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Natural and induced immune responses in oral cavity and saliva

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Abstract

This review comprehensively explores the intricate immune responses within the oral cavity, emphasizing the pivotal role of saliva in maintaining both oral and systemic health. Saliva, a complex biofluid, functions as a dynamic barrier against pathogens, housing diverse cellular components including epithelial cells, neutrophils, monocytes, dendritic cells, and lymphocytes, which collectively contribute to robust innate and adaptive immune responses. It acts as a physical and immunological barrier, providing the first line of defense against pathogens. The multifaceted protective mechanisms of salivary proteins, cytokines, and immunoglobulins, particularly secretory IgA (SIgA), are elucidated. We explore the natural and induced immune responses in saliva, focusing on its cellular and molecular composition. In addition to saliva, we highlight the significance of a serum-like fluid, the gingival crevicular fluid (GCF), in periodontal health and disease, and its potential as a diagnostic tool. Additionally, the review delves into the impact of diseases such as periodontitis, oral cancer, type 2 diabetes, and lupus on salivary immune responses, highlighting the potential of saliva as a non-invasive diagnostic tool for both oral and systemic conditions. We describe how oral tissue and the biofluid responds to diseases, including considerations to periodontal tissue health and in disease periodontitis. By examining the interplay between oral and systemic health through the oral-systemic axis, this review underscores the significance of salivary immune mechanisms in overall well-being and disease pathogenesis, emphasizing the importance of salivary mechanisms across the body.

Keywords Saliva, Immunity, Barriers, Host response, Microbiome, Host-microbial interactions

Background

The oral mucosa, the lining of the oral cavity, stands as a critical interface between the body's internal environment and the external world. It serves as a dynamic barrier, constantly challenged by a diverse array of microbes, food particles, and environmental insults. Saliva, a complex biofluid secreted by the salivary glands, plays a pivotal role in maintaining the integrity of this barrier and orchestrating the intricate balance between host defense and microbial tolerance.

Saliva's composition is a testament to its multifaceted functions. Beyond its primary constituents of water and electrolytes, saliva contains a rich repertoire of proteins, enzymes, mucins, and immunoglobulins that contribute

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to its lubricating, digestive, and antimicrobial properties. The constant flow of saliva not only facilitates the mechanical clearance of debris and microbes but also regulates the oral pH, buffering against the acidic byproducts of bacterial metabolism. This buffering capacity is essential for maintaining a healthy oral microbiome and preventing the demineralization of tooth enamel, a precursor to dental caries.

The oral cavity harbors a diverse and dynamic microbial community, collectively known as the oral microbiome. This intricate ecosystem plays a crucial role in both oral and systemic health. A balanced microbiome contributes to digestion, nutrient metabolism, and colonization resistance against opportunistic pathogens. However, disruptions in the microbial balance, often triggered by dietary habits, oral hygiene practices, or systemic diseases, can lead to dysbiosis. This imbalance can manifest as oral infections, periodontal disease, and even systemic complications.

Saliva serves as a critical mediator of host-microbial interactions in the oral cavity. Its array of antimicrobial peptides, such as lysozyme, lactoferrin, and histatins, provides a first line of defense against invading pathogens. Additionally, salivary immunoglobulins, particularly secretory IgA (SIgA), play a crucial role in neutralizing toxins, agglutinating bacteria, and preventing their adherence to mucosal surfaces. The interplay between these salivary components and the oral microbiome is essential for maintaining a healthy equilibrium. Clearly, the mechanisms by which saliva responds to disease are not well known in periodontitis, oral cancer, mucositis as illustrative examples. We will analyze the salivary changes associated with these conditions and discuss the potential of saliva as a diagnostic tool. Additionally, we explore the interplay between oral and systemic health by examining the oral-systemic axes.

We review intricate immune mechanisms within saliva, exploring its diverse cellular and molecular constituents and their roles in maintaining oral homeostasis and combating disease. We will examine the various cell types found in saliva, including epithelial cells, neutrophils, monocytes, dendritic cells, and lymphocytes, and discuss their individual contributions to both innate and adaptive immunity. We will further elucidate the protective functions of salivary proteins, cytokines, and immunoglobulins, emphasizing their interactions with the oral microbiome and their responses to various disease states.

By providing a comprehensive overview of the natural and induced immune responses in the oral cavity and saliva, this review aims to shed light on the critical role of saliva in maintaining oral and systemic health. Understanding the molecular mechanisms for developing innovative strategies to prevent and treat oral diseases, harness the diagnostic potential of saliva, and ultimately improve human health.

Saliva composition and function is regulated by immune cells

Saliva and the epithelial layer

Saliva acts as the lubrication of the mouth and interacts with every surface of the oral cavity. The mucous membrane is what lines the surfaces of the oral cavity and must fulfill a variety of different functions, from acting as an immune and physical barrier to maintain homeostasis. To properly function, the mucosal tissue has a high epithelial cell turnover which causes layers of epithelial cells to constantly shed into saliva. This constant shedding is what makes epithelial cells the primary type of cell found in saliva. Stratified squamous epithelial cells make up about 55–60% of all cell content found in saliva [1–3]. However, the number of epithelial cells in the host's saliva can vary based on the host's demographics [3]. The majority of epithelial cells found in saliva are non-keratinous, consisting of about 80% of the total epithelial cell content. This fraction accounts for the many non-keratinous surfaces within the oral cavity such as the inner cheeks and the floor of the mouth. The remaining content of the epithelial population is shed by the keratinous surfaces of the hard palate and parts of the gingiva. Lastly, about 1% of epithelial cell content are characterized as intermediate cells found below the superficial layer of the epithelium [2].

