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Review Article

Illuminating the oral microbiome: cellular microbiology

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Abstract

Epithelial cells line mucosal surfaces such as in the gingival crevice and provide a barrier to the ingress of colonizing microorganisms. However, epithelial cells are more than a passive barrier to microbial intrusion, and rather constitute an interactive interface with colonizing organisms which senses the composition of the microbiome and communicates this information to the underlying cells of the innate immune system. Microorganisms, for their part, have devised means to manipulate host cell signal transduction pathways to favor their colonization and survival. Study of this field, which has become known as cellular microbiology, has revealed much about epithelial cell physiology, bacterial colonization and pathogenic strategies, and innate host responses.

Keywords: epithelium, oral, invasion, signal transduction, periodontal, OSCC

Introduction

A significant component of the human microbiome resides on mucous membranes such as in the GI tract or oral cavity. Microbe-host interactions have coevolved to result, in most instances, in a harmonious co-existence. Innate immune mechanisms deployed by the epithelial cells that line mucosal surfaces restrict the dissemination of microbes which are well-adapted to thrive in the host microenvironment. Interestingly, the microbiome itself is important for the development and maturation of this innate immune system. Homeostasis at the mucosal barrier can break down following colonization by exogenous pathogens, or, as is more frequently the case in the oral cavity, when the microbial community transitions to a dysbiotic state often through the action of endogenous community inhabitants known as keystone pathogens. Pathogenic species or communities overcome inflammatory responses and nutritional immunity to breach their biogeographic containment and invade the epithelial tissue, either intra- or inter- cellularly, which in turn can be a staging post for systemic spread. Bacterial invasion is accomplished by a suite of effector molecules, many of which are secreted through specialized machinery, which mediate attachment and internalization. Remarkably, bacteria can seize control of host cell signal transduction pathways in an attempt to manipulate and subvert critical functions such as cytoskeletal architecture, proliferation, programmed cell death and immune competence. Epithelial cells respond in an attempt to minimize the impact of microbial intrusion, and the ensuing molecular struggle has relevance for inflammatory diseases such as periodontitis, and as is becoming increasingly recognized, cancers such as oral squamous cell carcinoma.

Characteristics of oral epithelial cells

(1) Stratification of oral epithelium

The oral epithelium is stratified and is broadly classified into two phenotypically distinct types: the keratinized and non-keratinized epithelial layers that line different areas of the oral cavity and separate it from the deeper underlying lamina propria or connective tissue. The lamina propia contains a dense matrix of vascular and lymphatic capillary networks and nerve bundles, and supports the epithelial layers. The non-keratinized regions include the junctional and sulcular epithelium, lining mucosa (alveolar mucosa at the floor of the mouth, soft palate, lip, and cheek), and specialized mucosa (dorsum of tongue and taste buds; Mackenzie et al. 1991). In contrast, keratinized squamous epithelial surfaces include the masticatory mucosa i.e. epithelial surfaces around the gingiva and hard palate, surfaces that are constantly subject to mechanical abrasion. The thickness, differentiation, and keratinization (discussed later) of the epithelial surface vary according to the specific site, its anatomical location, and function within the oral cavity (Nanci 2013, Moutsopoulos and Konkel 2018).

The keratinized epithelium is divided into four sub-layers or strata; stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (Fig. 1). After mitotic division, epithelial cells leave the lowermost layers (stratum basale) and progressively undergo biochemical and morphological changes as they differentiate and migrate toward the upper layers (spinous and granular layers), ultimately reaching the stratum corneum. This process of continuous renewal and passive migration toward the apical surface maintains the architectural integrity of the oral epithelium. Terminal differentiation of keratinocytes accompanies the formation of a dense lipid-protein matrix which forms an impenetrable barrier and the uppermost cornified layer/envelope (stratum corneum) in the keratinized epithelium (Nanci 2013). As the epithelial cells migrate they undergo marked structural changes characterized by the loss of organelles, pyknotic nuclei,

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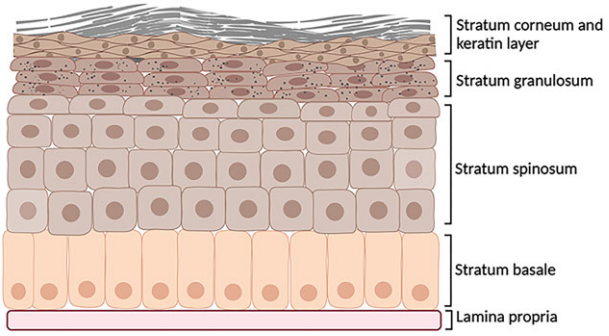

Figure 1. Sub-layers (strata) of oral keratinized epithelium: The keratinized oral mucosa is divided into four morphologically distinct sub-layers or strata. The actively dividing stratum basale is the lowermost layer. Cells undergoing mitotic division migrate towards the upper layers, i.e. the stratum spinosum, and stratum granulosum, undergoing biochemical and morphological changes characterized by progressive flattening, loss of organelles, and the production of keratohyalin granules as seen in the stratum granulosum layer. The uppermost layer, the stratum corneum, is comprised of flattened, often anucleate epithelial cells and extensive keratinization.

production of keratohyalin granules, and flattening, finally transforming into anucleate squamous cells. Keratins are a large family of filamentous proteins that are expressed in differentiating epithelial cells and provide resilience against mechanical stress while maintaining structural integrity. The keratohyalin granules located primarily within the cytoplasm of epithelial cells in the stratum granulosam release filaggrin, which enables the aggregation of keratin filaments and flattening of epithelial cells (keratinocytes). During cornification, the deposition of covalently linked, insoluble keratin occurs on the cytoplasmic face of the plasma membrane. Cytokeratin expression and topographical distribution in tissues vary depending on the epithelial site, stage of differentiation, and other underlying conditions such as inflammation, infections and oncogenic transformation (Magin et al. 2007, Dmello et al. 2019). Gingival tissues express keratins K1 and K10 within the upper layers, consistent with the expression in the cornified epithelium, while the suprabasal gingival layers express K2. The epithelial surfaces of the buccal mucosa express K4 and K13. However, the keratinization pattern of the attached gingiva can vary (Rosin et al. 2020). The junctional epithelium (JE), which maintains the direct attachment of the gingiva to the tooth surface, synthesizes K8, K13, K16, K18, and K19. The sulcular epithelium and other cells of the gingiva margin additionally express K4 (Mackenzie et al. 1991, Schroeder and Listgarten 1997, Pollanen et al. 2003). Changes in keratinization patterns and morphological characteristics of the oral epithelium can be assessed in tissue biopsy samples by immunostaining and by hematoxylin & eosin staining.

The permeability and polarity of the oral epithelium are reinforced by adhesive interactions that involve the formation of cellular junctions between epithelial cells. Broadly, cell junctions are classified into three groups known as tight junctions, gap junctions, and anchoring junctions. Anchoring junctions are further subclassified as adherens junctions, hemidesmosomes and desmosomes (Groeger and Meyle 2019). Tight junctions (TJs), also known as occluding junctions, are complex intercellular adhesion complexes that are apically located and form the border between the apical and basolateral surfaces of polarized epithelial cells. The main transmembrane proteins that mediate the formation of tight junctions in the gingival epithelium are members of the claudin family (claudin 1 and 4) and other junctional MARVEL domain proteins: occludin (MARVEL D1), tricellulin (MARVEL D2), MARVEL D3, and junctional adhesion molecules (JAMs). The extracellular domains of claudins and other transmembrane proteins from two interacting cells directly join one another and 'occlude' or obstruct the intercellular space. Polymerization of claudins and other transmembrane proteins is reinforced by scaffolding proteins such as zona occuldens-1 (ZO-1), which anchors the claudin strands to cytoskeletal actin. The main function of TJs is to form a diffusion barrier for the regulated paracellular transport of small molecules, ions, solutes, and water. Signaling proteins and actin cytoskeletal reassembly can all regulate the opening and rate of solute transfer across TJs (Harris and Tepass 2010, Zihni et al. 2016).

While TJs form a selective permeability barrier across sheets of epithelial cells, anchoring junctions (AJs) provide rigidity to cells by anchoring the cytoskeleton of one cell to the cytoskeleton of the adjacent cell or the extracellular matrix (ECM) (Takeichi 2014). The intracellular and junctional proteins that enable anchoring junction formation are functionally classified into two different families. Intracellular anchor proteins accumulate on the cytoplasmic face of the plasma membrane and anchor the cytoskeletal actin or intermediate filaments to the junctional complex. The transmembrane adhesion proteins bind the intracellular anchor proteins on the cytoplasmic face of the cell while their extracellular domains bind to the adhesion proteins from the neighboring cell or ECM proteins, thus creating a scaffold that imparts rigidity to epithelial cells. Various subtypes of AJs are described below (Alberts et al. 2002).

