health (also named symbionts) is known as dysbiosis. Growing evidence indicates that dysbiosis is associated with several diseases such as inflammatory bowel disease, obesity, diabetes mellitus, and periodontal disease (DeGruttola et al. 2016; Maruvada et al. 2017; Carding et al. 2015; Hajishengallis et al. 2011; Dutzan et al. 2018). Enhancers of dysbiosis include environmental (e.g., smoking, diet, antibiotics), host factors (e.g., immunosuppression), and some pathogens such as *Salmonella typhimurium* and *Helicobacter pylori* (Kau et al. 2011; Baumler and Sperandio 2016; Brawner et al. 2017).

*Porphyromonas gingivalis* is an oral gram-negative pathobiont with the ability to enhance oral dysbiosis and periodontal disease (Hajishengallis et al. 2011). The mechanisms by which *P. gingivalis* enhances oral dysbiosis remain not fully elucidated; nevertheless, evidence indicates that *P. gingivalis* could be manipulating the host immune response for its own benefit. For example, it has been demonstrated in humans and mice that *P. gingivalis* manipulates complement C5a receptor 1 (C5aR1) and Toll-like receptor 2 (TLR2) cross talk signaling to block phagocytosis through a suppression of phagolysosomal maturation and simultaneously promotion of inflammation (Maekawa et al. 2014; Wang et al. 2010). Another described mechanism involved in *P. gingivalis*-induced oral dysbiosis is a localized chemokine paralysis. This refers to the ability of *P. gingivalis* to suppress the expression of chemokines in oral epithelial cells (OECs) (e.g., CXCL8, CXCL9, CXCL10, and CXCL11) through a mechanism that involves negative regulation of NFκB by a serine phosphatase B secreted by *P. gingivalis*. As a consequence, decreased chemokine expression leads to a reduction in the number of immune cells normally

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recruited to the subgingival environment that are critical for immune surveillance and maintaining homeostatic host–bacteria interactions (Darveau et al. 1998; Jauregui et al. 2013; Takeuchi et al. 2013).

Epithelial cells, in addition to orchestrating the recruitment of immune cells through the production of chemokines in response to the microbiota, also produce antimicrobial factors such as human beta defensins (hBDs) that are critical to maintain host–bacteria homeostatic interactions (Dale 2002; Chung et al. 2007). It has been shown that OECs express several antimicrobial factors (e.g., hBD-2 and hBD-3, CCL20) in response to oral bacterial species, and these responses seem to be differentially induced by both oral pathogenic and commensal bacterial species (Dommisch et al. 2012; Hosokawa et al. 2006; Greer et al. 2013; Chung and Dale 2008; Ji et al. 2007).

Specific modulation of OEC antimicrobial responses by oral commensal and pathogenic bacteria could be a plausible mechanism contributing to the maintenance or disruption of the homeostatic host–microbe interactions during health and disease, respectively (Lamont et al. 2018). In fact, some oral bacterial species specifically induce the production of classical antimicrobial peptides (AMPs) such as hBDs by OECs, while exhibiting antimicrobial resistance to the AMPs. For example, *Fusobacterium nucleatum* has been shown to induce significant hBD2 expression in OECs and be resistant to hBD2 killing; however, *P. gingivalis* seems to be a weak hBD2 inducer and is more susceptible to killing by hBD2 (Ghosh et al. 2018). In contrast, *P. gingivalis* enhances a remarkable expression of the antimicrobial protein phospholipase A2-group IIA ($PLA_2$-IIA) by OECs, and is resistant, whereas *F. nucleatum* appears susceptible to $PLA_2$-IIA–induced killing (Al-Attar et al. 2018).

Whether *P. gingivalis*' ability to specifically modulate antimicrobial responses in OECs is a strategy to enhance oral dysbiosis remains unknown; however, the potentiation of the antimicrobial properties of OECs through upregulation of $PLA_2$-IIA seems to be a plausible mechanistic pathway as we have recently reported (Al-Attar et al. 2018). In this chapter, we will review the general properties and functions of $PLA_2$-IIA and postulate a hypothesis concerning its potential contribution to the pathogenesis of *P. gingivalis*-induced oral dysbiosis and periodontal disease.

## $PLA_2$-IIA, General Properties

$PLA_2$-IIA (also known as non-pancreatic secretory- or synovial secretory-$PLA_2$) belongs to a family of ten secreted $PLA_2$s (IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XIIA) identified in mammals and is the only isoform detectable in the blood. $PLA_2$-IIA is a highly cationic lipolytic enzyme, with catalytic activity (as for all secreted $PLA_2$s) that is calcium ($Ca^{2+}$) dependent (Murakami et al. 2011a, b). $PLA_2$-IIA mRNA has been detected in placenta, tonsil, skin keratinocytes, kidney, rheumatoid synovial cells, cartilage, colon, ileum, stomach, spleen, and lung tissues (Ishizaki et al. 1989; Kramer et al. 1989; Nevalainen and Haapanen 1993; Ilic et al. 2014). Among the cell types that have been shown to produce $PLA_2$-IIA in response to different stimuli such as pathogenic bacteria (e.g., *Pseudomonas aeruginosa*) or proinflammatory cytokines are hepatocytes, platelets, macrophages, and intestinal epithelial cells (i.e., Paneth cells) (Crowl et al. 1991; Okita et al. 2016; Mueller et al. 1993; Piris-Gimenez et al. 2005; Ouellette 2011; Pernet et al. 2014).

Given that $PLA_2$-IIA is secreted, it has been suggested that its primary targets should be extracellular non-cellular phospholipids including microvesicles, microbial membranes, and dietary phospholipids (Murakami et al. 2011a). Nevertheless, cell-associated functions have also been attributed to $PLA_2$-IIA in concert with other $PLA_2$s such as cytoplasmic $PLA_2$ isoforms that release arachidonic acid (AA) from cell membranes with the consequent production of inflammatory lipids (e.g., prostaglandins) (Murakami et al. 1996, 1997a). These observations remain controversial since $PLA_2$-IIA has low affinity for

phosphatidylcholine (PC), a major phospholipid in the outer leaflet of the plasma membrane. In contrast, due to its high cationic nature, $PLA_2$-IIA exhibits high affinity for phosphatidylethanolamine (PE), phosphatidylglycerol (PG), or phosphatidylserine (PS), which are enriched in bacterial membranes and apoptotic cells making them suitable targets for the enzyme (Atsumi et al. 1997; Bayburt et al. 1993; Nevalainen et al. 2008).

In addition to its enzymatic activities, it has been suggested that $PLA_2$-IIA could also modulate biological responses in an enzymatic-independent manner through interaction with cellular receptors. Particularly, the muscular-type receptor (M-type-R) or $sPLA_2$ mouse receptor ($PLA_2R1$) are type I transmembrane glycoproteins composed of multiple C-type lectin domains that can be activated by $PLA_2$-IIA (and other $PLA_2$s such as group IB and group X) leading to internalization into phagolysosomes, in which the enzyme is rapidly degraded. Thus, it could be a mechanism by which $PLA_2$-IIA can be physiologically cleared from fluids (Zvaritch et al. 1996; Cupillard et al. 1999; Murakami et al. 2014). Nevertheless, the lack of an intracellular tail of $PLA_2R1$ with cell signaling molecular features suggests that cellular responses mediated by this receptor could be linked to other receptors such as Toll-like receptors (TLRs) (Murakami et al. 2011b). $PLA_2R1$ expression has been shown in skeletal muscle, lung, liver, heart, and kidney as well as in myeloid cells such as neutrophils, macrophages, and eosinophils (Lambeau and Lazdunski 1999; Silliman et al. 2002; Granata et al. 2005; Triggiani et al. 2003). Interestingly, in vitro studies demonstrated that $PLA_2R1$ expression is increased during cellular senescence and regulates senescence in human fibroblasts through the p53 pathway (Augert et al. 2009). These findings were recently validated in vivo showing that $PLA_2R1$ mediates premature aging (Griveau et al. 2018).

In general, important regulatory roles of $PLA_2$-IIA in inflammation and infection have been demonstrated. Details about the potential mechanisms involved in these critical biological processes will be discussed in the following sections.

## Proinflammatory Properties of $PLA_2$-IIA

$PLA_2$-IIA was identified in humans in 1989 as an inducible enzyme during inflammatory responses isolated from rheumatoid arthritis synovial fluid (Kramer et al. 1989; Seilhamer et al. 1989). Since then, several studies have demonstrated that expression of $PLA_2$-IIA can be induced by proinflammatory cytokines including interleukin-1alpha (IL-1α), IL-1β, tumor necrosis factor-alpha (TNFα), as well as lipopolysaccharide (LPS) in macrophages. Consistently with these observations, elevated $PLA_2$-IIA levels in sera or inflammatory exudates are correlated with the severity of inflammation and infection (Gronroos et al. 2002; Tan and Goh 2017). On the other hand, glucocorticoids, anti-inflammatory cytokines, and growth factors (e.g., dexamethasone, transforming growth factor β, platelet-derived growth factor, and insulin-like growth factors I and II) are potent suppressors for the induction of $PLA_2$-IIA (Murakami et al. 1997b; Jacques et al. 1997).

It has been suggested that the proinflammatory properties of $PLA_2$-IIA would be related to its enzymatic activity participating in the release of AA and contributing to the production of eicosanoids such as prostaglandins, or independently of its enzymatic activity, modulating immune cell responses through the $PLA_2R1$. In general, several studies suggest that the role of $PLA_2$-IIA in AA release from cell membranes is minor and seems to be cell-type dependent when compared with other $PLA_2$ enzymes such as cytosolic $PLA_2$, whose role in AA production has been clearly demonstrated (Murakami et al. 2017; Fonteh et al. 1994; Kuwata et al. 1999; Marshall et al. 2000; Mounier et al. 1993).

Another proinflammatory mechanism is the activation of immune cells through the $PLA_2R1$. For example, macrophages stimulated with $PLA_2$-IIA showed activation of PI3K, AKT, ERK1/2, JNK, NFκB pathways resulting in up-regulation of inducible nitric oxide synthase (iNOS) and NO generation, as well as increased expression of cytokines/chemokines (Baek et al. 2000, 2001; Hernandez et al. 2002; Triggiani

et al. 2002). Similarly, in neutrophils, the activation of $PLA_2R1$ led to the release of elastase and changes in cell adhesion through activation of the p38-MAPK pathway (Silliman et al. 2002). Most recently, the ability of $PLA_2$-IIA to bind integrins (e.g., αvβ3 and α4β1) was demonstrated, which suggested that immune cell activation by $PLA_2$-IIA could also be mediated by this pathway (Fujita et al. 2015; Takada and Fujita 2017).

A most recent and plausible mechanism by which $PLA_2$-IIA could induce inflammation is through generation of inflammatory mediators such as lysophospholipids, fatty acids, and mitochondrial DNA as a product of the hydrolysis of the mitochondrial membrane. This is interesting given that platelets during inflammatory processes can release mitochondria that serve as a $PLA_2$-IIA substrate (Boudreau et al. 2014). Notably, neutrophils can internalize platelet microparticles (which contain transcription factors, nucleic acids, and mitochondria) in a mechanism mediated by $PLA_2$-IIA and platelet-type 12-lipoxygenase, which could also be contributing to pathologic inflammation in arthritis (Duchez et al. 2015).

A better understanding of the mechanisms by which $PLA_2$-IIA could be contributing to enhance inflammation is an evolving field. Future studies will be needed to clarify the role of $PLA_2$-IIA in inflammatory pathologies in which higher levels have been found locally and systemically.

## Antibacterial Properties of $PLA_2$-IIA

As indicated earlier in this chapter, the antibacterial properties of secreted $PLA_2$s have been broadly documented (Nevalainen et al. 2008; Koduri et al. 2002). Among all $sPLA_2$s, the antibacterial effect of $PLA_2$-IIA is the most potent (ng) among all secreted isoforms (Koduri et al. 2002).

It has been shown that $PLA_2$-IIA has a greater affinity for gram-positive than gram-negative bacteria. This seems to be due to the structural characteristics of the cell wall of these bacteria phyla, whereby multiple layers of peptidoglycan cross-linked with peptide interbridges also contain higher levels of teichoic and lipoteichoic acids in gram-positive bacteria. These components proscribe a negative charge to the bacterial wall that creates an ideal/complementary substrate for the cationic $PLA_2$-IIA (Foreman-Wykert et al. 1999; Weiss 2015; Dore and Boilard 2019). $PLA_2$-IIA is therefore able to penetrate and hydrolyze the plasma membrane of gram-positive bacteria (Beers et al. 2002). In contrast, gram-negative bacteria normally have a thinner peptidoglycan layer that is covered by lipopolysaccharide, which prevent the entry of $PLA_2$-IIA and subsequent bacterial killing (Beers et al. 2002). It is likely that for $PLA_2$-IIA to kill gram-negative bacteria, other host factors working in concert with the enzyme, such as complement, would be necessary (Madsen et al. 1996; Gronroos et al. 2005).

$PLA_2$-IIA seems to be a critical innate factor to protect against infections. For example, $PLA_2$-IIA sera levels are increased during group B streptococcus (GBS) infections and clinically relevant GBS strains are rapidly killed by $PLA_2$-IIA (Movert et al. 2013). Similarly, $PLA_2$-IIA has been shown to be an important innate factor that plays a protective role against *Bacillus anthracis* infections (Piris-Gimenez et al. 2005). Moreover, normal serum $PLA_2$-IIA concentrations effectively kill the commensal *Enterococcus faecium (*Paganelli et al. 2018*)*. Interestingly, some pathogenic strains of *Staphylococcus aureus* appear to modulate $PLA_2$-IIA expression at epithelial surfaces to avoid being killed and enabling disease processes (Pernet et al. 2015).

The potent antimicrobial properties of $PLA_2$-IIA at low concentrations are of particular clinical interest, given that $PLA_2$-IIA concentrations in body fluids under normal conditions have been shown to prevent some gram-positive bacterial infections. In addition, the bactericidal effect of acute phase serum seems to be determined mostly by the presence of elevated levels of $PLA_2$-IIA. For example, $PLA_2$-IIA serum levels from healthy individuals (1.5 ng/mL) or during sepsis (300 ng/mL) can kill *Listeria monocytogenes* or *S. aureus,* respectively, in 2 h (Gronroos et al. 2002). Similarly, $PLA_2$-IIA concentrations (50 ng/mL) in expectorations from adult cystic

fibrosis patients can efficiently kill *S. aureus* (Pernet et al. 2014). Likewise, higher levels of $PLA_2$-IIA have been detected in tears exceeding about 400 times those found in sera from healthy subjects, which suggests that this enzyme could play a significant role in the innate protective immunity of the ocular surfaces (Nevalainen et al. 1994).

This evidence suggests that $PLA_2$-IIA is an important innate antimicrobial factor that plays a significant role at mucosal surfaces maintaining homeostatic host–bacteria interactions as well as contributing to the control and eradication of different pathogens during the acute phase. It is tempting to hypothesize that increased $PLA_2$-IIA levels at mucosal surfaces may contribute to modify the normal abundance of bacterial species, especially reducing gram-positive bacterial numbers that could enhance a dysbiotic microbial ecology and disease.

## $PLA_2$-IIA, Role in Disease

$PLA_2$-IIA has been associated with several diseases such as atherosclerosis, rheumatoid arthritis, Alzheimer's disease, cancer, and inflammatory bowel disease. Interestingly, all these diseases have as a common denominator chronic inflammation, as well as microbial components that could be involved in the pathologic progression. Some of the main findings relating $PLA_2$-IIA with some of these diseases will be presented in this section.

### Atherosclerosis

The association between $PLA_2$-IIA and increased risk for atherosclerotic cardiovascular disease has been established (Sun et al. 2016). Clinical studies have indicated that elevated blood levels of $PLA_2$-IIA are an independent risk factor for coronary artery disease and predict clinical coronary events independent of other risk factors (Kugiyama et al. 1999). Similarly, higher circulation $PLA_2$-IIA levels and enzymatic activity were associated with an increased risk of incident and recurrent major vascular events such as cardiovascular death, myocardial infarction, and stroke. This association was similar to that observed between C-reactive protein and coronary artery disease (Boekholdt et al. 2005; Mallat et al. 2007; O'Donoghue et al. 2011).

It is well described that augmented levels of low-density lipoprotein (LDL) cholesterol particles increase the risk of coronary heart disease through stimulation of foam cell formation. A potential pro-atherogenic mechanism of $PLA_2$-IIA could involve induction of foam cell formation and LDL lipoprotein modification, both events associated with higher levels of $PLA_2$-IIA expressed in vascular smooth muscle cells from both medial and intimal layers and in macrophage-rich regions of atherosclerotic plaques (Menschikowski et al. 1995; Jaross et al. 2002). Consistent with these findings, it was shown that $PLA_2$-IIA is able to increase the adhesion and migration of monocytic cells, which could increase macrophage recruitment to endothelium during the atherogenic process (Ibeas et al. 2009). Evidence supporting the association between $PLA_2$-IIA with atherosclerotic disease has generated great interest in developing inhibitors that may help to reduce atherosclerosis (Magrioti and Kokotos 2010; Garcia-Garcia and Serruys 2009).

### Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune and inflammatory disease that causes inflammation and tissue damage of the joints leading to chronic pain and deformity (www.cdc.gov). The initial observations linking $PLA_2$-IIA and inflammation were performed in patients with RA and osteoarthritis, where characterization of $PLA_2$-IIA and detection of its enzymatic activity in sera and synovial fluids were determined (Vadas et al. 1985; Pruzanski et al. 1985). Further studies have documented that serum $PLA_2$-IIA concentrations are elevated in patients with RA and seem to correlate with severity of disease (Pruzanski et al. 1988, 1994; Kortekangas et al. 1994; Lin et al. 1996; Jamal et al. 1998). $PLA_2$-IIA has also been found in articular and extra-articular cartilage

from RA patients, which is consistent with the ability of human chondrocytes to synthesize and release $PLA_2$-IIA (Nevalainen et al. 1993; Pruzanski et al. 1990). Encouraged by these observations, several groups have investigated the potential role of $PLA_2$-IIA in the pathogenesis of RA. For example, injection of pure recombinant $PLA_2$-IIA elicited a dramatic inflammatory and arthritogenic response in rabbit joints characterized by hyperplasia of the synovial lining cells and prostaglandin production in comparison to pancreatic $PLA_2$, which had little activity in this model (Bomalaski et al. 1991). The authors suggested that given that proinflammatory cytokines can induce $PLA_2$-IIA synthesis and secretion, synovial $PLA_2$-IIA could play a role in acute episodes in chronic inflammatory conditions such as RA. Most recently, it was elegantly shown by Boilard's group that $PLA_2$-IIA could specifically potentiate inflammatory responses in vivo by acting with a type 12 lipoxygenase (12-LO) expressed by platelet extracellular vesicles (also called platelet microparticles-MP) to enhance internalization of those MP by neutrophils and promoting autoimmune inflammatory arthritis (Duchez et al. 2015).

## Cancer

Among the secreted $PLA_2$s, group IIA, III, and X enzymes are significantly dysregulated in various types of cancer such as lung, gastric, and colon. Several biological mechanisms involved in $PLA_2$s-induced pro-tumorigenic responses have been described such as stimulation of cell proliferation and cell survival (mediated by antiapoptotic effects), inflammation, and angiogenesis. Nevertheless, the role of $PLA_2$-IIA in cancer seems to exert either a pro- or anti-tumorigenic role, depending on the tissue and cancer type (Brglez et al. 2014). For example, patients with prostate (Jiang et al. 2002; Dong et al. 2010), esophageal (Menschikowski et al. 2013), and lung (Kupert et al. 2011) cancer exhibit elevated $PLA_2$-IIA levels compared with healthy controls. Of note, prolonged survival time was seen in patients with lower $PLA_2$-IIA levels (Menschikowski et al. 2013).

In contrast, the increased expression of $PLA_2$-IIA in gastric cancer cell lines and tumors appears to be associated with an anti-tumorigenic role of the enzyme, as it reduces cell migration and invasiveness in vitro, its expression correlates with longer survival and is a predictor of a favorable outcome for patients with gastric cancer (Minami et al. 1997; Ganesan et al. 2008; Wang et al. 2013). Interestingly, the expression of $PLA_2$-IIA mRNA was found to be elevated in primary, early-stage gastric tumors but decreased in late-stage metastatic tumors, suggesting that the loss of $PLA_2$-IIA activity may contribute to the development of more aggressive tumors (Ganesan et al. 2008).

The expression of $PLA_2$-IIA is also upregulated in colon cancer; however, its role in pathogenesis remains controversial with studies showing either pro- or anti-tumorigenic effects. This seems to be partly due to the difficulty of extrapolating results from mouse to human malignancies and inconsistent results in both mouse models and human tissues (Brglez et al. 2014; Mounier et al. 2008). A recent report demonstrated that intracellular $PLA_2$-IIA and $PLA_2$-X produced by Paneth cells are stem cell niche factors that, during homeostasis, inhibit Wnt signaling (pathway that has an anti-tumorigenic role); however, with inflammation $PLA_2$-IIA and -X can be secreted into the intestinal lumen, where the enzymes promote prostaglandin synthesis and Wnt signaling. Thus, these $PLA_2$ enzymes seem to be important genetic modifiers of inflammation and colon cancer (Schewe et al. 2016).

## Alzheimer's Disease

Expression of major groups of $PLA_2$ enzymes has been described in the central nervous system including $PLA_2$-IIA, which seems to be particularly associated with neurons and glial cells (Sun et al. 2004, 2010). Specifically, $PLA_2$-IIA and $PLA_2$-IVA seems to be contributors of neurodegeneration (Schaeffer et al. 2010; Sanchez-Mejia et al. 2008). As such, increased expression of

$PLA_2$-IIA mainly by astrocytes was found in Alzheimer's disease (AD) brains as compared to non-demented elderly brains. Likewise, $PLA_2$-IIA–positive astrocytes were associated with amyloid beta-containing plaques, which seem to play a major role in neuronal cell death and neuro-inflammation, two critical factors leading to neurodegeneration as the principal underlying cause of the cognitive decline seen in AD (Moses et al. 2006; Culmsee and Landshamer 2006). Supporting these observations, it has been demonstrated that $PLA_2$-IIA is highly cytotoxic for neurons and astrocytes, with proinflammatory mediators such as IL-1β and TNFα being major drivers for $PLA_2$-IIA expression in neurons and microglia (Schaeffer et al. 2010; Villanueva et al. 2012; Jensen et al. 2009; Thomas et al. 2000; Yagami et al. 2002). Most recently, a significant increase of secreted $PLA_2$ activity was observed in cerebrospinal fluid of AD patients, which presented the possibility of using secreted $PLA_2$ activity as a biomarker for neuroinflammation. Nevertheless, the specific contribution of $PLA_2$-IIA to these findings was not addressed (Chalbot et al. 2009).

## Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease is a group of conditions [i.e., Crohn's disease (CD) and ulcerative colitis (UC)] that are characterized by chronic inflammation of the gastrointestinal (GI) tract and leads to damage of the GI mucosal surfaces and the onset of clinical symptoms such as abdominal pain, persistent diarrhea, bloody stool, weight loss, and fatigue (www.cdc.gov). Although less evidence exists about a potential role of $PLA_2$-IIA in the pathogenesis of IBD, some studies have shown that increased $PLA_2$-IIA serum levels and activity are associated with CD and UC (Minami et al. 1994, 1997) and $PLA_2$-IIA activity decreased in parallel with clinical improvement and correlated with serum C-reactive protein and erythrocyte sedimentation rate (Minami et al. 1992). It seems like most attention has been placed on the inflammatory properties of $PLA_2$-IIA within the context of IBD, with little research exploring its role in intestinal microbial dysbiosis associated with its potent antimicrobial effects.

In general, the above evidence suggests that $PLA_2$-IIA is associated with several systemic diseases and seems to play an important role in their pathogenesis. Modulation of $PLA_2$-IIA expression could be an innovative strategy for preventing/treating them. For instance, the molecular structure of $PLA_2$-IIA was resolved, which has contributed to improve the understanding of its functions as well as the possibility to explore different strategies for identification and testing candidate chemical/drug inhibitors (Scott et al. 1991; Wery et al. 1991; Church et al. 2001; Thwin et al. 2007; Magrioti and Kokotos 2013). Interestingly, it has been shown that lipophilic tetracyclines such as minocycline and doxycycline inhibit $PLA_2$-IIA, probably by interaction with the substrate (Pruzanski et al. 1992).

Periodontal disease has also been associated as a risk factor for some of the chronic inflammatory diseases discussed above in which oral pathogenic bacteria such as *P. gingivalis* (a strong inducer of $PLA_2$-IIA) seem to contribute to their pathogenesis (de Pablo et al. 2009; Gibson 3rd et al. 2006; Velsko et al. 2015; Hayashi et al. 2010). It is tempting to hypothesize that *P. gingivalis* virulence factors (e.g., gingipains) may also be contributing to enhance atherosclerosis, joint inflammation, and central nervous system inflammation through stimulation of $PLA_2$-IIA production by tissue-specific cells (i.e., endothelial cells, articular fibroblasts and chondrocytes, as well astrocytes and microglia).

## Potential Role of $PLA_2$-IIA in *P. gingivalis*-Induced Oral Dysbiosis and Periodontitis

Although $PLA_2$-IIA has been associated with several chronic inflammatory diseases as described in the previous section, its role in the pathogenesis of periodontal disease remains surprisingly unexplored. Only two small clinical studies demonstrated that increased $PLA_2$-IIA levels and activity in gingival crevicular fluid

(GCF) and gingival tissue, respectively, were significantly elevated in periodontitis sites when compared with healthy sites; the levels returned to those comparable with health after periodontal treatment (Shinohara et al. 1995; Ishida et al. 1994). Consistent with these clinical findings, we have recently reported that gingival mRNA expression of $PLA_2$-IIA was significantly increased during initiation and progression of periodontal disease in non-human primates (NHPs), and interestingly, the $PLA_2$-IIA expression changes were concurrent with oral dysbiosis (Al-Attar et al. 2018). This is of interest given the clinically demonstrated proinflammatory and potent antimicrobial effects of $PLA_2$-IIA. Recent immunohistochemistry and immunofluorescence analysis of gingival tissues from NHPs and mice suggests that $PLA_2$-IIA is constitutively expressed by oral epithelium and some infiltrating inflammatory cells are also positive (Fig. 1a, b). Epithelial cells from the stratum basale and spinosum in NHP gingival tissue show the highest expression intensity for $PLA_2$-IIA, which seems to increase early after disease induction at 2 weeks and 1 month, with cells from outer layers (stratum granulosum and lucidum) showing also positive staining during disease progression (Fig. 1b).

The proposed hypothesis is that elevated oral local $PLA_2$-IIA levels could be contributing to early variations in the abundance of susceptible oral bacterial communities (i.e., oral dysbiosis) and gingival inflammation (Fig. 2). Supporting this hypothesis, we have recently demonstrated that *P. gingivalis* specifically increased the antimicrobial properties of oral epithelial cells through a remarkable induction of $PLA_2$-IIA expression via a mechanism that involved activation of the Notch-1 receptor, and some oral bacterial species (but not *P. gingivalis*) were differentially susceptible to the antimicrobial effects of recombinant $PLA_2$-IIA (Al-Attar et al. 2018). This is relevant given the key role that *P. gingivalis* seems to play in oral dysbiosis and periodontal disease (Hajishengallis et al. 2011; Maekawa et al. 2014).

While the role of $PLA_2$-IIA in the pathogenesis of periodontal disease in vivo remains to be determined, previous evidence supports the hypothesis that this enzyme may indeed play a significant role. For example, mouse strains (e.g., C57BL/6, 129/J) that have a natural mutation in the *PLA2G2A* gene (leading to the production of an inactive enzyme) are more resistant to *P. gingivalis*-induced periodontitis than mouse strains (e.g., BALB/c, DBA/2J) that express an active $PLA_2$-IIA enzyme (Baker et al. 2000; Kennedy et al. 1995). Therefore, a non-functional $PLA_2$-IIA in periodontitis-resistant mice strains may prevent potential *P. gingivalis*-induced antimicrobial and proinflammatory responses mediated by $PLA_2$-IIA that could lead to oral dysbiosis and periodontitis.

Ongoing preliminary mouse studies in our laboratory suggest that the oral microbiome of C57BL/6 transgenic mice (more resistant strain to *P. gingivalis*-induced periodontitis) expressing the human functional $PLA_2$-IIA (h$PLA_2$-IIA-Tg) has significant differences in the abundance of several bacterial species that belong to the *Firmicutes* and *Proteobacteria* phyla compared with their corresponding wild-type (WT) co-caged littermates (unpublished data). Notably, the oral microbiome of h$PLA_2$-IIA-Tg mice showed a significant decrease in the abundance of *Firmicutes* species in contrast to *Proteobacteria* species that were enriched. In particular, *Lactobacillus plantarum* and clostridia species were reduced in h$PLA_2$-IIA-Tg mice. *L. plantarum* is a gram-positive lactic acid bacterium currently used as a probiotic due to its anti-inflammatory and antioxidant activities, its positive effects in maintaining epithelial barrier integrity, and its ability to inhibit the growth of pathogens, which enhances the maintenance of intestinal microbial symbiosis (Jang et al. 2018; Wu et al. 2017; Yin et al. 2018; Wang et al. 2018). It has been shown that the distribution of oral lactobacilli species including *L. plantarum* seems to change with periodontal disease and could play an important role in the maintenance of the microbiologic balance in the oral cavity associated with their antimicrobial properties against periodontopathogenic bacteria such as *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Prevotella intermedia,* as well as their

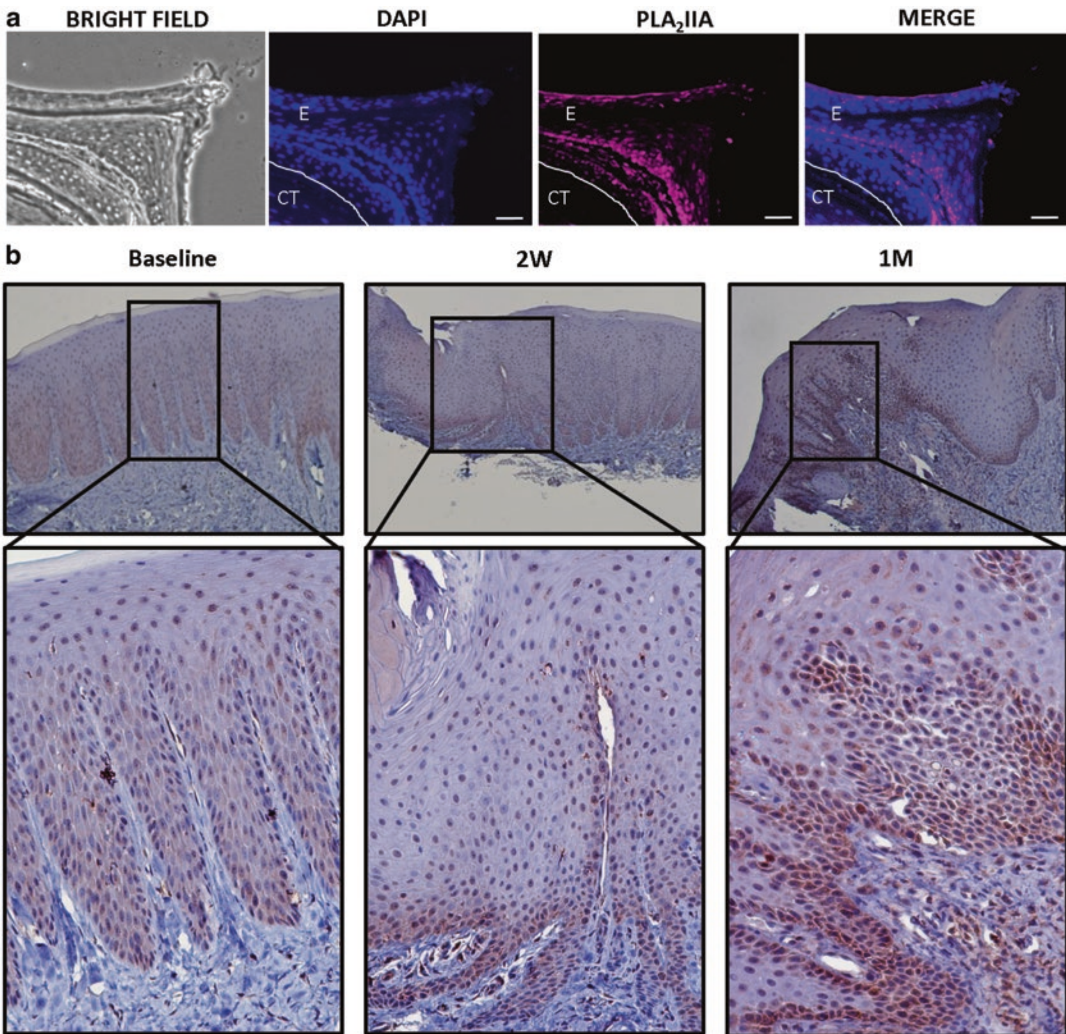


**Fig. 1** Patterns of $PLA_2$-IIA expression in gingival tissues from (**a**) mice (BALB/c) by immunofluorescence and (**b**) non-human primates (rhesus) by immunohistochemistry before ligature-induced periodontitis (baseline), 2 weeks (2 W) and 1 month (1 M) after disease induction. *CT* Connective Tissue; *E* Epithelium

anti-inflammatory effects (Schmitter et al. 2018; Koll-Klais et al. 2005; Teanpaisan et al. 2011; Khalaf et al. 2016).

In contrast, $hPLA_2$-IIA-Tg mice showed a significant enrichment in *Proteobacteria* species. Bacteria from this phylum are all gram-negative and have been broadly associated with mucosal inflammation and disease (Eom et al. 2018; Rizzatti et al. 2017). Likewise, some oral *Proteobacteria* species such as *A. actinomycetemcomitans, Campylobacter rectus, Eikenella corrodens*, *Desulfobulbus sp., Acinetobacter baumannii, and Escherichia coli* among others have been systematically isolated and associated with periodontal lesions (Kumar et al. 2005; Camelo-Castillo et al. 2015; Oliveira et al. 2016; Richards et al. 2015; Miller et al. 2018; da Silva-Boghossian et al. 2011; Perez-Chaparro et al. 2014). Based on this evidence, it is reasonable to hypothesize that elevation in the gingival $PLA_2$-IIA levels could impact the oral microbiome, decreasing the numbers of bacterial species critical for health and enhancing the growth of more pathogenic bacteria.