The main function of these cells is to prevent the initial stages of invasion of the oral tissues and thereby offer protection at a systemic level from invading pathogens. As the epithelium is shed, an average of about 100 individual bacterial organisms are removed from the surface of the oral cavity per epithelial cell [4]. Once in saliva, the pathogens attached to epithelial cells can be eliminated by gastric acid, after swallowing, or by the broad innate immune system within saliva [5]. In this way, saliva acts as the medium or vehicle in which pathogens attached to epithelial cells can be expelled or eliminated from the oral cavity.

Predominant innate immune cells protect oral mucosa

Polymorphonuclear neutrophils

Polymorphonuclear neutrophils (PMNs) are the second most common cell type, and the most abundant leukocyte in saliva acting as the primary cell of the innate immune system within the oral cavity. Neutrophils account for about 30–40% of the total cell count in saliva and make up about 95–98% of the total leukocyte population [1, 6]. Since saliva acts as a medium during the first step of eliminating pathogens and preventing infections, neutrophils act as the first point of contact and swiftly produce an immune response. In the presence of

pathogens, neutrophils can undergo various morphological changes to induce an innate immune response such as phagocytosis, degranulation, reactive oxygen species (ROS) production, and neutrophil extracellular traps (NETs). An ex vivo study found that in healthy participants the oral neutrophil population holds a larger percentage of late stage apoptotic/necrotic neutrophils than those found in venous blood at ~50% in comparison to ~10% of total neutrophil population, respectively. In the same study the isolated oral neutrophils were found to be far more active in comparison to neutrophils in circulating blood when stimulated by *F. nucleatum*, producing higher expression values for CD11b, CD63, CD66b, and ROS levels [7]. This suggests oral neutrophils are far more prepared to produce a rapid and robust inflammatory response in the presence of a pathogen which may lead to a stronger antimicrobial effect accompanied with tissue damage. However, their inflammatory responses can become dysregulated leading to pathogenic effects as seen with rheumatoid arthritis and systemic lupus erythematosus [8].

In healthy participants, flow cytometry and computational methods have determined sub-populations of neutrophils showing varying sizes and granularity, along with differing expression levels of CD markers, ROS production, and NET formation [9]. Two major and distinct populations of neutrophils were found in healthy saliva where they primarily differ in level of immune activity. The less active population has reduced expression levels for CD55 and CD63, but an increase in CD170 and CD16 when compared to the more active neutrophil population. The more active sub-population is responsible for most of the phagocytic, ROS production, and NET formation produced by saliva in the healthy oral cavity [10]. However, other studies have been able to determine 8 sub-populations using scRNAseq, showing high variation in the neutrophil population to account for many of the possible pathogens and immune system needs that may exist in saliva. In diseases, such as chronic periodontitis, there is a larger sub-population of inflammatory neutrophils, showing elevated levels of CD63, CD66, CD10, CD64, CD55, CD11b, and CD18, than in healthy counterparts. In addition, NETosis markers citrullinated histone H3 (CitH3) and myeloperoxidase (MPO) are found to be elevated in this population of neutrophils. Baseline measurements of ROS production in disease-state neutrophils are significantly higher than both sub-populations of neutrophils found in healthy. This overproduction of ROS and higher citH3 and MPO are signs of an over-inflammatory and dysregulated neutrophil activity that leads to excessive tissue damage [11, 12]. However, in an in *vitro* study inducing ROS production with phorbol myristate acetate (PMA), the more active population of neutrophils in healthy produce higher levels of ROS than in disease [10]. This could be an indication that neutrophil ROS production in the disease state could become exhausted and further disease progression or increase the susceptibility of the oral cavity to new pathogens.

Monocytes

Within saliva, $CD14^+$ monocytes account for about 1.3% of the total leukocyte population [1, 6]. Monocytes and their differentiated populations, such as macrophages and dendritic cells, originate from areas in the oral cavity with high populations of these cell types such as the salivary glands. The population of macrophages can be separated by their activated state termed as M1 and M2 where the shape, function, and some surface markers are unique to each state [13]. However, it is worth noting that these two profiles of macrophages are an oversimplification of the actual activated macrophage population. Within the subset of M1 or M2 macrophages there can exist vast differences and overlapping properties of their immunophenotype [14]. Nonetheless, these two categorizations of macrophages are used to determine whether the tissue environment leans toward a pro- or anti-inflammatory macrophage profile. The activation of macrophages into either the pro-inflammatory M1 state or anti-inflammatory M2 state depends on the location and environment of where the macrophage resides. M1 macrophages are considered to be "classically activated" by offering increased phagocytic activity, greater antigen-presenting capacity, and increased release of proinflammatory cytokines such as TNF-α and IL- 6 [15]. An in vitro study found that sterile human saliva influenced the activation of murine macrophages into an M1 state by detecting IL- 12 and IL- 6 expression [16]. This supports the idea that saliva promotes an environment that is pro-inflammatory to create an innate immune response that can handle constant exposure to pathogens. In cases of chronic periodontitis, the ratio of M1/M2 is much higher than what is found in healthy participants [17, 18]. This indicates that M1 macrophages may play a role in either the pathogenesis or the development of periodontitis.