Adherens junctions are intercellular structures that are formed by homophilic interactions of extracellular domains of transmembrane cadherins which are intracellularly linked to the cortical actin cytoskeleton by catenins and other acting bundling proteins such as alpha-actinin and vinculin (Shapiro and Weis 2009). The coupling of intercellular adhesion to the actin cytoskeleton allows AJs to be dynamic, and undergo remodeling during cellular growth or apoptosis. Adherens junctions often appear as 'belt-like structures' that can undergo contraction and other morphological changes (Takeichi 2014). Desmosomes form a strong 'rivet like' adhesion among cells. A dense plaque of the intracellular adhesion proteins plakoglobin and desmoplakin anchors intermediate filaments (sometimes keratins) to the transmembrane adhesion proteins (desmoglein and desmocollin). Hemidesmosomes, like desmosomes, bind to intermediate filaments but anchor the basal surface of cells to the basal lamina. Desmosomes and hemidesmosomes both impart tensile strength to epithelial sheets and result in strong adhesion (Alberts et al. 2002). The junctional epithelium attaches to the tooth via hemidesmosomes and shields the tooth from the external environment. Focal adhesions are another type of anchoring junctions that mechanically link cytosolic actin filaments to the ECM via integrins, heterodimeric molecules comprised of transmembrane alpha and beta subunits (Groeger and Meyle 2019). Extracellular domains of integrin bind fibronectin, collagen, laminin, and other ECM proteins, while the intracellular integrin domains bind actin filaments via vinculin, filamin, talin, and alpha-actinin, thus forming a cell-to-matrix adhesive complex. ECM protein- integrin- induced signaling can also provide various directional cues to epithelial cells that enable their survival, migration, spreading or proliferation. Thus, focal adhesions mechanically link cells to the ECM and activate intracellular signaling and mechanotransduction pathways. Oral epithelial cells also have gap junctions (GJs) which are specialized intercellular channels formed by the 'head-to-head docking' of two

connexin hexamers from neighboring cells. Connexins are four-pass transmembrane proteins, six of which polymerize to form a channel that connects two cells when aligned. Multiple GJs aggregate or cluster into plaques that connect to the apposing cell. GJs permit the direct transfer of ions, nutrients, and small metabolites between adjacent cells (Alberts et al. 2002). Several factors can impact the permeability of the oral epithelial barrier, and the destruction of cellular junctions by periodontal pathogen proteases is discussed below (Goodenough and Paul 2009, Groeger and Meyle 2015, Garcia et al. 2018, Groeger and Meyle 2019).

ii) Pattern recognition receptors and inflammasomes

Oral epithelial cells sense a multitude of exogenous stimuli via germline-encoded pattern recognition receptors (PRRs) which play an inductive role in localized immune responses by the recruitment and differentiation of immune cells (Table 1). Gingival epithelial cells express membrane-restricted Toll-like Receptors (TLRs), cytosolic PRRs, Nucleotide oligomerization domain like Receptors (NLRs), and Retinoic acid Inducible Gene I (RIG-I) like receptors (RLRs). Members of each receptor family recognize specific microbe-associated molecular patterns (MAMPs) and activate downstream signaling pathways that culminate in the release of antimicrobial peptides, chemokines, cytokines, growth factors, and various matrix metalloproteases (MMPs) (Wells et al. 2011, Belkaid and Harrison 2017).

TLRs can be localized in the cell membrane or endosomal membrane, where they recognize extracellular or endocytosed MAMPs, respectively. The extracellular regions of TLRs contain leucine-rich repeats (LRRs) which recognize specific ligands. The intracellular domain contains the Toll/IL-1 receptor-like domain that enables association with adaptor proteins and downstream signaling. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface, while TLR3, TLR7, TLR8, and TLR9 are localized within the endosomal compartment. With the exception of TLR2, which heterodimerizes with TLR1 or TLR6, all other TLRs form homodimers (Kawasaki and Kawai 2014). TLR2 heterodimers recognize lipoteichoic acids (LTA) from Gram-positive bacteria and zymosan from fungi; TLR4 recognizes lipopolysaccharides from Gram-negative bacteria, and TLR5 is activated upon binding of flagellin from flagellated bacteria. Endosomal TLRs play important roles in the recognition of nucleic acids. TLR3 recognizes double-stranded viral RNA (dsRNA), while TLRs 7 and 8 recognize short single-stranded RNA (ssRNA). TLR9 recognizes unmethylated CpG motifs that are prevalent in bacterial DNA. Upon ligand recognition, TLRs recruit adaptor proteins and kinases, and can also cooperate with other receptors such as CD36, integrins, and CD14 to activate downstream signaling that leads to the activation of transcription factors such as NF-kB, IRF3/7, AP1, and also various MAPKs (Fig. 2). Most TLRs, with the exception of TLR3, use the MyD88 adaptor protein to activate AP1 and NF-kB and induce proinflammatory cytokine expression. TLR3 and TLR4 recruit the TRIF adaptor protein. TLR3, 7, 9, and TLR4 can also activate interferon expression via IRF3/7 (Kawasaki and Kawai 2014, Li and Wu 2021).

The NLR family of PRRs has over 20 members and evolved to recognize intracellular pathogens, such as viruses or bacteria, that escape the phagosome (Lupfer and Kanneganti 2013). NLRs contain 3 major domains. The central nucleotide-binding domain (NBD) is shared by all members of the NLR family and is essential for nucleic acid binding and oligomerization of NLRs. NLRs have NACHT LRRs at the C-terminus, which enable ligand recognition; and an N-terminal effector domain, which mediates homotypic interactions with other proteins such as the caspase activation and recruitment domain (CARD) or the pyrin domain (PYD) containing proteins. The NLR family is further classified into four subgroups: NLRA, NLRB, NLRC, and NLRP according to the nature of their N-terminal domains comprising an acidic transactivation domain, a baculovirus IAP repeat (BIR), CARD, and the PYD, respectively. In the context of the oral epithelium, the most studied members belong to NLRCs and NLRPs families. The two earliest discovered members of the NLR family are NOD1 and NOD2, which are highly expressed in gingival epithelial cells (Sugawara et al. 2006). NOD1 senses peptidoglycan containing *meso*-diaminopimelic acid, and NOD2 detects muramyl dipeptide, both distinct substructures of bacterial peptidoglycan. Ligand recognition by LRR results in oligomerization via the NACHT domain and the exposure/activation of the N-terminal effector domain. N-terminal domains, upon activation, recruit effector proteins that bind via homotypic interactions. NOD1 and NOD2 proteins, after self-oligomerization, recruit the receptor-interacting serine/threonine-protein kinase 2 (RIPK2) through homotypic CARD-CARD interactions, and RIP2 subsequently activates NF-κB resulting in proinflammatory cytokine induction (Lupfer and Kanneganti 2013). NLR family of proteins, upon activation, are able to assemble the inflammasome complex. Oligomerization of NLRP or AIM2 proteins results in the recruitment of CARD domain-containing adaptor proteins such as apoptosis-associated speck-like protein containing a CARD (ASC), ultimately leading to the recruitment of procaspase 1. The formation of the oligomerized protein complex initiates self-cleavage of caspase 1, which then proteolytically activates pro-IL-1β and pro-IL-18, and induces their release via gasdermin pores (Devant and Kagan 2023). Concerted action of various PRRs can initiate inflammasome activation and/or augment its assembly. TLRs, and RLRs (discussed below) can also directly or indirectly promote inflammasome assembly leading to the proteolytic processing and release of proIL-1 and IL-18. DAMPs such as extracellular ATP, HMGB1, and mitochondrial macromolecules can also trigger inflammasome assembly in epithelial cells (Fig. 2; Fritz et al. 2006, Latz et al. 2013, Caruso et al. 2014).

The RLRs family members are cytoplasmic helicases that recognize viral RNA and induce interferon production, playing a critical role in antiviral immunity (Fig. 2). The three members of the RLR family are RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). All contain a central DexD/H helicase domain which has helicase and ATPase activity. RIG-I and MDA5 also have two N-terminal CARD domains and a C-terminal domain (CTD), which recognizes viral RNA. In addition, RIG-1 also contains a repressor domain (RD) which can inhibit the activation of the receptor (Rehwinkel and Gack 2020). Under homeostatic conditions, RIG-I is self-inhibited; however, during viral infection, the recognition of viral RNA via the CTD induces a conformational change, exposing its CARD domain resulting in multimerization. The exposed CARD domain also enables binding with the CARD domain found in the mitochondrial antiviral signaling protein (MAVS), which serves as an adaptor protein for RLR signaling. MAVS-mediated signaling activates IRF3 and TLR7 along with NF-κB, which together induce the transcription of Type I and Type III interferons. All 3 members of the RLR family differ structurally but work co-operatively in response to viral infection. MDA5 lacks a RD and cannot self-inhibit its activation. LGP2 lacks CARD domains and cannot recruit CARD domain binding proteins but plays a role in the negative regulation of other RLRs. (Jiang et al. 2011, Rehwinkel and Gack 2020).

Table 1. Epithelial pattern recognition receptors (PRRs).

PRR	LIGAND	PAMP/DAMP ORIGIN	LOCATION
	TLRs[1]		
TLR1/2	Triacyl lipoproteins; Zymosan	Bacteria; fungi	Cell surface
TLR2/6	Peptidoglycan, lipoteichoic acid; Zymosan	Bacteria; fungi	Cell surface
TLR3	dsRNA	Virus	Endosome
TLR4	Lipopolysaccharide (LPS); heat shock proteins*	Bacteria; host-derived DAMPS	Cell surface
TLR5	Flagellin	Bacteria	Cell surface
TLR7	ssRNA	Virus	Endosome
TLR9	Non-methylated CpG DNA	Bacteria; virus	Endosome
TLR10 (human)	?	?	Cell surface
TLR11 (mouse)	Microbial proteins: Flagellin (bacterial) and profilin (protozoa)	*Toxoplasma gondii*; bacteria	Endosome
TLR12 (mouse)	Microbial proteins: Flagellin (bacterial) and profilin (protozoa)	*Toxoplasma gondii*; bacteria	Endosome
TLR13 (mouse)	23 s ribosomal RNA		Endosome
	NLRs[2]		
NOD1	iE-DAP	Bacteria	Cytosol
NOD2	Muramyldipeptide	Bacteria	Cytosol
	RLRs[3]		
RIG-I	5′-triphosphorylated dsRNA	Virus	Cytosol
MDA5	5′-triphosphorylated dsRNA	Virus	Cytosol
LGP2	5′-triphosphorylated dsRNA	Virus	Cytosol
	DNA Sensors		
c-GAS/STING	Cyclic dinucleotides (CDNs)	Bacteria, virus, and mammalian	Cytosol
AIM2	dsDNA	Bacteria, virus, and mammalian	Cytosol
IFI16	DNA	Bacteria, virus, and mammalian	Cytosol

[1]Toll-like Receptors.
[2]Nucleotide oligomerization domain Like Receptors.
[3]Retinoic acid-inducible gene I (RIG-I)-like receptors.