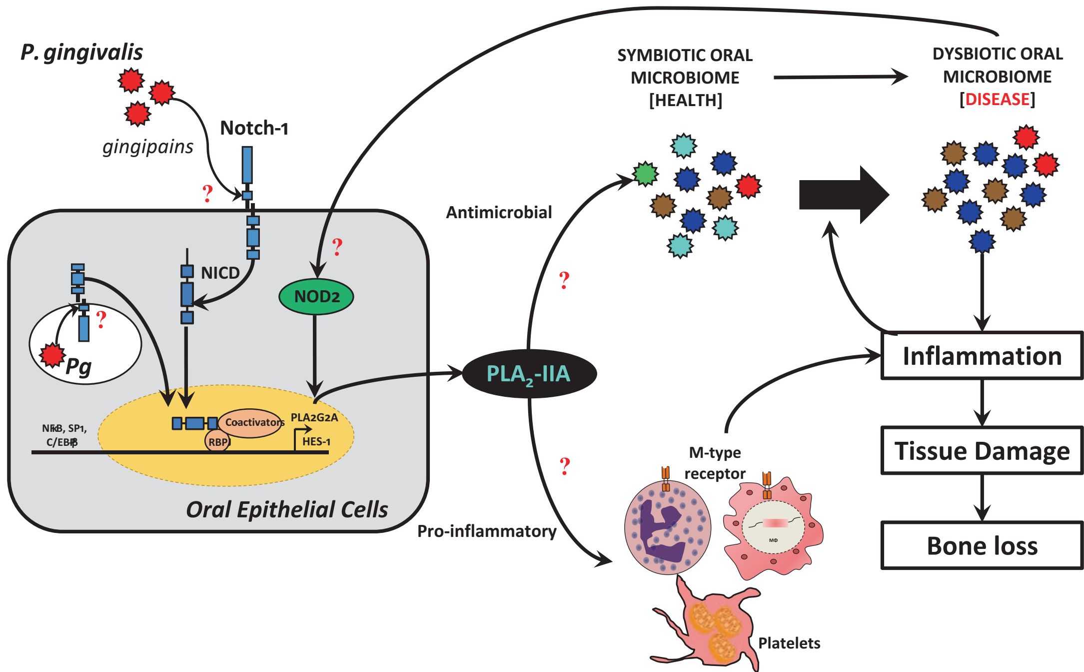


**Fig. 2** Hypothesis for the role of *P. gingivalis*-induced $PLA_2$-IIA through Notch-1 activation in oral epithelial cells as contributor to oral dysbiosis, inflammation, and tissue destruction

The above changes in the oral microbiome of h$PLA_2$-IIA-Tg mice are concurrent with variations in the expression of gingival innate genes. For example, down-regulation of genes associated with antimicrobial responses (e.g., S100a8, Defb1, B2m, Cxcl13) and recruitment of immune cells (e.g., MCP-1, MCP-3, MCP-5 and RANTES) have been identified in h$PLA_2$-IIA-Tg in comparison to wild-type (WT) co-caged mice. In contrast, increased expression of bacterial sensing genes such as nucleotide-binding oligomerization domain-containing protein 2 (NOD2) was seen in $PLA_2$-IIA Tg vs. WT mice (unpublished data). Of note, activation of NOD2-RIP2-NFkB pathway by peptidoglycan internalized via phagocytosis by alveolar macrophages also led to $PLA_2$-IIA expression (Pernet et al. 2015). Thus, up-regulation of NOD2 associated with a dysbiotic oral microbiome could also amplify $PLA_2$-IIA responses (Fig. 2). Whether these host innate immune changes are a consequence of a direct effect of $PLA_2$-IIA on specific cells or an indirect effect that is driven by a $PLA_2$-IIA–induced dysbiotic oral microbiome warrants further investigation.

Finally, our ongoing pilot clinical study suggests that $PLA_2$-IIA is detectable in unstimulated saliva, within a range from 7 to 100 ng/mL (unpublished data). These findings suggest that salivary $PLA_2$-IIA concentrations seem to be about 5–7 times higher than normal/healthy serum levels (1.5 ng/mL), which have been shown to kill pathogens like *L. monocytogenes* in about 2 h after exposure (Gronroos et al. 2002). This is relevant and suggests that elevations of local (salivary and gingival) $PLA_2$-IIA levels could contribute to modify the abundance of susceptible bacterial species in the oral microbiome leading to oral dysbiosis.

The early mechanisms associated with the breakdown of homeostasis between the resident microbiota and the periodontal tissues that results in progressing disease remain unclear. Elucidation of these processes will require novel approaches, likely evaluating unique cellular mechanisms that have evolved by the host to actively discriminate "friend from foe." Additionally, novel mech-

anisms employed by pathogenic bacteria to enhance their capacity to emerge and survive in the commensal autochthonous microbiota by "short circuiting" host cellular responses likely contribute to the observations of the capability of *P. gingivalis* to act as a keystone species and functionally hijack the microbial ecology to enhance chronic inflammation and destructive disease processes. This review presents an innovative hypothesis for a potential mechanism through intersecting functional pathways, by which *P. gingivalis* could accomplish this goal. Thus, the biological active molecule $PLA_2$-IIA with its targeted antimicrobial activity and ability to modulate immunoinflammatory responses, combined with the pathogen specifically activating the Notch-1 receptor pathway, makes this a new strategy, likely among others, used by *P. gingivalis* to modify the local ecology that starts disease.

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*Conflict of Interest*: The authors have no conflicts of interest.

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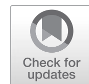

# *Helicobacter* spp. in Experimental Models of Colitis

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The colonization of body surfaces, notably of the intestine, by a complex microbiota is generally highly mutualistic, where vital functions are provided by the commensal microbiota to the host, including the synthesis of vitamins, the degradation of complex polysaccharides into small chain fatty acids (which are essential for the maintenance of the intestinal epithelial barrier), and, finally, the outcompetition of pathogens that accidentally gain access to the body ("colonization resistance") (Chow et al. 2011; Backhed 2005). However, under certain conditions, such as changes of environmental factors in a genetically predisposed host, some of these normally symbiotic bacteria may act as pathogens and induce pathologies. Hence, the term "pathobionts" was coined for these bacterial species with ambiguous biological properties (Round et al. 2009).

In the gastrointestinal tract, *segmented filamentous bacteria (SFP)*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Prevotella* spp., and particularly, members of the *Helicobacter* genus have been identified as frequent pathobionts (Chow et al. 2011).

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## *Helicobacter* spp. as Prototypic Pathobionts in Experimental Mouse Models of Inflammatory Diseases in the Gastrointestinal Tract

*Helicobacter* spp. are gram-negative, spiral bacteria, which normally grow under microaerophilic conditions (Owen 1998). The general interest in *Helicobacter* species as potential triggers of inflammatory disorders was spurred with the identification of *Helicobacter pylori* as the etiopathogenic agent of gastritis and peptic ulcer disease in patients in the early 1980s (Marshall and Warren 1984). Subsequently, an increasing number of *Helicobacter* species were isolated from different species, notably, also in laboratory animals and tested for their inflammation-inducing potential. Based on their tropism, *Helicobacter* spp. can be broadly divided into gastric (e.g., *H. pylori*) and enterohepatic species (e.g., *H. hepaticus, H. bilis, H. typhlonius*). To date, the genus includes at least 20 formally named *Helicobacter* spp., characterized on the basis of 16S rRNA analysis, complemented by biochemical, molecular, and morphological characteristics. At least 11 of these *Helicobacter* species have been described to colonize the lower gastrointestinal tract in laboratory mice (Whary et al. 2015). Several of these enterohepatic *Helicobacter* species are frequently used in experimental systems to study the regulation of host–pathobiont interactions that lead to

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histopathological alterations and clinical signs of disease, resembling those observed in patients with IBD (Fox et al. 2011).

We will first describe some of the characteristics of some of the *Helicobacter* spp. (*H. hepaticus, H. bilis,* and *H. typhlonius*), frequently used in mouse models of IBD-like disorders, with special emphasis on the deregulated host–pathobiont interactions that lead to exacerbated inflammation of the cecum and/or the large intestine (typhlitis, typhlocolitis, and colitis).

## *Helicobacter hepaticus*

In fully immunocompetent mice, colonization of the lower gastrointestinal tract with *H. hepaticus* does not induce any immunopathological alterations. Infection with *H. hepaticus* of lymphopenic scid mice colonized with a *Helicobacter*-free microbiota lead to a significant exacerbation of CD4 T-cell–induced colitis in these scid mice (Cahill et al. 1997; Kullberg et al. 1998). As a rare example within the ε-subgroup of proteobacteria, *H. hepaticus* uses the type 6 secretion system (T6SS) for translocating effector molecules across the bacterial membrane, whereas most other *Helicobacter* species including *H. bilis, typhlonius* (or the human pathogen, *H. pylori*), carry genes encoding a T4SS in their genomes. Surprisingly, however, loss-of-function mutants of genes in the T6SS gene complex of *H. hepaticus* were found to mediate enhanced internalization and propagation of *H. hepaticus* (Chow and Mazmanian 2010). This thus demonstrates that the T6SS complex of *H. hepaticus* does not solely exerts pathogenic functions. Similarly, the T6SS complex may even induce an anti-inflammatory gene profile in host cells, notably in the colonic epithelial cell line MODE-K, and appears to attenuate secretion of IL17A by CD4 T cells in *H. hepaticus*-colonized mice (Chow and Mazmanian 2010).

The complexity of the interaction of the host and *H. hepaticus* becomes obvious when mice deficient for IL10 signaling (either due to attenuated IL10 receptor signaling, or IL10 secretion) are colonized with *H. hepaticus*, which both lead to an exacerbating, IL17- and IL23-mediated inflammation of the colon and the cecum (Kullberg et al. 2001). Adding to the complexity of the pathobiont–host interaction, elegant work provided evidence that a soluble large polysaccharide derived from *H. hepaticus* induces in fully immunocompetent mice the secretion of IL10 by macrophages in a TLR2-/MSK/CREB-mediated manner to prevent the induction of an inflammatory response (Danne et al. 2017).

This mechanism resembles the effects described previously for the zwitterionic polysaccharide A (PSA) from *Bacteroides fragilis,* which causes an increased secretion of IL10 in plasmacytoid dendritic cells and regulatory T cells to prevent excessive PRR-mediated proinflammatory reactions (Mazmanian et al. 2008; Round et al. 2011). Hence, in the absence of appropriate IL10/IL10R-mediated inflammation attenuation, the interactions of *H. hepaticus*-derived inflammation-inducing PAMPs (pathogen-associated molecular patterns) with the respective receptors mainly on monocyte/macrophages exacerbate the intestinal inflammation, which is associated with an excessive IL23 response (Schiering et al. 2014).

A critical target cell type for the IL10-mediated attenuation of potential proinflammatory activities of *H. hepaticus*-mediated effect are the CD11b+, monocyte-derived CX3CR1$^{int}$ macrophages (Bain et al. 2018). These findings also nicely confirmed the concept that IBD-promoting macrophages represent relatively immature, recently recruited monocytes, which in the absence of IL10 signaling are prone to differentiate into potent proinflammatory macrophages. It is truly intriguing that *H. hepaticus* (and possibly also other *Helicobacter* spp., see below) evolved anti-inflammatory mechanisms by inducing potent IL10 secretion by host cells to counteract the bacterial PAMP-mediated proinflammatory effects on the immune system. It remains to be seen whether such an anti-inflammatory response initiated by *Helicobacter* species evolved to prevent induction of a hostile, proinflammatory environment, potentially leading to oxidative stress, which might otherwise affect the persistence of the pathobiont.

*H. hepaticus*-induced typhlocolitis in anti-IL10R–treated mice revealed a site-specific, differential IL-22 dependence of inflammation; while inflammation was reversed in the colon by treatment with a neutralizing anti-IL22 mAb, the cecal inflammation was not affected by anti–IL-22 treatment (Morrison et al. 2015). The underlying mechanisms for these site-specific differences are unclear: differential expression of IL22 binding protein, which acts as a natural IL-22 antagonist; IL22-induced generation of antimicrobial peptides, resulting in a local dysbiosis and reduced colonization resistance for pathobionts (Behnsen et al. 2014); and differential production of IL22, and/or differential cellular expression pattern of the IL22R receptor on the non-hematopoietic target cells, might contribute to these observations. Despite its host-protective effects in many inflammatory and infectious diseases, including its protective effects for epithelial cells and epithelial stem cells (Gronke et al. 2019), it is well established that IL22 also mediates immunopathologies (for review of pro-, and anti-inflammatory activities of IL-22: see: Rutz et al. 2013; Mizoguchi et al. 2018). The site-specific effects of IL22 are also reflected in the results of vaccination protocols against *H. pylori* in mice where the successful clearance of *H. pylori* from the gastric mucosa was clearly dependent on IL22-induced antimicrobial peptides, including RegIIIb (Moyat et al. 2017).

## *Helicobacter bilis*

*H. bilis* is not only a widespread pathobiont in laboratory mice but can also colonize the intestinal tract of humans and a rather wide range of other host species, such as dogs or sheeps (Whary et al. 2015). Similar to *H. hepaticus*, it colonizes in mice the biliary system, liver, and lower intestine. Shortly after its formal identification as a distinct *Helicobacter* species (Fox et al. 1995), its potential as a pathobiont was recognized. Administration of $10^8$ CFU of *H. bilis* by intraperitoneal injection of ASF-colonized scid mice resulted in the induction of moderate typhlocolitis, associated with an epithelial cell hyperplasia at the affected sites even in the absence of transferred colitogenic CD4 T cells (Shomer et al. 1997). This demonstrated that *H. bilis* can trigger an immunopathology even in the absence of a proinflammatory CD4 T-cell response.

Similarly, addition of *H. bilis* to immunocompetent mice, colonized with a commensal flora, exacerbated dextran sodium sulfate (DSS) (1.5%) induced colitis. In this model, the induction of an exacerbating colitis was associated with an enhanced Th17 CD4 T-cell immune response. The specificity of the elicited Th17 CD4 T cells, however, was mostly directed against the commensal microbiota rather than against the pathobiont (*H. bilis*) (Gomes-Neto et al. 2017).

Although the precise mechanisms operative in *H. bilis*-induced exacerbation of DSS colitis in the presence of a commensal flora are not completely understood, these observations are consistent with a hypothetical scenario where the priming of innate and/or adaptive immune cells by *H. bilis* (or *H. bilis*-derived metabolites/components) triggers a preferential differentiation of naïve CD4 T cells into highly colitogenic, functionally Th17 cells.

The observed absence of an evident pathobiont (*H. bilis*)-specific (proinflammatory) CD4 T-cell response is somewhat in contrast to the findings reported in *H. hepaticus*-mediated colonic inflammation using TCR transgenic (tg) CD4 T cells (Xu et al. 2018). These CD4 TCRtg T cells that recognize *H. hepaticus,* but also show some extent of cross-reactivity with the commensal microbiota, induced a strong colitis in *H. hepaticus*-positive, but not in *H. hepaticus*-negative recipients of CD4 T cells. The functional differentiation of the colitis inducing TCRtg CD4 T cells into Th17 cells was in this experimental system controlled by c-Maf (Xu et al. 2018). The functional differentiation into Th17 CD4 T cells, however, appears to be not a specific function of c-Maf: Subsequent reports demonstrated that c-Maf rather augments distinct, polarized, CD4 T-cell differentiation in a context-dependent manner (Gabryšová et al. 2018).

Intriguingly, despite their overlapping tissue distribution, *H. bilis* and *H. hepaticus* differ in

their inflammation-inducing potential depending on the genetic background of the mouse strain. While infection with *H. bilis* infection accelerates the development of colitis in multiple drug resistance-deficient (*mdr1a*$^{-/-}$) mice, infection of the same mouse strain with *H. hepaticus* delays onset of inflammation (Maggio-Price et al. 2002). The underlying mechanisms are, however, not fully understood. Different effects of a colonization with *H. bilis* vs. *H. hepaticus* on the composition of the intestinal microbiota or differential translocation of bacteria and/or metabolites to the intestinal mucosa in this particular mouse strain might be contributing factors.

## *Helicobacter typhlonius*

*H. typhlonius* was identified as a distinct *Helicobacter* species in 2001 (Franklin et al. 2001), and its complete genome was published in 2015. The genome of *H. typhlonius* comprises a putative pathogenicity island (PAI), containing components of the type IV secretion (T4SS) system, virulence-associated proteins, and cag PAI protein (Frank et al. 2015). In contrast to most other *Helicobacter* species, *H. typhlonius* (and also *H. rodentium*) is urease negative.

The potentially dramatic impact of a colonization with *H. typhlonius* on the outcome (and interpretation) of studies on intestinal inflammation in mice was best illustrated when *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ mice, which spontaneously develop colitis ("TRUC mice, *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ ulcerative colitis mice") (Garrett et al. 2007) were compared with another colony of mice with the same genetic background ("TRnUC mice, *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ non-ulcerative colitis mice"). These analyses revealed that the different propensity to spontaneously develop colitis (and also the differential capacity to induce colitis by fecal transplants) correlated with the presence vs. absence of *H. typhlonius* in the intestinal microbiota of the two mouse colonies (Powell et al. 2012).

As shown in Fig. 1, infection of *Rag2*$^{-/-}$ mice, colonized with a complex *Helicobacter*-free microbiota with *H. typhlonius*, substantially accelerates the kinetics of colitis induction. Furthermore, the appearance of clinical and histopathological signs of colitis becomes highly reproducible (and predictable) in the presence of this pathobiont (Brasseit et al. 2016). When *H. typhlonius*-positive and *H. typhlonius*-negative mice are analyzed at the time when they show comparable clinical and histopathological signs of CD4 T-cell–induced colitis, the inflammatory gene expression signature is comparable between the two groups (Fig. 1), indicating that the presence of *H. typhlonius* accelerates the onset of colonic inflammation but does not induce a qualitatively distinct mechanism of intestinal inflammation.

We are currently assessing the impact of *H. typhlonius* in SPF mice harboring a complex intestinal flora and in mice colonized with a highly defined bacterial consortia of 12 bacteria (sDMDMm2/OligoMM flora) (Brugiroux et al. 2016; Uchimura et al. 2016). Preliminary data support the notion that the composition of the non-*Helicobacter* indigenous microbiota substantially regulates the magnitude of the intestinal inflammation induced in immuno compromised mice, i.e., lymphopenic recipients of colitogenic CD4 T cells (Zysset, Kwong Chung, Faderl, et al., in preparation; Martin F. Faderl, PhD thesis, University of Bern 2018).

These findings clearly highlight the importance of also considering potential differences in the resident non-*Helicobacter* microbiota when pathobiont-specific effects are to be investigated. Disease-modulating effect of the indigenous microbiota on *Helicobacter* spp.-induced pathologies were also observed in other experimental systems, e.g., in a mouse model of *H. pylori* infection in FVB/N mice, transgenic for insulin-gastrin. Here, *H. pylori*-monoassociated mice showed an accelerated onset of gastritis and gastrointestinal intraepithelial neoplasia when compared with germ-free mice, yet, in comparison with *H. pylori*-infected mice colonized also with a complex gastric microbiota, the course of disease was highly attenuated (Ge et al. 2011).

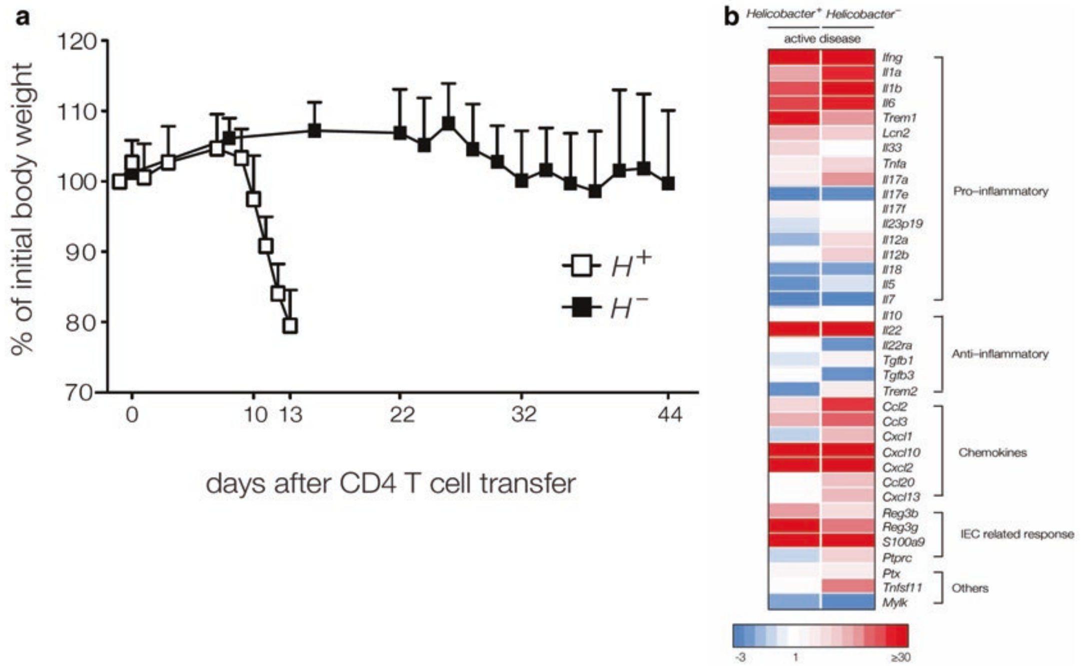


**Fig. 1** (**a**) Disease kinetics of T-cell transfer colitis in *Helicobacter typhlonius*-positive (*Helicobacter* +) and *H. typhlonius*-negative (*Helicobacter* −) lymphopenic mice in percent of body weight loss following colitis induction by adoptive transfer of CD4+ CD45 RB$^{hi}$ T cells. (**b**) Comparison of colonic gene expression profiles in *H. typhlonius*-positive (+) and *H. typhlonius*-negative (−) SPF Rag2$^{-/-}$ recipients of colitogenic CD4 CD45RB$^{hi}$ T cells at the time of active disease (approx. day 12 post CD4 T-cell transfer for *H. typhlonius*-positive recipients; d24 +/− 2d for *H. typhlonius*-negative recipients). RNA values were normalized to the endogenous control genes Gapdh and β2-microglobulin and presented as fold induction/reduction over CD4+ CD45RB$^{lo}$ T-cell transferred controls (modified from Brasseit et al. (2016) and reproduced with permission)

The underlying mechanisms for such a disease-modulating activity of the commensal flora in the presence of a pathobiont are not fully understood. Given the dramatic changes seen in the composition of the intestinal microbiota, but also at the colonic barrier, such as the degradation of the mucus layer during onset of intestinal inflammation (Fig. 2), the indigenous microbiota might also control the expansion and translocation of the *Helicobacter* spp. (or pathobiont-derived metabolites and components) to the intestinal mucosa during an ongoing inflammatory reaction, as an example, via an enhanced degradation of the mucus layer (Faderl et al. 2015).

## Current Open Questions and Difficulties in Defining Physiological vs. Pathological Functions of *Helicobacter* spp. in the Gastrointestinal Tract

Recent years have experienced a remarkable increase in our understanding of the biology of *Helicobacter* species as pathobionts. In particular, for several *Helicobacter* species, irrespective of the use of type IV vs. type VI secretion system, evidence was provided that in a fully immunocompetent host, *Helicobacter* spp. are able to mount both anti- and proinflammatory responses in the innate

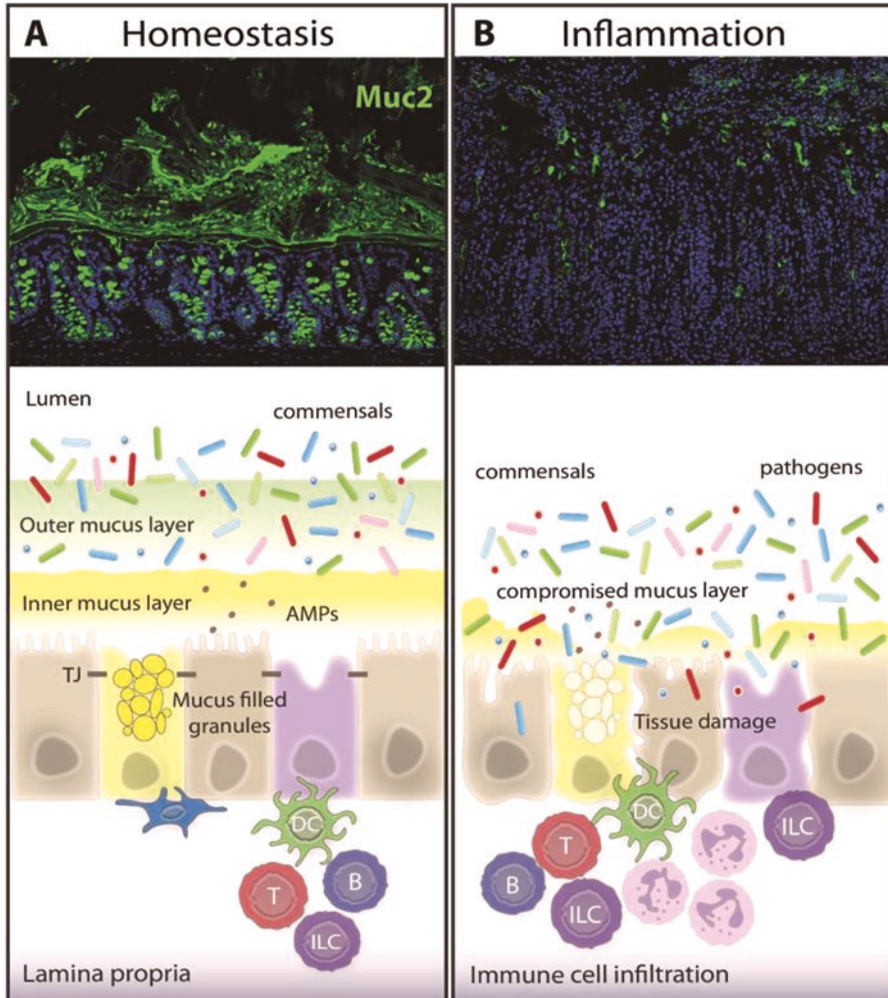


**Fig. 2** Dynamics of the colonic mucus layer and its effects on the segregation of luminal bacteria from the intestinal mucosa during homeostasis, inflammation, and resolution. (**a**) Under homeostatic conditions, the stratified mucus layer with its dense inner layer (stained with anti Muc-2, green) separates the luminal bacteria from the single layer of epithelial cells (containing numerous mucus-filled goblet cell) (blue: DAPI stained cell nuclei). (**b**) During inflammatory conditions, the mucus layer is disrupted, and luminal bacteria can get access to the epithelium and breach the epithelial barrier. The ensuing recruitment of inflammatory cells (e.g., neutrophils and inflammatory macrophages) to the lamina propria and the secretion of proinflammatory mediators, such as TNFα, may further damage the epithelial barrier. During active inflammation, the number of mucus-filled goblet cells is drastically reduced due to the accelerated release of mucin-containing granules. Note that the experimental administration of dextran sodium sulfate (DSS) in the drinking water for colitis induction leads to a change in the composition and the structure of the mucus layer, particularly in the colon, and may also have direct cytotoxic effects on intestinal epithelial cells, thus, further enhancing the intestinal permeability to cause intestinal inflammation. (Modified from Faderl et al. (2015), and reproduced with permission)

and adaptive immune system. The outcome of the constant interactions of pathobionts with their dual capacities and the host, i.e., either extended commensalism or onset of severe inflammation, likely depends on the genetic predisposition of the host; on the interactions of *Helicobacter* with the non-*Helicobacter* members of the complex intestinal microbiota; but also on further environmental factors such as host- and microbiota-derived metabolites (see Box 1 for a list of open questions).

**Box 1 *Helicobacter* spp. as a Pathobiont in the Intestinal Tract**

**Open questions:**

- Which are precisely the main inflammation-inducing mechanisms of *Helicobacter* spp. Are there differences among the different *Helicobacter* species?
- Is there a genetic evolution/selection of *Helicobacter* spp. over time in the infected host, with a differential expansion/contraction of distinct variants during mutualistic vs. pathological interactions within the host?
- How do *Helicobacter* spp. get access to the intestinal lamina propria and how is their distribution within the host regulated? Does the distribution of *Helicobacter* spp. differ under homeostatic, vs. inflammatory conditions?
- Is the induction of a *Helicobacter* spp.-specific CD4 T-cell response with a polarized differentiation into a colitogenic Th17 pathway critical for the transition from mutualistic to pathological host–pathobiont interactions?
- Do *Helicobacter* spp. primarily affect the local differentiation/recruitment of the monocyte/macrophage compartment, which may become further accentuated upon cognate interactions with activated CD4 T cells?
- Are the differential effects mediated by *Helicobacter* spp. also ascribed to interaction of soluble components of *Helicobacter* vs. intact, live bacteria?
- How do commensal, resident bacteria (and/or the adaptive and innate immune responses elicited against these commensals) affect the induction of a *Helicobacter* spp.-associated inflammatory response in the intestine?

Some of the difficulties that so far hamper a comprehensive understanding of the biology of *Helicobacter* spp. during mutualistic interactions with the host vs. induction of immunopathologies in the gastrointestinal tract are discussed below:

**Genetic changes in *Helicobacter* spp.** *Helicobacter* spp. in the host seem to be in a constant genetic flux: Genomic analyses of a *H. pylori* isolates done after a 6-year interval following the first isolation of *H. pylori* strain J99 from its human source patient revealed that this *H. pylori* strain underwent extensive genetic changes during chronic infections; several ORFs from loci scattered throughout the chromosome in the archival J99 strain did not hybridize with DNA from the strains isolated 6 years later, including multiple ORFs within the J99 plasticity zone (Israel et al. 2001). Subsequent work indicated that this continuous genetic flux of *H. pylori might* enable the colonization of the different parts of the stomach by distinct strains of *H. pylori* (Akada 2003).

The contribution of **genetic factors of the host** on the outcome of a colonization with *Helicobacter* spp. has been best documented for *H. hepaticus* in infected *Rag2*$^{-/-}$ mice. In contrast to the 129S6.*Rag*$^{-/-}$ strain, infection of C57BL/6.*Rag*$^{-/-}$ mice with *H. hepaticus* results in minimal disease in the cecum and no inflammation of the colon (Erdman et al. 2003), whereas *Rag*$^{-/-}$ mice on a 129SvEv genetic background spontaneously develop colitis (Maloy et al. 2003). These two *Rag2*$^{-/-}$ mouse lines notably differ genetically in a major locus, designated *Hiccs,* that confers susceptibility for *H. hepaticus*-induced colitis and associated cancer. Remarkably in the same region of chromosome 3, the *Hiccs region is also* associated with the susceptibility for colitis induction seen in the *Il10*$^{-/-}$, the *Gnai2*$^{-/-}$, and TRUC mouse models of chronic inflammation (Ryzhakov et al. 2018).

Interpreting experiments using **antibiotics** to prove/disprove a role for *Helicobacter* in disease process is difficult since even in the absence of live

*Helicobacter* bacteria, components (e.g., cell wall polysaccharide, but also MHC II restricted antigenic peptides) derived from *Helicobacter* may persist for extended time and, thus, impact the local immune response. Furthermore, extended periods of antibiosis may lead to persistence of a more pro-inflammatory phenotype among colonic macrophages, which may greatly affect quantitatively and qualitatively subsequent immune responses against luminal microbiota (Scott et al. 2018).

**Composition of the resident SPF flora in mice** As outlined above, the composition of the resident microbiota may substantially affect the outcome of an additional infection/colonization with one or more *Helicobacter* species at several levels. This includes the colonization efficiency *of Helicobacter* spp., the genetic stability of the pathobionts, or the extent of translocation of bacteria and bacterial products from the gut lumen to the intestinal mucosa. The latter may be affected by the composition and the density of the mucus layer or the differential induction of antimicrobial peptide production by the host. At present, it is unclear why monoassociation of mouse lines with *Helicobacter* spp., depending on the *Helicobacter* strain and system used, are able (*H. muridarum*, (Jiang et al. 2002)), or not (*H. hepaticus*, (Dieleman et al. 2000)) to induce intestinal pathologies.

## Outlook

Despite our improved understanding on the interactions of *Helicobacter* spp. with its host and the factors that lead to inflammation induction vs. maintenance of mutualistic interactions between the host and commensals, critical questions need to be addressed in the future. The use of defined gnotobiotic mouse models of disease and the extraordinary advances in omics-oriented technologies including (1) the metagenome and metatranscriptome analyses of the intestinal microbiota, (2) the transcriptomic analyses of the innate and adaptive immune cells on a single cell or population level, and (3) the epigenetic alterations in immune cells will likely contribute to an improved understanding of the critical factors, which dictate the behavior of a pathobiont, and how we can interfere with this process.

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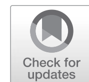


# T Helper 17 Cells as Pathogenic Drivers of Periodontitis

Nicolas Dutzan and Loreto Abusleme

## T-Cell Involvement in Periodontitis Pathogenesis

Periodontitis is a destructive-inflammatory disease that affects the tooth-supporting structures including gingiva, periodontal ligament, and alveolar bone (Pihlstrom et al. 2005). This condition is a significant cause of tooth loss in adults, diminishing the quality of life of patients (Bernabe and Marcenes 2010; Al-Harthi et al. 2013). Periodontitis affects 11% of the world population (Kassebaum et al. 2014; Tonetti et al. 2017), and its prevalence and severity are higher among low-income groups (Eke et al. 2012, 2015). Multiple reports have associated severe forms of this pathology with certain systemic conditions including atherosclerotic cardiovascular disease, adverse pregnancy outcomes, rheumatoid arthritis, and respiratory diseases (Hajishengallis 2015).

Alterations in the composition of oral resident microbial communities lead to dysbiosis and might increase the susceptibility to develop periodontitis (Hajishengallis et al. 2012). Indeed, recent studies have demonstrated that higher diversity, biomass, and richness of subgingival microbial communities are associated with periodontitis (Abusleme et al. 2013; Griffen et al. 2012). Furthermore, bleeding on probing, a widely used clinical sign of inflammation of the periodontal tissues, is associated with an increased bacterial burden (Abusleme et al. 2013). Low-abundance periodontitis-associated bacteria such as *Porphyromonas gingivalis (P. gingivalis)* have been postulated as triggers of periodontal dysbiosis. It has been proposed that *P. gingivalis* modulates the host's immune response to elicit the growth of dysbiotic bacterial communities (Hajishengallis et al. 2012). Ecological changes in the subgingival microbial communities are needed but not sufficient to induce periodontitis (Darveau 2010). Studies in germ-free animals have shown that the absence of live bacteria does not entirely abrogate bone loss, indicating a crucial role of host response in the destruction of periodontal tissues (Dutzan et al. 2017; Taubman et al. 1981; Baer and Newton 1959).