On the other hand, M2 macrophages are involved in producing an anti-inflammatory response that also positively influences tissue repair. For example, one murine study found that bone formation is dependent on the production of Cystatin C by M2 macrophages [19]. Another murine study found that injecting induced M2 macrophages into sites of inflammation caused by periodontitis actually suppressed both the excessive immune response and inhibited the differentiation of osteoclasts [20]. However, it is worth noting that the majority of the studies involving the use of M2 macrophages to dampen

the immune response in periodontitis are done in mice. Nonetheless, the importance of the ratio between M1/M2 macrophages is apparent, and further research in this area is required.

Oral dendritic cells (DCs)

DCs play an important role in bridging the innate response to the adaptive immune response. Their role falls into recognizing circulating antigen to produce an immune response that activates the downstream adaptive immune system. Dendritic cells can be organized into two classes based on their function and location before and after maturation. Conventional DCs are characterized by their function of patrolling tissue environments for foreign antigens. When reaching an antigen, these DCs will migrate to draining lymph nodes to undergo a maturation process where pathogen-derived peptides are presented to $CD4^+$ and $CD8^+$ T cells. On the other hand, the second class of DCs are considered unconventional as they circulate the bloodstream and enter inflamed tissues after infection [21]. Once found in tissue, unconventional DCs will activate T cells by the production of interferon (IFN).

Although not found in high numbers in saliva, Langerhans Cells (LCs) play an important role in responding to circulating pathogens found in saliva. LCs are part of conventional DCs that are characterized by residing on the epithelial layer of skin or mucosal lining to respond to circulating pathogens, migrate to nearby lymph nodes, and then induce an adaptive immune response. Although LCs are found in most tissues with an epithelial layer such as the skin and intestinal mucosa, oral LCs have a unique profile that differentiates these from other LCs. For example, oral LCs have higher expression levels for IgG (FcγRIII/CD16 and FcγRI/CD64) receptors which may imply these cells respond to pathogens coated with IgG antibodies much faster [22]. This finding also implies oral LCs have enhanced phagocytic capabilities to capture and process antigens and become antigen-presenting cells (APCs) thereby increasing the chances to induce an adaptive immune response. Increased IgG receptors could be explained by the need to recognize pathogens in an environment like the oral cavity, where cells are constantly exposed to these foreign pathogens. In addition to these receptors, oral LCs also have increased expression levels of major histocompatibility complex (MHC) I and II along with costimulatory CD40, CD80/B7.1, and CD86/B7.2 when compared to other LCs [22]. The high expression of both MHCs indicate that oral LCs are far better equipped to form into APCs and activate both $CD4^+$ and $CD8^+$ T cells. However, the higher expression of the three costimulatory molecules could indicate that oral LCs will tend to favor a $CD4^+$ T cell environment. In an environment like the oral cavity that depends on B cell activity for a strong humoral response against pathogens floating in saliva, it would make sense for oral LCs to favor $CD4^+$ T cells. Ultimately, dendritic cells, such as Langerhans cells of the oral mucosal lining, indirectly help saliva produce a strong innate immune response by favoring an adaptive immune cell environment that produces high levels of immunoglobulins that are free floating in saliva.

The adaptive immune responses in saliva

Lymphocytes

T and B cells make up the adaptive immune system and respond with high specificity and sustained protection [23]. However, to achieve such a high level of specificity the adaptive immune system must first undergo a primary adaptive response from a first infection. During a first infection, the innate immune system interacts with a pathogen that eventually leads to the formation of APCs that will migrate to the nearest lymph node [24]. Within the lymph nodes, naive T cells will activate through the recognition of antigenic material presented by APCs and mature into $CD4^+$ or $CD8^+$ T cells that in-turn begin the adaptive immune response and return to the site of infection. Since the adaptive immune response largely depends on antigen uptake and processing, lymphocytes have a slower reaction time to a pathogen than the immune cells of the innate immune system during a primary infection. This may explain the large differences in cell populations observed between innate immune cells and lymphocytes within mucosal barriers serving as the first line of defense, like the oral cavity. The population of lymphocytes can be generally categorized into $CD45^+CD3^+$ T cells and $CD45^+CD20^+$ B cells and flow cytometry studies have found cells at about 0.8% and 0.5% of the total leukocyte population, respectively [6]. The population of lymphocytes can be further subset based on their function. The activation of $CD8^+$ T cells, also known as Cytotoxic T cells (CTLs), begins the cell-mediated immune response to target infected cells and induce apoptosis. However, their role in oral disease progression is shown to be minimal when compared to other lymphocytes[cite study here]. Although these cells may not play a large role in the immune response of the oral cavity, some studies have shown $CD8^+$ T cells contribute towards the suppression of both inflammatory responses and bone loss by osteoclastogenesis [25, 26]. On the other hand, $CD4^+$ T cells, also known as T helper cells (Th-cells), drive the downstream process to activate the humoral response and begin the production of high-affinity and pathogen-specific antibodies. However, the subsets of Th cells can have drastic differences in their role and function and are primarily differentiated based on the surrounding environment. Some of the important Th cells in saliva, or the oral cavity as a whole, include Th1, Th2, and Treg cells.