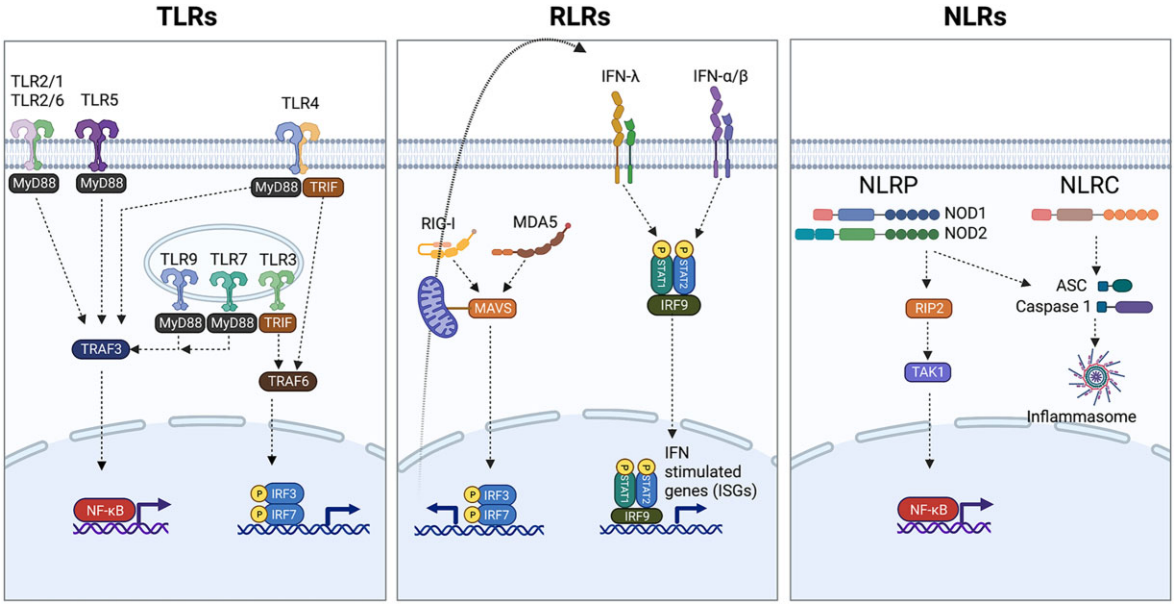

Figure 2. Epithelial PRRs: Three classes of PRR, Toll-like Receptors (TLRs), Rig-I-like Receptors (RLRs), and Nod-like Receptors (NLRs), sense extracellular or cytoplasmic microbe associated molecular patterns in epithelial cells. TLRs use MyD88 or TRIF as adaptor proteins to activate NF-kB or IRFs to activate proinflammatory cytokine and interferon (IFN) production, respectively. RLR sensing of viral ligands potently activates IFNs and downstream IFN-stimulated genes (ISGs). NLRs activate NF-kB via RIP2 kinase to induce proinflammatory cytokines. Separately NLRs (NLRPs and NLRCs) can activate ASC and caspase 1/11 to form the multimeric inflammasome essential for mature IL-1 beta production.

Another family of DNA sensors detects viral or bacterial DNA. The Cyclic AMP-GMP synthase (cGAS) is a nucleotidyltransferase that senses cytosolic DNA and catalyzes the synthesis of cyclical nucleotides such as cGAMP. cGAMP then serves as a secondary messenger to activate the endoplasmic reticulum residing adaptor protein STING (Stimulator of Interferon Genes) to induce interferons (Li and Wu 2021). Upon activation, STING relocalizes from the ER to the perinuclear space for TBK-1, IRF3, and NF-kB activation and interferon production. Other cytosolic DNA sensors are AIM2 and IFI16, which detect viral DNA and activate ASC and caspase-1-dependent inflammasome formation. Most PRRs have been extensively studied in myeloid cells, and comprehensive as-

sessment of their regulation and activation in the oral epithelium is still an understudied area (Crowl et al. 2017, Motwani et al. 2019, Li and Wu 2021).

Overall, PRRs regulate several aspects of oral epithelial cell signaling and impact immune homeostasis at the oral mucosal barrier. Homeostatic recognition of ligands from the oral microbiota by epithelial cells initiates a dialogue between microbial colonizers and also calibrates immune effector responses and differentiation of immune cells. Tonic sensing of microbial ligands causes the release of beta-defensins from oral epithelial cells that limits microbial burden within the oral tissue (Meade and O'Farrelly 2018). Tonic sensing or low activation of PRRs by microbial ligands or metabolites also results in neutrophil homeostasis and their regulated recruitment to the oral cavity (Darveau 2010, Deshmukh et al. 2014); maintains a natural state of viral resistance by basal expression of various interferon-stimulated genes (Moffatt and Lamont 2011, Forero et al. 2019, Winkler and Thackray 2019); boosts homeostatic IgA responses (Bunker and Bendelac 2018); and modulates epithelial cell growth and turnover (Meade and O'Farrelly 2018, Liu et al. 2022).

Models of bacteria-epithelial cell interactions

i) Internalization assays

Epithelial cells are not professionally phagocytic, yet one of the most dramatic outcomes of their interaction with bacteria is the internalization of the microbe within the host cell. A number of bacterial species can induce remodeling of the host cell cytoskeletal structure to create membrane invaginations that surround and engulf the bacteria. Intracellular bacteria can remain viable, initiate transcriptional reprogramming of the epithelial cell, and are capable of cell-to-cell spread (Lamont et al. 1995, Yilmaz et al. 2006, Takeuchi et al. 2016, Fitzsimonds et al. 2021). Consequences for the host cell include loss of control of cell cycle, proliferation, and programmed cell death pathways, as well as disruption of cytokine/chemokine production. There are two predominant technologies by which bacterial invasion of host epithelial cells is studied: antibiotic protection and fluorescent image analysis.

In the protection assay, an antibiotic is used to kill extracellular membrane-bound bacteria after infection, following which cells are lysed, and viable intracellular bacteria are enumerated by plate counting. The aminoglycoside gentamicin is usually the antibiotic of choice as it penetrates epithelial cells poorly. However, as active electron transport is required for aminoglycoside uptake into bacteria, gentamicin inherently lacks activity against anaerobic bacteria (Krause et al. 2016), including important oral organisms such as *Porphyromonas gingivalis*. Hence antibiotic protection assays for oral anaerobes often employ the anti-anaerobe antibiotic metronidazole along with high levels of gentamicin which may disrupt outer membrane stability through binding to LPS (Kadurugamuwa et al. 1993). It is important to emphasize that the antibiotic protection assay does not measure invasion per se, but rather is a survival assay which quantitates intracellular bacteria remaining viable after incubation and processing. Another consideration which may confound interpretation of this assay is the intracellular accumulation of even non-membrane permeable antibiotics, such as gentamicin, through micropinocytosis. The assay is also laborious and includes many steps which can expose anaerobic bacteria to air. Modifications of the antibiotic protection assay include differential labelling of internal and external bacteria before/after membrane permeabilization. Assays based on radioactive, fluorescent, luminescent, or chromogenic tags have been developed along with quantitation of labelled organisms by techniques such as flow cytometry. Additionally, intracellular bacteria can be quantitated by virtual colony counting, a high-throughput method which relates the time to reach a given absorbance threshold to the initial cell count using a series of calibration curves (Hoffmann et al. 2018). While these modifications can circumvent many of the issues inherent in the basic antibiotic protection assays, they have yet to be widely applied to oral organisms.

The limitations of the antibiotic protection assay have restricted its utility, and bacterial internalization is now more frequently measured by direct methods such as fluorescent image analysis. Bacteria can be engineered to express fluorescent proteins such as GFP or mCherry or labelled with specific fluorescent-tagged antibodies (primary or secondary), or with fluorescent dyes such as FITC or Texas Red. As GFP and its derivatives require oxygen for the formation of the chromophore, they are of limited use with oral anaerobes. However, the oxygen-independent flavin mononucleotide-based fluorescent proteins (FbFPs) have been employed successfully in *P. gingivalis* in some instances (Choi et al. 2011). Epithelial cell nuclei can be labelled with DAPI or Hoechst, the actin cytoskeleton with fluorescent phalloidin, and the membrane with fluorescent lectins. An increasing range of propriety stains for cell organelles and structures are also available commercially. Once bacteria and host cells are labelled, their association can be visualized by fluorescent microscopy. While internalization can be confirmed by differential antibody labelling before and after membrane permeabilization, a more powerful approach involves confocal laser scanning microscopy (CLSM). In CLSM, instead of illuminating the whole sample at once as in widefield microscopy, laser light is focused on a defined spot at a specific depth within the sample. Filtering techniques then eliminate out-of-focus light in specimens where the thickness exceeds the plane of focus. By scanning the specimen in a raster pattern, images of one single optical plane are created, and sequential optical planes can be assembled into a 3D image with deconvolution software. The spatial positioning of bacteria can thus be determined precisely, and trafficking as well as co-localization with subcellular compartments determined. Measurement of fluorescence levels also enables quantitation of intracellular or membrane-bound bacteria.

Both the antibiotic protection assay and fluorescent image analysis have generated significant information regarding the ability of oral bacteria to enter and traffic within oral epithelial cells. However, these techniques are at their most effective when combined with approaches which also interrogate bacterial or host cell functionality. These include bacterial isogenic mutants, inhibition of bacterial function with specific antibodies, pharmacological inhibition of epithelial cell functions, knockdown of host cell gene expression with siRNA, and use of activation specific phosphoantibodies or calcium binding dyes to evaluate signal transduction. Additionally, secretion of cytokines or other immune effector molecules can be measured by ELISA and related technologies.

(2) Cell lines

Epithelial cells originating from different anatomical sites have distinct properties, and thus study of the cellular microbiology of oral bacteria is more rigorous with epithelial cells derived from the oral cavity, and, in the case of periodontal organisms, crevicular or junctional epithelial cells. Early studies of oral bacteria

used KB or HEp-2 cell lines (Blix et al. 1992, Sandros et al. 1993) which were thought to have originated from oral epidermoid carcinomas. However, both these lines were later found to have been established via HeLa cell contamination, and therefore not representative of oral cells. An additional problem with any cell line is that as the cells are tumorigenic, study of cell life or death processes is compromised. Both of these issues were resolved when Oda et al. established conditions to reproducibly maintain primary gingival epithelial cells (GECs) in culture (Oda and Watson 1990, Oda et al. 1990). These cells can be recovered from tissue obtained following impacted third molar oral surgery and have a keratin expression profile consistent with that of junctional epithelium. Disadvantages of primary GECs include inter-patient variability, and the low cell numbers obtained prior to senescence. Hence Oda et al., and other groups, immortalized primary GECs using the E6/E7 genes from HPV, generating a line, which albeit immortalized and potentially tumorigenic, gives large numbers of cells with reproducible characteristics (Oda et al. 1996). Telomerase induced immortalization of GECs is a further refinement which generates high numbers of cells which, although immortalized, are not tumorigenic (Moffatt-Jauregui et al. 2013). Both immortalized and primary GECs can be cultured as organotypic multilayers, often of a substratum of collagen which may contain embedded fibroblasts, or fibronectin and gelatin, and which can be vascularized, to more closely model the 3D structure of the gingiva (Dickinson et al. 2011, Pinnock et al. 2014, Sasaki et al. 2021, Takeuchi et al. 2022, Zhang et al. 2022).