The cellular component of the immune system during periodontitis has been investigated for decades (Taubman et al. 2005; Hajishengallis 2014; Zappa et al. 1992; Kawai

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et al. 2007). A recent study determined using multiparametric flow cytometry that T cells are an essential type of patrolling cell during gingival tissue homeostasis and also in periodontitis. Indeed, T cells together with granulocytes constitute more than 70% of the inflammatory cells present in periodontitis lesions (Dutzan et al. 2016).

Research in murine models has revealed a key role for T cells in periodontitis immunopathology (Baker et al. 1999, 2001, 2002). Baker and collaborators have demonstrated that mice lacking T cells showed 70% less alveolar bone loss induced by *P. gingivalis* infection compared with wild-type control mice, indicating a modulatory role of T cells in pathological alveolar bone resorption. Further studies have demonstrated that T helper cells (Th) are critical orchestrators of alveolar bone destruction in periodontitis (Baker et al. 1999; Teng et al. 2000). Th cells are characterized by the expression the surface protein CD4 and by the secretion of cytokines that modulate the response of immune and stromal cells (O'Shea and Paul 2010). Among these cytokines, IL-17A secreted by the subset Th17 has been described as a crucial modulator of pathological processes during periodontitis (Dutzan et al. 2017, 2018; Eskan et al. 2012).

## Dual Role of Interleukin-17A in Periodontal Tissues

Interleukin-17A (IL-17A, also known as IL-17 and first described as CTLA-8) is a 155-aminoacid glycoprotein with a molecular weight of 30–35 kDa and encoded in the short arm of chromosome 6 (Rouvier et al. 1993; Moseley et al. 2003; Fossiez et al. 1996). Is secreted as a disulfide-linked homodimer but heterodimeric complexes are also generated with IL-17F, the most closely related member of the IL-17 family of cytokines. IL-17 family includes six cytokines (from IL-17A to IL-17F) that share amino acid sequence homology (Gaffen 2011). Mouse IL-17A displays 62% of structural homology with human IL-17A; both exhibit conserved glycosylation sites (Moseley et al. 2003).

IL-17 receptor (IL-17R) family is composed of five receptor subunits (IL-17RA through IL-17RE), all of which are single-pass transmembrane receptors with conserved structural features such as an extracellular fibronectin III-like and a cytoplasmic SEFIR (SEF/IL-17 receptor) domains (Gaffen 2009). IL-17A, IL-17F, and IL-17A-IL-17F heterodimers signal through the receptor subunits IL-17RA and IL-17C activating a cascade of intracellular events. These events include the recruitment of adaptor proteins such as Act1, which elicits the ubiquitination of TNFR-associated factor-6 (TRAF6) (Monin and Gaffen 2017). TRAF6 activates nuclear factor κB (NF-κB), mitogen-activated protein kinase (MAPK), and members of the CCAAT/enhancer-binding protein (C/EBP) pathways, triggering the transcription of IL-17A targeted gene (Monin and Gaffen 2017; Abusleme and Moutsopoulos 2016). Gene products induced by IL-17A vary depending on cell type and might include cytokines, chemokines, inflammatory effectors, matrix metalloproteinases (MMPs), or antimicrobial peptides (AMPs) (Onishi and Gaffen 2010; Amatya et al. 2017).

Non-hematopoietic cells are usually the primary responders to IL-17A (Amatya et al. 2017). Upon IL-17A stimulation, fibroblasts, epithelial, and endothelial cells produce inflammatory cytokines such as IL-6, IL-8, granulocyte-colony-stimulating factor (G-CSF), and prostaglandin E2 (PGE2) (Fossiez et al. 1996). Articular chondrocytes treated with IL-17A up-regulate nitric oxide production, cyclooxygenase-2 (COX-2), IL-1β, IL-6, and MMPs, which are associated with inflammation and cartilage degradation (Shalom-Barak et al. 1998). In osteoblasts, IL-17A stimulates COX-2–dependent PGE2 synthesis as well as the receptor activator of nuclear factor κB ligand (RANKL), a key factor for osteoclast differentiation and function (Kotake et al. 1999) (Fig. 1).

On the other hand, IL-17A is also a critical regulator of the integrity and protection of barrier sites (Veldhoen 2017). Intestinal epithelial cells depend on IL-17A to regulate the production of occludin and mucin-1, which are vital for maintaining barrier stability (Amatya et al. 2017; Lee

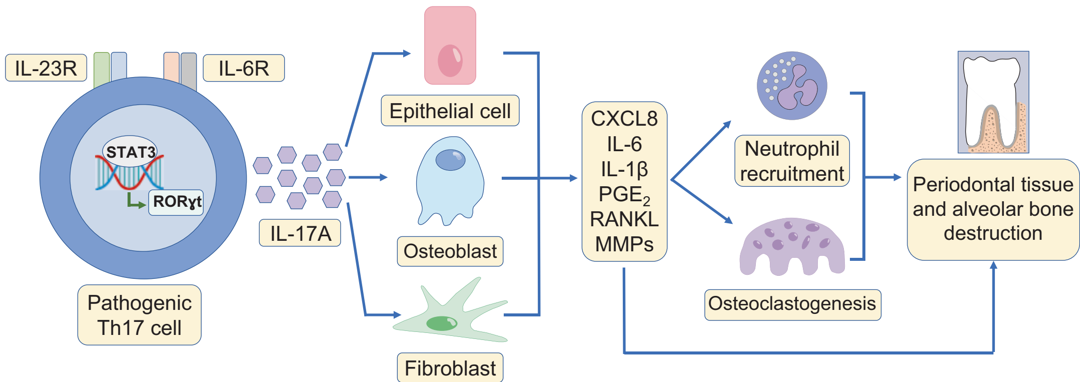


**Fig. 1** Current understanding of the role of Th17 cells in periodontitis immunopathology. Pathogenic Th17 cell development involves IL-6 and IL-23. Their intracellular signaling activates the transcription factors STAT3 and RORγt, triggering IL-17A production. Stromal cells such as epithelial cells, osteoblast, and fibroblast secrete inflammatory and tissue-destructive mediators after IL-17A activation. These molecules help to periodontal tissue and alveolar bone destruction directly and indirectly through neutrophil recruitment and osteoclast formation and activation. *IL-23R* interleukin-23 receptor, *IL-6R* interleukin-6 receptor, *STAT3* signal transducer and activator of transcription-3, *RORγt* retinoic acid-related orphan receptor-γt, *IL-17A* interleukin-17A, *IL-6* interleukin-6, *CXCL8* C-X-C motif chemokine ligand 8, *IL-1β* interleukin-1β, *$PGE_2$* Prostaglandin-E2, *RANKL* receptor activator of nuclear factor κB ligand, *MMPs* matrix metalloproteases

et al. 2015). Skin and mucosal epithelial cells regulate the production of AMPs, particularly β-defensins, lipocalin-2, and S100 proteins in response to IL-17A. These peptides contribute to prevent infections and control bacterial growth (Veldhoen 2017; Gaffen et al. 2014; Conti et al. 2011).

One of the main functions of IL-17A is the regulation of immune-cell recruitment, especially neutrophils. Chemokines such as CCL2, CCL7, CXCL1, CXCL2, CXCL5, and CXCL8, which mobilize monocytes and neutrophils, are secreted upon IL-17A stimulation (Abusleme and Moutsopoulos 2016; Veldhoen 2017; Gaffen et al. 2014). Production of CCL20, CXCL9, and CXCL10, essential for T cell chemotaxis, is also up-regulated by IL-17A (Veldhoen 2017; Gaffen et al. 2014). Other mechanisms of chemokine-independent cell recruitment are associated with IL-17A, including induction of several MMPs and PGE2 synthesis (COX-2-dependent). These molecules act on the extracellular matrix making it more accessible for migrating cells and promote vasodilatation among other actions (Veldhoen 2017; Gaffen et al. 2014). This regulation of cell migration underlines the immune-modulatory role of IL-17A.

Based on the evidence presented above, it is possible to conclude that IL-17A function is critical for protective immunity but also for the pathogenesis of diseases at barrier sites (Conti et al. 2011; Zenobia and Hajishengallis 2015; Jin and Dong 2013). This dual role of IL-17A is evident in the oral mucosa. IL-17A is vital to control *Candida albicans* infections, as patients with defects in IL-17/Th17 pathway are more susceptible to oropharyngeal candidiasis (Conti et al. 2011; Freeman and Holland 2010; Abusleme et al. 2018). On the other hand, dysregulation of IL-17A production in the oral cavity is involved in periodontitis immunopathology (Dutzan et al. 2018; Eskan et al. 2012; Tsukasaki et al. 2018). Studies in ligature-induced periodontitis have demonstrated up-regulation of both IL-17A (Dutzan et al. 2018; Eskan et al. 2012) and IL-17A–associated inflammatory mediators including neutrophil chemoattractants (Eskan et al. 2012). Pharmacological inhibition of IL-17A diminished bone destruction in models of spontaneous periodontal tissue and alveolar bone loss (Eskan et al. 2012; Moutsopoulos et al. 2014; Xiao et al. 2017) indicating a critical role of IL-17A in alveolar bone destruction during periodontitis. During experimental periodontitis,

IL-17A down-regulates the expression of the glycoprotein developmental endothelial locus 1 (Del-1) from endothelial cells, up-regulating the $\beta_2$ integrin LFA-1–mediated neutrophil recruitment and the subsequent periodontal bone loss (Eskan et al. 2012).

In patients, a positive correlation of local levels of IL-17A with disease severity and clinical parameters of periodontal destruction have been demonstrated (Zenobia and Hajishengallis 2015). In fact, IL-17A expression is higher in periodontitis than in gingivitis and it is almost undetectable in healthy control tissues (Moutsopoulos et al. 2012). Furthermore, single nucleotide polymorphism associated with increased expression of IL-17A was found to be more prevalent in patients with periodontitis than in control subjects (Correa et al. 2012). These studies, together with the proinflammatory and destructive properties of IL-17A, indicate that this cytokine might be an important player in the inflammatory-destructive events during periodontitis.

IL-17A is secreted by a broad spectrum of adaptive and innate immune cells including $CD4^+$ T cells, $CD8^+$ cytotoxic T cells, natural killer T cells (NKT), γδ T cells, innate lymphoid cells type 3 (ILC3), and lymphoid tissue inducer cells (LTi). Production of IL-17A by myeloid cells have also been reported, but its production by this type of immune cells is controversial (Jin and Dong 2013; Cua and Tato 2010). γδ T cells are characterized by their fast responsiveness and have been associated with the protective roles of IL-17A at barrier sites (Cua and Tato 2010). $CD4^+$ Th17 cells are one of the principal sources of IL-17A and, importantly, are key mediators in the pathogenesis of inflammatory and autoimmune diseases (Jin and Dong 2013; Harrington et al. 2005).

## Th17 Cell Differentiation and Its Unique Requirements at the Oral Mucosa

IL-17A was first described as a T-cell–produced cytokine with the capacity to elicit inflammatory responses in stromal cells (Rouvier et al. 1993; Fossiez et al. 1996; Gaffen et al. 2014). That discovery ignited a paradigm change in Th cell field where, at that time, Th1 and Th2 cells were the only described members (Mosmann and Coffman 1989). However, it was not until 2005 (12 years after the IL-17A cloning) that several studies demonstrated that Th cells producing IL-17 were a unique lineage that depended of specific transcription factors and were crucial mediators of autoimmunity (Gaffen et al. 2014; Harrington et al. 2005; Langrish et al. 2005). Th17 cells are characterized by the production of IL-17A, IL-17F, IL-22, and granulocyte-macrophage colony stimulating factor (GM-CSF) (Patel and Kuchroo 2015). Similar to innate IL-17A cell producers, Th17 cells express the chemokine receptor CCR6, the receptor for IL-23 (IL-23R), and the transcription factor retinoic acid-related orphan receptor-γt (RORγt) (Patel and Kuchroo 2015; Yang et al. 2014).

Th17 cell differentiation from naive T cells involves not only activation through T-cell receptor and co-stimulatory molecules but also a particular cytokine milieu. IL-6 is critical for early lineage commitment and transforming growth factor beta-1 (TGF-β1), IL-1β, and IL-23 participate in late stages of Th17 cell development (Patel and Kuchroo 2015; Yamane and Paul 2013). It is well documented that Th17 differentiation is tightly regulated by transcription factors such as signal transducer and activator of transcription-3 (STAT3) and RORγt (Ivanov et al. 2006; Harris et al. 2007). Patients with mutations in genes that encode these transcription factors have impaired Th17 differentiation and IL-17A production (Milner et al. 2008). Other transcription factors such as basic leucine zipper transcription factor/ATF-like (BATF) and interferon regulatory factor 4 (IRF4) also contribute to Th17 differentiation. These transcription factors (BAFT and IRF4) allow chromatin accessibility and together with STAT3 initiate a transcriptional program, which is continued and tailored by RORγt (Ivanov et al. 2006; Ciofani et al. 2012). Several other transcription factors participate in Th17 regulation. From these, recent studies have demonstrated an important role of c-Maf in controlling proinflammatory Th17 cells and their

adverse effects at mucosal surfaces (Xu et al. 2018; Aschenbrenner et al. 2018).

Th17 cell development and regulation have been studied at barrier sites such as skin, lower gastrointestinal (GI) tract, and recently at the oral mucosa (Dutzan et al. 2017). In the first two sites, Th17 regulation is dependent on commensal microbiota colonization (Naik et al. 2012; Hooper et al. 2012). At the small intestine, germ-free mice lack Th17 cells and specific bacteria (segmented filamentous bacteria—SFB) direct Th17 cell differentiation (Ivanov et al. 2008). In contrast to the lower GI tract, Th17 development in oral mucosa is independent of microbial colonization. Studies in germ-free mice found no differences in gingival Th17 cells numbers when they were compared with the numbers of cells in specific pathogen-free (SPF) animals (Dutzan et al. 2017).

Regarding the cytokine signals needed for Th17 cell differentiation in skin and GI tract, studies have demonstrated that IL-1β is fundamental in that process and its production is dependent on the presence of commensal microbiota (Naik et al. 2012; Shaw et al. 2012). This finding underscores, even more, the importance of commensal microbiota colonization in Th17 development at those barrier sites. In contrast to the IL-1β requirement for Th17 cell development in the skin and lower GI tract, gingival Th17 accumulation is IL-1β independent. Studies showed that IL-6, but not IL-1β or IL-23, is needed for homeostatic Th17 accumulation in gingival tissues (Dutzan et al. 2017). At this oral barrier site, IL-6 is mainly produced by epithelial cells upon mechanical stimulation. These results demonstrate that gingival mechanical stimulation during mastication is a constant and critical signal that drives Th17 cell accumulation in gingival tissues (Dutzan et al. 2017). These results highlight the individuality of immune regulation at the oral mucosa, particularly for Th17 cells.

## Heterogeneity and Pathogenicity of Th17 Cells

Evidence from animal models and clinical studies suggests that Th17 cells are not a homogeneous sub-population. These Th cells can have different transcriptional profiles and function, which depend on the cytokine signals, metabolism, receptor expression, and intracellular signaling (Lee et al. 2012).

Regarding the cytokine milieu in which Th17 cells are differentiated, T cells polarized under a microenvironment that includes IL-1β, IL-6, TGF-β3, and IL-23 appear to have a pathogenic profile as they induce inflammatory or autoimmune disease (Gaffen et al. 2014; Patel and Kuchroo 2015; Lee et al. 2012). On the other hand, Th17 cells polarized under the presence of TGF-β1 and IL-6 have demonstrated to have little or no capacity to trigger disease (Lee et al. 2012; Ghoreschi et al. 2010).

From these cytokines, IL-23 has been profiled as a key inducer of a transcriptional program that contributes to their pathogenicity (Gaffen et al. 2014). The pathogenic role of IL-23 becomes evident in pre-clinical and clinical studies, where pharmacological targeting of IL-23 inhibits inflammatory and autoimmune conditions associated with Th17 cells (Lee et al. 2015; Gaffen et al. 2014; Hawkes et al. 2018). T-cell reactivity to IL-23 is essential to the development of inflammation in experimental colitis (Ahern et al. 2010). IL-23 promotes intestinal Th17 accumulation and co-expression of IL-17A and INF-γ by Th17 cells. Moreover, it participates in T regulatory (Treg) cell inhibition and IL-10 downregulation (Ahern et al. 2010). These results are in agreement with studies in a model of periodontal inflammation and tissue destruction. This research determined that IL-6 signaling is vital for homeostatic Th17 accumulation, whereas both IL-23 and IL-6 are needed for Th17 cell expansion during inflammatory bone loss (Dutzan et al. 2018).

IL-6 has also been regarded as a critical cytokine signal for Th17 development. During experimental autoimmune encephalitis (EAE), IL-6 produced by dendritic cells (and not by other cell types such as monocytes or B cells) drives the generation of pathogenic Th17 cells (Heink et al. 2017). This pathogenic phenotype is triggered by a newly described signaling mode for IL-6 called "IL-6 cluster signaling" where IL-6 is loaded to the receptor IL-6Rα in intracellular compartments and then is transported as a complex to the DC membrane where interacts with the common subunit gp130 expressed by T cells (Heink et al. 2017). Additionally, the IL-6 secreted by other cell types is crucial to suppress Treg cells and prevent Th17 cells from converting into Treg cells, helping in perpetuating inflammation (Hunter and Jones 2015). In a model of autoimmune arthritis, studies have found that under inflammatory conditions, IL-6 secreted by synovial fibroblasts helps Treg cells (that classically express the transcription factor Foxp3) to lose its expression and become Th17 cells. These ex-Foxp3 Th17 cells have a more potent osteodestructive profile than naïve $CD4^+$ cells and conventional Th17 cells (Komatsu et al. 2014). Foxp3 instability, crucial for the generation of pathogenic Th17 cells, has been recently analyzed in a model of periodontitis. These cells were characterized by overexpression of *Rorc*, *Il17a*, *Il17f,* and *Tnfsf11* (RANKL) compared to conventional Th17 cells, and their adoptive transfer induced more periodontal bone loss than the transfer of conventional Th17 cells, indicating an important osteoclastogenic role of ex-Foxp3 Th17 cells during periodontal inflammation and destruction (Tsukasaki et al. 2018).

Th17 cells in human lesions of periodontitis are a heterogeneous population. Characterization of Th17 cells by multiparametric flow cytometry demonstrated that a subset of these cells co-produce IL-17A together with GM-CSF, INF-γ, or IL-22 (Dutzan et al. 2018). Besides, $CD4^+$-IL-17A producer cells were categorized under four different types of memory T cells, determined by CD69 (resident or non-resident T cells) and by CCR7 expression, which classifies them in central or effector memory (Dutzan et al. 2018; Sathaliyawala et al. 2013). Although the majority of Th17 cells were classified as resident effector memory T cells, IL-17A was also produced by the other three cell subtypes (Dutzan et al. 2018). These observations indicate that Th17 cells are not a homogeneous population and might have different pathogenic potential in inflamed gingival tissues.

Identifying pathogenic from non-pathogenic Th17 cells and the molecular mechanisms underlying their differentiation and function have direct clinical relevance. This identification becomes relevant in studies that showed the consequences of targeting IL-17A in diseases such as psoriasis, IBD, and rheumatoid arthritis (Hawkes et al. 2018; Davis Fred et al. 2015; Mease 2015; Miossec 2017). These studies have paved the way to a tailored inhibition of disease-associated Th17 cells, which could leave non-pathogenic Th17 cells and their functions intact. Such detailed characterization and further targeting might contribute to the development and use of custom-made compounds for the treatment of periodontitis patients, particularly benefiting those individuals that respond poorly to conventional treatment.

## Role of Th17 in Periodontitis Immunopathology

Th17 cells are among the main producers of IL-17A, and as such, share functions with this cytokine. Like IL-17A, these cells are well recognized as contributors to immune-surveillance and barrier homeostasis. However, they also have been identified as key drivers in the pathogenesis of inflammatory and autoimmune conditions including inflammatory bowel disease (IBD), psoriasis, ankylosis spondylitis, rheumatoid arthritis, multiple sclerosis, and periodontitis (Dutzan et al. 2018; Gaffen et al. 2014; Miossec and Kolls 2012).

Four years after the description of Th17 cells as a distinct cell lineage (Harrington et al. 2005; Langrish et al. 2005), direct evidence of the presence of this cell subtype in human lesions of periodontitis was established (Cardoso et al. 2009).

Over the years, several studies have documented a significant increase in Th17-related cytokines (such as IL-17A, IL-23, IL-21) in periodontitis lesions as well as up-regulation of proinflammatory and osteoclastogenic mediators also associated with Th17 cells such as IL-6 and RANKL (Cardoso et al. 2009; Dutzan et al. 2012; Vernal et al. 2005, 2006; Lester et al. 2007). The expression of the "master switch" transcription factor RORγt is also increased during periodontitis (Cardoso et al. 2009; Adibrad et al. 2012; Dutzan et al. 2009), and numbers of Th17 cells detected by flow cytometry positively correlate with disease severity (Dutzan et al. 2018). All these association studies, together with the inflammatory profile of IL-17A, have contributed to support the hypothesis of Th17 cells as drivers of periodontitis immunopathology.

Recent studies have contributed to understanding the role of Th17 cells in tissue destruction during periodontitis and proposed a targeted therapeutic approach to this condition (Dutzan et al. 2018). In this work, was established that during periodontitis dysbiotic microbial communities trigger Th17 cell expansion and their genetic ablation or pharmacological inhibition dramatically diminished alveolar bone destruction. In fact, when genes that encode the transcription factors STAT3 or RORγt were eliminated from αβ T cells, Th17 cells were not present in gingival tissues and ligature-induced bone loss was reduced up to 70%, suggesting that Th17 cells are significant drivers of periodontal immunopathology. These results were supported by the pharmacological inhibition of RORγt using a small-molecule compound (GSK805). The transcriptome analysis of small-molecule–treated gingival tissues detected inhibition of IL-17A–dependent pathways including neutrophil recruitment (Dutzan et al. 2018). Neutrophil accumulation in gingival tissues is one of the hallmarks of periodontitis (Dutzan et al. 2016), and their reduction significantly diminished periodontal bone loss in experimental periodontitis (Dutzan et al. 2018). These observations suggest a microbial-Th17-neutrophil axis as driver of periodontal tissue destruction during periodontitis (Fig. 1). These results obtained from animal models are supported by the evaluation of a group of patients with dominant-negative mutations on *STAT3* gene. These mutations lead to a rare congenital immunodeficiency known as Autosomal Dominant Hyper IgE Syndrome (AD-HIES) (Holland et al. 2007). AD-HIES patients exhibit a dramatic impairment in Th17 cell response explained by a reduced IL-6 and IL-23 function via STAT3, which leads to a reduction in RORγt expression and further impairment of Th17 differentiation and IL-17 production (Milner et al. 2008). Importantly, this patient cohort has reduced periodontal inflammation (bleeding on probing) and clinical attachment loss compared to healthy volunteers (Dutzan et al. 2018).

Future interventional studies are needed to conclusively implicate Th17 cells in the pathogenesis of human periodontitis. However, collectively, these findings support the idea of Th17 cells as pathogenic drivers of periodontitis and provide a solid mechanistic basis for local Th17 inhibition in human periodontitis.

## Concluding Remarks

The proinflammatory function of IL-17A together with the fact that Th17 cells are an important component of the immune cell infiltrate during periodontitis support the hypothesis of Th17 cells as conductors of tissue destruction during periodontitis. Recent mechanistic evidence demonstrated that in the absence of Th17 cells, tissue destruction dramatically diminished in experimental periodontitis. Also, these studies pointed toward a Th17-neutrophil axis as responsible for tissue damage. Observations in a unique cohort of patients with impaired Th17 differentiation supported the results obtained from animal studies, indicating a pivotal role of Th17 in driving inflammation and attachment loss in the oral cavity. Interventional studies are needed to conclusively demonstrate the role of these cells during periodontitis immunopathology. Taken together, the presented evidence supports the role of Th17 cells as drivers of periodontitis immunopathology with the advances in the development of pharmacological targets of Th17 responses, pro-

vide the foundations to consider Th17 cell inhibition as a promising therapeutic approach in periodontitis.

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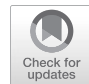


# *Candida*–Bacterial Biofilms and Host–Microbe Interactions in Oral Diseases

Thais de Cássia Negrini, Hyun Koo, and Rodrigo Alex Arthur

## Introduction

Oral cavity harbors a complex and highly diverse microbial community. The predominant taxa present in the oral cavity of healthy subjects belongs to genus *Streptococcus* followed by other bacterial species including *Neisseria, Haemophilus, Corynebacterium, Rothia, Actinomyces, Prevotella, Capnocytophaga, Porphyromonas,* and *Fusobacterium* (Zaura et al. 2009). Likewise, a diverse fungal community (up to 85 genera) can be found in the mouth, although *Candida* is by far the most abundant and frequently isolated in the oral mycobiome (Ghannoum et al. 2011). Many of these organisms are able to form biofilms on biotic (mucosal cells and teeth) and abiotic surfaces (restorative materials, prosthesis, and implants) forming polymicrobial communities with varying complexity and diversity depending on the site of colonization, host, and dietary factors (Simon-Soro and Mira 2015; Bowen et al. 2018).

Within diverse microbial communities, synergistic and antagonistic interactions among the counterparts of oral microbiome/mycobiome modulate the ecological stability at various sites in the oral cavity, helping to maintain a homeostatic state between microbiota and host (Marsh and Zaura 2017). However, once the stability is disturbed, the oral microbiome shifts to a pathogenic one posing soft and hard tissues to an increased risk for disease development (Lamont et al. 2018). As highlighted below, cross-kingdom interactions between *Candida* and oral bacteria are fundamental for allowing the co-existence of these organisms, which may also affect the course and the severity of biofilm-mediated diseases.

One of the well-known *Candida*–bacterial-mediated oral disease is denture stomatitis. This disease is characterized by a chronic inflammation of mucosa caused by biofilm-infected denture surface. It has a multifactorial etiology, including poor denture hygiene, continual and night-time wearing of denture, as well as the

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presence of *Candida*-formed biofilm covering the denture surfaces (Samaranayake et al. 2009; Gendreau and Loewy 2011). Although *Candida* has been historically considered as the main etiological factor of denture stomatitis (Budtz-Jørgensen 2000) (detected in up to 90% of the affected individuals), this fungus can be also found on denture biofilms of healthy individuals (Shi et al. 2016). A highly diverse bacterial community is also present on dentures, including *Actinomyces, Streptococcus, Veillonella, Capnocytophaga,* and *Neisseria*. While *Streptococcus gordonii* and *Streptococcus sanguinis* are more prevalent in denture biofilms of health individuals, *Fusobacterium* is often detected in high numbers in denture of individuals presenting stomatitis (Shi et al. 2016). In addition, *Staphylococcus epidemidis* and *Staphylococcus aureus* are commonly recovered from the dentures of individuals presenting denture stomatitis (Pereira et al. 2013). It is important to mention that even methicillin-resistant *S. aureus* may colonize denture surfaces (Lewis et al. 2015). It has been shown that co-cultivation between *S. aureus* and *C. albicans* results in up-regulation of several bacterial and fungal proteins related to carbohydrate and aminoacid metabolism, as well as glycolysis and cellular stress response, increasing overall virulence (Peters et al. 2010).

*Candida albicans* is typically found colonizing oral mucosal surfaces as a commensal organism (Dongari-Bagtzoglou et al. 2009; Diaz et al. 2012a; O'Donnell et al. 2015; Lohse et al. 2018). However, under predisposing factors, such as use of broad-spectrum antibiotics, corticosteroids, hyposalivation, and in immunocompromised individuals or in patients undergoing chemo- or radiotherapy, this organism can lead to severe mucosal infections often in partnership with bacteria (Samaranayake et al. 2009; Raber-Durlacher et al. 2010). Recent studies have suggested that a dysbiosis of mucosal microbiota during radiotherapy is associated with severity of mucositis and progression of mucosal lesions (Hou et al. 2018), while bacterial–fungal interactions appear to play a key role in exarcebating the severity of mucositis (Vasconcelos et al. 2016). In this context, previous findings have shown that synergistic cross-kingdom interactions between *C. albicans* and *Streptococcus oralis* enhanced the mixed-species biofilm virulence on mucosal surfaces (Xu et al. 2014a, 2016, 2017). *C. albicans* appears to increase the ability of *S. oralis* to colonize mucosal surfaces (Xu et al. 2014a), whereas *S. oralis* augments *C. albicans* invasion through epithelial junctions (Xu et al. 2016) leading to an increased mucosal inflammation response (Xu et al. 2014a). Co-cultivation of *C. albicans* with *S. oralis* or *Streptococcus gordonii* increases streptococcal biomass in mixed-species biofilms (Diaz et al. 2012b; Cavalcanti et al. 2016; Xu et al. 2017). Notably, *S. gordonii* and *C. albicans* in mixed-species biofilms display enhanced resistance to antifungals and antibiotics (Montelongo-Jauregui et al. 2016, 2018).

In addition to mucosal infections, severe early childhood caries (S-ECC) is another disease in which the role played by *Candida*–bacterial interactions has been increasingly recognized. Dental caries is a biofilm- and diet-dependent disease related to frequent intake of fermentable sugars (Sheiham and James 2015; Bowen et al. 2018). Sucrose is considered the most cariogenic (Paes Leme et al. 2006) since it serves as substrate for production of extracellular polysaccharides (EPS) and acids, providing the key virulence attributes of cariogenic biofilms (Bowen et al. 2018). Although the microbial etiology of dental caries has been traditionally associated with bacteria (Wolff et al. 2013; Thomas et al. 2012; Simon-Soro and Mira 2015), *C. albicans* has been frequently isolated in plaque biofilm from children affected by severe childhood caries (Hajishengallis et al. 2017; Jean et al. 2018; Xiao et al. 2018a). Furthermore, a recent clinical study showed that the presence of *C. albicans* alters the oral bacteriome, particularly by enhancing the levels of *Streptococcus mutans* in plaque of S-ECC (Xiao et al. 2018b). Previous in vivo studies have shown that *C. albicans* synergistically interacts with *S. mutans*, which is found in high levels in S-ECC. The presence of *Candida* boosted mixed cross-kingdom biofilm accumulation with *S. mutans*, enhancing microbial carriage in vivo and leading to rampant and severe

caries lesions (similar to S-ECC) in a rodent model (Falsetta et al. 2014; Hwang et al. 2017). Initial mechanistic studies revealed mutually beneficial interactions whereby *C. albicans* tooth colonization, co-adhesion, and mixed biofilm development are facilitated by bacterial EPS. Once together, cross-feeding and signaling interactions enhance *C. albicans* growth and metabolism, while fungal-derived metabolites induce EPS production and *S. mutans* growth boosting biofilm accumulation with increased acidogenicity (Falsetta et al. 2014; Ellepola et al. 2017; Kim et al. 2017; He et al. 2017; Xiao et al. 2018b). Acidification at the biofilm/tooth interface is a hallmark for the onset of dental caries as it disrupts the mineral equilibrium between the enamel and the surrounding environment inducing loss of calcium and phosphate from the tooth surface (Dawes 2003). These findings are consistent with clinical observations, indicating an active role for *C. albicans* in cariogenic biofilms (Xiao et al. 2018a, b).

*Candida*–bacterial interactions have been also associated with other diseases and infections including periodontitis, peri-implantitis, and endodontic infections. Several bacterial groups have been known to be periodontitis-associated, such as *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Fusobacterium nucleatum, Prevotella intermedia,* and others (Perez-Chaparro et al. 2014). However, greater abundance of *C. albicans* is found in patients with periodontitis (Peters et al. 2017), while higher density of *Candida* in subgingival biofilm has also been linked with the severity of chronic periodontitis (Canabarro et al. 2013). Interestingly, *C. albicans* appears to promote *P. gingivalis* ability to invade host cells (Tamai et al. 2011). Moreover, increased expression of genes related to *P. gingivalis* growth, division, and peptidoglycan biosynthesis is observed in *C. albicans–P. gingivalis* mixed-species biofilms (Sztukowska et al. 2018) suggesting that co-cultivation may enhance *P. gingivalis* virulence. In addition to the higher infectivity, the consumption of oxygen by *Candida* may create an oxygen tension that favors *P. gingivalis* growth (Sztukowska et al. 2018).

Although *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, *P. intermedia,* and *T. denticola* are the most prevalent microorganisms found in peri-implantitis (Ting et al. 2018), *C. albicans* has been also recognized as part of peri-implantitis-associated biofilms (Mombelli and Decaillet 2011). Regarding endodontic infections, *Enterococcus faecalis* is usually found in unsuccessfully treated root canals (Bouillaguet et al. 2018), and there is increasing evidence that *C. albicans* may also be found in polymicrobial biofilms related to root canal infections (Mergoni et al. 2018; Peerson et al. 2017). Although *Candida* have been found in periodontitis, peri-implant, and root canal system infections, the pathogenic role played by *Candida*–bacterial interactions remains unclear requiring additional mechanistic and in vivo studies.

## *Candida*–Bacterial Interactions

Available evidences suggest a synergistic interaction between *C. albicans* and oral bacteria that potentially increase the virulence of polymicrobial biofilms (O'Donnell et al. 2015; Koo et al. 2018; Montelongo-Jauregui and Lopez-Ribot 2018) (Table 1 and Figs. 1 and 2).