Th1 cells are involved in the bone loss characterized in chronic periodontitis by stimulating osteoclasts to reabsorb bone matter [27]. Th2 and Treg cells have a stronger role in the regulation of the immune response and its inflammatory effects in the affected tissue including the inhibition of bone loss in periodontitis [28].

B cell activation at the presence of an antigen begins the humoral response responsible for the high specificity and affinity of the adaptive immune system towards a recognized and targeted pathogen. Naive B cells can activate to become memory B cells and plasma cells which will lead to the production of antibodies. B cell activation, in the presence of APCs and mediated by Th cells, induces the proliferation and differentiation into plasma cells. In turn, these short-lived plasma cells respond by producing antibodies, such as IgA, that are secreted outside of the tissue and into saliva [29]. On the other hand, memory B cells do not immediately produce some response, but instead act as the reservoir of humoral responses to the same pathogen by proliferating into plasma cells in subsequent infections. In cases of chronic or aggressive periodontitis, the saliva levels of the B cell activating factor (BAFF) become elevated when compared to the healthy control [30]. This could be an indication that B cells play some type of role in the progression of periodontitis. Additionally, B cells also play a prominent role in autoimmune diseases such as Sjogren's syndrome. Part of the characterization of primary Sjogren's syndrome is the increase of $CD27^+$ memory B cells into the salivary glands where downstream effects eventually lead to the production of autoantibodies thereby damaging the surrounding tissue [31]. In healthy tissue, B cells exhibit tolerance towards auto-antigens, but if this tolerance is exacerbated autoimmune diseases can arise. Although the level of lymphocytes is small in saliva and the oral cavity, these cells play an important role in providing an immune response with high specificity and affinity to invading pathogens and any subsequent infections. Additionally, changes in the population ratios of these cells could serve as strong indicators for disease in the oral cavity.

The oral immune system tolerance

The healthy oral cavity is constantly introduced to foreign antigens, and yet the immune system can tailor its response to address insults from pathogenic microorganisms while remaining tolerant to non-pathogenic bacteria and food. This is, in part, explained by the complex interplay and communication between oral epithelial cells (OECs), tolerogenic DCs, and Treg $CD4^+$ cells found in the oral cavity. Based on the integrity of the cell barrier, epithelial cells will send molecular signals to circulating DCs to either maintain them in a tolerogenic state or induce a proinflammatory response [32]. An in vitro study found that OECs will signal DCs to maintain a tolerogenic state and decrease the frequency of CD80 and CD86 used to activate the downstream proliferation of T cells. Additionally, in the presence of OECs, DCs were found to have decreased production of inflammatory cytokines such as TNF-α and IL- 12. This same study suggests that OECs can dampen $CD4^+$ T cell activity when in close proximity, as seen by the suppression of IFN-γ and TNF-α when $CD4^+$ T cells are incubated with OECs [33]. In a subsequent study by the same researchers, they found that OEC's also directly inhibit inflammatory T cell activity even after activation through DC's or anti-CD3/CD28. The research group determined that T cell expression of inflammatory markers, T-bet and IFNγ, was largely controlled by blocking the synthesis of prostaglandin E2 (PGE2) or its binding ability to EP2/EP4 receptors [34]. In addition, other immune cell interactions using CD40/CD40L, CD58/CD2, and PD-L1/PD- 1 were not involved in mediating T cell activity. These findings suggest that PGE2 is the primary chemical mediator between OEC's and T cells for the suppression of inflammatory activity, but only in a healthy oral environment. In a later section of this review, we further discuss the role of molecules like PGE2 in periodontitis and how the detection of a viral insult blocks T cell suppression by OEC. These findings highlight the complex communication between OECs, immune cells, and the local microbial environment.

DCs also play a role in properly responding to the external stimuli through the detection of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) using pattern-recognition receptors (PPRs). In the presence of PAMPs, dendritic cells, as such other innate immune cells, can recognize molecular signatures of pathogens and bacteria. This mechanism allows dendritic cells to recognize local tissue damage and adequately respond to external stimuli. As previously stated, as part of their response, DCs are involved in the activation of T cells of a specific phenotype. In the presence of non-harmful antigens, DCs can activate the pathway to produce mature T reg cells which in turn limit the proliferation of inflammatory T cells. Studies have found that the ligation of Toll-like receptors (TLRs) in dendritic cells, such as TLR4 in oral Langerhan cells, after coming in contact with commensal bacteria will induce an anti-inflammatory environment. In this scenario, LCs release IL- 10 while also inducing the production of IL- 10, TGF-β1, FOXP3, IFN-γ, and IL- 2 in T cells [35]. Additionally, another paper found that DCs of the oral cavity, after stimulation with an antigen such as ovalbumin, will induce $CD4^+$ T cells with anti-inflammatory markers such as IL- 10 and IFN-γ [36]. In the same study, researchers were able to show an increase in ovalbumin specific Th1 cells after multiple rounds of sublingual immunotherapy [36]. Ultimately, DCs have a

unique role of properly responding to circulating stimuli, whether pathogenic or not, to determine what type of signal should be sent to downstream effectors of the immune system.