(3) Epithelial-specific knockout mice

Transgenic mice provide valuable insights into the interrogation of gene function in various cell types, tissues, and disease models. The allelic deletion of genes is accomplished by the use of various genome editing technologies that employ nucleases such as Zinc Finger Nucleases (ZFN), or transcription activator-like (TALs) effector nucleases (TALENs) to edit genomic sequences to delete, conditionally induce or inactivate genes. More recently, the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated proteins (Cas-9) has exploded as it has several advantages over the older approaches, such as shorter timelines, higher success rates, lower costs, and the ability to target multiple genes simultaneously (Gupta and Musunuru 2014). The CRISPR system uses short guide RNAs (gRNAs) that target Cas9 (endonuclease) to the specific region in the genome creating precise double-strand breaks in DNA. Another significant difference between CRISPR compared to older technologies is the ability to delete genes in the fertilized zygote (fertilized oocyte) rather than embryonic stem cells.

Conventional or global knockout mice completely lack gene expression in all tissues, and thus could have adverse phenotypes, including embryonic lethality, depending on the gene. The tissue-specific or conditional knockout mice enables a more precise deletion of a gene in specific tissues or stages of life. Conditional knockout mice have become an important tool for modeling host-pathogen interactions in vivo. Ablation of a gene in the oral epithelium specifically allows the role of the corresponding gene product to be assessed in an epithelial context. LoxP-Cre systems allow for potent and selective recombination that enables the conditional deletion or expression of genes in a cell lineage-specific manner. The Cre protein is a site-specific recombinase that targets a specific sequence (LoxP site) and catalyzes DNA recombination between two loxP sites. The loxP site is a 34 bp sequence comprising of two 13 bp inverted and palindromic repeats. To generate mutant mice, two elements are needed. First, a loxP flanked gene and a Cre-driver strain in which Cre recombinase is expressed by an endogenous specific promoter. Various tissue-specific Cre lines have been generated that selectively target gene expression in epithelial cells. The keratin 13 cre (K13-Cre) enables gene manipulation specifically in oral tissues (superficial oral, esophageal epithelium) as Keratin 13 is preferentially expressed in the suprabasal layers of the oral epithelium in mice (Presland and Dale 2000). While this in vivo tool not has not yet been fully exploited for the study of bacterial-epithelial interactions, it has been used to examine the role of the IL-17 receptor in oropharyngeal candidiasis (Conti et al. 2016).

The bacterial intracellular lifestyle

(1) Adherence and invasion

A variety of oral bacteria are capable of inducing their uptake into epithelial cells; however, we shall focus here on *P. gingivalis* which has been most extensively studied. *Porphyromonas gingivalis* attaches to and invades GECs rapidly and in high numbers (Belton et al. 1999). As *P. gingivalis*, along with other characterized oral anaerobes, lacks the machinery of the type 3 secretion system; to effectuate invasion the organism relies on a more limited number of functionally versatile invasins. The intimate association of *P. gingivalis* with GECs begins before even attachment has occurred. A haloacid dehalogenase (HAD) family serine phosphatase (SerB) is secreted by the organism and taken up by host cells whereupon it dephosphorylates and thus activates the actin depolymerizing molecule cofilin (Tribble et al. 2006, Moffatt et al. 2012). This results in a transient loss of actin cytoskeletal structure, which is a prelude to wholescale actin remodeling required for creation of membrane invaginations that engulf the bacteria. Cytoskeletal rearrangements are also mediated by the FimA fimbrial adhesin engaging β1-integrin receptors on the GEC surface (Yilmaz et al. 2002). Signaling cascades that are induced include phosphorylation and activation of FAK, paxillin and JNK (Watanabe et al. 2001, Yilmaz et al. 2002), and an increase in cytoplasmic Ca^{2+} concentration following release from intracellular stores (Izutsu et al. 1996) along with Ca^{2+} oscillations in nuclear and cytoplasmic spaces due to influx through Ca^{2+} channels (Belton et al. 2004). Information flow through these pathways ultimately results in nucleation of actin filaments which form microspike-like protrusions, and long stable microfilaments are distributed throughout the cells (Yilmaz et al. 2003). In addition to actin microfilament restructuring, the tubulin cytoskeleton of GECs is involved in *P. gingivalis* invasion. Inhibition of microtubule assembly/disassembly with nocodazole suppresses *P. gingivalis* internalization (Lamont et al. 1995), and this function is also mediated by the SerB phosphatase of *P. gingivalis* (Tribble et al. 2006).

While invasion following FimA-mediated adherence appears to be the predominant means of internalization by *P. gingivalis*, nevertheless mutants deficient in FimA production remain capable of invasion, albeit at significantly reduced levels (Yilmaz et al. 2003). Additional *P. gingivalis* outer membrane proteins which can trigger invasion include OmpA2 (Naylor et al. 2017), and the gingipain proteases (Park and Lamont 1998). The extent to which these invasion mechanisms converge on the pathways regulated by FimA remains to be determined.

(2) Intracellular trafficking

Once inside the epithelial cell, *P. gingivalis* remains viable for extended periods and also prolongs survival of the host cell

(discussed in more detail below). Indeed, proteomic analysis of intracellular *P. gingivalis* shows a decrease expression of gingipain proteases (Hendrickson et al. 2009), which may serve to reduce damage to the host cell. Intracellular *P. gingivalis* can adopt a variety of trafficking strategies with differing consequences for exit and spread. A subpopulation of *P. gingivalis* remain in the cytoplasm, predominantly in the perinuclear region (Belton et al. 1999). The cytoplasm is generally anoxic (Riemer et al. 2009) which, along with the abundant source of proteins, will favor the metabolism of *P. gingivalis*. In contrast, transmission electron microscopy with tomographic reconstruction has shown a population of intracellular *P. gingivalis* trafficking to endoplasmic-rich autophagosomes where they can remain viable (Lee et al. 2018). In both cases, *P. gingivalis* may spread directly from cell to cell, without exposure to the extracellular milieu, through actin-based membrane protrusions (Yilmaz et al. 2006). Alternatively, *P. gingivalis* can exit epithelial cells through a recycling pathway (Takeuchi et al. 2011, Takeuchi et al. 2016). Following trafficking to early endosomes, there is recruitment of VAMP2, a vesicle-associated protein involved in docking and fusion, and Rab4A, a GTPase that regulates membrane dynamics. These orchestrated endosomal developmental events allow *P. gingivalis*-containing vesicles to fuse with the cytoplasmic membrane and escape the cell (Takeuchi et al. 2016). The parameters that determine which intracellular option is adopted by *P. gingivalis* are unknown. Individual cells or subpopulations could follow distinct trafficking routes, which may depend on the initial mechanism of entry. Moreover, there are reports that *P. gingivalis* populations can comprise bistable subtypes with differing invasive abilities (Suwannakul et al. 2010), which may be reflective of intracellular trafficking pathways. Flexibility in this regard may facilitate *P. gingivalis* growth, dissemination, and avoidance of host immune effectors depending on the microenvironmental context.

(3) Intracellular survival

While on the one hand, an intracellular location is beneficial for bacteria through the availability of nutrients and sequestration from immune attack, on the other, intracellular bacteria must finds ways of avoiding antibacterial activities of epithelial cells. One major means by which *P. gingivalis* overcomes epithelial cell defenses to ensure intracellular survival is by targeting the ectonucleotidase CD73 (Lee et al. 2020). *Porphyromonas gingivalis* increases the surface expression and activity of CD73, thus negatively regulates the production of eATP-coupled reactive oxygen species (ROS) which are toxic to *P. gingivalis*. Additionally, elevated CD73 action can reverse the increased secretion of IL-6 induced by *P. gingivalis*, and as IL-6 can inhibit intracellular *P. gingivalis* survival by ROS induction, this will favor intracellular persistence of the organism. Enhanced CD73 activity significantly increases *P. gingivalis* intracellular growth in the presence of substrate-AMP, and simultaneously acts as a negative regulator of reactive oxygen species (ROS) generation upon eATP treatment. Intracellular ROS may represent the predominant antibacterial mechanism relevant to *P. gingivalis* survival, as the organism adopts several strategies to prevent ROS accumulation. A nucleoside-diphosphate kinase (NDK) enzyme produced by *P. gingivalis* can antagonize ROS production through inhibition of the eATP/P2X$_7$ pathway, which reduces both NADPH oxidase-, and mitochondria- mediated ROS generation (Choi et al. 2013, Roberts et al. 2017) *P. gingivalis* can also inhibit ROS action by stimulating synthesis of the antioxidant glutathione (Choi et al. 2013). After resisting the onslaught of intracellular killing mechanisms, *P. gingivalis* has sophisticated strategies to thrive and ensure persistence. GECs express adenosine receptors, including the high affinity A2a (Spooner et al. 2014) and *P. gingivalis* can utilize A2a receptor coupled danger signal adenosine signaling to increase intracellular levels of cAMP, a potential energy source for proliferation of intracellular organisms. Signaling through cAMP can also suppress inflammatory responses, another contributory factor to the persistence of *P. gingivalis* (Lee and Yilmaz 2021).