### When the Cell–Cell Direct Contact Matters: Adhesion Between *C. albicans* and Oral Bacteria by Physical Contact

Oral streptococci express many cell surface adhesins (such as polypeptides antigens I/II) that enable adhesive interactions with mucin-like salivary proteins (allowing them to adhere to available biotic and abiotic sites in the oral cavity) but also mediating co-adhesion/aggregation with different microorganisms (Jenkinson and Demuth 1997). In this context, *S. gordonii* co-adhere with *C. albicans* via SspA/SspB (members of antigen I/II polypeptides), and co-cultivation results in increased biofilm biomass compared to that formed by each of the organisms grown separately (Bamford et al. 2009). By interacting with

**Table 1** Main findings for *Candida*–bacterial interactions

| Interaction | Features and induced phenotype |
|---|---|
| *C. albicans* and *S. gordonii* | • Cell–cell direct physical contact mediated by fungal hyphal cell wall ALS3 adhesins and bacterial cell surface antigens I/II (SspA/SspB);<br>• Bacterial AI-2 and produced $H_2O_2$ induce hyphae formation;<br>• Competence-stimulating peptide inhibits fungal biofilm formation;<br>• Increased resistance to antifungals (fluconazole, amphotericin B, and caspofungin) and antimicrobials (clindamycin); |
| *C. albicans* and *S. oralis* | • Cell–cell direct physical contact mediated by fungal hyphal cell wall ALS1 adhesins and bacterial cell surface antigens I/II;<br>• Up-regulation of *Candida egf1* gene induced by *S. oralis quorum sensing* leading to hyphal formation;<br>• Increased *S. oralis* biofilm formation;<br>• Increased tissue invasion by *C. albicans;*<br>• Increased severity of mucosal infection; |
| *C. albicans* and *S. mutans* | • Fungal–bacterial interaction is mostly mediated by EPS-mediated interaction:<br>– GtfB adheres to mannan residues at hyphal cell wall synthesizes alfa-glucans that mediates adhesive interaction with *S. mutans*;<br>– GtfB adheres to fungal-secreted beta-glucans and synthesizes alfa-glucans, which in turn mediates interaction with *S. mutans*;<br>• Higher expression of fungal genes HWP1, ALS1, and ALS3;<br>• Higher expression of bacterial *gtfB* and *gtfC* genes;<br>• Enhanced fungal and bacterial biofilm formation;<br>• Mixed-species biofilms is highly virulent leading to increased severity of dental caries;<br>• Mixed-species biofilms induce enhanced *Candida* drug resistance against fluconazole;<br>• Bacterial trans-2-decenoic signaling acid, mutanobactin-A, and competence-stimulating peptide (under *ComC* gene) inhibit yeast-to-hyphae formation;<br>• Enhanced *gtfB* expression induced by fungal-derived farnesol; |
| *C. albicans* and *Actinomyces* | • Cell–cell direct contact is independent of *Actinomyces* fimbriae;<br>• Increased biomass of both *Candida* and *Actinomyces* in mixed-species biofilms;<br>• Increased acidogenicity of biofilms;<br>• Increased dissolution of hydroxyapatite; |
| *C. albicans* and *P. gingivalis* | • Cell–cell direct contact mediated by fungal hyphal cell wall ALS3 adhesins and bacterial cell surface protein InIJ;<br>• Enhanced *P. gingivalis* tissue invasion;<br>• Up-regulation of *P. gingivalis* virulence genes related to growth, division, and peptidoglycan biosynthesis;<br>• Reduced hyphae formation;<br>• Down-regulation of *sap4* and *hwp1* fungal genes. |
| *C. albicans* and *F. nucleatum* | • Cell–cell direct contact mediated by fungal hyphal cell wall Flo9 adhesins and bacterial cell surface adhesin RadD;<br>• Increased *C. albicans* resistance to phagocytosis;<br>• Reduced inflammatory response against *F. nucleatum*;<br>• Inhibition of hyphae formation by bacterial *quorum sensing* under FsrB transcriptional regulation;<br>• Decreased fungal biofilm formation by EntV bacteriocin; |

| *C. albicans* and *S. aureus* | • Cell–cell direct contact mediated by fungal hyphal cell wall ALS3 adhesins and multiple bacterial cell surface adhesins, specially FnpB, SasF, and Atl<br>• *S. aureus* is able to penetrate into mucosal tissues using *Candida* hyphae as port of entry;<br>• Up-regulation of fungal and bacterial proteins related to carbohydrate and aminoacid metabolism, as well as glycolysis and cellular stress response;<br>• Down-regulation of *S. aureus* transcriptional repressor of virulence factor CodY protein;<br>• Up-regulation of *S. aureus* Ldh1 (L-lactate dehydrogenase 1), which increases protection against host-derived oxidative molecules<br>• Reduced bacterial susceptibility to vancomycin induced by farnesol; |
|---|---|
| *C. albicans* and polymicrobial bacterial biofilms (*C. albicans* + *S. oralis* + *A. oris* OR *C. albicans* + *S. mutans* + *S. sanguinis* + *A. viscosus* + *A. odontolyticus* OR *C. albicans* + *S. sanguinis* + *S. mutans*) | • Increased streptococcal/*Actinomyces* biomass on biofilms;<br>• Up-regulation of *Candida* virulence genes *epa1*, *als3*, *sap6*, and *hwp1* leading to increased hyphae formation;<br>• Greater tissue invasion by *Candida*;<br>• Greater tissue damage;<br>• Greater inflammatory response |

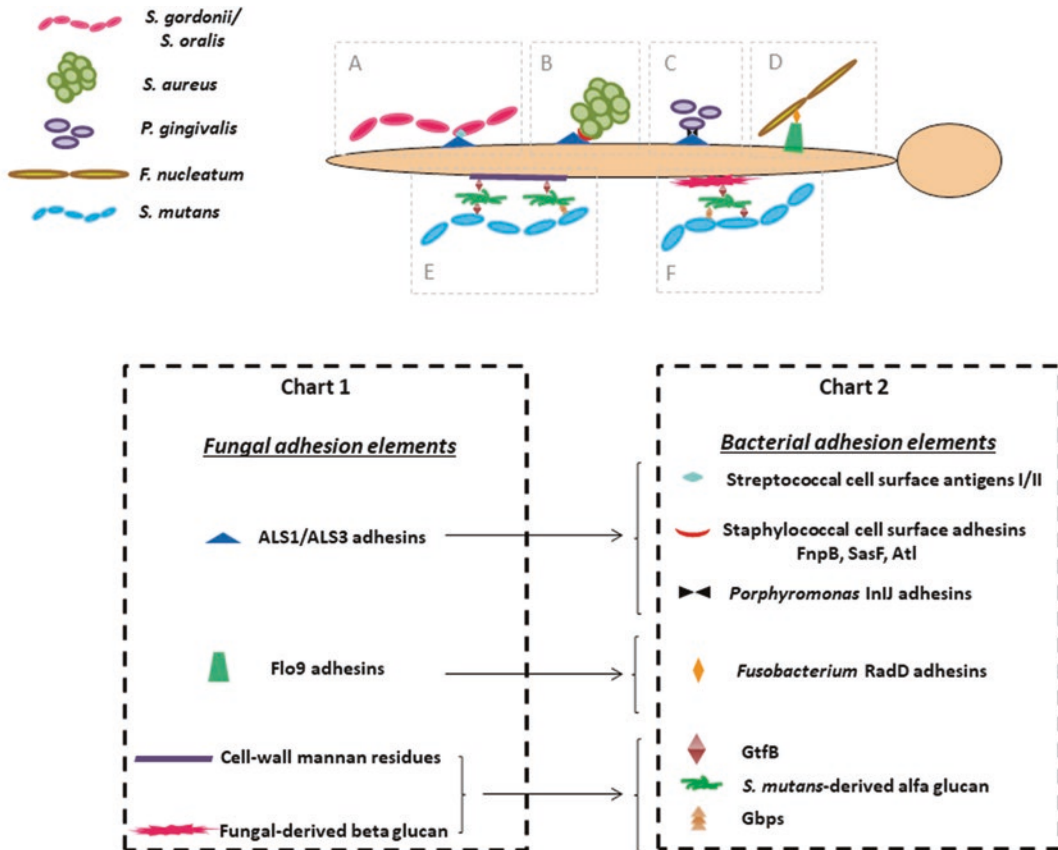


**Fig. 1** Schematic representation of both fungal and bacterial adhesion elements (Chart 1 and 2). Interaction of *C. albicans* with *S. gordonii*/*S. oralis* (A), *S. aureus* (B), *P. gingivalis* (C), *F. nucleatum* (D), and with *S. mutans* (E and F)

*C. albicans, S. gordonii* also promotes hyphae formation while being aggregated around hyphal filaments (Bamford et al. 2009) (Fig. 2A). Further studies revealed that the cell–cell co-adhesion is mediated specifically between *S. gordonii* SspB cell surface adhesin and *C. albicans* ALS3 hyphal wall protein (Silverman et al. 2010) (Fig. 1A). It is likely that this same mechanism of Ssp/ALS-mediated adhesion governs the interaction between *Candida* and other mitis group streptococci, such as *S. oralis* (Xu et al. 2014b) (Fig. 2B). Recent evidences suggest that *S. mutans* cell surface antigens I/II may also play a role in mediating the interaction with *C. albicans* ALS1 and ALS3 adhesins even under high-sucrose availability (Yang et al. 2018). *Actinomyces* species are also able to interact with *C. albicans* (Arzmi et al. 2015; Deng et al. 2019), and although the exact mechanism behind the interaction is still unknown, it has been suggested that this interaction is *Actinomyces*-fimbriae independent (Cavalcanti et al. 2017).

ALS3 hyphal cell wall adhesin also plays a role mediating the adhesion between *C. albicans* to *S. aureus* or to *P. gingivalis*. Binding of *S. aureus* to ALS-3–deficient *C. albicans* is reduced (Peters et al. 2012), and although the interaction with ALS3 is mediated by multiple staphylococcal adhesins, it seems that fibronectin binding protein B (FnpB), *S. aureus* surface protein F (SasF), and a putative *N*-acetylmuramoyl-L-alanine amidase (Atl) are the most important ones (Schlecht et al. 2015) (Fig. 1B). Additionally, in contrast to a poor binding to yeast cells, *P. gingivalis* adheres well to hyphal elements in a fimbriae-independent mechanism since no differ-

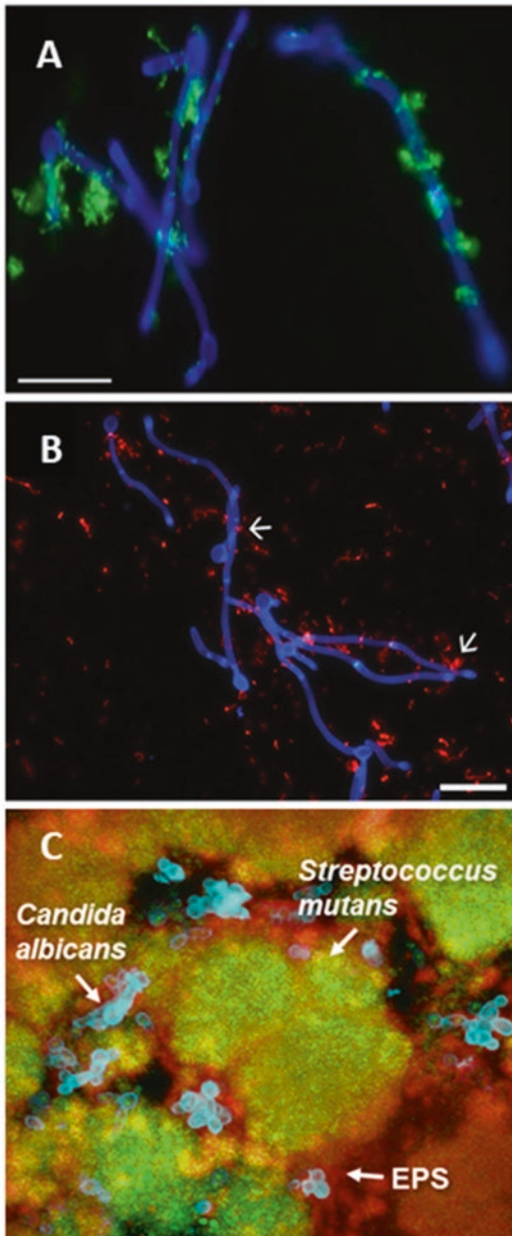


**Fig. 2** Fluorescence laser microscopy images of *C. albicans*–oral streptococci interactions. (**A**) *Streptococcus gordonii* (green) bound along hyphal filaments and as forming microcolonies (scale bar 5 μm); (**B**) *Streptococcus oralis* (red color) adhered principally to hyphal filaments being clusters of streptococci associated with hyphal filaments (arrows) (scale bar 50 μm); *C. albicans* was stained with fluorescent brightener Calcofluor (blue color). (**C**) Confocal fluorescence microscopy images of *C. albicans*–*S. mutans* mixed biofilms, illustrating the spatial relationship between *C. albicans* (blue), *S. mutans* (green), and exopolysaccharides (red). Original images (**A**) from Dutton LC et al., Mol Oral Microbiol 2014;31(2):136–161; (**B**) from Cavalcanti I et al., Pathogens and Disease, 2016,74(3). pii: ftw002; (**C**) from Koo et al., PLoS Pathog 2018; 14(12): e1007342

ence on binding to *C. albicans* is found between *fimA*-deficient *P. gingivalis* and the wild-type strain. Instead, a *P. gingivalis* cell surface protein (InIJ) involved in biofilm formation (Capestany et al. 2006) is shown to mediate the interaction with *Candida* ALS3 protein (Sztukowska et al. 2018) (Fig. 1C). Moreover, ALS3 may also have a role in mediating the interaction between *C. albicans* and *Rothia dentocariosa* (Uppuluri et al. 2017).

It is noteworthy how the hyphal cell wall structure interferes with *Candida*–bacterial interaction. Although *O*-mannosylation-defective *C. albicans* (deletion of alfa-1,2-mannosyltransferase genes; MNT) is able to form hyphal structures, these are not recognized by *S. gordonii.* MNT genes catalyze the addition of mannose residues to mannoproteins found on the fungal outer cell wall layer (Munro et al. 2005). ALS3 protein found on hyphal cell wall of *O*-mannosylation-defective *C. albicans* might have its conformational structure changed preventing it to be recognized by *S. gordonii* SspB adhesin (Dutton et al. 2014). Similarly to the importance of fungal cell wall structure for the cell–cell contact between *C. albicans* and *S. gordonii*, it is also suggested that interactions between *P. gingivalis* and *C. albicans* is also dependent on *Candida* cell wall composition. *P. gingivalis* peptidylarginine deiminase (PPAD) exoenzyme is responsible for converting positively charged protein arginine residues to neutral citrulline residues (Zhao et al. 2017). In mixed-species biofilms, PPAD-deficient *P. gingivalis* presents decreased adherence to hyphal cells compared to wild-type strain. Proteomics analysis reveals that the *Candida* cell surface proteins are modified by PPAD activity being this modification an important step for mediating the interaction between these organisms (Karkowska-Kuleta et al. 2018).

*Fusobacterium nucleatum* is another bacteria that directly binds to *C. albicans* via cell–cell interaction. RadD adhesin present at the *F. nucleatum* cell surface appears to mediate adherence between *F. nucleatum* and oral streptococci (Kaplan et al. 2009). Flo9 is an adhesin-like mannoprotein found at *C. albicans* surface. A reduc-

tion on the number of *F. nucleatum* attached to hyphal elements is found in the presence of Flo9-defective *C. albicans*. Furthermore, RadD-deficient *F. nucleatum* also presented impaired ability to bind to *C. albicans* hyphal cells (Wu et al. 2015) (Fig. 1D). Unlike the importance of *C. albicans* cell wall *O*-mannosylation for the *Candida*–*S. gordonii* interaction (Dutton et al. 2014), it seems that no interference for co-aggregation is found between *N*-mannosylation-defective *C. albicans* and *F. nucleatum*. Instead, *N*-glycosylation-defective *C. albicans* presents an impaired co-aggregation with *F. nucleatum*, in a mechanism that might also be related to conformational changes of cell wall protein (in this case Flo9) that impairs its recognition by *F. nucleatum* adhesin (Wu et al. 2015).

## Getting Together Using Alternative Mechanisms: EPS-Mediated *C. albicans* and *Streptococcal* Interactions in Mixed-Species Biofilms

Alternative to the direct cell–cell *Candida*–bacterial adhesion, available data suggest that EPS-mediated interaction via exoenzymes occur between *C. albicans* and *S. mutans* as well as between *C. albicans* and *S. gordonii*. Instead of interacting directly via cell–cell physical contact, the co-adhesion appears to be largely mediated by glucosyltransferase (Gtf) enzymes that adhere to *C. albicans* yeast and hyphal cell surfaces (Gregoire et al. 2011; Falsetta et al. 2014; Ricker et al. 2014). The GtfB is secreted extracellularly by *S. mutans* and produces EPS alpha glucans using sucrose as substrate. The Gtf binds avidly to the fungal cell surface in active form producing large amounts of EPS in situ when sucrose is available, which in turn promotes co-adhesion with *S. mutans* (Gregoire et al. 2011) (Fig. 2C). The EPS produced directly on the fungal surface provides binding sites for *S. mutans* via bacterial cell-associated glucan binding proteins (Gbps). This is an important mechanism that facilitates cross-kingdom co-adhesion and colonization to the tooth surface and EPS-rich matrix formation, which ultimately boosts mixed-species biofilm formation (Gregoire et al. 2011; Falsetta et al. 2014; Bowen et al. 2018) (Fig. 1E). *C. albicans* also contributes to the biofilm matrix build-up by secreting its own beta-glucans during biofilm formation. These *C. albicans*-derived glucans contribute to the bulk and scaffolding of the EPS matrix, acting as a bridge between *C. albicans* and the alfa-glucans synthesized by *S. mutans* (Falsetta et al. 2014) (Fig. 1F).

The importance of cell wall bound GtfB and its products for *C. albicans* and *S. mutans* interaction was confirmed by Hwang et al. (2017) showing that either *S. mutans* defective in GtfB or GtfB binding defective *C. albicans* was severely impaired of forming mixed-species biofilms. By using single-molecule force spectroscopy with atomic force microscopy technique to measure the magnitude of adhesion force and stability of GtfB binding to *C. albicans* surface, Hwang et al. (2015) demonstrated that the binding strength and binding stability of GtfB on *C. albicans* surfaces are higher than observed for the same enzyme on *S. mutans* surfaces. Furthermore, they also found that GtfB bound to *C. albicans* produces more EPS than GtfB bound to *S. mutans* surfaces. Further analyses revealed that mannans present on *C. albicans* cell wall provide specific GtfB binding sites and mediate this cross-kingdom interaction between *C. albicans* and *S. mutans* (Hwang et al. 2017). GtfB binding was nearly abrogated in *C. albicans* mutants lacking either *N*- or *O*-linked mannans located on the outer most layer of the fungal cell wall. In turn, *C. albicans* mannoprotein-defective mutants were severely impaired of developing mixed-species biofilms with *S. mutans* (Hwang et al. 2017), to *F. nucleatum* and *P. gingivalis* (Dutton et al. 2014; Wu et al. 2015; Karkowska-Kuleta et al. 2018). The findings of Hwang et al. (2017) also highlights that the interaction between *C. albicans* and *S. mutans* is dependent of *Candida* cell wall structure.

It is important to note that the dietary sugar is an essential factor that directly modulates the *Candida*–*S. mutans* interaction. Deletion of

well-known fungal biofilm regulator genes, such as Egf1 (involved with hyphae transition) and Bcr1 (biofilm development regulator), do not impact the ability of *C. albicans* to form mixed-species biofilms with *S. mutans* under a sucrose-rich condition (Hwang et al. 2017). Expression of well-known *Candida* virulence genes, such as *hwp1* (hyphae wall protein), *als1* and *als3* (agglutinin-like sequence) (Nobile et al. 2006; Liu and Filler 2011), is up-regulated in the presence of *S. mutans* and upon cell wall GtfB binding leading to enhanced fungal biofilm formation (Ellepola et al. 2017). These findings show that GtfB and their glucan products-mediated association is a distinctive *Candida*–bacterial interaction whereby the EPS mediates co-adhesion instead of direct cell-cell interactions. The EPS also enhances microbial adhesion to the tooth surface while enmeshing the microorganisms providing cohesion and protection to the biofilm structure, making them difficult to treat or remove from surfaces (Kim et al. 2018). Furthermore, the EPS alters the diffusion properties of the biofilms favoring the diffusion of sugars while retarding buffer neutralization and retaining acids produced within the biofilm structure (Bowen et al. 2018). These biochemical properties contribute to the development of highly cariogenic biofilms.

Interestingly, *S. gordonii* and *C. albicans* also appear to use similar interaction mechanisms based on Gtfs activity. Mixed-species biofilms formed in the presence of saliva by *S. gordonii*-defective GtfG mutants and *C. albicans* present lower biovolume compared to mixed biofilms formed in the presence of *S. gordonii* wild type. It has been hypothesized that *S. gordonii* GtfG may bind to salivary components coating *C. albicans* cell wall, or directly to β-glucan or mannan found at the fungal cell wall promoting the cross-kingdom interaction (Ricker et al. 2014), but the exact mechanism responsible for this interaction has not been elucidated. Interestingly, the interaction between *C. albicans* and *S. gordonii* seems to be facilitated by soluble alfa-glucans synthesized by GtfG in the presence of sucrose (Ricker et al. 2014).

## Once Together: A Cross-Kingdom Relationship via Chemical Interactions

The physical interaction between *Candida* and oral bacteria promotes co-adhesion and close proximity within biofilms, enabling them to develop chemical interactions that support co-existence.

### Sugar and Acid Metabolism-Based Interactions

The co-cultivation between *C. albicans* and *S. mutans* also induces changes on each other's metabolic activity. A transcriptome analysis of mixed-species biofilms revealed an up-regulation of *S. mutans* genes related to carbohydrate transport and metabolic/catabolic pathways suggesting enhanced sugar utilization (Sztajer et al. 2014; He et al. 2017). It is important to emphasize that biofilm formation by *C. albicans* is directly dependent on the available carbon source. Sparse *C. albicans* biofilm formation is found in the presence of sucrose; however, enhanced *Candida* biofilm growth is found when this organism is co-cultivated with *S. mutans* under sucrose availability. In this condition, *S. mutans* is able to break down sucrose (poorly metabolized by *Candida*), providing glucose and fructose residues that can be more readily utilized for *C. albicans* metabolism and growth (Kim et al. 2017). However, competition for available carbohydrates in mixed-species biofilms may also result in niches of carbohydrate limitation that induces both the up-regulation of *S. mutans* galactose metabolism pathway and up-regulation of some pyruvate metabolism encoding genes that are related to pyruvate-formate lyase and pyruvate-dehydrogenase complex pathways. Ultimately, the increased amount of formate on the supernatant of mixed-species biofilms as a result of the enhanced pyruvate pathway may lead to competitive advantage for both organisms over acid-sensitive ones by keeping a localized and more acidified environment (He et al. 2017; Kim et al. 2017). These data suggest that the modulation of *S. mutans* metabolism might be a key factor that allows co-existence

of both microorganisms in mixed-species biofilms, enhanced sugar utilization and environmental acidification, which potentialize the biofilm virulence and the severity of carious lesions (Falsetta et al. 2014; Xiao et al. 2018b). Metabolism-based chemical interactions also occur between *C. albicans* and *S. aureus*. While glucose and fructose are rapidly metabolized by both microorganisms, it seems that the pentose-phosphate pathway is enhanced in mixed-species biofilms (Weidt et al. 2016).

### Quorum Sensing and Other Molecules-Based Interactions

Several interactions based on cross-kingdom signalling are also found between fungal–bacterial mixed-species biofilms. Farnesol is a *C. albicans*'s quorum sensing molecule well-known for governing yeast-to-hyphae transition and *Candida* biofilm formation (Polke et al. 2018). While exerting an antibacterial effect in concentrations higher than 50 μM, evidences suggest that at a concentration of 40 μM, farnesol induces oxidative stress in *S. aureus* enhancing the expression of efflux pump genes, which directly reduce the bacteria susceptibility to vancomycin (Kong et al. 2017). A similar farnesol concentration-dependent effect is found for *S. mutans*. At high concentrations (higher than 100 μM), it is able to damage *S. mutans* cell surface affecting bacterial viability and decreasing both biofilms biomass and the amount of EPS (Koo et al. 2003; Kim et al. 2017). However, at a concentration between 25 and 50 μM, farnesol enhances *S. mutans* growth and microcolonies formation as well as *gtfB* gene expression. Conversely, filamentation is induced when *C. albicans* is co-cultivated with *S. gordonii*. *S. gordonii* appears to block or inactivate farnesol receptors and/or induce intracellular signaling in *C. albicans* that overrides the farnesol signal (Bamford et al. 2009). A recent study reported that the up-regulation of *Candida egf1* gene by *S. oralis* quorum sensing-dependent mechanisms may lead to increased hyphae formation (Xu et al. 2017). In contrast, a quorum sensing-mediated inhibitory effect on hyphae formation was observed via *E. faecalis* FsrB transcriptional regulation (Nakayama et al. 2001), whereas this effect was dampened in the presence of *fsrB*-defective strains (Cruz et al. 2013).

Autoinducer-2 (AI-2) is a universal signaling molecule produced by *luxS* gene of many oral bacteria that might regulate virulence factors, metabolic pathways, and, therefore, bacterial pathogenicity (Vendeville et al. 2005). *Candida* hyphae formation were induced in mixed-species biofilms formed with *S. gordonii,* whereas *luxS*-defective *S. gordonii* was unable to produce robust mixed-biofilms with less and shortened hyphal forms, suggesting a synergistic effect mediated by *S. gordonii*-derived AI-2 molecule (Bamford et al. 2009). In contrast, *A. actinomycetemcomitans*-derived AI-2 inhibits both hyphae and fungal biofilm development (Bachtiar et al. 2014). Differences in the chemical structure of AI-2 produced by *S. gordonii* and *A. actinomycetemcomitans* may help explain why *Candida* reacts differently to this signaling molecule. Hyphae formation appears to be inhibited when in close proximity of *S. mutans* or in the presence of *E. faecalis.* Fatty acid signaling trans-2-decenoic acid and mutanobactin-A synthesized by *S. mutans* are able to inhibit yeast-to-cell transition (Joyner et al. 2010; Vilchez et al. 2010). A competence-stimulating peptide (CSP) under *comC* gene activity that is synthesized by *S. mutans* at the early stages of co-culture (up to 4-h-old cultures) also inhibits *Candida* hyphae formation (Jarosz et al. 2009), whereas it seems that *S. gordonii*-derived CSP inhibits *C. albicans* biofilm development, either by affecting fungal attachment or by promoting cell dispersal, without affecting hyphae formation (Jack et al. 2015). Moreover, a bacteriocin produced by *E. faecalis*, named EntV, also decreases *C. albicans* biofilm formation (Graham et al. 2017). Conversely, *S. gordonii* can create localized areas of high $H_2O_2$ concentration within biofilm structure that may induce stress response on *C. albicans* leading to the hyphal development (Bamford et al. 2009).

Taking together, all these findings may implicate the importance of cross-kingdom interactions, synergistic at times and antagonistic at

others, for balancing microbial diversity and pathogenesis of *Candida*–bacterial biofilms.

## Immune Response Against Oral Fungal–Bacterial Biofilms

Although the oral cavity is highly colonized by microorganisms, an equilibrium exists between the microbiota and host immune responses (Rijkschroeff et al. 2018). Commensal microbial cells tend to live in symbiosis with the host. Breakdown of the dynamic and friendly interactions between host and commensal microbial cells may lead to chronic inflammatory disorders, including autoimmunity, allergies, and metabolic syndromes (Crump and Sahingur 2016; Belkaid and Harrison 2017). This way, a coordinated set of immune responses against microbial cells and innocuous antigens is of utmost importance for the maintenance of host survival and equilibrium. Available evidences have suggested that microbial dysbiosis may lead to overgrowth of *Candida* and commensal bacterial associated to mucosal sites, such as intestinal-gastric mucosa, vaginal mucosa, and oropharyngeal mucosa. In these specific polymicrobial environments, commensal bacteria may modulate *C. albicans* virulence impacting how the host responds to that mixed-species infection (Ranjan and Dongari-Bagtzoglou 2018). The host immune response to *Candida*–bacterial-mediated mucosal infection is niche-specific due to the specificity of each mucosal/epithelial tissue and the local microbiota (Ranjan and Dongari-Bagtzoglou 2018). Therefore, what mediates the interaction between oral *Candida*–bacterial biofilms and host defense cells and the impact of these interactions on disease onsets needs to be individually assessed for each desired site. Therefore, a wide variety of responses might be needed to control each type of infection (Turvey and Broide 2010). Understanding how the host orchestrates an immune response against *Candida*–bacterial biofilms remains a challenge for the scientific community and a key step for dissecting the pathogenic mechanisms and developing new therapeutic strategies.

## Does the Co-infection with Candida–Bacterial Biofilms Alter Immune Response in the Oral Cavity?

Many studies have been done assessing immune response against oral polymicrobial biofilms in which the role of *Candida*–bacterial interactions on the onset of the diseases has not been well established, such as periodontitis and endodontic infections (Ebersole et al. 2017; Gomes and Herrera 2018). Therefore, most of the evidences about host response to classical *Candida*–bacterial oral mixed-species biofilms comes from oropharyngeal/oral mucositis models.

By using a murine oral mucosal co-infection model, it has been shown that the co-cultivation between *C. albicans* and *S. oralis* enhances the ability of *S. oralis* to grow as biofilm on tongue mucosa, which also led to increased tissue invasion by *C. albicans.* While being present as mucosal surface biofilms with some invasion into cell layers as *C. albicans* mono-infection, co-infection results in entire *C. albicans* biomass invading through mucosal cell layers being hyphae largely present within extracellular spaces (Xu et al. 2014a). This greater invasiveness may also be related to the fact that increased levels of epithelial cells derived calpain (able to cleavage cell junctions) is found by the interaction between mucosal cells and mixed-species biofilms highlighting an important cross-kingdom microbial interaction that compromises the integrity of oral mucosa allowing deeper fungal tissue colonization and penetration (Xu et al. 2016). In the presence of both *C. albicans* and *S. oralis*, the severity and frequency of tongue thrush lesions are also greater than those induced by *C. albicans* as monospecies biofilms. This co-infection also lead to a marked induction of neutrophil-activating cytokines (IL-17, CXCL1, MIP-2/CXCL2, TNF, IL1α, IL-1β), implying a higher neutrophil response/chemotaxis generating an exaggerated inflammatory response that was dependent on Toll-like receptor 2 (TLR2) proinflammatory signaling (Xu et al. 2014a). The reason for increased levels of TLR2 in oral tissues during co-infection is likely to be associated to tissue infiltration by neutrophils cells, which

express high levels of this receptor. Yet, epithelial cells of buccal mucosa may also be a possible source of TLR2 since the expression of these receptors is higher in the presence of *C. albicans* (Xu et al. 2014a; Zhang et al. 2004).

On a three-dimensional model of the buccal mucosa formed by immortalized keratinocytes on a fibroblast-embedded collagenous matrix for the growth of *Candida-S.oralis* biofilms, it has been shown that reduced moisture on the surface of the oral mucosa promotes fungal invasion and tissue damage, independently of antimicrobial proteins derived from the host or the amount of nutrients present. In addition, it is important to note that there is an increase in the growth of *S.oralis* when co-cultivated with *C. albicans,* which facilitates and promotes fungal invasion, tissue damage, and mucosal penetration by *Candida* (Bertolini et al. 2015) in a similar way to that described by Xu et al. (2014a). Based on these data, it is clear that *S. oralis* play an important role on the etiology of oropharyngeal mucositis by enhancing the virulence of the mixed-species biofilm. It is important to highlight that the infective behavior of *S. oralis* in relation to oral mucosa is only evident when these microorganisms are associated with *C. albicans* in a mixed-species biofilms (Diaz et al. 2014; Xu et al. 2014a, b).

Besides the role of *Candida–S. oralis* on mucosal immune response, it also seems that co-cultivation of *C. albicans* and *S. aureus* creates a port of entry allowing *S. aureus* to deeply penetrate into tissue. Under this condition, dissemination of *S. aureus* as a secondary infection is seen in mice with oral candidiasis leading to high mortality (Kong et al. 2015). Moreover, co-cultivation of *C. albicans* and *F. nucleatum* appears to make *C. albicans* more resistant to phagocytosis by murine-derived macrophage. The immune-inflammatory response may also be down-regulated since mixed-species biofilms induce lower expression of *F. nucleatum*-induced MCP-1 and TNFα by these macrophage cells. Ultimately, this mutual inhibition could limit the innate immune response allowing long-term persistence of these organisms within the host (Bor et al. 2016).

The interaction between biofilms, specially polymicrobial ones, and immune defense cells might induce different responses compared to single-species biofilms. This way, expression of IL-18 by epithelial cells in contact to polymicrobial *Candida*–bacterial biofilms is greater than the expression induced by single-species biofilms (Cavalcanti et al. 2015). Moreover, it has been shown that the in vivo capacity of epithelial cells in producing Human-derived antimicrobial peptides (HDP/AMP) and interleukins is directly dependent on species abundance on polymicrobial biofilms (Langfeldt et al. 2014). This highlights the importance of studies designed to assess host response to mixed-species biofilms.

The immune response against microbial cells is also species dependent. As an example from intra-abdominal infections, it seems that the presence of other *Candida* strains such as *C. glabrata* or *C. dubliniensis* associated to *S. aureus* provides a protection against further infection induced by *C. albicans*. Whether this same behavior happens in fungal–bacterial biofilms-mediated oral diseases remains unclear and deserves further investigation (Lilly et al. 2018). Moreover, as reviewed by Noble et al. (2017), *C. albicans* possesses a remarkable plasticity related to yeast-to-hyphae transition. This fungus may present at least six distinct morphotypes, which are similar to yeast form cells being this behavior niche-specific. While hyphae and pseudohyphae predominate in most virulence models, newly developed elongated yeasts may be more specialized for commensalism (Noble et al. 2017). It is interesting to note that co-cultivation of *C. albicans* and *S. aureus* in an intra-abdominal infection rat model leads to 100% mortality. However, this mortality is associated with exacerbated local and systemic inflammation, without the need for *C. albicans* yeast-to-hyphae transition since co-infection with *C. albicans* yeast-locked or hyphae-locked mutants showed similar mortality (Nash et al. 2014). Thus, the role played by these different *Candida* morphotypes on immune response deserves to be further investigated.

Even though the host possesses a very complex and intricate response against microbial cells, microorganisms developed several mecha-

nisms for evading the host immune response allowing infection persistence. Since a discussion about microbial evasion is not the main focus of this chapter, only some escape strategies at both bacterial and fungal single-species levels are presented aiming to illustrate the mechanisms used for host immune evasion. By promoting post-translational citrullination of arginine residues, the PPAD enzyme secreted by *P. gingivalis* decreases chemotaxis to neutrophils making this microorganism able to escape from internalization by phagocytes (Bielecka et al. 2018; Stobernack et al. 2018). A self-lipolysis of cell wall lipoproteins induced by *S. aureus*-secreted glycerol ester hydrolase lipase masks the microbial recognition by innate immune cells (Chen and Alonzo 2019). Resistance of *S. mitis, S. oralis*, *S. sanguinis, S. gordonii,* and *S. mutans* to opsonization by host complement system has also been reported (Negrini et al. 2012; Alves et al. 2019). Additionally, *C. albicans* intact biofilms impair reactive-oxygen species production and phagocytosis by neutrophils. In turn, the killing effect of neutrophils against biofilms formed by mutant strains lacking EPS (mutants for genes related to matrix alfa-mannan synthesis) is enhanced, suggesting that phagocytosis inhibition is related to the concealment of cell surface ligands by EPS (Johnson et al. 2016). Similar EPS role on the impairment of phagocytosis may be present in *Candida*–bacterial mixed biofilms found in the oral cavity.

Considering the versatility of microbial cells in modulating virulence and escape mechanisms, more studies are needed to understand the role of *Candida*–bacterial interactions in host immune evasion, which may lead to more efficacious strategies toward clinical success in the treatment of polymicrobial infectious diseases.