Research on the topic of immune cell tolerance has to be studied more in the oral mucosa since this plays a pivotal role in ensuring an adequate defense against new foreign organisms constantly introduced in the mouth. However, mechanisms of immune tolerance can also be observed and studied in other mucosal tissue where host cells constantly interact with microbes. For example, epithelial cells of the gut will signal to circulating immune cells about a pathogenic insult to elicit an immune response [37]. Another study found that in the presence of *Salmonella*, intestinal epithelial cells had higher levels of gene expression involved in anti-bacterial pathways [38]. Research on the tolerance of the immune system and its mediators should be an area of research where more focus should be placed. This could help answer questions as to why patients have different levels of an immune response when introduced to the same pathogenic insult.

Salivary immune molecules

Oral immunoglobulins

Antibodies, also known as immunoglobulins (Ig), are a method of protection utilized by our immune systems to eliminate foreign pathogens. Immunoglobulins can vary in type, structure, and function. While all these antibodies are produced by B cells differentiated into plasma cells, some can take different forms in different parts of the body to carry out various protective and immune activities. The most studied and abundant immunoglobulin isotype in our bodies is immunoglobulin G (IgG), which is produced, primarily, in the secondary immune response to pathogens and can activate the complement system [39]. With 4 subclasses, IgG provides a wide range of protection from within the systemic immune pathways, as IgG makes up around 80% of the immunoglobulins found circulating in the bloodstream. Immunoglobulin M (IgM) has a pentameric structure, making it the largest of the immunoglobulins, and is mostly produced in the primary immune response to infectious agents and antigens [39], allowing for an initial response while other antibodies are produced over time. In mucosal surfaces, immunoglobulin A (IgA) is the predominant isotype at 90% (Fig. 1). It is locally produced in a dimeric composition from plasma cells and, as it is secreted through the epithelium into the mucosa, gains a protective secretory component (SC) to create its known form, secretory IgA (SIgA). The added SC contributes to stability of dimeric IgA, as monomeric forms are connected with a heavy J chain, as well as contributing to its increased ability to competitively inhibit binding of pathogens [40]. SIgA comes into two forms, SIgA1 and SIgA2, which differ slightly in structure where SIgA2 has a shorter hinge, resulting in a difference in planarity [41]. The most relevant consequence of this structural difference is predicted to be differences in antigen interaction at the Fab regions, with SIgA1 binding to various antigens in a variety of orientations and SIgA2's more rigid, planar structure allows for its binding on antigens that are on relatively fixed surfaces [42]. In saliva, SIgA contributes to more than 80% of the salivary IgA pool and concentrations from unstimulated whole saliva stand at ~0.19 mg/ml, compared to serum where IgA concentration is ~2.2 mg/ml [43]. SIgA2 is, overall, found to be at the same concentrations, if not greater, than SIgA1 although, the levels of SIgA1 versus SIgA2 vary across secretion and mucosal sites, indicating selective advantages to one subclass for specific immune mechanisms [42]. Its prevalence at these mucosal sites has much to do with SIgA's immune response potential and the natural defense mechanisms the antibody has, due to its structure.

Secretory IgA in saliva

The secretory IgA is largely utilized in physically protective ways such as "coating, cross-linking, agglutination, and enchained growth of mucosal antigens" [44]. The cross-linking capabilities are important in fluids like saliva because it allows the SIgA to act as a physical barrier before pathogens can even reach the tissues of the body. Specifically secreted from epithelial cells within the salivary glands, thymic stromal lymphopoietin (TSLP), aids in host defense due to its antimicrobial properties. Existing in a long form (lfTSL) and a short form (sfTSLP), researchers have found that both forms have antimicrobial functions, likely due to its C-terminal that can disrupt cell membranes. In healthy control participants, sfTSLP is dominant and produced constitutively, with detection of lfTSLP significantly increasing over sfTSLP in inflammatory conditions [45, 46]. A variety of cytokines with a variety of functions, from immune cell signaling to specific microbe clearing abilities, are present in saliva and all contribute to the oral cavity's overall immune abilities. A method called agglutination is when IgA will bind to pathogenic particles, or antigens, using both of its binding sites, effectively clumping particles together, which prevents adherence to tissue surfaces, such as oral epithelium or teeth, and creates a large target for immune cells to come and destroy. This all happens without any signaling involved and is a product of the structure and binding abilities of the isotype. SIgA can also bind to any pathogenic toxins or enzymes that are released from a pathogen, effectively neutralizing those secreted toxins. These physical means of eliminating pathogenic threats by cross-linking or binding is known as immune exclusion as the IgA is not actively setting out to eliminate/