The epithelial cell response to bacterial interlopers

Adhesion and invasion of host epithelial cells by many oral microbes initiate signaling cascades that regulate gene expression. These host-pathogen dynamics are of elevated importance in parts of the human body such as the oral cavity, where the polymicrobial community is diverse, shifting in composition, and highly abundant with constant contact alongside multiple epithelial barriers (i.e. buccal, palatal, tongue, and gingiva). It is well established that polymicrobial effects on host cells and tissues in the oral cavity contribute to the development of the progression of periodontitis and various secondary inflammatory diseases. Additionally, disruption of host cell signalling and the inflammatory microenvironment can influence the development and progression of cancers in the oral cavity and other sites such as the GI tract that can be colonized by oral organisms. Extensive characterization of host cell responses to infection with periodontal bacteria can reveal the underlying mechanisms of inflammation in gingival or other epithelial cells, as well as resident and recruited immune cells (Hajishengallis and Chavakis 2021).

The 'omics' revolution has fundamentally changed our understanding of interactions between host cells and the oral microbiota. RNA-Seq is a frequently utilized approach for global analysis of host cell responses to oral microbes. RNA-Seq utilizes high-throughput sequencing to quantitate levels of RNA in cells, and coupled with secondary verification by techniques such qRT-PCR, is a reliable and reproducible technique to measure gene transcriptional activity. However, many caveats must be considered in gene expression data as genes may be post-transcriptionally regulated, and protein interactions and functions are further impacted by a multiple array of post-translational modifications. Additionally, a major challenge for transcriptomics studies is to dissect the massive amount of data generated to provide insight into the biological impact of host-microbe interactions. Pathway analysis and gene ontology analysis are frequently utilized approaches to organize and categorize differential expression data by biological functions. In the context of signaling, its critical to consider many signaling pathways are parallel, interconnected, and can each influence expression of overlapping subsets of genes. Nonetheless, analysis of transcriptional signatures following bacterial challenge have provided significant insight into the cellular responses and the molecular tug-of-war between eubiotic bacteria that promote homeostasis or more pathogenic organisms that contribute to dysbiosis and immunopathology.

The impact of organisms such as *P. gingivalis* and *Fusobacterium nucleatum* reverberates extensively through host epithelial cells, often involving pathways that control epithelial cell life and death decisions, growth and differentiation, to the extent that both organisms are associated with unregulated growth and a tumorigenic phenotype. In addition, the inflammatory microenvironment and epithelial barrier function can be extensively recalibrated by oral bacterial challenge. We shall continue to focus

mainly on *P. gingivalis* (Fig. 3) before turning our attention to the broader periodontal microbiome.

(1) Programmed cell death

In addition to ensuring its own survival, *P. gingivalis* has also developed mechanisms to prevent programmed cell death of GECs and sustain its eukaryotic host. Indeed, *P. gingivalis* devotes considerable effort to suppressing apoptosis through several host pathways. Anti-apoptotic mechanisms activated by *P. gingivalis* generally revolve around intrinsic mitochondrial-mediated cell death, and include those involving JAK-STAT and PI3K-Akt pathways (Yilmaz et al. 2004, Mao et al. 2007). Increased expression of Bcl2, along with a decrease in pro-apoptotic factors such as Bax and Bad, alters the ratio of these interacting proteins and promotes stabilization of the mitochondrial membrane and inhibition of cytochrome c release, and consequently, activity of the downstream caspases including caspase-9 and the executioner caspase-3 is suppressed (Nakhjiri et al. 2001, Mao et al. 2007, Yao et al. 2010). The multipurpose transcriptional regulator Forkhead Box O1 (FOXO1), activated by *P. gingivalis* through dephosphorylation of serine residues, induces antiapoptotic programs in GECs (Wang et al. 2015). NDK produced by *P. gingivalis* and can phosphorylate heat-shock protein 27 (HSP27), which suppresses apoptosis through binding to cytochrome c and prevents caspase 9 activation (Lee et al. 2018). As the $P2X_7$ pathway is pro-apoptotic, antagonism by NDK also contributes to the suppression of apoptosis.

MicroRNAs provide another facet to host cell regulation, and *P. gingivalis* can modulate the expression of multiple miRNAs (Chen et al. 2016, Olsen et al. 2017). miR-203, up-regulated by *P. gingivalis*, stimulates STAT3 signaling that positively regulates the expression of anti-apoptotic factors such as Bcl-2 (Moffatt and Lamont 2011). At the same time, miR-203 over-expression by *P. gingivalis* inhibits the pro-apoptotic factor SOCS3 (Moffatt and Lamont 2011). Conversely, *P. gingivalis* suppresses miR-205–5p and consequently activates JAK-STAT signaling in GECs (Li et al. 2020).

In contrast to apoptosis, *P. gingivalis* can induce programmed cell death through other mechanisms. Necroptosis, is a recently recognized type of regulated cell death associated with massive release of damage-associated molecular patterns (DAMPs) and mediated by the receptor-interacting protein serine-threonine kinases-3 (RIPK3)/mixed lineage kinase domain-like protein (MLKL) (Tang et al. 2023). *Porphyromonas gingivalis* LPS can induce necroptosis in oral epithelial cells and the resulting release of DAMPs can inhibit M2 macrophage polarization (Geng et al. 2022). Similarly, *P. gingivalis* LPS can induce gingival fibroblasts to undergo death by ferroptosis (Qiao et al. 2022) which is characterized by mitochondrial shrinkage, increased iron and ROS, eventually producing lipid peroxidation in the cell membrane (Tang et al. 2023). Pyroptosis, dependent on the activation of caspase-1 can also be induced in gingival epithelial cells by *P. gingivalis* LPS (Li et al. 2021).

(2) Cell cycle and proliferation

Epithelial cells infected with *P. gingivalis* show enhanced expression of genes involved in proliferation as well as an accelerated progression through the cell cycle (Kuboniwa et al. 2008, Geng et al. 2017, Zhang et al. 2019). An antibody array analysis showed that this occurs through modulation of pathways involving cyclins, p53, and PI3K (Kuboniwa et al. 2008). Interestingly, cell cycle phase also influences invasion, as *P. gingivalis* preferentially interacts with GECs in the S phase (Al-Taweel et al. 2016). GEC proliferation can be controlled through FimA interactions with surface receptors (Kuboniwa et al. 2008), and a low molecular weight tyrosine phosphatase, Ltp1, which is secreted within gingival epithelial cells and translocates to the nucleus where it transcriptionally upregulates RGCC (Regulator of Growth and Cell Cycle) (Liu et al. 2021). Phosphoarray technology and siRNA showed that Ltp1 controls RGCC expression through Akt, which is activated by phosphorylation on S473. Akt activation is opposed by PTEN, and *P. gingivalis* decreased the amount of PTEN in epithelial cells by dephosphorylation at Y336, which leads to increased proteasomal degradation (Liu et al. 2021).

RNA-Seq analysis of GECs challenged with *P. gingivalis* revealed significant induction of olfactomedin 4 (OLFM4). OLFM4 enhances STAT3-mediated cell proliferation and survival, cell migration, and oncogenic transformation (Fitzsimonds et al. 2021). In this context, OLFM4 is antiapoptotic; however, OLFM4 was not found to impact the apoptosis of GECs following challenge with *P. gingivalis*. Rather, OLFM4 promoted cell proliferation and migration through Matrigel. Induction of OLFM4 is dependent on elevated gene expression of the Notch receptor and Jagged-1 in coordination with *P. gingivalis* gingipain activity that activates Notch signaling (Fitzsimonds et al. 2021).

(3) Epithelial mesenchymal transition (EMT)

EMT is a process whereby epithelial cells lose tight junctions and polarization, and acquire mesenchymal properties including motility and a stem cell-like phenotype. As such, EMT is important in activities such as embryogenesis and wound healing. However, dysregulated EMT can contribute to tumor metastasis (Lamouille et al. 2014). EMT is controlled by a complex network that includes a series of transcription factors, such as Zinc Finger E-box Binding Homeobox (Zeb) 1 and 2, Snail Family Transcriptional Repressors 1 and 2, and Twist. *Porphyromonas gingivalis* impacts the activity of several pathways that control EMT in epithelial cells and induces at least a partial shift toward the mesenchymal state (Ha et al. 2015, Sztukowska et al. 2016, Geng et al. 2017, Lee et al. 2017, Abdulkareem et al. 2018, Ohshima et al. 2019). Zeb1 is regulated through FimA-dependent signaling, whereas Zeb2 is elevated by gingipain processing and activation of beta-catenin, along with dephosphorylation and activation of Forkhead Box O1 (Sztukowska et al. 2016, Ohshima et al. 2019). *Porphyromonas gingivalis* infection also increases cancer stem cell markers, such as CD44 and CD133, and enhances epithelial cell migration (Ha et al. 2015, Ha et al. 2016, Sztukowska et al. 2016, Lee et al. 2017, Abdulkareem et al. 2018). Degradation of extracellular matrix and loss of epithelial barrier function, as discussed below, will also facilitate the spread of mesenchymal-like cells with a stem cell-like phenotype that are potentially capable of seeding tumors at remote sites. Moreover, *P. gingivalis*, along with *Treponema denticola* and *F. nucleatum*, enhance epithelial cell migration, invasion, stemness, and tumor aggressivity, independent of proliferation, via crosstalk between integrin/FAK and TLR/MyDD88 signaling pathways (Kamarajan et al. 2020). *F. nucleatum* can also induce an EMT program in oral epithelial cells through elevated TGF-β, TNFα, and EGF signaling (Abdulkareem et al. 2018). Upregulation of long noncoding (lnc)RNA MIR4435-2HG by *F. nucleatum* can also contribute to EMT in oral epithelial cells through a miR-296-5p/Akt2/Snai1 signaling pathway (Zhang et al. 2020).