## Strategies for Combating *Candida*–Bacterial Biofilms Associated to Diseased Sites

Considering the role played by *Candida*–bacterial biofilms in oral diseases, interventions that target this cross-kingdom biofilms may lead to new and more effective treatments. Tolerance to antimicrobial agents is an important property of biofilm lifestyle. In addition to contributing to protection against antimicrobials and microbial evasion from immune response, EPS also act as a physical scaffold that provides adhesion, cohesion, and mechanical stability for microbial attachment and growth. The presence of mixture of bacterial and fungal-derived EPS enhances the drug-trapping effect of the matrix, which substantially decreases drug efficacy of existing antibacterial and antifungal agents against cross-kingdom biofilms (Fleming et al. 2016; Kong et al. 2016; Mitchell et al. 2016; Kim et al. 2018). To circumvent these important aspect, the use of enzymes able to degrade bacterial and fungal-derived EPS might be an interesting approach to weaken the biofilm three-dimensional structure while increasing antimicrobial drug efficacy. Considering the concern that the dispersion of biofilms may overload the host immune system and release microbial cells able to colonize other sites, the concomitant use of EPS-degrading enzymes and antimicrobial agents has been encouraged (Koo et al. 2017; Fleming and Rumbaugh 2018).

Propidium iodine (PI) is able to disrupt EPS matrix by inhibiting GtfB-driven EPS synthesis on *C. albicans* and *S. mutans* mixed-species biofilms, which leads to an enhanced fungal susceptibility to fluconazole. Importantly, the combined PI/fluconazole treatment is able to deplete the carriage of fungal cells on mixed-species biofilms formed in vivo. Alternatively, the use of glucanohydrolases to digest EPS-derived α-glucan-matrix produced by GtfB bound onto *C. albicans* surface also enhances the antifungal effect of fluconazole against *C. albicans* (Kim et al. 2018). Moreover, cell wall β1,3-glucan secreted by *C. albicans* has been shown to prevent the penetration of vancomycin through biofilm matrix enhancing *S. aureus* drug tolerance (Kong et al. 2016). By exposing the mixed-species biofilms to caspofungin, an inhibitor of β1,3-glucan synthesis, increased *S. aureus* susceptibility to the antimicrobial was observed. Moreover, the use of glucanase reduced resistance of *S. aureus* to the tested antimicrobial

agent (Kong et al. 2016). Additionally, since extracellular DNA may also contribute to biofilm EPS formation, the use of deoxyribonucleases might also be an additional tool aiming at biofilm dispersal (Fleming and Rumbaugh 2017). In this context, an increased antifungal effect of miconazole has been shown by exposing mixed-species biofilms formed by *C. albicans* and *S. aureus* to DNAse (Kean et al. 2017). Chemical structure of EPS as well as the biofilm EPS concentration depend on several factors, including, but not limited to, the microbial composition of the polymicrobial biofilm and host factors, including diet. The cross-kingdom and inter-species metabolic pathways and interactions may lead to the synthesis of EPS that are structurally more complex and chemically diverse. This highlights the importance of studies designed to better understand and characterize EPS from polymicrobial biofilms, which ultimately would allow the development of more specific and effective EPS-degrading enzymes.

Besides EPS-degrading strategies coupled to antimicrobials/antifungals, synthetic chlorhexidine-carrier nanosystems based on iron-oxide magnetic nanoparticles and chitosan have been suggested as preventive or therapeutic agent for treatment of biofilm-mediated oral diseases (Vieira et al. 2019). These nanocarriers are able to reduce *C. albicans* and *S. mutans* mixed-species biofilm growth providing similar or superior anti-biofilm effect compared to chlorhexidine (Vieira et al. 2019). Additionally, mixed-species biofilms formed in the presence of farnesol showed reduced biomass and metabolic activity (Fernandes et al. 2016). Moreover, by disrupting cell membrane, eugenol has been shown to inhibit mixed-species biofilm formation by multidrug resistant *S. mutans* and *C. albicans* strains (Jafri et al. 2019).

New antimicrobial compounds or devices have also been efficacious in controlling mixed-species biofilms. Synthetic guanylated polymethacrylate compound, an analog of (HDP/AMP), has shown antimicrobial effect against mixed-species *C. albicans* and *S. aureus* (Qu et al. 2016). Combinations of 2-aminobenzimidazole and curcumin has been highly efficacious in controlling fungal–staphylococcal biofilm growth by exerting both antimicrobial and anti-adhesion effects (Tan et al. 2019). Gradual release of carvacrol by poly-lactic acid electrospun membranes reduces *S. aureus* and *C. albicans* mixed-species biofilm formation (Scaffaro et al. 2018). Furthermore, the use of bacterial-derived enzymes may also play a role in controlling mixed-species fungal–bacterial biofilm growth. Staphylokinase is a natural protein synthesized by *S. aureus* that inhibits the antimicrobial effects of human defensins as well as facilitates bacterial tissue penetration (Pietrocola et al. 2017). An increased susceptibility to both fluconazole and vancomycin is found when *S. aureus* and *C. albicans* mixed-species biofilms are treated with *S. aureus*-derived staphylokinase. It seems that enzyme is also able to reduce both fungal and bacterial viability at the early stages of biofilm growth (Liu et al. 2019). Furthermore, natural products extracted from various sources have also showed the ability to inhibit *Candida–E. faecalis*, *Candida–S. aureus,* or *Candida–S. mutans* mixed-species biofilms (Oliveira et al. 2014; Sangalli et al. 2018; de Oliveira et al. 2017).

Photodynamic therapy (PDT) may also be able to control mixed-species fungal–bacterial biofilms growth. When erythrosine is used as a photosensitizer under green LED, both counts of *S. sanguinis* and *C. albicans* are decreased on mixed-species biofilms. Both microbial cells were more resistant to PDT in mixed-species biofilms compared to their counterparts monospecies biofilms, being *S. sanguinis* more sensitive to this therapy than *C. albicans* (Palma et al. 2018). Additionally, the use of curcumin as photosensitizer under LED light is able to reduce metabolic activity and biomass of multispecies biofilms formed by *C. albicans*, *C. glabrata,* and *S. mutans* (Quishida et al. 2016). Moreover, tetracationic porphyrin sensitized by white light reduces viability of both *S. aureus* and *C. albicans* in mixed-species biofilms, being *C. albicans* more resistant to PDT than *S. aureus* (Beirão et al. 2014). Furthermore, the counts of *S. mutans, C. albicans,* and *S. aureus* in three-species biofilms are decreased by methylene blue sensitized

by a low-power indium-gallium-aluminium-phosphide laser (Pereira et al. 2011).

Considering that α 1-6 mannans and β1-6-glucans are the main polysaccharide components of *Candida* spp. biofilms (Zarnowski et al. 2014), the use of mannosidase and glucanase disrupts *Candida* biofilm matrix increasing fungal susceptibility to fluconazole (Dominguez et al. 2019). Similarly, concomitant use of a combination of dextranase (α-(1→6) glucanase) and mutanase (α-(1→3) glucanase) followed by essential oil use is able to completely digest the EPS matrix of *S. mutans* biofilm leading to an enhanced and more precise antimicrobial effect in mixed biofilm settings (Ren et al. 2019). Moreover, in vivo models based on the colonization of urinary and vascular catheters and dentures show that biofilms of *C. albicans* incorporate host-derived proteins and cells (Nett et al. 2015). By blocking fungal interaction with host-derived fibronectin, *C. albicans* biofilm formation is decreased (Nett et al. 2016). Therefore, it is likely that biofilms could be controlled by targeting specific host-derived proteins found at the biofilm EPS (Nett et al. 2016). Since EPS seems to play an important role on the microbial evasion from host immune response (Kernien et al. 2017, 2018), EPS-degrading enzymes coupled with antifungals/antimicrobials might also be an interesting approach allowing a more effective host immune response against *Candida*–bacterial-related oral diseases.

Furthermore, it has been well-understood that *Candida* hyphae formation is an important virulence factor that is related to optimal fungal biofilm formation and tolerance to antimicrobials, as well as that affecting fungal recognition by host immune response (Mukaremera et al. 2017; Sharma et al. 2019). Therefore, the modulation/impairment of *Candida* morphological transition is desirable (Jacobsen et al. 2012), which could bring some benefit in terms of controlling *Candida*–bacterial polymicrobial biofilm development. By using a library screening, it has been suggested that a biaryl amide molecule presents the ability to reduce *Candida* filamentation and inhibit biofilm formation, as well as decreasing the in vivo severity of oral candidiasis (Romo et al. 2017). A synthetic analog of *E. faecalis* bacteriocin EntV has been shown to increase the antifungal activity of macrophages and reduce both the severity of infection by *C. albicans* and fungal burden in a murine model of candidiasis (Graham et al. 2017). Strategies focused on the development of *Candida* protease inhibitors, specifically related to the secreted aspartyl protease, might also be a feasible target for antifungal drug therapy development (Monika et al. 2017). Considering that β-glucans are important pathogen-associated molecular patterns (PAMPs) that are masked on the fungal cell wall, search for drugs that could unmask these glucans leading to a more efficient recognition of these microorganisms by cells of the immune system is also an interesting therapeutic approach (Chen et al. 2019a).

Due to the increased rate of microbial drug resistance, HDP/AMP have been proposed as an attractive alternative to antimicrobial agents for controlling biofilm growth and microbial virulence (Pletzer et al. 2016; Nuti et al. 2017). These HDP/AMP present broad-spectrum antimicrobial activity and exert their antimicrobial effect by disrupting cell membrane or by interacting with microbial intracellular components (Bechinger and Gorr 2017). Synthetic fragments of human β-defensin-3 and LL-37 have shown promising anti-biofilm effects against *S. mutans*, *E. faecalis,* and *C. albicans* (Ahn et al. 2017; Caiaffa et al. 2017; Silva et al. 2017; Yoo et al. 2017; Chen et al. 2019b). In addition to these fragments, synthetic peptides derived from histatin-5 present antimicrobial effect against *P. gingivalis* leading to inhibition of alveolar bone loss in a rat periodontitis model (Wang et al. 2017). Although there are some ongoing clinical phase II studies assessing the efficacy of synthetic HDP/AMP on the treatment of oral candidiasis and mucositis in immunocompromised or in patients undergoing chemoradiation for the treatment of neck/head cancer (Greber and Dawgul 2017), there is a lack of clinical studies testing the use of these HDP/AMP for management of oral diseases mediated by *Candida*–bacterial biofilms.

## Concluding Remarks and Perspectives

This chapter highlights the potential role played by cross-kingdom *Candida*–bacterial polymicrobial biofilms in oral diseases, such as dental caries, periodontitis, peri-implantitis, root canal infections, and denture-associated stomatitis. While direct cell–cell contact mediates the interaction between *Candida* and most of oral bacteria (such as *S. gordonii, S. oralis* and *S. sanguinis*, *Actinomyces*, *Porphyromonas, Aggregatibacter, Fusobacterium, Staphylococcus,* and *Rothia*), exoenzymes (such as GtfB, and their bioproducts EPS) seems to optimize the interaction between *Candida* and *S. mutans*. Quorum sensing and other molecules (such as *Candida*-derived farnesol, streptococcal-derived autoinducer AI-2, bacteriocins and competence-stimulating peptides) play an important role in mediating cross-kingdom *Candida*–bacterial interactions that can be stimulatory or inhibitory depending on host and environmental factors. Thus, a tightly regulated cooperative and antagonistic balance through stimulus-inhibition mechanisms appear to mediate bacterial–fungal co-existence and survival within biofilms, which can become synergistic when conditions are conducive for disease.

Host immunity plays a key role in modulating the virulence of microbial cells by maintaining homeostasis. Based on evidences from oropharyngeal/oral mucositis models, both *C. albicans* and *S. oralis* act synergistically enhancing the virulence of mixed-species biofilms leading to an exacerbated inflammatory response. Taken that the immune response elicited against polymicrobial biofilms is different compared to that elicited against monospecies biofilms, it is important to study the role of mixed-species interactions on the host immune response.

Among the available approaches for combating polymicrobial biofilms, it appears that EPS-degrading enzymes coupled with antimicrobial/antifungals might bring benefits in terms of controlling biofilm growth and modulating the immune response. The EPS can provide protection to microorganism while playing a role as an evasion mechanism from host immune response. Furthermore, the heterogeneity and chemical diversity of EPS may also affect these protective mechanisms. To this end, studies need to be designed to better understand and characterize EPS from polymicrobial biofilms, which ultimately would allow the development of more specific and effective EPS-degrading enzymes. The development of new drug nanocarriers that are able to deeply penetrate into the biofilm and whose effects are triggered by specific environmental factors are encouraged. Taken the complexity of biofilm composition and structures, it is likely a combination of different therapies aiming at both bacterial and fungal targets might provide a long-lasting and more efficacious cross-kingdom biofilm disruption.

Although the interaction of *Candida* and oral bacteria in oral mucosal infection has been well-described in the literature, the knowledge about the pathogenic mechanisms of *C. albicans*–bacterial interactions in dental caries as well as their role in periodontitis, peri-implant, and endodontic infections needs further elucidation. Mechanistic, in vivo, and clinical longitudinal studies are needed to clarify and to address the role played by cross-kingdom *Candida*–bacterial interactions on the pathogenesis of these oral diseases.

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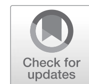

# Comparative Analysis of Gene Expression Patterns for Oral Epithelium-Related Functions with Aging

J. L. Ebersole, L. Orraca, M. J. Novak, S. Kirakodu, J. Gonzalez-Martinez, and O. A. Gonzalez

## Introduction

The majority of agents that cause infections in humans gain access through the mucosal surfaces of the body. As such, the epithelium and epithelial cells have evolved to provide an array of features to protect from pathogenic challenge. These include barrier functions in which the epithelial cells rapidly mature and are sloughed from the surface while maintaining tight junctions enhancing exclusion of deleterious agents at luminal surfaces (Parrish 2017; Yu et al. 2012). In addition to these mechanical barriers, recent evidence has supported the capacity of epithelial cells to constitutively synthesize an array of innate immune protective molecules, as well as a range of cell communication factors providing an "early warning system" to the host inflammatory and immune armamentarium (Pardo-Camacho et al. 2018; Partida-Rodriguez et al. 2017; Ahluwalia et al. 2017). Moreover, a number of these protective signaling molecules are induced through engagement of microbial-associated molecular patterns (e.g., MAMPs, PAMPs) and danger-associated molecular patterns (DAMPs) (Rajaee et al. 2018; Patel 2018; De Lorenzo et al. 2018; Stocks et al. 2018; Walsh et al. 2013; Olive 2012). The functions of the epithelial cells continue to emerge as critical determinants of maintaining host integrity from challenge with pathogenic bacteria, viruses, and fungi, which includes receptor recognition and engagement resulting in specific intracellular signaling pathways leading to antimicrobial activities in the local mucosal environment (Jin and Weinberg 2018; Guncu et al. 2015; Sukhithasri et al. 2013; Ho et al. 2013; McCormick and Weinberg 2010).

The oral cavity is somewhat unique in the properties of its epithelium. While other mucosal

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sites in the body consider it a substantial benefit to maintain the integrity of the barrier function, in the oral cavity, the epithelium is routinely deliberately breached from about 6 months to 21 years of age with eruption of the deciduous and permanent dentition. This developmental anomaly of innate immune protection has fostered the development of a unique junctional epithelium that covers connective tissue cells and a collagen matrix that attaches the erupted teeth to the underlying alveolar bone. This junctional epithelial lining of the subgingival sulcus, in health, is attached to the cementoenamel junction of the teeth (Tsukamoto et al. 2012; Hatakeyama et al. 2006; Bosshardt and Lang 2005). Interestingly, in health, this junctional epithelium is somewhat leaky and allows that passage of a low protein fluid transudate in the gingival crevice that mechanically aids in rinsing colonizing bacteria into the saliva, which is swallowed approximating 1 L/day. Accompanying accumulation of bacterial deposits supra- and subgingivally, the gingival tissue reacts with an inflammatory response with the classic signs of acute inflammation. This inflammation, termed gingivitis, is considered a reversible process that responds rapidly to removal of the bacterial insult (Tonetti et al. 2015; Chapple et al. 2015). An inability to clear this stimulus can lead to a persistent immunoinflammatory lesion, i.e., periodontitis, with ulceration of the epithelium, influx of an array of inflammatory cells, breakdown of connective tissue and collagen, vasculitis, and net resorption of alveolar bone at the localized site of the microbial challenge (Tonetti et al. 2015). While substantial strides are being made in the area of tissue regeneration to reestablish normal function for the periodontium following disease, periodontitis remains considered as irreversible once tissue destruction has occurred.

Age-dependent variations in epithelial barrier function have been previously described in different tissues (e.g., skin, lung, intestine, and kidney) of humans and animal models. A common finding is an impaired cell–cell adhesion mediated by tight junctions consistent with aging-increased permeability (Parrish 2017). Additionally, this decline in epithelial barrier function and repair seems to be associated with an alteration in epithelium stem cells niches (Doles et al. 2012; Moorefield et al. 2017). Nevertheless, the molecular mechanisms associated to these observations remain unclear. Thus, as the epithelial cells and functions of the epithelium are critical to the health of the oral cavity, we used a nonhuman primate model to profile the transcriptome of gingival tissues in health across the lifespan. It was hypothesized that in younger animals, epithelial genes related to functions of a more rigid, less developmentally flexible tissue would be decreased, enabling these young animals to respond to the microbial burden by enhanced signaling pathways associated with rapid wound healing, anti-inflammatory/inflammation resolution, maintaining an effective barrier. In contrast, in older animals, these patterns would differ creating epithelial cells highly responsive to the surrounding environment and less able to modulate and resolve the noxious challenge from the bacteria in the absence of some collateral damage of the periodontal tissues and enhancing the long-term risk for initiation and progression of periodontitis.

## Methods

### Nonhuman Primate Model and Oral Clinical Evaluation

Rhesus monkeys (*Macaca mulatta*) ($n$ = 23; 10 females and 13 males) housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, Puerto Rico, were used in these studies. Healthy animals (5–7/group) were distributed by age into four groups: ≤3 years (young), 3–7 years (adolescent), 12–16 years (adult), and 18–23 years (aged). The nonhuman primates are typically fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad). The diet is supplemented with fruits and vegetables, and water is provided ad libitum in an enclosed corral setting.

A protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico enabled anesthetized animals to be examined for clinical

measures of periodontal including probing pocket depth (PD) and bleeding on probing (BOP) as we have described previously (Ebersole et al. 2008).

### Tissue Sampling and Gene Expression Microarray Analysis

A buccal gingival sample from healthy tissues from the premolar/molar maxillary region of each animal was taken using a standard gingivectomy technique and maintained frozen in RNAlater solution. Total RNA was isolated from each gingival tissue using a standard procedure as we have described, and tissue RNA samples submitted to the microarray core to assess RNA quality analyze the transcriptome using the GeneChip® Rhesus Macaque Genome Array (Affymetrix) (Meka et al. 2010; Gonzalez et al. 2011). Individual samples were used for gene expression analyses.

### Data Analysis

Normalization of values across the chips was accomplished through signal intensity standardization across each chip using Affymetrix PLIER algorithm. The GeneChip® Rhesus Macaque Genome Array contained matched and mismatched pairs allowing the MAS 5 algorithm to be used. For each gene, we first determined differences in expression across the groups using ANOVA (version 9.3, SAS Inc., Cary, NC). The healthy-aged tissues were then compared among the age groups using a *t*-test and accepting a *p*-value $\leq 0.05$ for significance. Because of the cost of these types of nonhuman primate experiments and availability of primates of the various ages, we did not have sufficient samples to identify if the relationship between age and gene expression could be treated using a linear model; thus, the subjects were classified and ANOVA was used for analysis. Correlations with aging and clinical parameters in healthy tissues were determined using a Spearman Rank correlation analysis. A *p*-value $\leq 0.05$ was used to evaluate the significance of the correlation. The data have been uploaded to http://www.ncbi.nlm.nih.gov/geo/info/submission.html.

## Results

### Epithelium Gene Transcriptome in Healthy Gingival Tissues

Using the microarray results, we examined 336 genes that are linked to epithelium and epithelial cells functions (Table 1). The set of genes were categorized into 9 broad functional groups: extracellular matrix and cell structure; extracellular matrix remodeling enzymes; cell adhesion molecules, cytoskeleton regulation; inflammatory response; growth factors; kinases/cell signaling; cell surface receptors; junction associated molecules; autophagy/apoptosis; antimicrobial peptides; and transcription factors.

Figure 1a–d summarizes the level of expression of genes in which the normalized signal was >100 in gingival tissues from any of the 4 groups of animals that included 255 genes. From these data, we identified a group of genes that were altered in younger and aged animals when compared to expression levels in the adult tissues, which included selected extracellular matrix components (e.g., KRT2, KRT4, MMP1, MMP9, TIMP1, F13A1, SERPINF1, CTSK, FBN1, LAD1, CHI3L1), cytoskeleton regulators (e.g., ACTN1, TAGLN, ZYX, DES), cell surface receptors and adhesion molecules (e.g., SELL, ICAM2, ITGAL, SPP1, ITGB2, ITGA8, SELP, ITGAM, ICAM1, ITGAX), and host response genes (e.g., PPBP, CAMP, DEFB4, CXCL11).

### Aging Effects on Epithelium Gene Transcriptome

Within the subset of 255 genes, Fig. 2a–c provides volcano plot visualization of the distribution of altered responses and significant differences in the young, adolescent, and aged animals versus healthy adult levels that were considered to be the normal expression level. From these analyses, it appeared that a lower number of genes were significantly different in the young

**Table 1** Targeted gene for functions of epithelium

| Gene ID | Product, Fxn Group | Gene ID | Product, Fxn Group | Gene ID | Product, Fxn Group |
|---|---|---|---|---|---|
| COL17A1 | Collagen, ECM structure | ITGB1 | Integrin, cell adhesion | RIPK1 | Ser/thr, kinases |
| COL1A1 | Collagen, ECM structure | ITGB2 | Integrin, cell adhesion | VPS13A | Vacuolar protein, kinases |
| COL1A2 | Collagen, ECM structure | ITGB3 | Integrin, cell adhesion | GSK3B | Glycogen synthase, kinases |
| COL3A1 | Collagen, ECM structure | ITGB4 | Integrin, cell adhesion | AGER | Glycation end products, receptors |
| COL5A1 | Collagen, ECM structure | ITGB5 | Integrin, cell adhesion | CD36 | Scavenger, receptors |
| COL7A1 | Collagen, ECM structure | ITGB6 | Integrin, cell adhesion | CD44 | Hyaluronic acid, receptors |
| FBLN5 | Fibulin, ECM structure | LGALS3 | Galectin, cell adhesion | CD59 | C′ mediated lysis, receptors |
| FBN1 | Fibrillin, ECM structure | MSN | Moesin, cell adhesion | EGFR | Epidermal growth factor, receptors |
| FN1 | Fibronectin, ECM structure | PVRL1 | Poliovirus receptor related, cell adhesion | ESR1 | Estrogen, receptors |
| HSPG2 | Heparin sulfate proteoglycan, ECM structure | PVRL2 | Poliovirus receptor related, cell adhesion | F2R | Thrombin, receptors |
| KRT1 | Keratin, ECM structure | PVRL3 | Poliovirus receptor related, cell adhesion | IL9R | Interleukin, receptors |
| KRT10 | Keratin, ECM structure | PVRL4 | Poliovirus receptor related, cell adhesion | PECAM1 | Platelet/endothelial receptors |
| KRT12 | Keratin, ECM structure | SELL | Selectin, cell adhesion | PROCR | Protein C, receptors |
| KRT13 | Keratin, ECM structure | SELP | Selectin, cell adhesion | THBD | Thrombomodulin, receptors |
| KRT14 | Keratin, ECM structure | SPP1 | Secreted phosphoprotein, cell adhesion | TNFRSF1A | TNF family, receptors |
| KRT15 | Keratin, ECM structure | VTN | Vitronectin, cell adhesion | TNFRSF6B | TNF family, receptors |
| KRT16 | Keratin, ECM structure | VWF | Von Willebrand factor, cell adhesion | TRAF1 | TNF associated, receptors |
| KRT17 | Keratin, ECM structure | ACTN1 | Actin, cytoskeleton regulators | TRAF2 | TNF associated, receptors |
| KRT18 | Keratin, ECM structure | ACTN2 | Actin, cytoskeleton regulators | CDSN | Corneodesmosin, junction proteins |
| KRT19 | Keratin, ECM structure | ACTN3 | Actin, cytoskeleton regulators | DSC1 | Desmocollin, junction proteins |
| KRT2 | Keratin, ECM structure | ACTN4 | Actin, cytoskeleton regulators | DSC2 | Desmocollin, junction proteins |
| KRT20 | Keratin, ECM structure | ATP2C1 | ATPase secretory pathway, cytoskeleton regulators | DSC3 | Desmocollin, junction proteins |
| KRT23 | Keratin, ECM structure | ATP2C2 | ATPase secretory pathway, cytoskeleton regulators | DSG1 | Desmoglein, junction proteins |
| KRT24 | Keratin, ECM structure | CCDC19 | Cilia/flagella associated protein, cytoskeleton regulators | DSG2 | Desmoglein, junction proteins |
| KRT25 | Keratin, ECM structure | DNM1 | Dynamin, cytoskeleton regulators | DSG3 | Desmoglein, junction proteins |
| KRT27 | Keratin, ECM structure | ENTPD1 | EctoATPase, cytoskeleton regulators | DSP | Desmoplakin, junction proteins |
| KRT28 | Keratin, ECM structure | FLNA | Filamin, cytoskeleton regulators | EVPL | Envoplakin, junction proteins |
| KRT3 | Keratin, ECM structure | FLNB | Filamin, cytoskeleton regulators | F11R | F11 receptor, junction proteins |

| | | | | | |
|---|---|---|---|---|---|
| KRT35 | Keratin, ECM structure | PDGFRB | Platelet-derived growth factor receptor, cytoskeleton regulators | GJA1 | Gap junction, junction proteins |
| KRT37 | Keratin, ECM structure | RAC1 | Ras family GTPase, cytoskeleton regulators | GJA3 | Gap junction, junction proteins |
| KRT38 | Keratin, ECM structure | SMURF1 | Ubiquitin ligase, cytoskeleton regulators | GJA4 | Gap junction, junction proteins |
| KRT4 | Keratin, ECM structure | STX5 | Syntaxin, cytoskeleton regulators | GJA5 | Gap junction, junction proteins |
| KRT5 | Keratin, ECM structure | TAGLN | Transgelin, cytoskeleton regulators | GJA8 | Gap junction, junction proteins |
| KRT6A | Keratin, ECM structure | TIAM1 | T-cell lymphoma invasion/metastases, cytoskeleton regulators | GJB1 | Gap junction, junction proteins |
| KRT6B | Keratin, ECM structure | TLN1 | Talin, cytoskeleton regulators | GJB2 | Gap junction, junction proteins |
| KRT6C | Keratin, ECM structure | TLN2 | Talin, cytoskeleton regulators | GJB3 | Gap junction, junction proteins |
| KRT7 | Keratin, ECM structure | VCL | Vinculin, cytoskeleton regulators | GJB4 | Gap junction, junction proteins |
| KRT71 | Keratin, ECM structure | WAS | Wiscott-Aldrich syndrome, cytoskeleton regulators | GJB5 | Gap junction, junction proteins |
| KRT72 | Keratin, ECM structure | WASF1 | Wiscott-Aldrich syndrome, cytoskeleton regulators | GJC2 | Gap junction, junction proteins |
| KRT73 | Keratin, ECM structure | WASL | Wiscott-Aldrich syndrome, cytoskeleton regulators | GJC3 | Gap junction, junction proteins |
| KRT74 | Keratin, ECM structure | ZYX | Zyxin, cytoskeleton regulators | GJD2 | Gap junction, junction proteins |
| KRT75 | Keratin, ECM structure | ALOX5 | Lipoxygenase, inflammation | JAM2 | Junctional adhesion, junction proteins |
| KRT76 | Keratin, ECM structure | APOH | Apolipoprotein, inflammation | JAM3 | Junctional adhesion, junction proteins |
| KRT77 | Keratin, ECM structure | CCL2 | MCP-1, inflammation | JAM3 | Junctional adhesion, junction proteins |
| KRT78 | Keratin, ECM structure | CCL5 | RANTES, inflammation | JUP | Plakoglobin, junction proteins |
| KRT79 | Keratin, ECM structure | CCL7 | MCP-3, inflammation | MAGI1 | Guanylate kinase, junction proteins |
| KRT8 | Keratin, ECM structure | CXCL10 | IP-10, inflammation | MAGI2 | Guanylate kinase, junction proteins |
| KRT80 | Keratin, ECM structure | CXCL11 | I-TAC, inflammation | OCLN | Occludin, junction proteins |
| KRT84 | Keratin, ECM structure | CXCL17 | DC/monocyte chemokine, inflammation | PKP1 | Plakophilin, junction proteins |
| KRT85 | Keratin, ECM structure | CXCL2 | MIP-2α, inflammation | PKP2 | Plakophilin, junction proteins |
| KRT9 | Keratin, ECM structure | CXCL5 | ENA-78, inflammation | PKP3 | Plakophilin, junction proteins |

(continued)

**Table 1** (continued)

| Gene ID | Product, Fxn Group | Gene ID | Product, Fxn Group | Gene ID | Product, Fxn Group |
|---|---|---|---|---|---|
| LAD1 | Ladinin, ECM structure | IKBKB | NFαB inhibitor, inflammation | PKP4 | Plakophilin, junction proteins |
| LAMA3 | Laminin, ECM structure | IL1RN | IL-1 receptor antagonist, inflammation | PLEC1 | Plectin, junction proteins |
| LAMA5 | Laminin, ECM structure | IL23A | Cytokine, inflammation | PNN | Pinin, junction proteins |
| LAMB3 | Laminin, ECM structure | LIF | Leukemia inhibitory factor, inflammation | PPL | Periplakin, junction proteins |
| LAMC2 | Laminin, ECM structure | NFKB1 | NFκB, inflammation | TJAP1 | Tight junction associated, junction proteins |
| PRELP | Prolargin proteoglycan, ECM structure | NFKBIA | NFκB, inflammation | TJP1 | Tight junction, junction proteins |
| SPARC | Osteonectin, ECM structure | OSM | Oncostatin M, inflammation | TJP2 | Tight junction, junction proteins |
| VCAN | Versican, ECM structure | PTGS2 | Cox2, inflammation | ATG10 | Autophagy/apoptosis |
| VIM | Vimentin, ECM structure | TNF | Tumor necrosis factor, inflammation | ATG12 | Autophagy/apoptosis |
| CHI3L1 | Chitinase, ECM remodeling | ARHGEF2 | Microtubule regulated, growth factors | ATG13 | Autophagy/apoptosis |
| CTSG | Cathepsin, ECM remodeling | BMP1 | Bone morphogenetic protein, growth factors | GABARAP | ATG8A, autophagy/apoptosis |
| CTSK | Cathepsin, ECM remodeling | BMP2 | Bone morphogenetic protein, growth factors | GABARAPL2 | ATG8C, autophagy/apoptosis |
| ELA2 | Elastase, ECM remodeling | BMP7 | Bone morphogenetic protein, growth factors | BCL2 | Apoptosis regulator, autophagy/apoptosis |
| F13A1 | Coagulation factor XIII, ECM remodeling | CTGF | Connective tissue, growth factors | BIRC2 | Apoptosis inhibitor, autophagy/apoptosis |
| F3 | Thromboplastin, ECM remodeling | EGF | Epidermal, growth factors | BIRC3 | Apoptosis inhibitor, autophagy/apoptosis |
| LOX | Lysyl oxidase, ECM remodeling | FGF10 | Fibroblast, growth factors | CASP3 | Caspase, autophagy/apoptosis |
| MMP1 | Matrix metalloproteinase, ECM remodeling | FGF7 | Fibroblast, growth factors | DAXX | Death domain, autophagy/apoptosis |
| MMP2 | Matrix metalloproteinase, ECM remodeling | GNG11 | G-protein, growth factors | FAS | Death receptor, autophagy/apoptosis |
| MMP7 | Matrix metalloproteinase, ECM remodeling | PPBP/CXCL7 | Connective tissue, growth factors | PERP | TP53 effector, autophagy/apoptosis |
| MMP9 | Matrix metalloproteinase, ECM remodeling | PPP2CA | Microtubules, growth factors | CAMP | Cathelicidin, AMPs |
| PLAT | Plasminogen activator, ECM remodeling | PTEN | Tumor suppressor, growth factors | DEFA1 | Defensins, AMPs |
| PLAU | Plasminogen activator, ECM remodeling | PTP4A1 | Phosphatase, growth factors | DEFA4 | Defensins, AMPs |

| | | | | | |
|---|---|---|---|---|---|
| PLAUR | Plasminogen activator receptor, ECM remodeling | RHOA | Ras homolog, growth factors | DEFA5 | Defensins, AMPs |
| PLOD1 | Lysyl hydroxylase, ECM remodeling | TGFB1 | Transforming, growth factors | DEFA6 | Defensins, AMPs |
| PLOD2 | Lysyl hydroxylase, ECM remodeling | TGFB2 | Transforming, growth factors | DEFB1 | Defensins, AMPs |
| SERPINE1 | PAI-1, ECM remodeling | TGFB3 | Transforming, growth factors | DEFB103A | Defensins, AMPs |
| SERPINF1 | Alpha-2 antiplasmin, ECM remodeling | TMEFF1 | EGF-like, growth factors | DEFB104A | Defensins, AMPs |
| SERPINF2 | Alpha-2 antiplasmin, ECM remodeling | TSPAN13 | Tetraspanin, growth factors | DEFB105A | Defensins, AMPs |
| TIMP1 | Metallopeptidase inhibitor, ECM remodeling | VEGFA | Vascular, growth factors | DEFB106A | Defensins, AMPs |
| CAV1 | Calveolin, cell adhesion | WISP1 | Connective, growth factors | DEFB108B | Defensins, AMPs |
| CAV2 | Calveolin, cell adhesion | WNT5B | Adipogenesis, growth factors | DEFB118 | Defensins, AMPs |
| CAV3 | Calveolin, cell adhesion | AKT1 | Ser/thr, Kinases | DEFB119 | Defensins, AMPs |
| CDH1 | Cadherin, cell adhesion | CHUK | IKK-α, kinases | DEFB121 | Defensins, AMPs |
| CDH2 | Cadherin, cell adhesion | CSNK2A1 | Casein, kinases | DEFB122 | Defensins, AMPs |
| CDH3 | Cadherin, cell adhesion | CSNK2A2 | Casein, kinases | DEFB123 | Defensins, AMPs |
| CDH4 | Cadherin, cell adhesion | DBF4 | Zinc finger, kinases | DEFB125 | Defensins, AMPs |
| CDH5 | Cadherin, cell adhesion | JAG1 | Notch signaling, kinases | DEFB126 | Defensins, AMPs |
| CTNNA1 | Catenin, cell adhesion | MAP2K1 | Mitogen activated, kinases | DEFB127 | Defensins, AMPs |
| CTNNA2 | Catenin, cell adhesion | MAP2K3 | Mitogen activated, kinases | DEFB129 | Defensins, AMPs |
| CTNNA3 | Catenin, cell adhesion | MAP2K4 | Mitogen activated, kinases | DEFB132 | Defensins, AMPs |
| CTNNAL1 | Catenin, cell adhesion | MAP2K6 | Mitogen activated, kinases | DEFB4 | Defensins, AMPs |
| CTNNB1 | Catenin, cell adhesion | MAP2K7 | Mitogen activated, kinases | GZMA | Granzyme, AMPs |
| CTNNBIP1 | Catenin, cell adhesion | MAP3K1 | Mitogen activated, kinases | PLA2G2A | Phospholipase, AMPs |
| CTNNBL1 | Catenin, cell adhesion | MAP3K14 | Mitogen activated, kinases | PLUNC | Palate/lung/nasal, AMPs |
| CTNND1 | Catenin, cell adhesion | MAP3K5 | Mitogen activated, kinases | CEBPA | Leu zipper, transcription factors |
| CTNND2 | Catenin, cell adhesion | MAPK1 | Mitogen activated, kinases | EHF | ETS homologous, transcription factors |