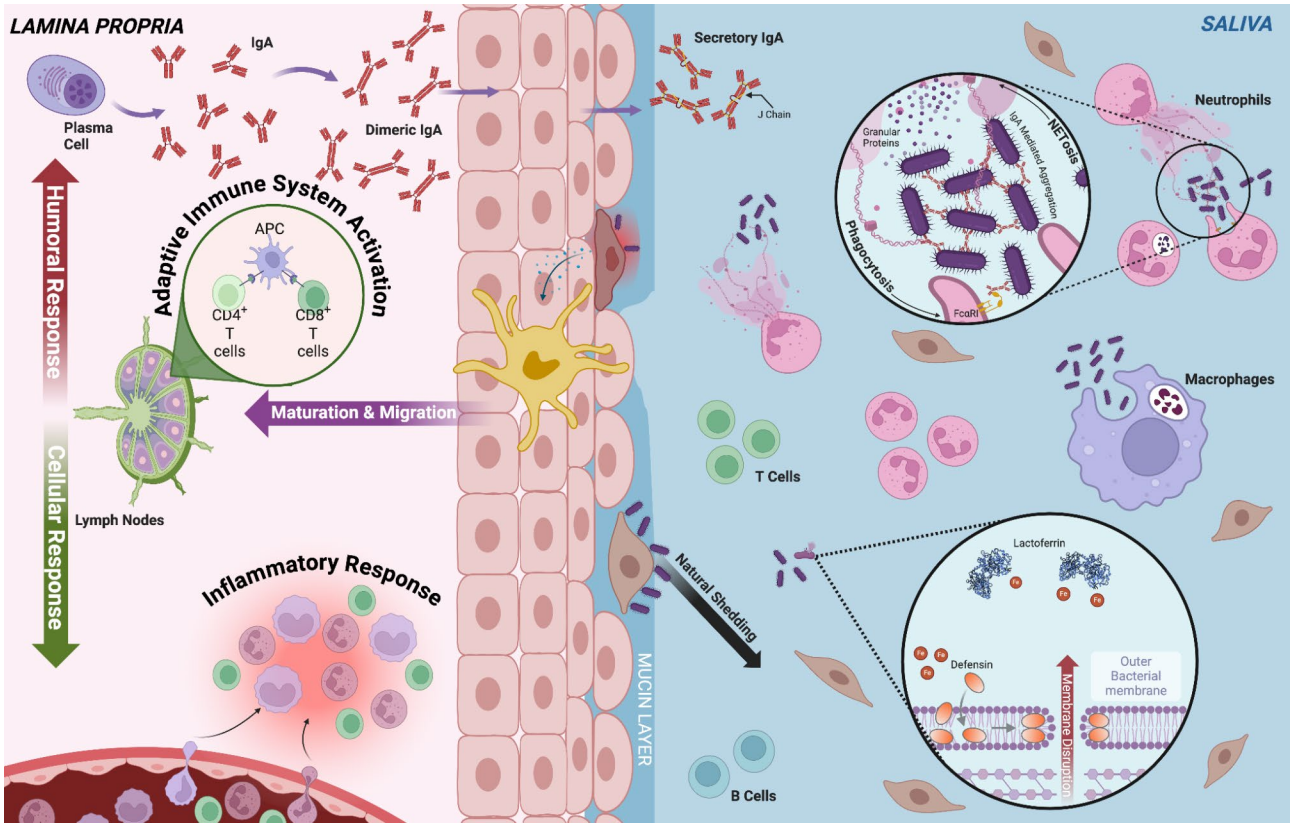

Fig. 1 Salivary and Mucosal Barriers and Responses. Free-floating cells and proteins found within saliva will prevent microbes from disrupting the mucosal epithelial layer. At the moment of infection or cell damage within the epithelial layer, the targeted cell will send signals out to dendritic cells that will then migrate to the lymph nodes and mature in the form of APCs. These APC's will then activate subsets of T cells ($CD8^+$ or $CD4^+$) responsible for the activation of the humoral or cellular response which can either alleviate infections or exacerbate inflammation

kill pathogens, but rather preventing them from operating within our bodies. However, SIgA is not alone in immune defense and can also help present antigens to immune cells to communicate which particles are pathogenic and should be targeted by fellow immune cells. SIgA has specific effector mediators that exist on many different immune cells such as macrophages, neutrophils, eosinophils, and monocytes that, when the mediators are activated, can trigger elimination processes in the forms of phagocytosis, cytokine release, or antibody dependent cell-mediated cytotoxicity [41]. The FcαRI receptor found on various immune effector cells is where SIgA will bind to signal inflammatory cytokines or inflammatory degranulation. Pathogen recognition by SIgA will alter the structure of the immunoglobulin to increase binding to the FcαRI host cell receptor [47]. Considering the duality of the defense mechanism methodology, SIgA would fall into the innate and acquired immune system categories, producing a robust physical barrier defense as well as recruiting and directing immune cells to carry out their own defensive mechanisms.

The role of SIgA healthy vs. disease

With SIgA as the first barrier within the oral cavity, it stands as a primary physical line of defense against pathogens entering the bloodstream where serum IgA is found to have a monomeric composition and is associated more with signaling inflammatory reactions [48]. There are observed differences in the glycosylation of these different IgAs [43], illustrating that they circulate within two distinct immune pathways and can use them to detect different kinds of diseases, mainly those where pathogens infiltrate via mucosal pathways. Salivary IgA cannot be used as a proxy for serum IgA, however, it can be used distinctly to identify infection and disease where serum IgA is less impactful in immune response. As a response to pathogen invasion in the oral cavity, or disease originated from the oral or other bodily areas, SIgA production increases to combat these foreign invaders, compared to a healthy person, from which there is no need for an elevated immune response. An evaluation of IgA from saliva compared to serum, from patients who were infected with SARS-CoV- 2, illustrated a higher salivary IgA response in acute infection phase compared to healthy levels [49]. Another study had examined IgA

levels from saliva in patients infected with SARS-CoV-2 compared to non-infected patients, finding a three-fold higher response in infected patients (13.8 AU/mL) against healthy (4.2 AU/mL) [50]. These studies were able to utilize enzyme-linked immunosorbent assays (ELISAs) for a highly customizable and specific method of measuring IgA. In these studies, SARS-CoV- 2 specific antigens were used, to which IgA showed high binding affinity, proving the efficacy of IgA against pathogenic particles. Bio-fluids other than saliva can be and were evaluated. While blood holds a higher concentration of antibodies, saliva is partial to IgA dominance and can elicit a more specific defense. In paired serum and saliva samples, patients in the convalescent phase of COVID- 19 had a more robust response to SARS-CoV- 2 S1, S2, and Nucleoprotein in their saliva compared to their paired serum ($p < 0.0001$, $p = 0.0036$, and $p = 0.0009$, respectively) [49]. This antibody specificity and difference is indicative of the functional pathways in which salivary IgA operates.