(4) Innate immune responses

The epithelial cells of the gingival crevice continually surveil the colonizing microbiota, assess the pathogenic potential and respond with a tailored cytokine/chemokine and antimicrobial pep-

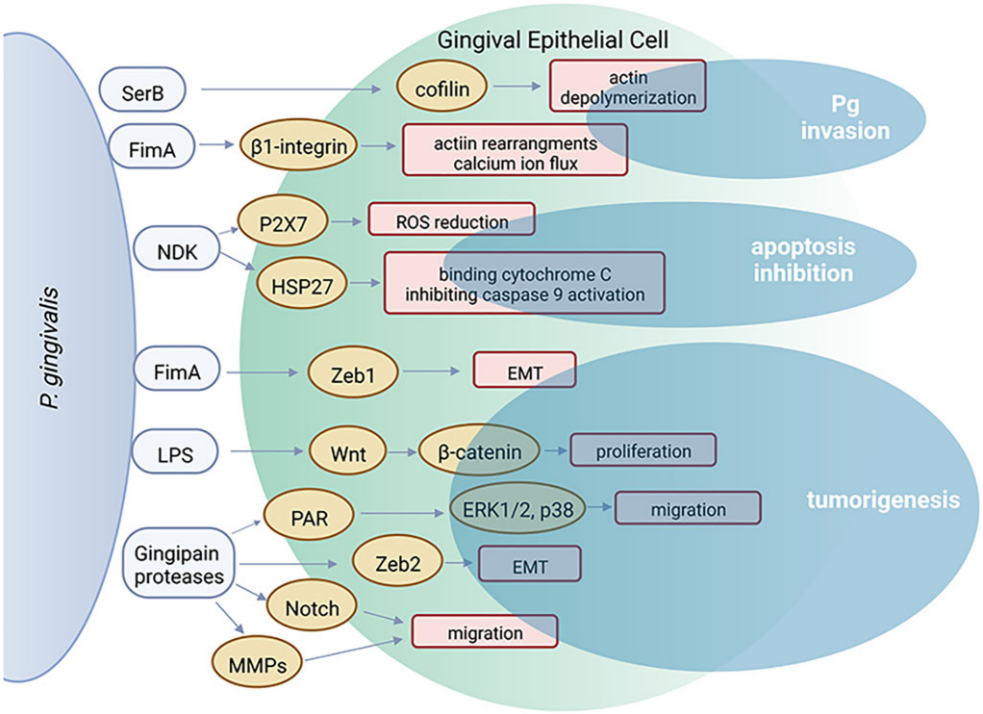

Figure 3. Overview of *P. gingivalis* interactions with gingival epithelial cells: Shown are major *P. gingivalis* effector molecules (blue), their epithelial cell molecular targets (yellow), and the pathways (pink) and phenotypes impacted.

tide response to maintain homeostasis. Bacteria with pathogenic potential can disrupt epithelial cell homeostatic responses and facilitate overgrowth of the microbial community as a whole. As a host-adapted inflammophilic organism, *P. gingivalis* displays a remarkable capacity to selectively suppress specific components of innate immunity while at the same time exploiting the products of inflammatory responses for growth (Hajishengallis 2014, Lamont et al. 2018). A variety of proinflammatory cytokines are secreted by epithelial cells in response to *P. gingivalis* (Darveau 2010, Sahingur and Yeudall 2015, Uemura et al. 2022). These include IL-1β and IL-18 following activation of the NLRP3 inflammasome, which can be achieved by the LPS of *P. gingivalis* (Guo et al. 2015, Atanasova and Yilmaz 2022). However, inflammasome activation is tempered by the action of the *P. gingivalis* NDK enzyme, which by degrading extracellular (e) ATP suppresses eATP-mediated activation of NLRP3 through the purinergic receptor $P2X_7$ (Lee and Yilmaz 2021). NDK functions on more than one level as it also attenuates the release of High mobility group box 1 protein (HMGB1), which synergizes with eATP in inflammasome activation (Johnson et al. 2015). Remarkably, NDK is released by intracellular *P. gingivalis* and secreted by epithelial cells through pannexin 1, which is also the pathway for eATP release (Atanasova et al. 2016). Moreover, initial mobilization of NDK is stimulated by eATP (Atanasova et al. 2016). The ability of *P. gingivalis* to co-ordinate mobilization and secretion of an effector molecule with its host target in indicative of a longstanding evolutionary relationship between *P. gingivalis* and GECs, a theme that recurs often in the interrelationship between these two cell types.

In addition to NDK, *P. gingivalis* also releases SerB intracellularly (Takeuchi et al. 2013). SerB dephosphorylates the p65 subunit of the NF-κB transcription factor. Translocation of NF-κB-p65 homodimers into the nucleus is thus reduced and IL-8 (CXCL8) gene transcription is diminished, even in the presence of otherwise stimulatory organisms such as *F. nucleatum* (Darveau et al. 1998, Takeuchi et al. 2013). In this manner, *P. gingivalis* can target with precision the neutrophil chemokine IL-8, which, while one of the many gene products controlled by NF-κB, is promoted predominantly by p65 homodimers. Disruption of neutrophil recruitment, even if transient as suggested by in vivo observations (Hajishengallis et al. 2011), could have a debilitating effect on immune surveillance during the development of a dysbiotic microbiome. Indeed, defective neutrophil recruitment into the gingival crevice could allow overgrowth of pathobionts and disruption of homeostasis. This phenomenon, known as localized chemokine paralysis (Darveau et al. 1998) extends to the T-helper 1 (Th1) chemokines CXCL10 (IP-10), CXCL9 (Mig) and CXCL11 (ITAC), which are suppressed through downregulation of IRF-1 and STAT1 (Jauregui et al. 2013). As with IL-8, this inhibition supersedes the ability of pro-inflammatory organisms such as *F. nucleatum* to incite production of these chemokines. *Porphyromonas gingivalis* may therefore reduce Th1 development, which plays an important role in cell-mediated immunity against periodontal bacteria (Gemmell et al. 2007, Hajishengallis 2014). A potential consequence is that Th17-mediated inflammation will flourish, a concept supported by the induction of Th17-promoting cytokines IL-6 and IL-23, but not the Th1-related IL-12, in antigen-presenting cells by *P. gingivalis* (Moutsopoulos et al. 2012).

Heterogeneity in epithelial cell characteristics can also impact innate immune responses. For example, GECs heterozygous for the TLR4 polymorphism Asp299Gly were found to be functionally hypo-responsive to *P. gingivalis* in terms of cytokine and chemokine production (Kinane et al. 2006). Reduced TLR2 expression can occur in GECs when there is increased CpG methylation in the promoter region. Moreover, prolonged challenge with *P. gingivalis* in vitro or in vivo increases DNA methylation in GECs

(Benakanakere et al. 2015), suggestive of another mechanism by which *P. gingivalis* can induce immune dysbiosis.

(5) Antiviral immunity

Anatomical barrier sites such as the oral mucosa are subject to frequent viral infection. Antiviral immunity at these surfaces is regulated to a large extent by the production of interferons that function as critical antiviral cytokines by restricting viral infection, replication, and release. *Porphyromonas gingivalis* uniquely can induce broad interferon (IFN) paralysis in oral epithelial cells by shutting down the production of types 1 (IFN alpha/beta), II (IFN-gamma), and III (IFN lambda) (Jauregui et al. 2013, Rodriguez-Hernandez et al. 2021). Type I and III IFNs play important roles in antiviral immunity by inducing the expression of several IFN-stimulated genes (ISGs) that restrict viral replication. *Porphyromonas gingivalis* blocks the transcription of multiple ISGs and represses type III and I IFN production by inhibiting IRF3/7 and NF-kB activation. Furthermore, *P. gingivalis* blocks IFN receptor activity by proteolytically cleaving IFN receptors, making cells refractory to exogenous IFN stimulation, and also proteolytically degrading STAT1 making the cells susceptible to viral infection (Fig. 4). *P. gingivalis*-mediated degradation of IRF1 severely impairs antibacterial responses by shutting down IFN-gamma signaling and priming of innate immune effector responses. Dampened IFN contributes to diminsihed production of chemokines CXCL-9, 10, and 11, which regulate immune cell (T cells, NK cell, and macrophage) migration and can also regulate their differentiation and activation by binding to CXCR3 (Tokunaga et al. 2018).

(6) Barrier function

In addition to intracellular invasion, *P. gingivalis* can penetrate the gingival epithelium by a paracellular or intercellular route (Dickinson et al. 2011, Ji and Choi 2020, Zheng et al. 2021). This process is facilitated by disruption of epithelial barrier function as measured by transepithelial cell electrical resistance (TER) which is directly related to the integrity and function of the paracellular occluding barrier (Groeger and Meyle 2019). Alternatively, barrier function can be assessed microscopically using fluorescent beads of specific sizes, or other fluorescently-labeled molecules such as LPS or peptidoglycan. The gingipain proteases, in particular Kgp, are major effector molecules of barrier disruption and can degrade junctional adhesion molecule (JAM1), coxsackievirus and adenovirus receptor (CXADR a JAM family protein), occludin, E-cadherin, and β1-integrin (Katz et al. 2000, Katz et al. 2002, Takeuchi et al. 2019, Takeuchi et al. 2021). Gingival epithelial cell-cell connections may also be damaged by the LPS of *P. gingivalis* which can trigger caspase-1 activation and pyroptosis (Li et al. 2021). LPS and *P. gingivalis* cells can inhibit expression of the GRHL2 transcription factor which in turn reduces levels of adherens junction and tight junction proteins (Chen et al. 2019).

Host matrix metalloproteinase enzymes (MMPs), which degrade extracellular matrix and basement components are important in the regulation of tissue homeostasis and barrier function. *Porphyromonas gingivalis* can increase production of several MMPs, including 1, 2, 7, 9, and 10, from oral epithelial cells (Inaba et al. 2014, Ha et al. 2015, Sztukowska et al. 2016, Lee et al. 2017). Moreover, *P. gingivalis* gingipains stimulate proteinase-activated receptors 2 and 4, which increases signaling through ERK 1/2-Ets1, p38/HSP27, and NF-κB pathways, consequently elevating MMP-9 proenzyme (pro-MMP-9) expression (Inaba et al. 2014, Inaba et al. 2015). Activation of pro-MMP-9 then occurs by gingipain processing.

Barrier function is also regulated epigenetically by *P. gingivalis*. Challenge with *P. gingivalis* was found to increase methylation of genes encoding CDH1, PKP2, and TJP1 and reduce their expression (Barros et al. 2020). TJP1 (ZO-1) is an adaptor protein in tight junctions; CDH1 (E-cadherin) comprises adherens junctions; and PKP2 (plakophilin 2) is part of the desmosomes. Thus, epigenetic mechanisms compromise major components of epithelial barrier function.