(continued)

**Table 1** (continued)

| Gene ID | Product, Fxn Group | Gene ID | Product, Fxn Group | Gene ID | Product, Fxn Group |
|---|---|---|---|---|---|
| DES | Desmin, cell adhesion | MAPK13 | Mitogen activated, kinases | ETS1 | Proto-oncogene, transcription factors |
| ICAM1 | Intracellular adhesion, cell adhesion | MAPK14 | Mitogen activated, kinases | JAK3 | Janus kinase, transcription factors |
| ICAM2 | Intracellular adhesion, cell adhesion | MAPK3 | Mitogen activated, kinases | JUNB | Proto-oncogene, transcription factors |
| ITGA1 | Integrin, cell adhesion | MAPK8 | Mitogen activated, kinases | KRAS | Proto-oncogene, transcription factors |
| ITGA2 | Integrin, cell adhesion | NOTCH1 | Transmembrane EGF, kinases | MITF | Melaninogenesis, transcription factors |
| ITGA3 | Integrin, cell adhesion | NOTCH2 | Transmembrane EGF, kinases | RAF1 | Proto-oncogene, transcription factors |
| ITGA4 | Integrin, cell adhesion | NOTCH3 | Transmembrane EGF, kinases | TCF3 | Ig, transcription factors |
| ITGA5 | Integrin, cell adhesion | NOTCH4 | Transmembrane EGF, kinases | TWIST1 | bHLH, transcription factors |
| ITGA6 | Integrin, cell adhesion | PIK3C3 | Lipid, kinases | ZEB1 | Zinc finger homeobox, transcription factors |
| ITGA7 | Integrin, cell adhesion | PIK3R4 | Lipid, kinases | ZEB2 | Zinc finger homeobox, transcription factors |
| ITGA8 | Integrin, cell adhesion | PRKAA1 | AMP activated, kinases | | |
| ITGA9 | Integrin, cell adhesion | PRKAA2 | AMP activated, kinases | | |
| ITGAL | Integrin, cell adhesion | PRKCG | PKC, kinases | | |
| ITGAM | Integrin, cell adhesion | PRKCZ | PKC, kinases | | |
| ITGAV | Integrin, cell adhesion | PRKD1 | PKD, kinases | | |
| ITGAX | Integrin, cell adhesion | PTK2 | Tyr, kinases | | |

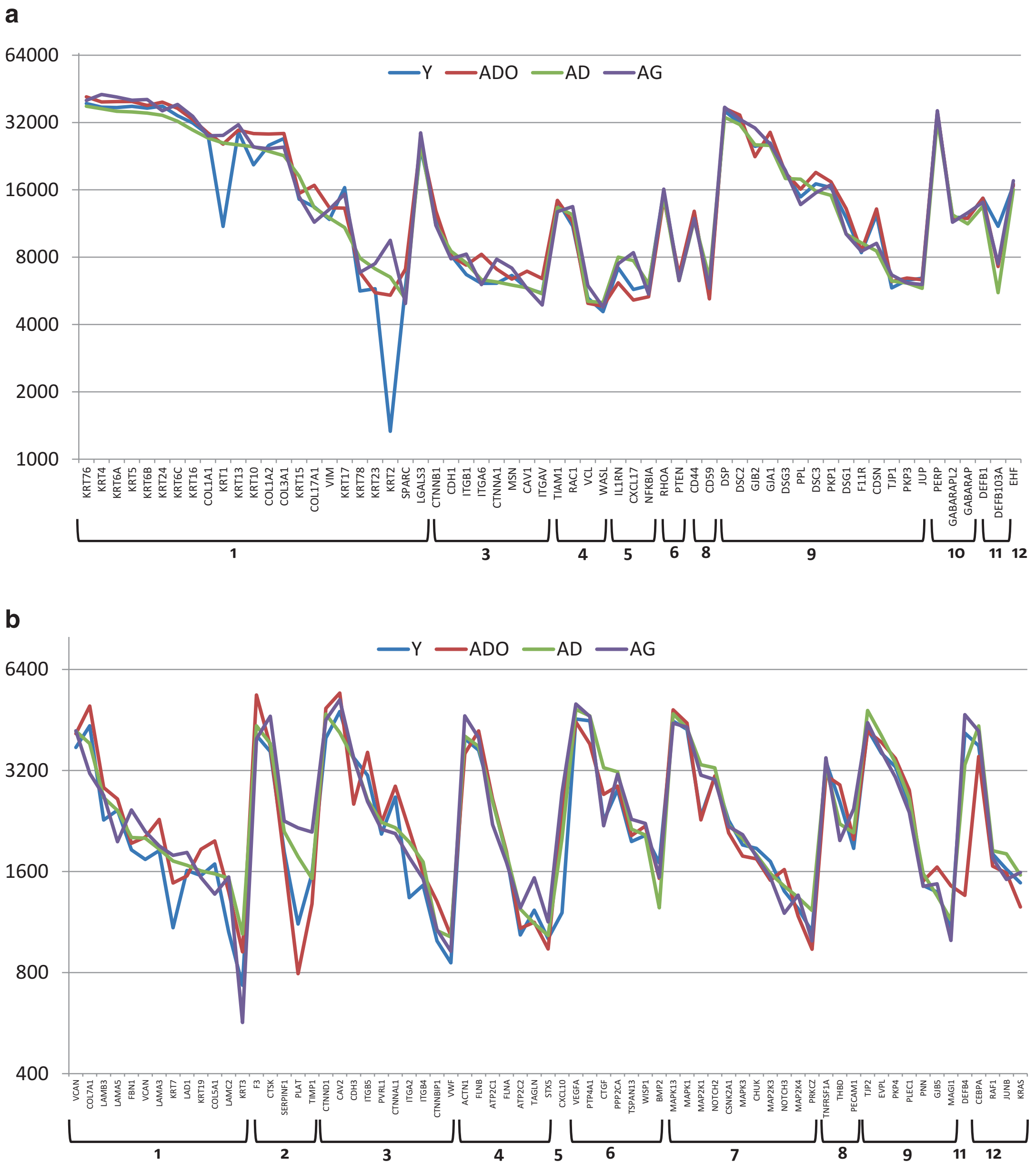


**Fig. 1** (**a**–**d**): Gene expression levels in gingival tissues reflecting epithelium/epithelial cell functions. The lines represent the mean normalized signal level for each age group on animals. The genes are stratified into general functional categories and grouped in the graphs based upon the magnitude of signal (1: extracellular matrix components; 2: extracellular matrix enzymes; 3: cell adhesion molecules; 4: cytoskeleton regulators; 5: inflammatory cytokines/chemokines; 6: growth factors; 7: kinases/cell signaling; 8: cell surface receptors; 9: junction associated proteins; 10: autophagy/apoptosis; 11: antimicrobial molecules; 12: transcription factors)

animals versus the other groups, where 10–20% of the genes varied from healthy adult tissues.

Interrogating this dataset more specifically, Fig. 3 provides a heatmap representation of the fold increase or decrease in gene expression in healthy young, adolescent, and aged tissues compared to adults. Additionally, the genes were classified into 9 categories across their range of functions for the epithelium and epithelial cells. The results showed that the extracellular matrix

**Fig. 1** (continued)

and cell structure, cell adhesion molecules, and cell surface receptor categories appeared to be most frequently different. Several extracellular matrix structural and cell adhesion molecules were elevated in the aged tissues, with generally decreased levels in the young tissue samples. The cell surface receptors were, generally, increased across the different age groups versus the adult levels. Also of interest was the lack of effect on the array of molecules related to epithelium junctions, transcription factors, kinases, and genes linked to autophagy/apoptosis. Of note, specific genes such as SPP1 and PPBP showed and aging-related increase. Table 2 provides a pathway analysis to assess biologic processes that were enriched in the set of genes that were significantly

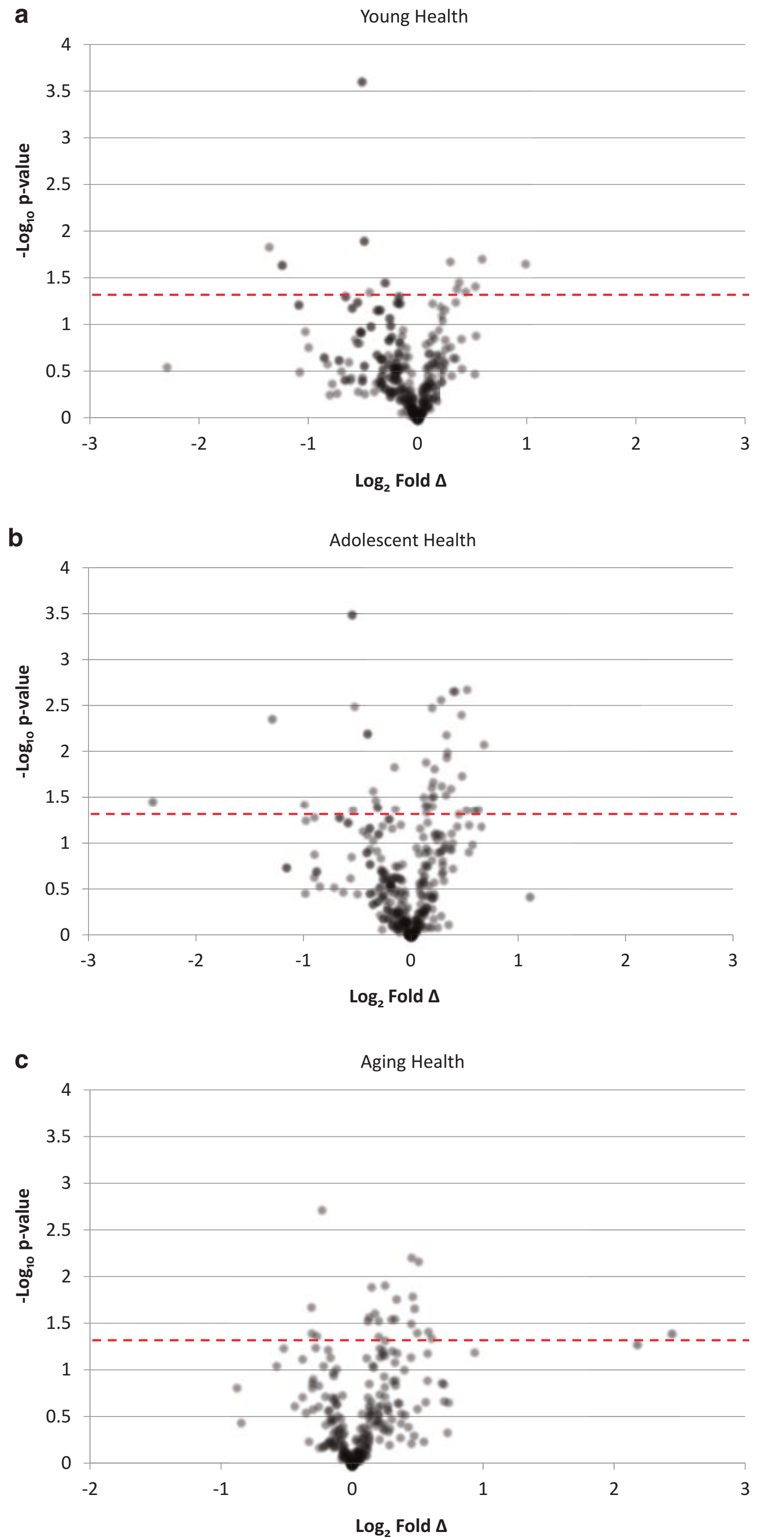


**Fig. 2** (**a**–**c**) Volcano plots of gene expression levels in young, adolescent, and aged animals compared to the healthy adult tissue levels. Each point denotes a gene related to fold and statistical difference from adult levels. The red dashed line signifies a *p*-value <0.05

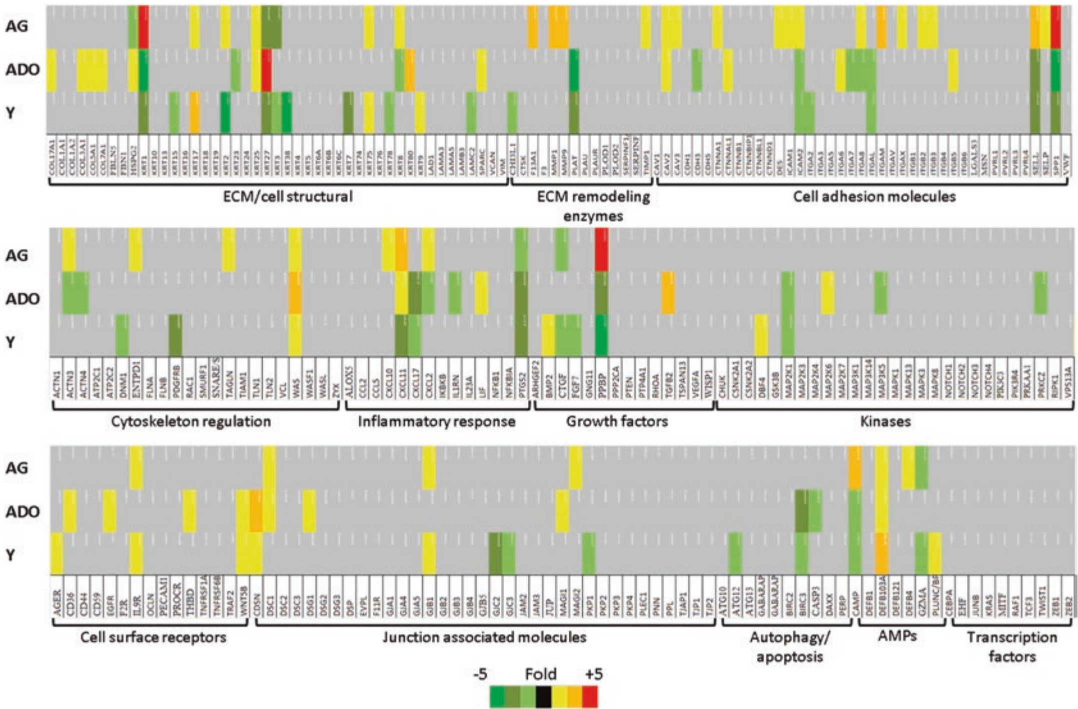


**Fig. 3** Heatmap of fold differences in gene expression in young, adolescent, and aged animals compared to health adults. Genes are grouped into the 9 major categories, and the coloration reflects the mean differences in gene levels for the age group

**Table 2** Major pathways of epithelial gene up-regulation in healthy gingival tissues

| PANTHER biological processes | *M. mulatta* Genome # | Tissue # | Expected | Fold enrichment | Raw*P*-value | FDR |
|---|---|---|---|---|---|---|
| Cell–matrix adhesion | 46 | 5 | 0.16 | 31.16 | 9.18E−07 | 5.06E−05 |
| Cell adhesion | 308 | 18 | 1.07 | 16.75 | 4.50E−17 | 1.10E−14 |
| Cell–cell adhesion | 143 | 7 | 0.50 | 14.03 | 9.09E−07 | 7.39E−05 |
| MAK cascade | 333 | 7 | 1.16 | 6.03 | 1.79E−04 | 5.47E−03 |
| Signal transduction | 2088 | 21 | 7.28 | 2.88 | 6.37E−06 | 3.11E−04 |
| Cell communication | 2399 | 21 | 8.37 | 2.51 | 5.16E−05 | 1.80E−03 |
| Regulation of phosphate metabolic processes | 509 | 8 | 1.78 | 4.51 | 4.24E−04 | 1.15E−02 |
| Cell differentiation | 503 | 7 | 1.75 | 3.99 | 1.96E−03 | 4.78E−02 |
| Developmental process | 1412 | 16 | 1.93 | 3.25 | 2.54E−05 | 1.03E−03 |

and/or >1.25-fold-regulated. As was seen in the heatmap, cell–matrix, cell–cell adhesion, and differentiation were enriched. While the heatmap did not provide a clear visualization of alterations in MAPK signaling pathway genes, these were enriched in the pathway analysis evaluation.

Figure 4a and b focuses on the details of altered expression of the array of keratins that are critical for epithelial cell functions. The results showed that approximately 20 of the keratins were expressed at high levels in the gingival tissues. Keratins 2, 5, 6B, 13, 16, 17 were all significantly increased in healthy-aged tissues versus adults. In contrast, keratins 1 and 2 were significantly decreased and keratin 17 increased in tissue from young animals compared to healthy adults. An additional set of molecules critical for communication of the epithelial cells are the

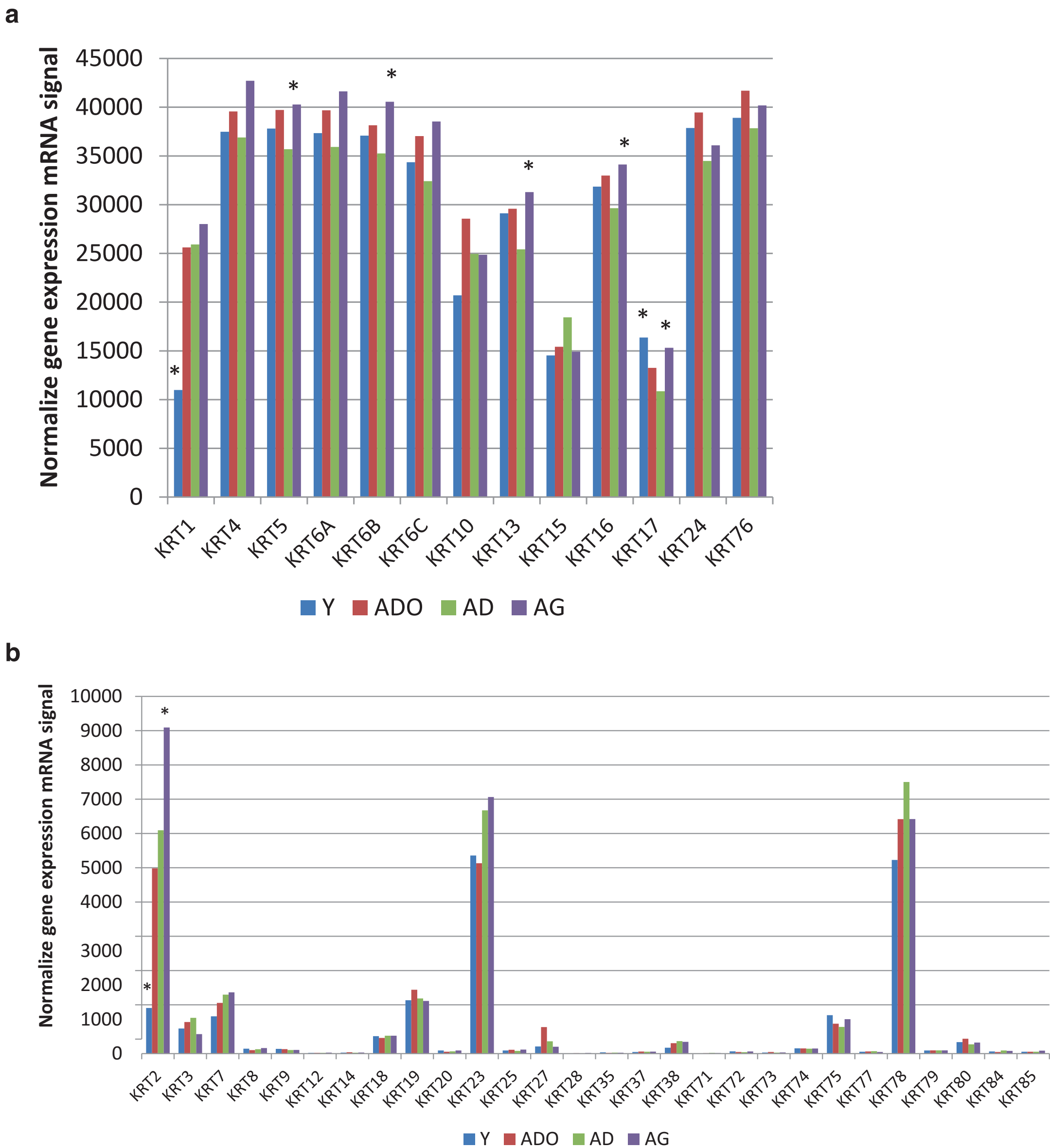


**Fig. 4** (**a** and **b**) Normalized gene expression levels for keratins in the 4 age groups. The bars denote mean group levels. The asterisk (∗) signifies statistically different than other groups at $p < 0.05$

array of integrin surface receptors. Figure 5 provides an overview of these response profiles across the age groups. Approximately 15 of these integrins are highly expressed in the gingival tissues across the age groups. Only ITGA8, ITGAM (CD11b), and ITGB2 were significantly increased in the aged tissues compared to adults, with no difference in the younger animals. ITGB2 is a component portion of integrins that bind ICAMs, VCAM, and even complement components. ITGAM/ITGB2 is particularly implicated in interactions of monocytes, macrophages, and granulocytes and the uptake of complement-coated particles. Thus, while these integrins can be related to epithelial cell biology, their role in these complex oral tissues may be more related to

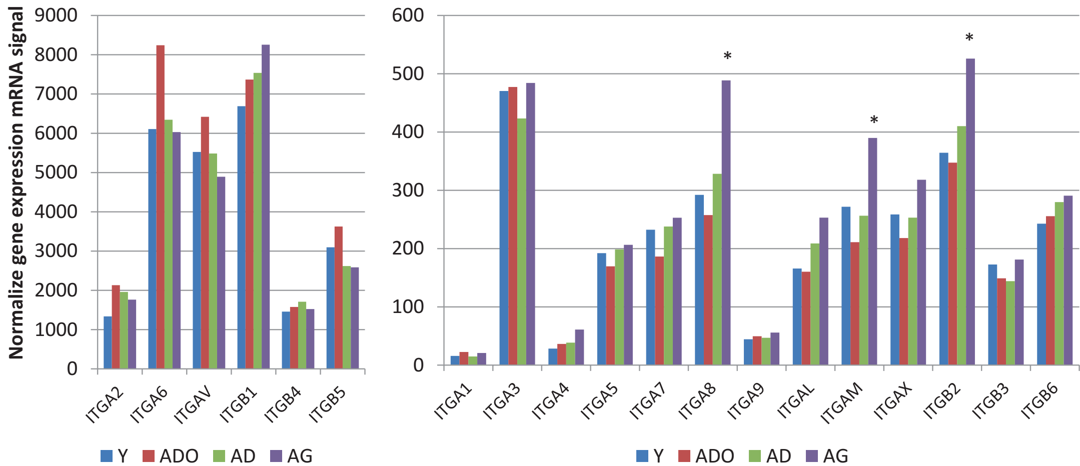


**Fig. 5** Normalized gene expression levels for integrins in the 4 age groups. The bars denote mean group levels. The asterisk (∗) signifies statistically different than other groups at $p < 0.05$

the physiologic inflammation of the gingiva and reflect tissue maintenance by inflammatory cell responses in these tissues. Lastly, we focused on the array of biomolecules related to epithelial junctions including desmosomal and hemidesmosomal proteins (Fig. 6a, b). As was noted from the heatmap, few of these proteins were significantly altered across the age groups, with only CDSN (corneodesmosin) being increased in younger animals versus adults, and COL7A1 (collagen) and LAMA5 (laminin) decreased in the aged animal tissues.

The data were also analyzed beyond an age categorization (young, adolescent, adult, aged) by evaluating correlations of the gene expression profiles with age as a continuous variable (Fig. 7a). The results demonstrated about 10% of the genes demonstrated significant correlations ($p < 0.01$) with similar numbers positively and negatively correlated. While those positively correlated genes represented a range of functions, of interest was the number of collagen and integrin genes that were significantly decreased with aging even in healthy tissues. Figure 7b, c provides a similar type of assessment, relating gene profiles to clinical features of the periodontium in the healthy animals (bleeding on probing—BOP; mean probing pocket depth—PPD). In contrast to the correlations with age, fewer relationships were observed with either of the clinical parameters, with only PLAU, SMURF1, and MAP 3 K5 genes positively correlated and KRT17 and BMP2 negatively correlated with both BOP and PPD.

## Discussion

Within the paradigms of gingivitis and periodontitis that affect the global population, there remain some observations that have yet to be understood at the molecular level. First, while gingivitis is generally considered to presage to periodontal lesions, identified populations have long-standing, florid gingival inflammation and never progress to periodontitis (Loe et al. 1986; Lang et al. 2009). Second, many cases of localized aggressive periodontitis that tend to occur in younger individuals associated with infection with *Aggregatibacter actinomycetemcomitans* demonstrate substantial rapid localized bone loss in the absence of gross inflammatory changes in the gingival tissues (Kinane and Hodge 2001; Jenkins and Papapanou 2001; Bimstein et al. 2002). Third, in children and adolescents, there is a high incidence of gingivitis that increases in prevalence and severity through puberty, in the absence of progressing to periodontitis (Albandar and Tinoco 2002; Modeer and Wondimu 2000; Bimstein et al. 2013; Bimstein and Ebersole 1989).

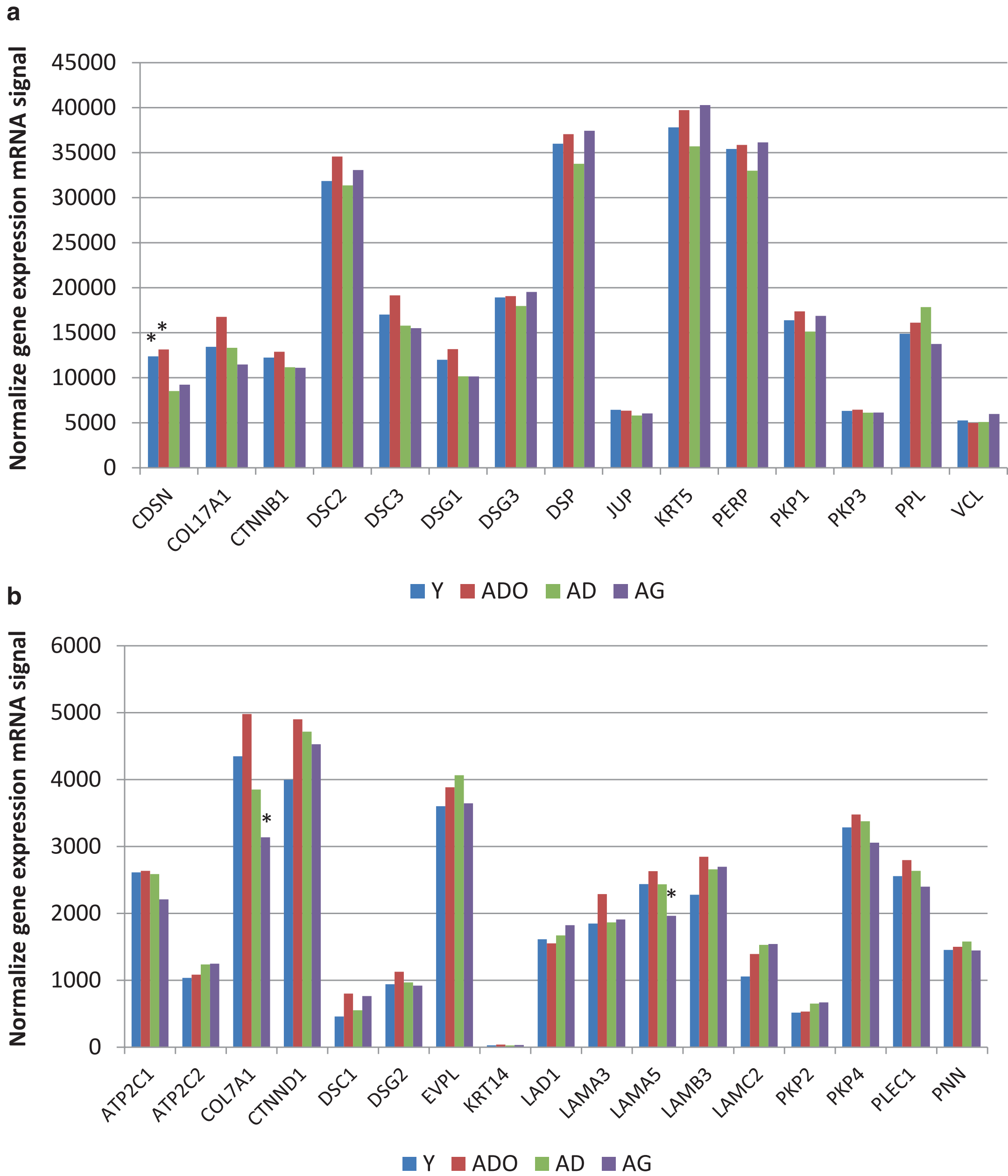


**Fig. 6** (**a** and **b**) Normalized gene expression levels for desmosomal and hemidesmosomal genes involved in cell junction formation in the 4 age groups. The bars denote mean group levels. The asterisk (∗) signifies statistically different than other groups at $p < 0.05$

Fourth, during pregnancy, subsets of women can develop rather severe pregnancy-associated gingivitis that has been suggested to be linked to hormonal changes that could influence the oral microbial ecology, although there remains sparse data documenting the molecular features of this unique gingivitis that does not progress to periodontitis (Gumus et al. 2016; Gursoy et al. 2014; Barak et al. 2003). Finally, periodontitis has long been described as a disease of aging with sub-

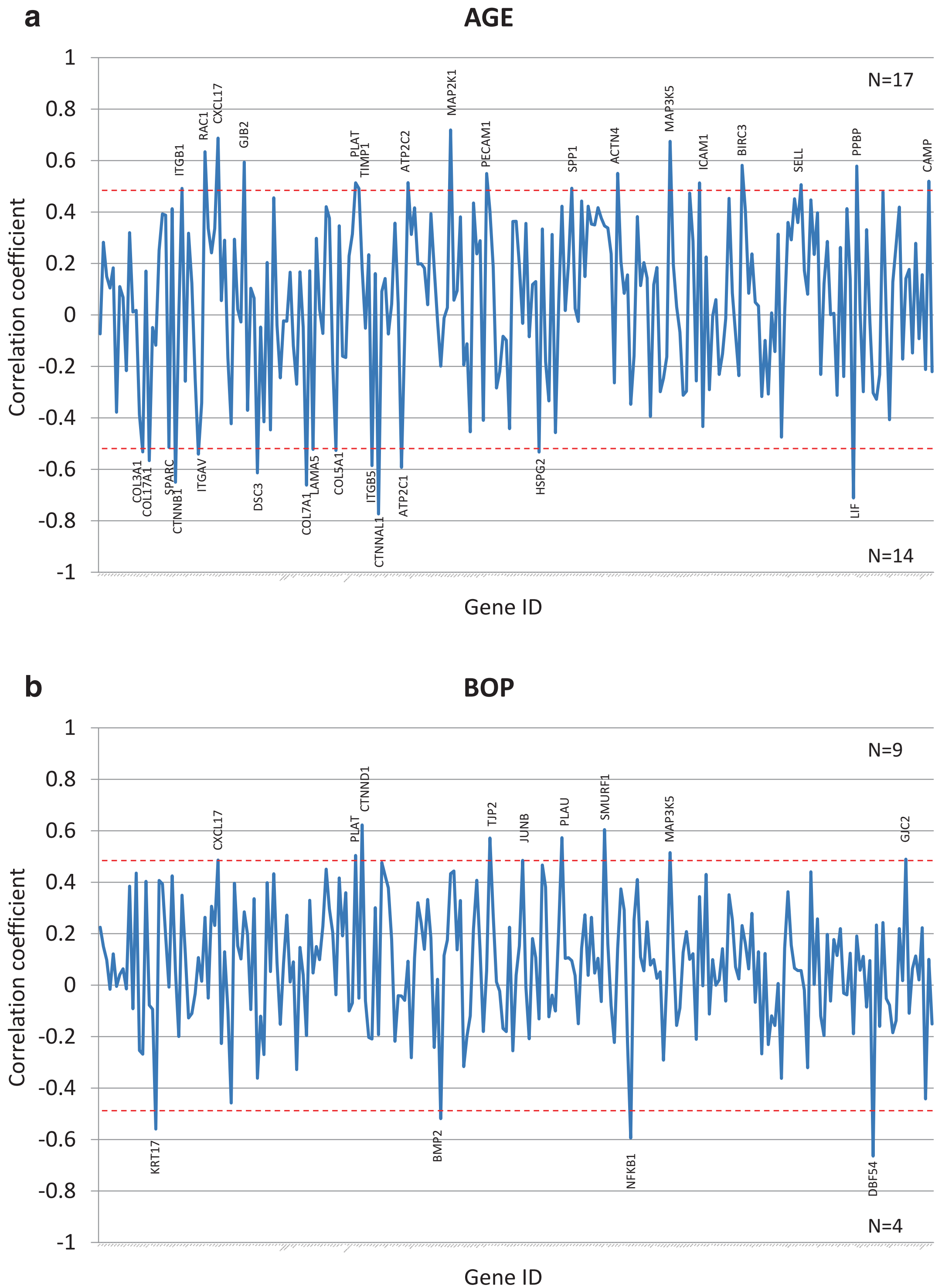


**Fig. 7** Correlation analysis of gene expression levels with age (**a**) and clinical parameters of mean bleeding on probing (**b**) and mean probing pocket depth (**c**). The red dashed lines denote significance level at $p < 0.01$

stantial increases in incidence and severity in aging populations, and thought to be related to a lifetime accumulation of noxious challenge to the gingival tissues (Papapanou and Susin 2017; Wu et al. 2016; Lamster et al. 2016; Hajishengallis 2014; Huttner et al. 2009). Thus, there remains a need to better understand the underlying molecular biology of the range of cells in gingival tissues and how their functions can dictate variation in disease expression across the lifespan.