The cytokine responses in oral fluids

Cytokines are signaling proteins that play an important role when mounting any kind of immune response. Secreted mainly by immune cells, cytokines of all different types and functions direct cellular immune response and regulate inflammation. Immune cells such as macrophages, neutrophils, lymphocytes, and others release cytokines as well as cells in various tissues throughout the body, such as epithelial cells and cells within connective tissues. This allows for high interconnectivity throughout the body with cytokines being able to bind locally, affecting cells at an infection or damage site, and distally, traveling through the bloodstream to bind to a specific target receptor. Specific kinds of cytokines can have different functions; chemokines are responsible for directing immune cells towards sites of infection, interferons signal cells to put up cellular defenses against pathogen invasion, tumor necrosis factors (TNF) help to regulate inflammation and can signal to immune cells that kill tumor cells, interleukins stimulate communication and coordination between WBCs, and colony-stimulating factors signal hematopoietic stem cells to develop into specific immune cell types.

These mediators play a significant role in regulating the inflammatory response to invaders and tissue damage. Inflammation is an immune system defense mechanism that initiates immune cell recruitment to the site of invasion, induces fever, and increases vascular permeability. Regulating and knowing when to inhibit inflammation is just as important as stimulating the immune system because, if left unchecked, inflammatory mechanisms can result in tissue damage. The potential for cell signaling is almost limitless. With so many different groups and factors, various origins and target effector cells, differing and overlapping functions, beneficial inflammation walks a very fine line. As signaling markers, cytokines, and other markers, are in constant communication to either produce a higher inflammatory response or to inhibit induction and start tissue repair. Some cytokines can fall into either a pro-inflammatory or anti-inflammatory category, or both. The cytokine families of interleukin-1 (IL- 1) and interleukin- 6 (IL- 6) can be categorized as pro-inflammatory, while IL- 1 receptor antagonist (IL- 1RA), IL- 4, and IL- 10 can be characterized as anti-inflammatory, suppressing and dampening inflammation signaling and overall immune response [51]. In inflammation or diseased states, detection of cytokines often increases as the immune system mounts a defense and these signaling molecules begin to differentiate T cells, thus involving a cascade of increased inflammation and recruitment of immune cells to sites of infection. Cytokines are major factors in modulating this response and when it reaches the point of chronic inflammation or prolonged disease, the balance shifts to where inflammatory inhibitors are no longer being produced to dampen or counteract the inflammation damage.

Functional signaling proteins in saliva

The pathways involving these signaling molecules are complex and interconnected but are being increasingly studied in research to see specific mechanisms of inflammation when different diseases or infections manifest. As T lymphocytes play a major role in adaptive immunity, the types of Th cells that cytokines release from and act upon can be examined at heightened specificity. For example, the lymphocytes that develop into Th1 cells produce vast amounts of inflammatory cytokines such as IL-2, IFN-γ, and TNF-α, lending to a cell-mediated immune response while Th2 cells produce IL- 4, IL- 5, IL- 10, and IL- 13, lending to B lymphocyte antibody responses [52, 53]. These maturation pathways are regulated by IL- 6 and IL- 4 [53, 54], creating a somewhat circular feedback loop that is carefully mediated by the anti-inflammatory mechanisms of IL- 10 and IL- 13. The tenuous cyclic relationship involving all these factors becomes faulty and imbalanced in cases of chronic inflammation. There are various immunoassays to test for the presence and concentrations of a variety of different cytokines, 45 of which can be found categorized and briefly summarized in Table 1. Many studies evaluate cytokine levels in infectious diseases (Human Herpesvirus- 6 and Epstein Barr Virus [55]), oral diseases (caries [56], gingival inflammation [57], and periodontitis [58]), and autoimmune diseases (Sjogren's Syndrome [59]) to find, in many cases, increased concentrations of cytokines in diseased patients over controls. This increased detection in diseased patients can have diagnostic potential for

Table 1 Cytokines and their general main functions for immunity and inflammation. List of cytokines tested from saliva based on cellular mechanism pathways influenced on or by the cytokine. Pathways include Th1/Th2 cellular maturation and effects, Th9/Th17/Th22/Treg cellular maturation and effects, inflammatory cytokines, chemokines, and growth factors. Includes descriptions of their general functions for immunity and inflammation. Many of these analytes are detectable in saliva, especially when testing in diseased patients