Once in the tissues, intercellular *P. gingivalis* becomes a more accessible target for the innate immune system (Hajishengallis 2015). We shall confine our comments here to antimicrobial peptides (AMPs), small cationic molecules with broad-spectrum antimicrobial activity which play an important role in periodontal tissues (McCrudden et al. 2013) and in controlling the total bacterial load (Wang et al. 2015). *Porphyromonas gingivalis* appears to be substantially resistant to human beta-defensins, which are produced primarily by epithelial cells (Shelburne et al. 2005), likely due to degradation by gingipains. Similarly, gingipains can degrade LL-37, a member of the cathelicidin family of AMPs (Gutner et al. 2009), and additionally, OmpA homologs of *P. gingivalis* also confer resistance to LL-37 action (Horie et al. 2018). *Porphyromonas gingivalis* can suppress the production of LL-37 from GECs by the induction of IL-33, which inhibits transcription of the *LL-37* gene (Tada et al. 2017). Conversely, calprotectin, a S100 calcium binding protein, can protect epithelial cells in culture against binding and invasion by *P. gingivalis* (Nisapakultorn et al. 2001).

The periodontal microbiome

While most research has focused on *P. gingivalis*, other periodontal organisms similarly engage epithelial cells in an intricate dialog. *Fusobacterium nucleatum* can invade GECs, albeit at lower levels than *P. gingivalis*, at least in vitro (Han et al. 2000), and moreover *P. gingivalis* can suppress invasion of *F. nucleatum* in polymicrobial infections (Jung et al. 2017). Increased epithelial cell proliferation through the Ku70/p53 pathway can result from *F. nucleatum* infection. *Fusobacterium nucleatum* has the ability to induce nuclear localization of NF-κB in GECs while increasing secretion of IL-1β via activation of the NLRP3 inflammasome and caspase 1 (Bui et al. 2016). Release of endogenous secondary DAMPs such as ASC and HMGB1, further amplifies inflammation. p38 is also activated by *F. nucleatum*, which leads to increased secretion of MMP-9 and MMP-13 (Uitto et al. 2005, Groeger et al. 2022). Human beta-defensins are produced in oral epithelial cells in response FAD-I interactions with TLR1/2 and TLR2/6 (Bhattacharyya et al. 2016).

Fusobacterium nucleatum also provides an instructive example of a more recently recognized homeostatic mechanism involving small RNAs. The organism triggers exosome-mediated release of host transfer RNA–derived small RNAs (tsRNAs), tsRNA-000794 and tsRNA-020498, by oral epithelial cells (He et al. 2018). Both tsRNAs can inhibit the growth of *F. nucleatum* by interfering with protein synthesis.

Aggregatibacter actinomycetemcomitans can invade epithelial cells, although many studies have utilized HeLa cells which may not provide an accurate readout of the invasive potential of the organism. The trimeric autotransporter protein ApiA is involved in both binding and internalization (Asakawa et al. 2003, Cugini et al. 2018), as is Omp29 which can remodel the F-actin cytoskeleton through FAK-RhoA signaling (Kajiya et al. 2011). On the epithelial membrane, integrin α5 may be a receptor for ApiA, and initiate a signaling cascade which reduces cell adhesion by decreasing the expression of integrins (Kochi et al. 2017). A proinflammatory phenotype is induced in part through ApiA and Omp29, which can in-

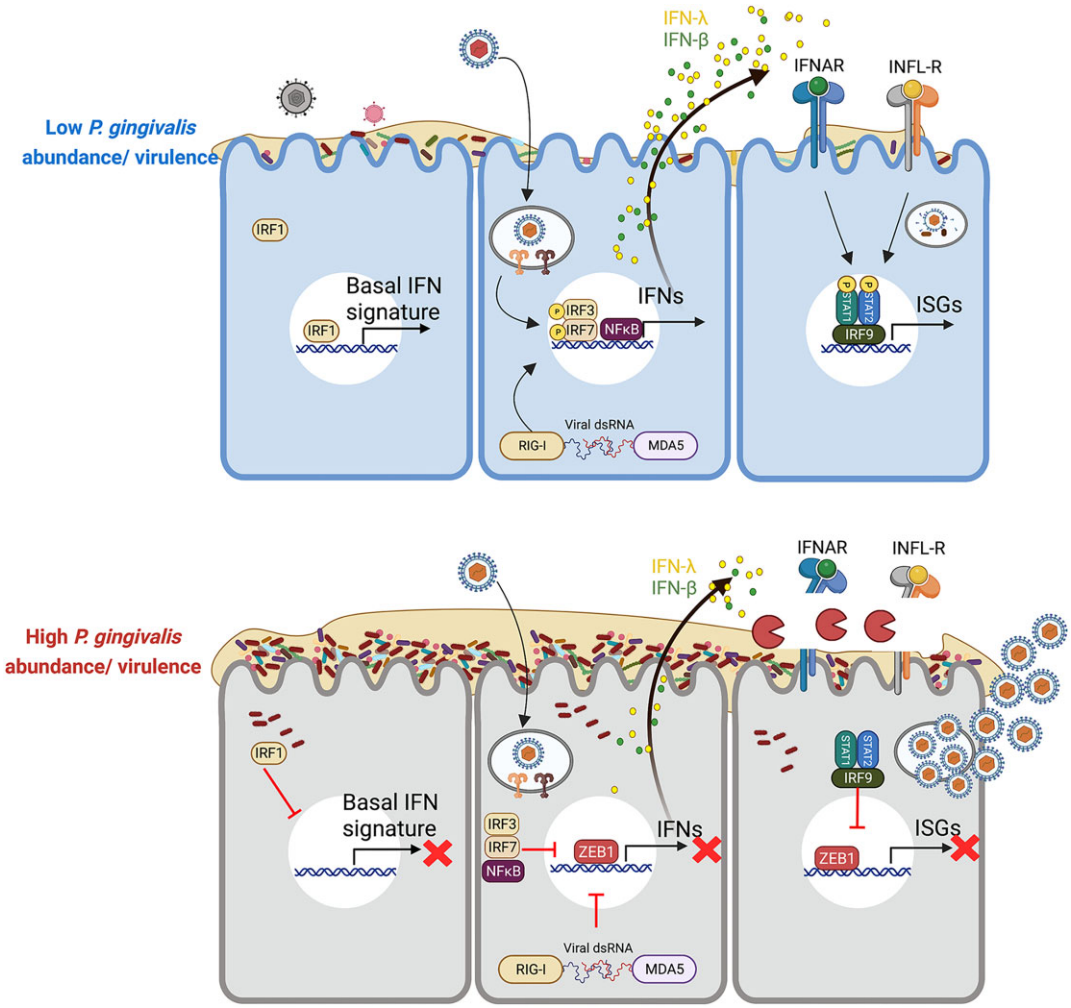

Figure 4. *P. gingivalis* infection induces interferon paralysis in GECs: IRF1 maintains basal or tonic expression of ISGs in GECs. During viral infection, GECs preferentially induce Type III IFNs (IFN-lambda) and, to a lower extent, Type I IFN (alpha/beta), which signals via cognate receptors to amplify ISG expression. Upon binding to IFN, IFNs receptors phosphorylate STAT1, STAT2, and IRF9 (known as IFN stimulate gene factor 3 complex, ISG3), which translocates to the nucleus and binds to the IFN stimulated response element (ISRE) to induce transcription of multiple ISGs. ISGs inhibit viral replication and reinforce antiviral immune responses. *Porphyromonas gingivalis* (Pg) uses multiple strategies to subvert epithelial antiviral immunity. Pg proteolytically degrades IRF1 resulting in a compromised basal ISG signature. Pg infection also inactivates PRR-induced IFN production by (i) inhibiting NF-kB phosphorylation and nuclear translocation; (ii) transcriptional downregulation of IRF3/7; and (iii) upregulation of ZEB1, which represses the IFN-lamba promoter. *Pg* derived proteases, gingipains, cleave IFN receptors, shutting down ISG3 activity and inducible ISG expression.

crease expression of IL-6 and IL-8 from GECs (Asakawa et al. 2003, Kishimoto et al. 2008, Umeda et al. 2012, Ando-Suguimoto et al. 2020). However, Omp29 has also been reported to inhibit IL-8 production (da Silva et al. 2022), indicating that a complex relationship exists between GECs and *A. actinomycetemcomitans* which may be strain dependent. Indeed, strains of *A. actinomycetemcomitans* can differ with respect leukotoxin production and the expression of fimbriae which are lost upon laboratory subculture (Scannapieco et al. 1987, Rosan et al. 1988, He et al. 1999).

Unlike *P. gingivalis*, *A. actinomycetemcomitans* can stimulate GEC apoptosis through Omp29 binding to fibronectin (Fn), which activates Fn/integrinβ1/FAK signalling-dependent TGF-β release from the ECM, thereby inducing apoptosis via a TGF-βR/Smad2 pathway (Yoshimoto et al. 2016). Another mechanism through which *A. actinomycetemcomitans* can induce apoptosis in GECs is through the ATM-checkpoint kinase 2 pathway, which is engaged by the cytolethal distending toxin (Cdt) (Alaoui-El-Azher et al. 2010). Cdt primarily triggers DNA damage with resultant growth arrest at the G2/M interphase of the cell cycle which will further contribute to breakdown of the gingival epithelial barrier by *A. actinomycetemcomitans*, an effect exacerbated by the ability of Cdt to disrupt cell junctions (DiRienzo 2014).