This study used a nonhuman primate model to focus in the biology of the epithelium and epithelial cells in gingival tissues to test a hypothesis that alterations in the transcriptome representing a range of functions of these cells/tissues would be altered with aging even in clinically healthy sites. We had previously reported on rather dramatic changes in various immune and inflammatory cells in gingival tissues in this model. There were clear alterations even in healthy-aged tissues with regard to lymphocyte classes (Ebersole et al. 2014, 2016a), apoptosis (Gonzalez et al. 2011, 2013), macrophage function and antigen recognition and presentation (Gonzalez et al. 2014, 2015, 2018), hypoxia (Ebersole et al. 2018), and inflammasome characteristics (Ebersole et al. 2016b). However, while there were some differences in the epithelial-related gene expression profiles in periodontal health with aging, the number of genes affected with a fold-change >1.25 was only about 30% and only 8% >1.5-fold. These alterations were also focused on a more limited functional activity of the epithelium/epithelial cells with extracellular matrix structural components, cell adhesion molecules, and cell surface receptors appearing to be most greatly affected.

Drilling down into these categories, multiple collagen and keratin gene levels were lower in young versus aged tissues, which were confirmed with correlation analysis related to aging. These findings suggested that these altered structural components in healthy aging could either reflect a physiological adaptation with aging that helps to maintain healthy tissues, or potentially these changes reflect altered epithelium characteristics that could increase the risk for initiation of periodontitis. Clear histopathological results demonstrate a breakdown in epithelium integrity accompanying the chronic inflammation of periodontitis (Bosshardt and Lang 2005; Dale 2002; Van der Velden 1984). It is accepted that these microulcerations enhance access of the microbiome components (e.g., bacteria, bacterial structures) into deeper tissues contributing to activating the local inflammatory response responsible for tissue destruction. Additionally, this process is considered as part of the feature allowing bacteria to traverse the gingival tissues and enter into the systemic circulation (Cardoso et al. 2018; Abbayya et al. 2015; Maddi and Scannapieco 2013; Kumar 2013). However, examination of the genes related to cell–cell interactions and cell–matrix interactions (desmosomes, hemidesmosomes) did not show a substantial impact of aging on the expression of these molecules. Thus, how these patterns reflect aging processes in health and risk for disease remains ill-defined, and further studies will be required to discriminate between these options.

An array of genes for cell adhesion molecules including cadherins, integrins, caveolins, and selectins were increased with aging. These molecules are critical for maintaining homeostasis of the epithelium in the septic environment of the oral cavity. Thus, since the tissue samples were from clinically healthy sites in the aged animals, this type of response profile may signify an effective healthy aging process in the tissues from these animals. Of note, remarkably elevated gingival expression levels of SPP (osteopontin) and PPBP (pro-platelet basic protein:CXCL7:NAP-2) were observed with aging. SPP has been shown to play important roles in wound healing seemingly through inhibiting apoptosis and modulating the expression of MMPs (Icer and Gezmen-Karadag 2018). From an epithelial cell function viewpoint, PPBP as a heterodimer with other chemokines is involved in glycosaminoglycan interactions with cells via the CXCR2 receptor (Brown et al. 2017). It is a chemoattractant for neutrophils and has some antimicrobials activities. This chemokine has been associated with the pathogenesis of chronic diseases, such as cancer and arthritis (Yeo et al. 2016; Desurmont et al. 2015). It is also identified as one of a group of

platelet-associated chemokines that were systemically elevated in patients with antiphospholipid syndrome (Patsouras et al. 2015), which has also been linked to the microbiome in periodontitis (Schenkein et al. 2003). Finally, a recent study by Shusterman et al. (2017) combining data from murine studies and an existing human dataset identified a gene cluster of platelet factor 4 (PF4:CXCL4)/PPBP/CXCL5 (neutrophil activating peptide 78: ENA-78) being significantly associated with aggressive periodontitis. These variations are consistent with previous reports demonstrating the persistence of inflammatory cells in diseased gingiva that may results from decreased apoptotic responses and/or enhanced transmigration of neutrophils into the inflammatory lesion with aging (Gonzalez et al. 2013; Wael Youssef 2018; Xia et al. 2017; Zhang et al. 2016; Jang et al. 2015; Sakai et al. 1999). Since this study showed elevations in "clinically healthy" aging tissues, there is a potential that this profile describes an enhanced risk of exhibiting disease initiation in the aged individuals.

While considerable effort has been delivered in attempting to delineate the microbiome and host response parameters that drive the disease process, there remains much less information defining, at the molecular level, what tissue responses are required to help maintain health. Recently, understanding the characteristics of the bacteria that constitute a healthy microbiome and the metabolic functions for these commensal bacteria has come under increasing scrutiny as both an explanatory variable in determining the population variation in disease and as a potential therapeutic target for more biologically oriented treatment strategies (Nassar et al. 2017; Ebersole et al. 2017; Hajishengallis and Lamont 2016; Lamont and Hajishengallis 2015; Wade 2013). However, much less is known regarding the host features controlling the periodontal microbiome in health. As an example, there is limited literature that the expression of various epithelial genes/proteins can be regulated by microbial biofilms and that members of the "red complex" can alter components of the epithelial junctions, particularly desmosomal components (Belibasakis et al. 2015). However, if age-associated alterations in these epithelial functions can affect the characteristics of the subgingival microbiome in moving from health to disease related remains unknown.

As noted, our previous examination of the gingival transcriptome in healthy nonhuman primates with aging, as well as with naturally occurring periodontitis demonstrated significant differences in gene profiles that supported innate and adaptive immune responses, inflammation, and cellular senescence changes occur in aging gingival tissues even when clinically healthy. These findings suggested that a basis for increased periodontitis in the human population with age may be linked to inherent changes in the biology of the gingival tissues during aging decreasing the capacity of the tissues to respond to local environmental changes, including alterations in the pathogenic capacity of the microbiome (Belibasakis 2018). The findings from this study suggested some changes in the functional activities of the epithelium and epithelial cells with aging; however, these differences were considerably less that noted with aging effects on immune system components. These more marginal changes in healthy aging will need to be evaluated in the context of the changes taking place in naturally occurring periodontitis, as well as the dynamics of epithelial responses in the gingival during ligature-induced periodontitis using this human-like disease model. Therefore, a more clear understanding of the fundamental biologic responses of the epithelium should provide insight into disease variation related to increased susceptibility or resistance to periodontitis across the population.

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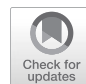


# Neutrophil Interaction with Emerging Oral Pathogens: A Novel View of the Disease Paradigm

Irina Miralda, Aruna Vashishta, and Silvia M. Uriarte

## Introduction

Periodontitis is a multifactorial chronic inflammatory disease that affects the integrity of the periodontium (composed of the gingiva, periodontal ligament, cementum, and alveolar bone). The prevalence of the disease is high, affecting 42% of adults 30 years or older in the USA alone (Eke et al. 2018). Treatment includes deep cleaning, antibiotics, and in severe cases surgery (Douglass 2006). Unfortunately, these treatments are only efficacious in the short term because the infection almost always returns. This disease has also been associated with several comorbidities including rheumatoid arthritis and cardiovascular disease among other inflammatory conditions (Kebschull et al. 2010; Bingham and Moni 2013; Hajishengallis 2015). Novel culture-independent techniques have facilitated the identification of new bacterial species at periodontal lesions and induced a reappraisal of the microbial etiology of periodontitis (Costalonga and Herzberg 2014).

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The current model describing the etiology and pathogenesis for periodontitis is the polymicrobial synergy and dysbiosis (PSD) model (Hajishengallis 2013; Hajishengallis and Lamont 2012). The PSD model indicates that a perturbation of the symbiotic microbial community, associated with periodontal health, results in an increase in the diversity and microbial burden. This results in a dysbiotic microbial community that can adapt to and take advantage of the inflammatory environment to enhance bacterial fitness (Hajishengallis and Lamont 2012).

The host inflammatory response against this dysbiotic microbial community causes the destruction of the periodontium, and human neutrophils are the main phagocytic cell recruited to the periodontal pocket (Scott and Krauss 2012; Uriarte et al. 2016). Based on the published evidence, neutrophils control the indigenous microbial community by mounting an acute inflammation to preserve a healthy gingival tissue. However, neutrophil response against the dysbiotic microbial community results in a dysregulated inflammatory, response causing disease progression (Darveau 2009, 2010). Therefore, to maintain periodontal health, a fragile equilibrium between neutrophil recruitment and activation at the gingiva is essential.

The advance of new high-throughput technology has revealed that the etiology of periodontitis is more complex than the initial paradigm. High numbers of fastidious and “yet-to-be cultivated”

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taxons with strong correlation with disease progression have been identified by human oral microbiome studies (Kumar et al. 2003, 2005). Characterization of the pathogenic potential of these newly appreciated oral pathogens is just beginning to emerge (Hajishengallis and Lamont 2016). The ability of these emerging oral pathogens—as members of the dysbiotic community—to flourish at the gingiva suggests that the microorganisms developed mechanisms to evade or disable the innate immune system. In this chapter, we highlight the clinical studies that opened the Pandora box of the oral mucosa and revealed the presence of newly appreciated periodontitis-associated bacteria, basic description of neutrophil effector functions, and discuss the studies that describe the interactions between neutrophils and the newly appreciated oral pathogens.

## Dysbiosis in Periodontitis: Revealing the Presence of Underappreciated Bacteria

The microbial shift observed in the gingival crevice from a symbiotic oral microbiota to a dysbiotic polymicrobial community is currently the most accepted paradigm to describe the onset and progression of periodontitis (Hajishengallis 2013; Hajishengallis and Lamont 2016; Lamont and Hajishengallis 2015). Initial studies at the turn of the twentieth century used the microbiological techniques available at that time to begin characterizing the etiological agents responsible for periodontitis (Socransky and Haffajee 1994). Those initial studies discovered *spirochetes*, *fusiforms,* and *streptococci* (Socransky and Haffajee 1994; Feres et al. 2004) present in periodontal pockets. Later, using microscopic techniques, Listgarten et al. (Listgarten 1976; Listgarten and Hellden 1978) showed presence of morphological differences in the composition of subgingival microbiota in subjects with different stages of oral diseases. The development of immunological techniques and DNA probes helped to identify the microbial species present in the subgingival pockets (Tsai et al. 2003). However, high-throughput analysis of microbial communities present in subgingival pockets became possible with molecular DNA-based technologies. Using the community fingerprinting techniques, like restriction fragment length polymorphism (Deng et al. 2008), or denaturing gradient gel electrophoresis (Anderson and Cairney 2004), variation and shift in the composition of the microbial community in periodontitis could be identified. The development of DNA–DNA checkerboard hybridization helped elucidated the specific association of oral bacteria with health and disease (Dahlen and Leonhardt 2006). Socransky et al. (1998) described the presence of five microbial communities in subgingival biofilms associated with health and different stages of periodontitis. The 16S rRNA approach has revolutionized the identification of bacterial taxa by classifying whether they are cultivable or "yet-to-be-cultivated," in a mixed population and showed diversity of the oral microbiota (Paster et al. 2001, 2006). Next-generation sequencing technologies further reformed the study of oral microbial diversity by providing the existence of underappreciated periodontal pathogens (Kumar et al. 2006; Dewhirst et al. 2010; Abusleme et al. 2013; Aas et al. 2005; Griffen et al. 2012). The pioneer traditional studies together with the more advanced 16S rRNA gene comparative analysis identified presence of approximately 700 predominant taxa in oral microbiome of which approximately 1/3 is "yet-to-be-cultivated" (Krishnan et al. 2017). About 400–500 taxa were reported in the subgingival crevice alone (Paster et al. 2001; Aas et al. 2005).

The initial dogma in periodontitis was that the microbial community shifted from gram-positive aerobic to gram-negative anaerobic phenotype as disease progressed (Berezow and Darveau 2011; Marsh 1994). The plaque analysis from periodontitis-diseased sites revealed the presence of *Porphyromonas gingivalis*, *Tannerella forsythia,* and *Treponema denticola* related with disease severity (Socransky et al. 1998; Socransky and Haffajee 2005) and the presence of *Aggregatibacter actinomycetemcomitans* associated with aggressive periodontitis (Feres et al. 2004). However, examination of

the microbial composition present in the gingival crevice by the use of more advance techniques, revealed the complexity of the microbial communities and challenged the dogma of disease progression associated with a shift from gram-positive to gram-negative. For example, high numbers of gram-positive bacterium, *Filifactor alocis,* are present in disease periodontal pockets while increased number of gram-negative uncultivated *Veillonella sp* oral clone X042 are found in healthy periodontal pockets have been reported (Kumar et al. 2006). Furthermore, a recent review compared 41 published studies, from 1999 till 2013, that showed an association between emerging periodontal organisms and periodontitis and concluded that 17 newly identified species that includes five gram-positive (*Eubacterium saphenum*, *Mogibacterium timidum*, *Peptostreptococcus stomatis*, *F. alocis* and *Enterococcus faecalis*), eight gram-negative, and four not-yet-cultivable group have a moderate association with the etiology of periodontists (Perez-Chaparro et al. 2014).

The biofilms associated with different stages of periodontitis have substantial overlap in the microbial composition, and the phylogenetic profile of active and non-progressing lesions have similar microbial communities, raising the question whether just composition of microbes or the functional activity of microbial community leads to dysbiosis (Yost et al. 2015; Solbiati and Frias-Lopez 2018). Recent metatranscriptome studies of the subgingival plaque were able to identify changes in functional activities linked to progression of periodontitis (Yost et al. 2015; Jorth et al. 2014; Duran-Pinedo et al. 2015). For example, alteration in potassium ion transport increased the virulence of the oral community and altered the immune response of gingival epithelium (Yost et al. 2015; Duran-Pinedo et al. 2015). The metagenome and metatranscriptome analysis of subgingival plaques revealed the presence of underappreciated periodontal pathogens (Solbiati and Frias-Lopez 2018; Dabdoub et al. 2016). In order to better comprehend the role of these emerging organisms in the onset and progression of the disease, it is essential to study their interaction with other microbes in the community as well as with host cells. In this book chapter, we will focus on the published studies of two organisms classified as putative periodontal pathogens, *F. alocis* and *Peptoanaerobacter stomatis*. We will briefly introduce the current microbiological knowledge about *F. alocis* and *P. stomatis*, followed by what we know about their interaction with neutrophils.

## *Filifactor alocis*

*F. alocis* was first identified in 1985 from the gingival sulcus in gingivitis and periodontitis patients and named as *Fusobacterium alocis* (Cato et al. 1985). In 1999, this bacterium was reclassified in genus *Filifactor* (Jalava and Eerola 1999). *F. alocis* is a gram-positive obligate anaerobic rod, non-spore forming, asaccharolytic, utilizing specific amino acids including arginine, and a slow growing bacteria (Jalava and Eerola 1999; Aruni et al. 2015). Multiple studies have shown the high prevalence of *F. alocis* in subgingival plaques of periodontitis patients compared to absence or low number detected at healthy sites (Kumar et al. 2003, 2005, 2006; Perez-Chaparro et al. 2014). The incidence of *F. alocis* has been reported also in endodontic infections (Zhang et al. 2012; Siqueira and Rocas 2003; Siqueira et al. 2009) and peri-implantitis (da Silva et al. 2014). A study focusing on co-occurrence of oral pathogens in disease sites showed a positive correlation between *F. alocis* and eight oral pathogens including *P. gingivalis* and *T. forsythia,* suggesting possible synergistic interactions among them (Chen et al. 2015). It has been established that *F. alocis* can form biofilm close to apical and middle thirds of the gingival pockets in close proximity to soft tissue (Schlafer et al. 2010). In vitro studies have shown that *F. alocis* could interact with numerous oral bacteria and play significant role in community formation (Aruni et al. 2011; Wang et al. 2013). *F. alocis* co-cultured with *P. gingivalis* enhanced the biofilm formation (Aruni et al. 2011) forming heterotypic biofilm with enhanced *P. gingivalis* growth (Wang et al. 2013) and it also form biofilm with *Fusobacterium nucleatum* and *A. actinomycetemcomitans* (Wang et al. 2013).

*F. alocis* has an exceptional property of resistance to oxidative stress, with its growth stimulated under this condition (Aruni et al. 2011) providing an advantageous attribute to survive in the inflammatory environment that is a hallmark of periodontitis. Moreover, as an asaccharolytic bacterium, *F. alocis* amino acid requirement for its growth is likely fulfilled by the byproducts of the disrupted host tissue produced as a consequence of the chronic periodontal inflammation (Hajishengallis 2013; Aruni et al. 2011, 2015). Another important virulence property of *F. alocis* is the presence of different proteases, most of these proteases are membrane bound but an important collagen peptidase was identified in extracellular fraction that could play critical role in tissue destruction in periodontitis (Aruni et al. 2012). In vitro studies with epithelial cells show that *F. alocis* can adhere and invade in monoculture and this attribute is enhanced in presence of *P. gingivalis* (Aruni et al. 2011; Moffatt et al. 2011). *F. alocis* induces secretion of proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α from epithelial cells and promotes apoptosis by suppression of MEK1/2 and activation of caspase 3 (Moffatt et al. 2011). Thus, *F. alocis* has a wide array of potential virulence factors to survive in the hostile periodontitis environment.

### *Peptoanaerobacter stomatis*

*P. stomatis* is a newly recognized perio-pathogen cultured from gingival plaques and is the first cultivable member of human oral taxon 081 (Sizova et al. 2012). It is classified as a novel genus and species within the family of *Peptostreptococcaceae* (Sizova et al. 2015). *P. stomatis* is a gram-positive, motile peritrichous rod often occurring in chains (Sizova et al. 2015). This organism is a strict anaerobe, non-spore forming, with a diameter of 0.5 to 0.7 μm and a length of 1.0 to 2.3 μm (Sizova et al. 2015). *P. stomatis* is present in high numbers in periodontal patients' oral biofilms (Kumar et al. 2005; Murphy and Frick 2013) and also reported in dentoalveolar abscesses and endodontic infections (Downes and Wade 2006). Characterization of the pathogenic potential of *P. stomatis* is in its infancy; future in vitro and in vivo studies will determine what role this organism plays in the dysbiotic microbial community present in periodontitis.

## The Veil Disappeared and Revealed a Different Battlefield: Neutrophils coping with Emerging Oral Pathogens

### The Neutrophil

If the integrity of the host is compromised, the first immune cell to respond and be recruited in vast numbers to the site of infection is the neutrophil (Ryder 2010). As primary effector cells of the innate immune system, neutrophils possess numerous strategies to locate, detain and kill microbes (Amulic et al. 2012; Kolaczkowska and Kubes 2013). Neutrophils have multiple killing mechanisms to eliminate both intracellular as well as extracellular microorganisms (Kolaczkowska and Kubes 2013; Nauseef 2007). In the context of oral mucosal immunity, neutrophils are found in large numbers in the gingival crevice and epithelium, entitling them as the major effector cell of the periodontium. The acute and regulated innate immune response, driven by neutrophils, is of critical importance to the maintenance of periodontal health in the host. Dysregulated recruitment of neutrophils to the gingiva, on the other hand, is associated with disease progression (Hajishengallis et al. 2016). In the inflamed periodontal tissue, chemotactic factors such as IL-8, as well as formylated bacterial-derived peptides such as fMLF, will be abundant and guide neutrophils from the blood vessels through the gingival tissue toward the periodontal pocket (Uriarte et al. 2016). Upon encounter with the offending microbe, the phagocytic process is initiated followed by high oxygen consumption through a process known as the respiratory burst with the generation of reactive oxygen species (ROS) within the bacteria-containing phagosome (Nauseef 2007). Activation of the NADPH

oxidase, a multicomponent enzyme, is responsible for ROS production (Babior et al. 2002; Belambri et al. 2018). Neutrophils have the capacity to tailor their response depending on the type of stimuli they encounter. Stimulation of neutrophils by a soluble stimuli, such as fMLF, triggers assembly and activation of the NADPH oxidase at the plasma membrane and release of superoxide anions towards the extracellular space. In contrast, if neutrophils encounter a particulate stimuli, for example a bacterium, assembly and activation of the NADPH oxidase will take place at the membrane of the bacteria-containing phagosome with release of superoxide anions inside the phagosome (Nauseef 2007, 2014; Babior et al. 2002).

As a member of the granulocyte family of leukocytes, neutrophils contain four different types of granules, which are differentiated based on their density, protein content, and on their functional response (Borregaard and Cowland 1997; Borregaard 2010). The different neutrophil granule subtypes can either be recruited to the bacteria-containing phagosome or stimulated to undergo exocytosis and release their matrix content to the extracellular environment (Borregaard et al. 2007). The hierarchical mobilization of neutrophil granules is driven by the strength of the stimuli required for its exocytosis. The weak stimuli induces mobilization of secretory vesicles, and increasing stronger stimulation is required to mobilize gelatinase, specific and azurophil granules, respectively (Nauseef and Borregaard 2014). The diverse repertoire of proteins and receptors present at the membrane of each granule subtype as well as within the granule lumen highlights the important role each granule plays in the different neutrophil responses during inflammation (Uriarte et al. 2008; Lominadze et al. 2005; Rørvig et al. 2013). The antimicrobial efficacy of neutrophils on intracellular as well as extracellular microorganisms is enhanced by the ability to combine both oxygen-dependent and independent mechanisms.

In 2004, Brinkmann et al. described a novel mechanism deployed by neutrophils to trap and kill microbes—neutrophil extracellular traps (NETs)—in which neutrophils extrude decondensed chromatin decorated with neutrophil granule proteins (Brinkmann et al. 2004). NETs can trap bacteria, due to electrostatic charge interactions, and have microbicidal effects due to the high concentration of localized antimicrobial peptides (Parker et al. 2012; White et al. 2016; Yipp et al. 2012; Fuchs et al. 2007; Brinkmann and Zychlinsky 2012). Depending on the stimuli that neutrophils encounter, production of intracellular ROS may or may not be required for NET formation (Parker et al. 2012; Fuchs et al. 2007; Vitkov et al. 2009; Remijsen et al. 2011; Pilsczek et al. 2010). Whether NET formation has beneficial or detrimental effects to the host in the presence of infection remains controversial (Brinkmann and Zychlinsky 2012; Barrientos et al. 2013; Cheng and Palaniyar 2013; Simon et al. 2013). In the context of periodontitis, both excessive and ineffective NET formation has been associated with disease progression (Cooper et al. 2013). Degradation of DNA, by the production of DNases, is a successful strategy utilized by microbial pathogens to escape trapping and killing by NETs (Vitkov et al. 2009; Buchanan et al. 2006; Beiter et al. 2006; Sumby et al. 2005; Porschen and Sonntag 1974; Rudek and Haque 1976; Dahlen et al. 1983). However, crevicular exudate outflow may inhibit optimal functioning of the bacterial DNases, and work in concert with NETs to clear pathogens from the oral cavity and prevent development of periodontitis (Vitkov et al. 2009). For a deeper understanding of neutrophil antimicrobial functions, the reader is referred to these review articles (Amulic et al. 2012; Nauseef 2007; Belambri et al. 2018; Borregaard 2010; Ley et al. 2018).

The traditional view of neutrophils as “pathogen busters” of innate immunity has changed over the past 20 years. Neutrophils role as regulators of the inflammatory and immune responses is well established by their capacity to transcribe, perform de novo synthesis, and release different cytokines and chemokines (Cassatella 1999; Tamassia et al. 2018; Tecchio and Cassatella 2016), as well as by exocytosis of granule proteins, primarily stored in azurophilic granules, with chemotactic activity toward neutrophils as well as other leuko-

cytes (Chertov et al. 2000). Neutrophils will produce and release an array of different cytokines and chemokines depending on the type of stimulation they encounter. The contribution of neutrophil-derived cytokines and chemokines, which are released at the local inflamed site, is significant in the amplification loop of the local immune response. For example, the release of CCL2 and CCL20 from neutrophils stimulated with IFN-γ and LPS promotes chemotaxis of Th17 cells in vitro (Pelletier et al. 2010). We can extrapolate those in vitro findings and speculate that infiltration of Th17 cells into the gingival tissue could be mediated, in part, by the chemokines locally produced by neutrophils exposed to the dysbiotic microbial environment. The ability of neutrophils to engage into cross talk relationships with other leukocytes highlights the primordial role that this professional phagocyte plays in modulation of the immune response.

Another example of pathogen manipulation of neutrophil effector functions during the inflammatory response is the activation of the triggering receptor expressed on myeloid cells 1 (TREM-1). Activation of TREM-1 by periodontal pathogens with concomitant increase levels of the soluble form sTREM-1 has been associated with enhanced cytokine production and periodontitis severity (Bostanci et al. 2013a). *P. gingivalis*, through its Lys and Arg-gingipains, induces activation of TREM-1 in human neutrophils and regulates both the release of sTREM-1 as well as its degradation according to the bacterium needs during the different stages of periodontitis progression (Bostanci et al. 2013b). More, in vivo studies are needed to gain better insights into the neutrophil-centered regulatory role during chronic inflammation to develop more efficacious therapies to combat chronic inflammatory diseases such as periodontitis. To deepen the knowledge of neutrophils as effector cells of the immune response, reading the following articles by Cassatella's group is recommended (Cassatella 1999; Tecchio and Cassatella 2016; Scapini and Cassatella 2014).

## Neutrophils Coping with Two Emerging Gram-Positive Oral Pathogens

In chronic inflammatory infectious diseases, such as periodontitis, pathogenic microorganisms must subvert the immune response to survive in the human host. This outcome leads to disease due to the ability of the pathogenic bacteria to alter the normal neutrophil turnover by triggering neutrophil lysis, NETosis, or delaying neutrophil apoptosis after phagocytosis. As a result of this host–pathogen interaction, the tissue-damaging molecules released by neutrophils contribute to the generation of tissue breakdown products, which supports the nutritional needs of the asaccharolytic oral pathogens. The high number of *F. alocis* and *P. stomatis* found in subgingival pockets of periodontitis disease patients will suggest that these two organisms can subvert neutrophil antimicrobial mechanisms and successfully grow under inflammatory conditions.

Using the subcutaneous chamber model, *F. alocis* infection triggered a quick recruitment of neutrophils to the site of infection, the production of proinflammatory cytokines, and infected distal sites causing acute kidney damage primarily to the tubular epithelial cells (Wang et al. 2014). This in vivo study begins to point out the pathogenic profile of *F. alocis* and the ability of this organism to colonize other organs. In support of our initial observations, a recent study identified high content of *F. alocis* on the pleural fluid of a 65-year-old male patient hospitalized for thoracic empyema (Gray and Vidwans 2019). This case report study is the first one to show *F. alocis* presence outside the oral cavity and its association to another disease other than periodontitis. Our earlier report in mice and this recent case report in humans reveal that *F. alocis* can colonize distal organs outside the oral cavity.

In 2012, the pioneer study of Aruni et al. (Aruni et al. 2012) characterize the proteome map of *F. alocis* and described the presence of several putative virulence factors such as proteases, neutrophil-activating factor, and collagenases. In the diseased periodontal pocket, high

concentrations of complement proteins and its derivative peptides are found (Hajishengallis et al. 2019). Established periodontal pathogens evolved different complement evasion strategies to survive in the periodontal pocket (reviewed in (Hajishengallis and Lambris 2012, 2016). It has been described that *F. alocis* activates all the complement pathways and that C3b deposition is induced primarily by the alternative pathway and enhanced when the bacterium is grown in the planktonic form compared to biofilm growth (Jusko et al. 2016). Interestingly, *F. alocis* has a cytoplasmic enzyme, FACIN, that once present on the bacterial surface can inhibit activation of complement component 3 (C3) convertases (Jusko et al. 2016). FACIN also functions as an important enzyme involved in key metabolic pathways for bacterial growth, which will limit the possibility to generate an isogenic mutant. However, there is a need to perform genetic manipulation of *F. alocis* to generate isogenic mutants, since it will enable to gather better insight of the role that different putative virulence factors might play in microbial interaction and host response.

Manipulation of the host innate immune response allows periodontal pathogens, such as *P. gingivalis*, to drive in the periodontal pocket. *P. gingivalis* evades neutrophil antimicrobial killing by promoting a cross talk between TLR2/1 and C5a receptor that dismantles the antimicrobial signaling pathway while promotes the production of inflammatory mediators (Maekawa et al. 2014). The detection of high numbers of *F. alocis* in disease sites is an indication that this organism is able to subvert innate immunity. The initial characterization, from our laboratory, about how *F. alocis* interacts with neutrophils reveals that this putative oral pathogen can modulate neutrophil effector functions. *F. alocis* is recognized by TLR2 and triggers exocytosis of three of the four neutrophil granule subtypes through activation of both p38 MAPK and ERK1/2 (Armstrong et al. 2016). In addition, neutrophils that were challenged with either live or heat-killed *F. alocis* showed enhanced random and directed migration toward IL-8, an effect that was dependent on the bacteria-induced granule exocytosis (Armstrong et al. 2016). Human neutrophils efficiently internalized *F. alocis,* but the organism manages to subvert the antimicrobial response by inducing a minimal respiratory burst response and preventing phagosome maturation (Edmisson et al. 2018). In co-infection studies with either *Staphylococcus aureus* or *P. stomatis*, only the minimal ROS production will be localized in the *F. alocis*-containing phagosomes (Edmisson et al. 2018). Furthermore, only live *F. alocis* is able to inhibit granule trafficking to the phagosome to prevent phagosome maturation, which allows *F. alocis* to survive in neutrophils. *F. alocis,* independent of bacterial dose or time, does not induce NET formation, but pre-treatment of neutrophils with *F. alocis* significantly inhibits PMA-induced NETs (Armstrong et al. 2018). We recently reported that *F. alocis* signals primarily by TLR2/6 heterodimers and promotes release of neutrophil-derived cytokines and chemokines but to a lesser extend compared to *P. stomatis* (Vashishta et al. 2019). Figure 1a shows *F. alocis* ability to modulate neutrophil effector functions to promote bacterial survival.

In stark contrast, *P. stomatis* interaction with neutrophils results in hyperactivation of the professional phagocyte (Fig. 1b). Contrary to the response seen with *F. alocis,* when suspension neutrophils were exposed to *P. stomatis,* they display low phagocytic capacity toward this emerging oral pathogen. However, the low percent of *P. stomatis* that was engulfed by neutrophils was killed primarily by oxygen-independent mechanisms (Jimenez Flores et al. 2017). *P. stomatis* interaction with neutrophils results is both a strong respiratory burst response and significant exocytosis of the four neutrophil granule subtypes. In addition, *P. stomatis* is a strong priming agent of neutrophil respiratory burst response. Pre-treatment of neutrophils with *P. stomatis* significantly enhances fMLF-induced respiratory burst response similar to levels achieved by TNFα—a well-established neutrophil priming agent (Jimenez Flores et al. 2017). The ability of *P. stomatis* to prime neutrophils and to promote release of granule content will contribute to tissue breakdown and chronic inflammation, both features associated with periodontitis. Furthermore,

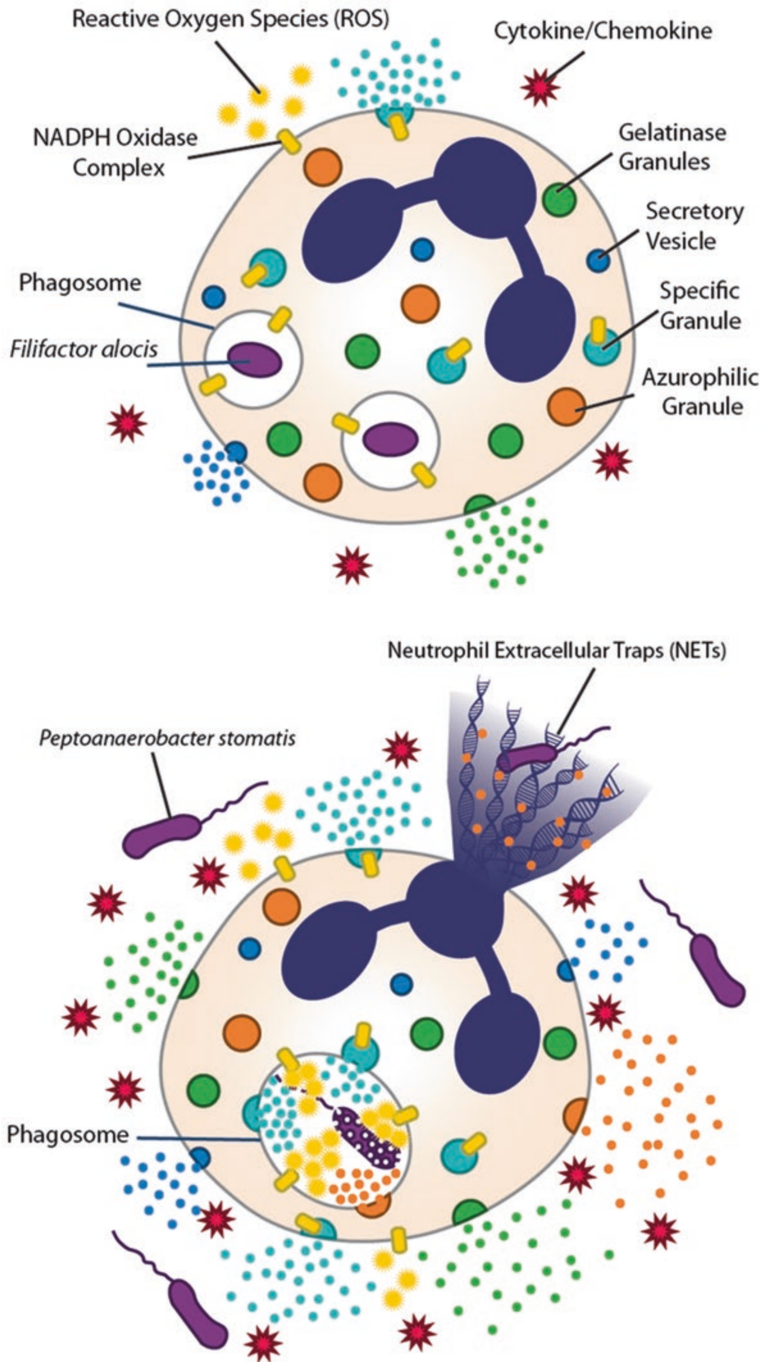


**Fig. 1** Neutrophil interaction with *F. alocis* and *P. stomatis*. (**a**) Human neutrophils can efficiently phagocytize *F. alocis*. However, the oral pathogen is able to persist within the neutrophils by inducing minimal production of intracellular reactive oxygen species (ROS) and minimizing the fusion of antimicrobial granules with the bacteria-containing phagosome. Simultaneously, *F. alocis* stimulates a proinflammatory response from neutrophils by causing the moderate exocytosis of granules, release of proinflammatory cytokines, and small production of extracellular ROS. (**b**) Contrastingly, *P. stomatis* prevents phagocytosis by human neutrophils because once internalized is promptly eliminated. At the *P. stomatis* phagosome, there is robust generation of ROS and efficient fusion of the microbicidal specific and azurophilic granules to the phagosome. Despite its proficient killing, *P. stomatis* induces an extremely proinflammatory response form neutrophils. Upon *P. stomatis* challenge, neutrophils show an exaggerated exocytosis of all four granule subtypes, robust release of cytokines and chemokines, and induced the formation of neutrophil extracellular traps (NETs). Additionally, *P. stomatis* robustly primes neutrophils to produce an even greater extracellular ROS production in response to secondary stimulation

*P. stomatis* has the ability to promote NET formation, albeit to a lesser extent compared to *S. gordonii* (Armstrong et al. 2018). We recently reported that both *F. alocis* and *P. stomatis* trigger TLR2/6 signaling pathways, but the modulation of neutrophil-derived cytokines and chemokines by the two organisms is very different. Only *P. stomatis* challenge of human neutrophils, but not *F. alocis* or *P. gingivalis*, induced the release of active neutrophil-derived chemokines to promote both neutrophil and monocyte migration (Vashishta et al. 2019).