Treponema denticola has the ability to invade into and survive within GECs, which is dependent of activity of dentilisin, a chymotrypsin-like surface protease (Inagaki et al. 2016). In periodontal ligament cells (PDLs), dentilisin can induce RAS4 upregulation resulting in an increase in MMP-2 expression and activity which can cause actin reorganization (Malone et al. 2021). Intracellular *T. denticola* can survive intracellularly for long periods of time by resisting endolysosomal degradation (Shin and Choi 2012). As a highly motile spirochete, *T. denticola* can penetrate gingival multilayers by dentilisin-mediated degradation of tight junctions (Ellen et al. 2000, Lux et al. 2001, Chi et al. 2003). Additionally, an RNA-Seq study of GEC responses to *T. denticola* infection revealed robust induction of MMPs, with ECM remodeling one of the most enriched gene ontology terms, including elevated expression of MMPs-1, -2, -3, -9, -10, -12, -13, and -28 (Hinson et al. 2022). Similarly, gene ontology analysis of *T. denticola*-infected human PDLs

revealed significant dentilisin triggered up-regulation of ECM reorganization, degradation of collagen and ECM, and ECM-receptor interactions (Ganther et al. 2021). *T. denticola*-mediated activation of TLR2 and MyD88 promoted activation and nuclear translocation of the Sp1 transcriptional regulator, and led to elevated expression of MMP-2, -14, -17, and -28 (Ganther et al. 2021). RNA-Seq interrogation also found that the IL-1 superfamily member, IL-36γ, was the most differentially expressed cytokine (Hinson et al. 2022). Stimulation of TLR2/6 but not TLR2/1 or TLR4 led to RelA-dependent regulation of IL-36γ gene expression. MAPK and PI3K/Akt activation also regulated IL-36γ expression. IL-36 cytokines are thought to contribute to periodontal destruction (Cloitre et al. 2019), possibly by acting as an alarmin which induces proinflammatory signaling in a MyD88-dependent manner (Swindell et al. 2018). Interestingly, *P. gingivalis* can also increase expression of IL-36γ in epithelial cells through upregulation of IRF6 expression (Huynh et al. 2016). IL-36γ may therefore function as a critical inducer and amplifier of gingival inflammation and subsequent alveolar bone loss. *T. denticola* can also exhibit targeted stealth properties with regard to neutrophil chemotaxis and immune activation, as not only can dentilisin degrade IL-8, but challenge of GECs with *T. denticola* does not result in secretion of IL-8 or IL-6, and the cells do not produce ICAM-1 (Brissette et al. 2008).

Fusobacterium nucleatum and colorectal cancer

Fusobacterium nucleatum, while normally found in the oral cavity and generally a core member of the oral microbiome with high abundance in both periodontal health and disease, can colonize secondary sites and is associated with pathologies such as inflammatory bowel disease and adverse pregnancy outcomes. *Fusobacterium nucleatum* is also an emerging oncomicrobe that is highly associated with colorectal cancer (CRC) and, to a lesser extent, breast cancer, head and neck cancers, and esophageal cancer (Groeger et al. 2022). The association of *F. nucleatum* with CRC has attracted considerable interest in recent years and hence we shall summarize this emerging field.

The FadA adhesin, which is necessary for attachment and subsequent invasion of epithelial cells by *F. nucleatum* (Xu et al. 2007), may play a major role in CRC by binding to E-cadherin on CRC cells, activating β-catenin signaling and thus regulating inflammatory and oncogenic responses (Rubinstein et al. 2013). The FadA-E-cadherin axis also increases production of annexin A1, which regulates Wnt/β-catenin-based proliferation (Rubinstein et al. 2019). Localization of *F. nucleatum* in the gastrointestinal tract may depend on another fusobacterial adhesin, Fap2, which binds to Gal-GalNac, abundant on CRC cell surfaces (Abed et al. 2016). Fap2 can also immunosuppress tumor-infiltrating lymphocytes, by binding and activating the inhibitory immunoreceptor TIGIT, which is expressed by T and natural killer cells (Gur et al. 2015). Additionally, *F. nucleatum* activates the human inhibitory receptor CEACAM1, which also suppresses the activities of T and natural killer cells (Gur et al. 2019).

Host transcriptome and epigenomic analyses have revealed that *F. nucleatum* downregulates expression of METTL3, the major N6-methyladenosine (m^6A) methyltransferase, leading to significantly fewer m^6A modifications in CRC cells and promoting CRC aggressiveness (Chen et al. 2022). *Fusobacterium nucleatum* interactions with CRC cells induce Hippo/YAP signaling, inhibiting FOXD3 transcription factor expression, and influencing METTL3 expression which promotes KIF26b mRNA stability, a driver of *F. nucleatum*-induced metastasis (Chen et al. 2022). *Fusobacterium nucleatum* also impacts epigenetic gene regulation by repressing the expression of numerous chromatin-modifying enzymes (Despins et al. 2021). Gene ontology analysis demonstrated significant regulation of histone 3 methylation, histone acetylation, along with regulation of transcription factor binding and activity in primary human epithelial and endothelial cells. Genes commonly induced in CRC (EFNA1 and LIF) were significantly induced during *F. nucleatum* infection, as were genes related immune migration and inflammatory processes (Despins et al. 2021).

Another means by which *F. nucleatum* can promote CRC metastasis is by upregulating the expression of the lncRNA EVADR (Lu et al. 2022). EVADR acts as a modular scaffold for the Y-box binding protein 1 (YBX1) to increase translation of EMT transcription factors, such as Snail, Slug, and Zeb1.

Heterotypic communities of organisms

While challenge of epithelial cells with singles species has revealed much about cellular microbiology, in vivo epithelial cells are usually faced with a polymicrobial community. To address this issue, models have been developed in which large numbers of organisms assembled into a community interact with epithelial cells simultaneously. Multispecies communities were found to downregulate desmosomal junction proteins in multilayers of gingival epithelial cells, and interestingly this effect was independent of the presence of potential pathogens such as *P. gingivalis*, *Tannerella forsythia* or *T. denticola* (Belibasakis et al. 2015). Production of IL-8 in this model system is also increased; however, this effect is enhanced when the pathogens are present (Belibasakis et al. 2013). Similarly, epithelial monolayers increase production of IL-1β, IL-6, IL-8, TNF, and MMP-8 when challenged with dysbiotic biofilms containing higher levels of *A. actinomycetemcomitans, P. gingivalis*, and *P. intermedia* (Herrero et al. 2018). However, such responses are likely to be highly context dependent as Shang et al. (2019) reported that biofilms containing higher proportions of pathogens stimulated weaker immune responses from reconstituted human gingiva compared to communities associated with health.

Interactions among organisms can have a significant impact on epithelial cell responses. For example, when oxygen is available *S. gordonii* produces hydrogen peroxide as a metabolic byproduct. A reducing environment is required for activity of *P. gingivalis* gingipains, in order to maintain the cysteine in the catalytic domain, and hence, peroxide produced by *S. gordonii* inactivates gingipains and antagonizes activation of Notch signaling by *P. gingivalis* (Fitzsimonds et al. 2021). *S. gordonii* can also antagonize *P. gingivalis* indirectly, using epithelial cells as an intermediary. *S. gordonii* challenge of GECs activates the TAK1-NLK pathway, which prevents *P. gingivalis*-mediated dephosphorylation of FOXO1, and FOXO1-mediated induction of ZEB2 gene expression (Ohshima et al. 2019). In this context, therefore, *S. gordonii* can act as a homeostatic commensal mitigating the protumorigenic effect of *P. gingivalis* on epithelial cells. Indeed, a comparison of global transcriptional responses to *P. gingivalis* and *S. gordonii* together, compared to each organism individually, shows that *S. gordonii* blocks the action of *P. gingivalis* to a significant degree. Hence, one role for oral commensals may be to maintain homeostatic inflammation and impede dysbiotic epithelial cell responses to pathogens. However, community dependent roles for the vast majority of species considered commensal remain to be investigated.

Bacterial interactions with epithelial cells observed *in vivo*

The collective outcome of a wide range of in vitro studies paints a picture of *P. gingivalis* as adept at survival in intra- and intercellular locations. The relevance of these studies is supported by in vivo observations. The localization of bacteria in tissue derived from the periodontium can be revealed by a variety of techniques including bacteria-specific staining, in situ hybridization, immunohistochemistry and RNA Scope (Ji and Choi 2020, Lee et al. 2020, Rodriguez-Hernandez et al. 2021). *P. gingivalis*, along with other important periodontal organisms such as *T. denticola*, *T. forsythia*, *F. nucleatum*, *Filifactor alocis*, and *A. actinomycetemcomitans* can be found intracellularly in the junctional and sulcular epithelium, and extracellularly in inflamed areas of the epithelium and lamina propia. Moreover, fluorescent in situ hybridization (FISH) with 16S specific probes has shown that both *P. gingivalis* and *F. alocis*, which can co-localize, are metabolically active in tissues and thus likely to contribute to tissue destruction (Lee et al. 2020). Interestingly, IL-8 concentrations are lower in the gingival crevicular fluid of patients with chronic or aggressive periodontitis, compared to healthy controls (Mathur et al. 1996, Chung et al. 1997, Ozmeric et al. 1998, Jin et al. 2000), supporting the relevance of the chemokine paralysis properties of *P. gingivalis* identified in vitro.

Conclusions

The oral epithelial barrier is continuously challenged by an abundant and complex community of microorganisms which have co-evolved over a longstanding association. Epithelial cell-microbe interactions, i.e. cellular microbiology, thus define, to a large extent, the homeostatic or dysbiotic state of the gingival tissues. Interestingly, despite the intensive microbial challenge, gingival health, involving a mild and tightly controlled gingival inflammation, is the most common outcome. Disease, as defined by tissue destruction, will only ensue if there is disruption of homeostatic inflammatory responses, usually precipitated by the action of a subset of organisms that exert an influence disproportionate to their number, i.e. the keystone pathogens. Comparison of epithelial responses associated with health/disease or commensals/pathogens can provide insights into the molecular events that define these states; however, the complexity arising from multiple levels of host cell regulation and multidimensional interactions among oral bacteria renders such interpretations challenging. Nonetheless, significant advances in the spatial relationship between host and microbe (adherence, invasion, intracellular trafficking), signal transduction systems activated in both microbes and host cells, and the transcriptional landscape underlying the interplay, provide a strong foundation for such aspirations. As technology for simultaneous RNA-Seq of microbe and host cells (dual RNA-Seq), high throughput proteomics, and high-resolution imaging of live cells becomes more facile, increased precision in unraveling health or disease-associated outcomes can be anticipated. Moreover, machine learning approaches can help deconvolute signal from noise, particularly with regard to the identification of biomarkers of disease.

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