Based on the current knowledge about the interactions between neutrophils and the two emerging oral pathogens, *F. alocis* and *P. stomatis*, a model of the battlefield in the periodontal pocket is depicted in Fig. 2. Periodontal pathogens use different strategies to subvert and evade neutrophils killing while supporting inflammation. In the case of the emerging oral pathogens, *P. stomatis* and *F. alocis,* we see the story of the tortoise and the hare at play. When neutrophils encounter *P. stomatis*, they directly cause intense inflammation through the exocytosis of all four of their granules and production of ROS and NETs. *P. stomatis* also incites inflammation through the robust release of neutrophil-derived cytokines and chemokines. The chemokines will recruit more neutrophils and monocytes to the tissue, which can add to the inflammatory response. On the other hand, the cytokines and other inflammatory products like dead-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) will prime naïve neutrophils and heighten their response to any secondary stimulus, which will further magnify the inflammatory response. Ultimately, the hyperactive neutrophil response to the bacterial challenge results in the death of both *P. stomatis* and the neutrophil. Contrastingly, neutrophils' encounter with *F. alocis* is more moderate. Direct tissue damage still takes place through the *F. alocis*-induced exocytosis of three out of the 4 neutrophil granules with minimal ROS production, but since neutrophils are activated to a lesser degree, fewer cytokines and chemokines are released. In comparison to *P. stomatis, F. alocis* primes neutrophils to a lesser degree and does not recruit as many immune cells. The key to *F. alocis* pathogenesis is that it can remain viable within neutrophils and prolongs their lifespan (*I. Miralda, A. Vashishta, C. K. Klaes, R. J. Lamont, S. M. Uriarte, unpublished observations*). This outcome results in a delay in neutrophil clearance by macrophages, through efferocytosis, and inhibition of the resolution of inflammation.

## Conclusion and Future Directions

High number of activated neutrophils are recruited to the gingival tissue but fail to clear the infection and instead contribute to sustain a dysregulated inflammation that provides a favorable environment for "inflammo-*philic*" pathogens to outgrow and promote immunopathology (Hajishengallis 2014). Significant advances have been made regarding the characterization of the complex microbial composition both in healthy as well as in disease sites. In the past decade, the metagenomics studies applied to subgingival plaque derived from periodontitis disease sites revealed the complex microbial composition. Furthermore, metatranscriptomic studies shed light into the complex functional interactions that occur within the periodontitis microenvironment. All these "omic" studies opened the door to a new field of the "yet-to-be cultivated" taxons, with high abundance in disease sites compared to healthy sites. The challenge now is to define what role these emerging oral pathogens play in disease progression and how they interact with the host immune response. What are the bacterial relationships between the established periodontal pathogens and the uncultivable or emerging oral bacteria? Do some of the emerging oral bacteria play a bystander role while others could be key players in promoting the shift in the bacterial composition? What are the potential virulence characteristics of these emerging oral organisms; have they evolved to evade or manipulate the host immune response? These are some of the open questions that will require investigation to advance our knowledge and understanding of this complex multifactorial chronic inflammatory disease.

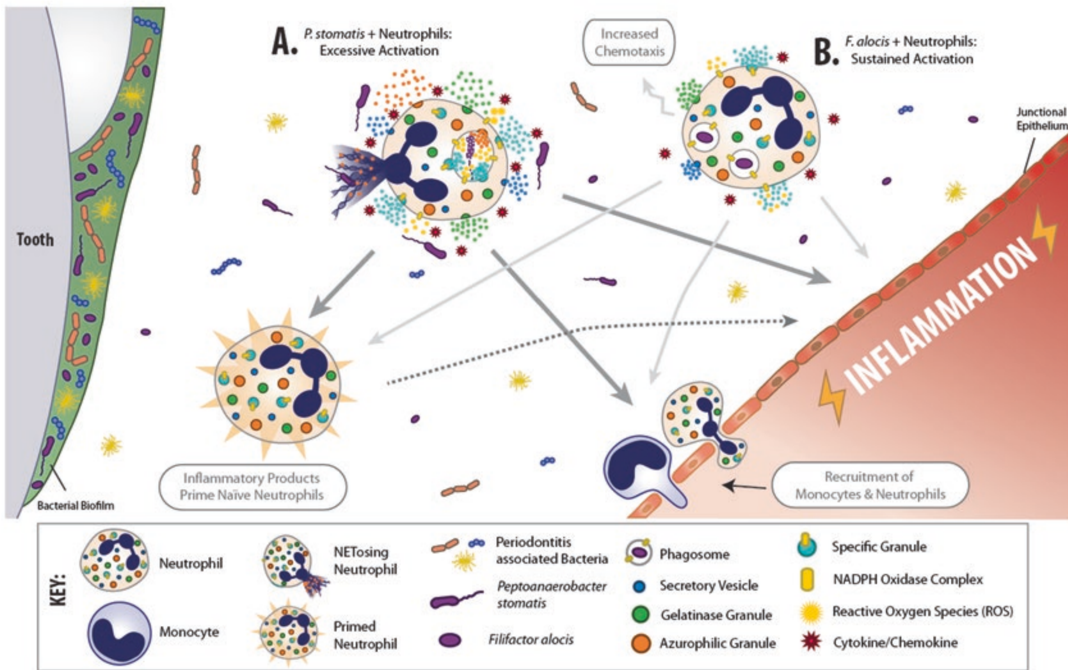


**Fig. 2** The war zone of the gingival pocket: While neutrophils are essential to maintain homeostasis in the oral cavity, they can also drive inflammation and tissue destruction. Periodontal pathogens have different modus operandi against neutrophils to promote inflammation and contribute to the disease progression. (**a**) When neutrophils encounter *P. stomatis*, they directly cause intense inflammation through the exocytosis of all four of their granules and production of ROS and NETs. Furthermore, *P. stomatis* promotes robust release of neutrophil-derived cytokines and chemokines, which will recruit more neutrophils and monocytes to the tissue, inciting inflammation. Ultimately, the intense response provoked by the encounter results in the death of both *P. stomatis* and the neutrophil. (**b**) In stark contrast, neutrophils' encounter with *F. alocis* is more moderate. Direct tissue breakdown damage still takes place through the exocytosis of three out of the 4 neutrophil granule subtypes with minimal ROS production, but since neutrophils are activated to a lesser degree, fewer cytokines and chemokines are released. However, *F. alocis* increases the migration of neutrophils that have phagocytized the organism. The key to *F. alocis* pathogenesis is that the organism has developed strategies to subvert killing and remains viable within neutrophils. The effects of *P. stomatis*, *F. alocis*, and primed neutrophils are delineated with the bold dark gray arrows, the thin light gray arrows, and the dotted arrow, respectively

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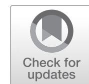


# Biologically Defined or Biologically Informed Traits Are More Heritable Than Clinically Defined Ones: The Case of Oral and Dental Phenotypes

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## Introduction

Most people worldwide are affected by common oral diseases and impairments—periodontitis, dental caries, and tooth loss persist as clinical and public health problems (Frencken et al. 2017). Traditional oral health promotion approaches, relying on individual behavior change and dental office-based modalities, have been moderately successful in preventing the onset of oral disease and have failed at reducing health disparities (Kay and Locker 1996; Watt 2007). Addressing risk factors that are shared with other health conditions, such as smoking and cardiometabolic diseases, is a logical and efficient approach to improve oral health in large segments of the population (Sheiham and Watt 2000). However, population-level risk factors and public health approaches (i.e., focusing on social determinants of health), while optimal on average, may not be suitable for guiding the care of individuals (Rockhill 2001; Rose 2001). The advent of precision health care is expected to ameliorate this issue by accelerating the development and application of optimal care according to individuals' susceptibilities, lifestyles, and environment (Collins and Varmus 2015).

The surge of scientific, biological, and technological advances during the last two decades has created promise and expectation that better preventive and therapeutic solutions are possible in dentistry. The development of precision health is based upon the premise that the current classification of people into diagnostic, susceptibility,

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and treatment taxonomies can be improved (Divaris 2017). Traditional symptom-based and clinically defined categories are certainly foundational and valuable in health care—most diagnostic classification systems, therapies, and outcome assessment are based on them. Nevertheless, these classifications do typically fail to measure the disease process and rather enumerate its clinically manifest results. On the other hand, refined health and disease traits that are informed by the underlying biology are better reflections of the disease process and thus more likely to be “precise” (Chen and Snyder 2013). Examples of biologically informed research strategies have emerged in several domains of health care including, among others, cardiovascular disease (Leopold and Loscalzo 2018), neurology and psychiatry (Gibbs et al. 2018), and asthma care (Chung and Adcock 2019). Clinical or biological, phenotypic homogeneity is desirable for the development of optimal diagnostic, risk assessment, and disease management applications and is likely better suited for interrogations of molecular context of health and disease (Robinson 2012; Divaris 2019a).

Above and beyond the necessary development of better disease taxonomies, comprehensive knowledge of individual susceptibility and factors that influence it are key elements for precision health. Environmental influences and genomics are considered as major determinants of disease susceptibility (Belsky et al. 2009; Rutter 2007). In the oral health domain, the genomics evidence base is growing but remains insufficient to inform care for the common, non-syndromic forms of periodontitis and dental caries (Divaris 2019b). This is in contrast with early and consistent observations that considerable variation in oral disease is attributable to heritable factors (Morelli et al. 2019). The genetic basis of oral health has long been theorized but little evidence exists on specific genetic factors underlying common oral and dental disease traits. For example, most reports of the heritability of periodontitis indicate that up to a third of the population variance of periodontitis is due to genetic factors, with more severe disease forms being more strongly genetically controlled (Nibali et al. 2019). Despite this evident heritability, the number of consensus genes linked to periodontitis is less than a handful (Morelli et al. 2019; Shungin et al. 2019).

The advent of high-throughput genotyping and genome-wide association studies (GWAS) have helped the generation of massive phenotype–genotype association data (Buniello et al. 2019). This genomic information can be used to efficiently screen various phenotypes for “heritable content” (Zaitlen and Kraft 2012; Manolio et al. 2009) and aid inferences regarding disease subtypes that may be more genetically driven than others. In this paper, we report the results of a systematic examination of the heritable variance in 30 oral and dental traits explained by high-density genotypes generated in a GWAS among over 4000 middle-age European American adults. The premise of this investigation is that oral and dental traits with high heritability are more likely to be biologically (versus environmentally) driven and may be optimal candidates for formal interrogations of genomic context and precision health applications.

## Methods

The individuals used for all estimations of heritability were Americans of European descent, ages 53–74 years, and participants of the Atherosclerosis Risk in Communities (ARIC) study (The ARIC Investigators 1989). This prospective cohort study was designed to examine cardiovascular disease risk using individuals living in four communities: Forsyth County, North Carolina; Jackson, Mississippi; Minneapolis, Minnesota; and Washington County, Maryland. An ancillary dental study (dental ARIC) in which a comprehensive oral, periodontal, and dental exam [including enumeration of missing teeth, probing pocket depth (PD), gingival recession, clinical attachment level (CAL), etc.] was performed between 1996 and 1998 during the fourth visit of the ARIC study (Beck et al. 2001). The visit also included an examination for dental caries experience and the collection of gingival crevicular fluid (GCF), as well as subgingival

microbial plaque samples in a subset of participants. DNA was extracted from blood samples collected during the study visit, and genotyping was subsequently performed using the Genome-Wide Human SNP array 6.0 by Affymetrix containing 906,600 genetic markers (single nucleotide polymorphisms, SNPs). Additional SNPs were imputed using HapMap Phase II CEU build 36. Quality control procedures and criteria for all genotyping and imputation steps, analytical approaches including specific methods and software pipelines for the GWAS, have been reported previously (Divaris et al. 2012, 2013; Agler et al. 2019).

Data collected via the clinical information, a follow-up telephone survey, and the biospecimens from the dental component of ARIC have been used to generate several oral/dental phenotypes. For the purposes of this study, we examined the following trait domains: number of remaining natural teeth at the baseline examination, incident tooth loss [>2 teeth over a 10-year period, assessed via a telephone survey; (Naorungroj et al. 2017)]; dental caries [DMFS index; (Wang et al. 2012) and tooth morbidity, $DM_TFS$ index (Morelli et al. 2019; Shungin et al. 2019)]; periodontitis [defined using the CDC/AAP chronic periodontitis definition (Page and Eke 2007); the 2017 world workshop classification (WW17; Papapanou et al. 2018); extent probing depth and attachment loss scores (Carlos et al. 1986); mean interproximal attachment loss (Sanders et al. 2017)]; periodontal profile classes (PPC; Morelli et al. 2017, 2018); subgingival pathogen colonization (Divaris et al. 2012); gingival crevicular fluid (GCF) interleukin (IL)-1β expression (Offenbacher et al. 2018); and periodontal complex traits (PCT) (Offenbacher et al. 2016). The PPC classification (Morelli et al. 2017) entails an empirically derived 7-category taxonomy that accounts for intraoral patterns of clinical parameters and tooth loss, whereas the 6 PCTs (Offenbacher et al. 2016) are essentially vectors of "biological variance" [i.e., subgingival colonization patterns and host inflammatory response (GCF IL-1β), combined with clinical parameters] in periodontitis. Of note, WW17 Stages 3 and 4, both having interdental CAL ≥ 5 mm, were differentiated in the ARIC dataset based on the existence of tooth loss due to periodontitis (≥5 teeth) or < 20 remaining teeth (i.e., 10 opposing pairs).

The heritable variance ($h^2$) in these traits that could be attributable to the human genome was estimated using Visscher's Genome-wide Complex Trait Analysis (GCTA) (Yang et al. 2011, 2013). The GCTA approach allows the estimation of heritability using the common SNPs available on most genotyping arrays and unrelated individuals (the same data used to perform most GWAS). This is done by, first, estimation of a genetic relationship matrix (GRM) using all genotyped individuals. In a second step, the GRM is carried forward to restricted maximum likelihood (REML) analysis to obtain the proportion of trait variance explained by all the SNPs. The estimated variance explained may be influenced by the unknown linkage disequilibrium (LD) structure of all GWAS SNPs with causal variants—which is generally unknown or unobservable. Therefore, we used two different sets of SNPs for the computation of the GRM: all genotyped SNPs ($n$ = 656,292; considered to be the highest quality SNP set) and all genotyped and imputed SNPs with imputation quality score ($R^2$) ≥ 0.6 ($n$ = 2,104,905; considered to be the most inclusive SNP set). SNPs with minor allele frequency (MAF) <5% were excluded from the creation of both GRMs. We computed and report $h^2$ and standard errors (se) for both SNP sets, as well as using crude [i.e., considering only ancestry, adjusted using 10 population stratification principal components (PC; Price et al. 2006)] and adjusted genetic models [i.e., including terms for 10 PCs, as well as age (in years) and sex (binary)].

## Results

Heritability estimates for the "traditional," clinically defined oral and dental traits are presented in Table 1. Considering the high-quality set of SNPs and adjusted models, severe chronic periodontitis was the periodontal trait with the highest $h^2$ (0.22), followed by extent probing depth ≥ 4 mm and mean interproximal attach-

**Table 1** Phenotypic variance explained for "traditional," clinically defined oral and dental traits by all genotyped and imputed autosomal SNPs available among the European American participants of the Dental Atherosclerosis Risk In Communities study ($n = 4504$)

| | Genotyped SNPs | Imputed[a] SNPs |
|---|---|---|
| Exclusion filters: | MAF < 0.05 | MAF < 0.05, $R^{2\,b} < 0.6$ |
| *n* (SNPs)[c]: | 656,292 | 2,104,905 |
| | Variance explained (se) | Variance explained (se) |
| **Number of remaining teeth** | | |
| + 10 PCs for population structure | 0.115 (0.07) | 0.065 (0.06) |
| + 10 PCs, sex, age | 0.114 (0.07) | 0.064 (0.06) |
| **Incident tooth loss**[d] | | |
| + 10 PCs for population structure | 0.064 (0.11) | 0.006 (0.09) |
| + 10 PCs, sex, age | 0.055 (0.11) | 0.001 (0.10) |
| **DMFS** (decayed, missing, filled tooth surfaces due to caries) | | |
| + 10 PCs for population structure | 0.126 (0.08) | 0.057 (0.06) |
| + 10 PCs, sex, age | 0.131 (0.08) | 0.066 (0.06) |
| **Tooth morbidity** (DMFS including teeth lost due to all causes or $DM_TFS$) | | |
| + 10 PCs for population structure | 0.256 (0.07) | 0.184 (0.06) |
| + 10 PCs, sex, age | 0.242 (0.07) | 0.171 (0.06) |
| **Any periodontitis**[e] (moderate/severe versus health/mild) | | |
| + 10 PCs for population structure | 0.012 (0.07) | 0.039 (0.06) |
| + 10 PCs, sex, age | 0.018 (0.08) | 0.049 (0.06) |
| **Moderate periodontitis**[e] (versus health/mild) | | |
| + 10 PCs for population structure | 0.006 (0.14) | 0.068 (0.12) |
| + 10 PCs, sex, age | 0.000 (0.14) | 0.066 (0.12) |
| **Severe periodontitis**[e] (versus health/mild) | | |
| + 10 PCs for population structure | 0.175 (0.19) | 0.083 (0.16) |
| + 10 PCs, sex, age | 0.220 (0.19) | 0.127 (0.16) |
| **Mean interproximal attachment loss** | | |
| + 10 PCs for population structure | 0.161 (0.07) | 0.122 (0.06) |
| + 10 PCs, sex, age | 0.152 (0.08) | 0.106 (0.06) |
| **Extent of attachment loss ≥ 3 mm** (proportion of sites) | | |
| + 10 PCs for population structure | 0.113 (0.08) | 0.073 (0.06) |
| + 10 PCs, sex, age | 0.075 (0.08) | 0.037 (0.06) |
| **Extent of attachment loss ≥ 4 mm** (proportion of sites) | | |
| + 10 PCs for population structure | 0.092 (0.07) | 0.053 (0.06) |
| + 10 PCs, sex, age | 0.076 (0.07) | 0.035 (0.06) |
| **Extent of probing depth ≥ 4 mm** (proportion of sites) | | |
| + 10 PCs for population structure | 0.167 (0.07) | 0.143 (0.06) |
| + 10 PCs, sex, age | 0.190 (0.07) | 0.150 (0.06) |

*MAF* minor allele frequency, *se* standard error, *PCs* principal components
[a]Imputed using HapMap II-CEU
[b]Imputation quality score
[c]Number of SNPs that were used to estimate the genetic relationship matrix after exclusions, among the study participants as a first step in the GCTA prior to conducting REML
[d]Incident tooth loss was derived from a follow-up telephone survey in a subset of participants ($n = 2771$) and defined as >2 teeth lost during the last decade
[e]Defined according to the CDC/AAP chronic periodontitis taxonomy (Page and Eke 2007)

ment loss (0.19 and 0.15, respectively). The tooth morbidity index (i.e., $DM_TFS$ is the traditional DMFS with the addition of teeth/surfaces lost due to all causes) showed almost double the heritability of the dental caries experience DMFS index (0.24 versus 0.13). Of note, comparison of crude versus adjusted (i.e., including age and sex) model did not reveal any substantial differences, whereas directly genotyped SNP sets generally resulted in higher $h^2$ estimates compared to those obtained by the larger set including both genotyped and imputed SNPs.

Results of heritability estimation for PPC and WW17 derived contrasts, representing two "contemporary" clinical classification systems, are presented in Table 2. With regard to the WW17 classification, our sample included a very small number of participants classified as Stage 1; therefore, we treated Stages 1 and 2 as one group and contrasted it with combinations of participants in Stages 3 and 4. Stage 4 versus Stages 1 and 2 was the contrast producing the highest $h^2$ estimate, essentially double than what was obtained for Stage 3 (0.15 versus 0.08). When considering the PPC classification, we examined 7 contrasts, i.e., various disease categories versus health. The "high gingival index" group characterized by extensive gingival inflammation (PPC-C, as presented in Morelli et al. 2017), produced by far the highest heritability: $h^2 = 0.52$ (se = 0.19), and followed by "severe periodontal disease" [PPC-G; $h^2 = 0.26$ (se = 0.19)]. A joint contrast of the "severe periodontal disease" and the "severe tooth loss" (characterized by an average of 6–8 remaining teeth with disease, PPC-F) groups compared to health resulted in an $h^2 = 0.26$ (se = 0.15).

The estimates of phenotypic variance explained in the biologically defined (i.e., subgingival pathogen colonization and GCF IL-1β expression) and biologically informed (i.e., PCTs) are presented in Table 3. Both microbial traits showed high heritability, with the red complex demonstrating $h^2 = 0.53$ (se = 0.31) and the orange complex demonstrating $h^2 = 0.46$ (se = 0.32). High (top quartile) GCF IL-1β showed only modest heritability of 0.16. With regard to PCTs, the heritability of 2 complex traits [PCT-1 (explaining 46% of the variance, representing a uniform positive loading with all periodontal pathogens and borderline elevated GCF-IL1β) and PCT-4 (a mixed bacterial community without *A. actinomycetemcomitans* with moderately positive loading of GCF-IL1β)] was estimated as near zero. In contrast, PCT-3 (the "*A. actinomycetemcomitans* trait" according to Offenbacher et al. 2016) showed the highest heritability estimate in our analysis, $h^2 = 0.72$ (se = 0.32), followed by PCT-6 (i.e., the "smoking/diabetes trait"), $h^2 = 0.40$ (se = 0.35).

## Discussion

In this paper, we present the results of a comprehensive exploration of the heritability of 30 oral and dental traits, as approximated by phenotypic variance explained by two large sets of GWAS SNPs, among a sizeable sample of middle-aged European Americans. Our results indicate substantially higher variance explained by the genome for biologically defined (i.e., biomarker levels) and biologically informed traits (i.e., combinations of biomarker and clinical data) compared to clinically defined ones. Importantly, traits representing high levels of inflammation and severe disease (i.e., severe periodontitis, severe tooth loss, and high "red complex colonization") were the ones encompassing the most genetically explainable (i.e., heritable) variance. These results support our thesis regarding the suitability of homogeneous and biologically informed oral and dental traits for genetic investigations and, downstream, precision health applications.

A plausible interpretation of our findings revolves around the notion of "endophenotypes" (Ghiassian et al. 2016)—biological intermediates of clinical disease (e.g., serum lipids in the context of cardiovascular disease), typically considered as biological vectors underlying a proportion of cases. Because these traits are closer to biological and physiological parameters reflecting the disease process, their genomics interrogation may be both fruitful (i.e., a genetic association signal may be easier to detect versus for a hetero-

**Table 2** Phenotypic variance explained for contemporary classifications of periodontitis by all genotyped and imputed autosomal SNPs available among European American participants of the Dental Atherosclerosis Risk In Communities study

| | | Genotyped SNPs | Imputed[a] SNPs |
|---|---|---|---|
| Exclusion filters: | | MAF < 0.05 | MAF < 0.05, $R^2$ [b] < 0.6 |
| *n* (SNPs)[c]: | *n* | 656,292 | 2,104,905 |
| | | Variance explained (se) | Variance explained (se) |
| **Any PPC[d] (disease) vs. healthy** | 4504 | | |
| + 10 PCs for population structure | | 0.084 (0.07) | 0.064 (0.06) |
| + 10 PCs, sex, age | | 0.073 (0.07) | 0.056 (0.06) |
| **PPC[d]: "High gingival index" vs. healthy** | 1621 | | |
| + 10 PCs for population structure | | 0.479 (0.19) | 0.397 (0.17) |
| + 10 PCs, sex, age | | 0.517 (0.19) | 0.454 (0.17) |
| **PPC[d]: "Posterior disease," "severe disease," "tooth loss," "severe tooth loss" vs. healthy** | 3532 | | |
| + 10 PCs for population structure | | 0.041 (0.09) | 0.046 (0.08) |
| + 10 PCs, sex, age | | 0.038 (0.09) | 0.047 (0.08) |
| **PPC[d]: "Posterior disease," "severe disease," "tooth loss," "severe tooth loss" vs. healthy** | 2705 | | |
| + 10 PCs for population structure | | 0.155 (0.12) | 0.120 (0.10) |
| + 10 PCs, sex, age | | 0.149 (0.12) | 0.126 (0.10) |
| **PPC[d]: "Severe disease" vs. healthy** | 1699 | | |
| + 10 PCs for population structure | | 0.272 (0.19) | 0.158 (0.17) |
| + 10 PCs, sex, age | | 0.257 (0.19) | 0.142 (0.17) |
| **PPC[d]: "Severe disease," "severe tooth loss" vs. healthy** | 2174 | | |
| + 10 PCs for population structure | | 0.245 (0.15) | 0.152 (0.13) |
| + 10 PCs, sex, age | | 0.261 (0.15) | 0.171 (0.13) |
| **PPC[d]: "Tooth loss," "severe tooth loss" vs. healthy** | 2467 | | |
| + 10 PCs for population structure | | 0.103 (0.13) | 0.079 (0.11) |
| + 10 PCs, sex, age | | 0.106 (0.13) | 0.096 (0.11) |
| **Periodontitis WW17[e] stage 3 vs. 1/2** | 3414 | | |
| + 10 PCs for population structure | | 0.062 (0.10) | 0.108 (0.08) |
| + 10 PCs, sex, age | | 0.076 (0.10) | 0.123 (0.08) |
| **Periodontitis WW17[e] stage 3/4 vs. 1/2** | 4504 | | |
| + 10 PCs for population structure | | 0.079 (0.07) | 0.103 (0.06) |
| + 10 PCs, sex, age | | 0.084 (0.07) | 0.116 (0.06) |
| **Periodontitis WW17[e] stage 4 vs. 1/2** | 3047 | | |
| + 10 PCs for population structure | | 0.175 (0.11) | 0.139 (0.09) |
| + 10 PCs, sex, age | | 0.146 (0.11) | 0.125 (0.09) |

*MAF* minor allele frequency, *se* standard error, *PCs* principal components
[a]Imputed using HapMap II-CEU
[b]Imputation quality score
[c]Number of SNPs that were used to estimate the genetic relationship matrix after exclusions, among the study participants as a first step in the GCTA prior to conducting REML
[d]Periodontal profile class (PPC). PPC monikers are introduced and defined in Table 1 of Morelli et al. (2016)
[e]2017 world workshop on the classification of periodontal and peri-implant diseases and conditions (Papapanou et al. 2018)

**Table 3** Phenotypic variance explained for 2 bacterial colonization and 6 biologically informed complex periodontal traits (PCT) by all genotyped and imputed autosomal SNPs available, among European American participants of the Dental Atherosclerosis Risk In Communities study

| | | Genotyped SNPs | Imputed[a] SNPs |
|---|---|---|---|
| Exclusion filters: | | MAF < 0.05 | MAF < 0.05, $R^{2\,b}$ < 0.6 |
| *n* (SNPs)[c]: | *n* | 656,292 | 2,104,905 |
| | | Variance explained (se) | Variance explained (se) |
| **High[d] orange complex colonization** | 978 | | |
| + 10 PCs for population structure | | 0.464 (0.32) | 0.318 (0.30) |
| + 10 PCs, sex, age | | 0.456 (0.32) | 0.310 (0.29) |
| **High[d] red complex colonization** | 986 | | |
| + 10 PCs for population structure | | 0.565 (0.31) | 0.424 (0.29) |
| + 10 PCs, sex, age | | 0.534 (0.31) | 0.405 (0.29) |
| **High[e] GCF IL-1β expression** | 4907 | | |
| + 10 PCs for population structure | | 0.156 (0.08) | 0.155 (0.08) |
| + 10 PCs, sex, age | | 0.155 (0.08) | 0.156 (0.08) |
| **PCT[f]1** | 975 | | |
| + 10 PCs for population structure | | <0.0001 | <0.0001 |
| + 10 PCs, sex, age | | <0.0001 | <0.0001 |
| **PCT[f]2** | 975 | | |
| + 10 PCs for population structure | | 0.18 (0.36) | 0.01 (0.31) |
| + 10 PCs, sex, age | | 0.14 (0.36) | <0.0001 |
| **PCT[f]3** | 975 | | |
| + 10 PCs for population structure | | 0.71 (0.32) | 0.57 (0.30) |
| + 10 PCs, sex, age | | 0.72 (0.32) | 0.57 (0.31) |
| **PCT[f]4** | 975 | | |
| + 10 PCs for population structure | | <0.0001 | <0.0001 |
| + 10 PCs, sex, age | | <0.0001 | <0.0001 |
| **PCT[f]5** | 975 | | |
| + 10 PCs for population structure | | 0.16 (0.35) | 0.15 (0.31) |
| + 10 PCs, sex, age | | 0.18 (0.35) | 0.17 (0.31) |
| **PCT[f]6** | 975 | | |
| + 10 PCs for population structure | | 0.19 (0.36) | 0.19 (0.31) |
| + 10 PCs, sex, age | | 0.40 (0.35) | 0.35 (0.31) |

*MAF* minor allele frequency, *se* standard error, *PCs* principal components
[a]Imputed using HapMap II-CEU
[b]Imputation quality score
[c]Number of SNPs that were used to estimate the genetic relationship matrix after exclusions, among the study participants as a first step in the GCTA prior to conducting REML
[d]Defined as the highest quintile versus the lower 4 quintiles, as quantitated by checkerboard DNA–DNA hybridization (Divaris et al. 2012)
[e]Defined as the top quartile versus the lower 3 quartiles (Offenbacher et al. 2018)
[f]Periodontal complex trait, introduced by Offenbacher et al. (2016)

geneous clinical outcome) and impactful—genetic discoveries for biological disease intermediates may provide clues for "druggable" pathways or other mechanistic interventions (Ramanan and Saykin 2013).

Our finding of the highest heritability for PCT-3, a composite trait including an aggressive periodontal pathogen (*A. actinomycetemcomitans*), the local inflammatory host response (GCF IL-1β), combined with high levels of clinically manifested disease, is unsurprising (Lopez et al. 2015). Similarly, our finding of over 50% heritability for the "high gingival index" PPC trait points to a possibly latent hyper-inflammatory trait that may be expressed above and beyond clinical attachment loss and tooth loss. Our group has recently reported an association between the *ASIC2* locus (Zhang et al. 2016) and severe gingival inflammation. Of note, individuals in this "high gingival index" latent class had the second highest stroke incidence rate among all ARIC study participants—with those in the "severe disease" group having the highest (Sen et al. 2018). This finding is of significance because stroke has an underlying inflammatory component and these two disease subtypes are the "most inflamed"—likely pointing to a shared biological etiologic component.

Our findings of near zero variance explained by the genome for PCT-1 and PCT-4 do not imply that heritability for these traits is exactly zero, as GCTA typically captures the lower bound of heritability; however, we would argue that, based on the obtained $h^2$ estimates, the heritable variance explained by GWAS SNPs for these traits is smaller than what we found for the other PCTs. We must acknowledge that, despite this low estimated heritability, our group has identified and followed-up several genome-wide significant loci for PCT-1, including *IFI16* and *AIM2* (Marchesan et al. 2017), as well as for PCT-4 using gene-centric analyses (Offenbacher et al. 2016; Rhodin et al. 2014). Moreover, one of the complex periodontal traits that showed high heritability (PCT-6; $h^2 = 0.40$) has the interesting feature of being the only PCT significantly associated with sex, age, smoking, and diabetes (Offenbacher et al. 2016), which are known important risk factors for periodontitis.

It is important to acknowledge that there is ongoing dialogue regarding the merits and limitations of the GCTA method (Krishna Kumar et al. 2016; Yang et al. 2016). The method has been used extensively for the interrogation of several traits, producing results that have been validated both empirically and mathematically (Lee and Chow 2014). In general, we consider that GCTA computes that lowest bound of true heritability under common assumptions for common-complex diseases and traits (Lee and Chow 2014), because it typically disregards low-frequency variants, variants' postulated causal or functional roles, as well as any interactions that may be part of the so-called "missing heritability" (Manolio et al. 2009). Of note, in a previous study (Divaris et al. 2013), we found that the inclusion of a genome-wide interaction with smoking (treated as a binary variable; ever/never) in the genetic model increased the explained variance in severe chronic periodontitis to over 50%. It is likely and to some degree expected that different heritability estimates might be obtained in a different population with different age distribution, ancestral background, environmental or behavioral risk factors, disease prevalence, etc. However, in this paper, we consider $h^2$ estimates relative to each other and not in absolute terms.

In sum, our results indicate that genome-wide sets of polymorphisms explain modest levels of the observed variance in clinical oral and dental measures, those traditionally used to define and classify disease. Subgingival bacterial colonization and complex phenotypes that encompass both bacterial colonization and local inflammatory response had the highest heritability, suggesting that these biologically informed traits are promising targets for genomics investigations, because they arguably contain most genetic signal and are more homogeneous than clinically defined disease taxonomies. These features are highly desirable for developing precision health solutions because they enable the re-classification of individuals to more homogeneous health and disease groups with the hopes of optimizing prevention, diagnosis, and therapy (Insel and Cuthbert 2015). Based on our findings, we posit that new, further enriched biological phenotypes

(i.e., based upon additional data on microbial colonization, as well as biomarker mediators and effector molecular pathways) can be used to define new disease subtypes and enable us to better understand the molecular basis of oral diseases, thereby facilitating the development of precision prevention and therapies.

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