	Biological Functions	Presence in Saliva
Th1/Th2	Relating to the stimulation $CD4^+$ cells to mature into Th1 or Th2 cells; or downstream effects of their activation [60]	
GM-CSF	Hematopoietic growth factor and immune modulator; proangiogenic, proinflammatory cytokines; produced by variety of cell types and tissue types, induced by IL- 1, IL- 6, and TNF-α [61, 62]	Yes
IFN-γ	Effector molecule in macrophage and inflammatory activation, important in inherent immunity, specifically tumor control, viral infection, and intracellular bacteria [63]	Yes
IL- 1β	Pro-inflammatory cytokine released upon activation of inflammasome; Immune cell recruitment and activation, modulates adaptive immunity [61, 64]	Yes
IL- 2	Non-specific antigen proliferative factor for T lymphocytes, regulating inflammation and tumors; during inflammation, secretes pro-inflammatory cytokines IL- 1, TNF-α, and TNF-β [65, 66]	Yes
IL- 4	Immunomodulatory cytokine in adaptive immunity; activates B and T cells, humoral immune response, and reduces pathological inflammation [63]	Yes
IL- 5	Driver of maturation and maintenance of eosinophils and antibody secreting B cells; implication in mast cells of asthmatic airways-lung and saliva axis [67, 68]	Yes
IL- 6	Pleiotropic cytokine influencing antigen-specific immune responses and pro-inflammatory reactions [61, 67]	Yes
IL- 8	Pro-inflammatory cytokine regulating PMN activity, inducing chemotaxis, cellular shape change respiratory burst, and inducing adhesion of PMN to endothelial cells [69, 70]	Yes
IL- 12p70	Cytokine produced by immune cells in response to antigenic stimulation; stimulates growth and function of T cells; associated with autoimmune and inflammatory conditions [66, 71]	Yes
IL- 13	Anti-inflammatory cytokine implicated in muscle metabolism mediation, cancer invasion, and progression of paracrine and autocrine signaling in tumor microenvironments [72]	Yes
IL- 18	Proinflammatory cytokine that regulates autoimmune and inflammatory diseases; signals to CD4 T cells; also implicated in generating and activating Th17 cells [73]	Yes
TNF-α	Inflammatory cytokine that activates endothelial and fibroblast cell, leading to upregulation of adhesion molecules; upregulation is implicated in periodontitis [61, 74]	Yes
Th9/Th17/Th22/Treg	Relating to the stimulation $CD4^+$ cells to mature into Th9, Th17, Th22 or Treg cells; or downstream effects of their activation [75]	
IL- 9	Pleiotropic cytokine with pro-allergic inflammation capabilities, immunity mechanisms against extracellular parasites [76]	Yes
IL- 10	Anti-inflammatory cytokine released to suppress activation of IL- 6, IL- 1, and TNF-α [77]	Yes
IL- 17 A (CTLA- 8)	Pro-inflammatory cytokine to activate B cells and macrophages for antibody and more pro-inflammatory signaling; activate various non-immune cells to produce antimicrobials; mediates occurrence/development of chronic immune-mediated inflammatory diseases [78, 79]	Yes
IL- 21	Pleiotropic pro-inflammatory cytokine targeting broad range of immune cells and exaggerates host-immune response; suppresses Th2 differentiation/development and anti-inflammatory IL- 13 [80]	Yes
IL- 22	Regulates mucosal responses to invasion and damage, promoting tissue repair and supporting epithelial growth and health [81]	Yes
IL- 23	Important for Th17 cell survival and maintenance; acts as bridge between nonspecific and specific immunity [82]	Yes
IL- 27	Potentially pro- and anti-inflammatory; induces development of Th1 and T follicular helper (Tfh); inhibit differentiation of Th2 and Th17 cells; promote virus-specific CD4 T cells [83]	Yes
Inflammatory Cytokines	Cytokines involved in immune regulation and inflammation, either promoting or inhibiting inflammation [84]	
IFN-α	Antiviral cytokines produced in response to viral infection; can modulate genes encoded for proteins for inflammation, apoptosis, and other immune responses [85, 86]	No
IL- 1-α	Pro-inflammatory cytokine similar to IL- 1β but exists in homeostatic environments preformed; can act as an alarmin, dual function intra and extracellularly to promote inflammation [87, 88]	Epithelial and endothelial cells, parotid saliva

Table 1 (continued)

	Biological Functions	Presence in Saliva
IL- 1RA	Anti-inflammatory by competitive inhibition with IL- 1 onto the IL- 1 receptors [88, 89]	Yes
IL- 7	Non-hematopoietic-derived cytokine that plays an essential role in supporting normal T cell development and homeostasis; upregulation enhances effector T cell response and is associated with autoimmune disorders [90]	Yes
IL- 15	Induces proliferation of natural killer cells and cytotoxicity, participation in early innate immune cytokine response [91]	Yes
IL- 31	Pleiotropic cytokine mainly implicated in pruritus and other itchy skin conditions; also implicated in pathogenesis of allergic inflammation in the airways [92, 93]	Oral mucosa
TNF-ß	Pro- and anti-inflammatory capabilities; initiating early immune response but can inhibit proliferation and cytokine production functions, even promote apoptosis [94, 95]	Yes
Chemokines	Chemokines involved mainly in migration of cells and maintaining homeostasis of the immune system [96]	
Eotaxin (CCL11)	Protein to stimulate eosinophils and recruit them to inflammatory sites; implicated in CNS [97]	No
GRO-α (CXCL1)	Chemotactic for neutrophils, angiogenesis in cutaneous wound healing, oral keratinocytes proliferation and migration increase with stimulation from other cytokines [98]	Yes
IP- 10 (CXCL10)	Stimulated by cytokine upregulation, specifically IFN-γ; induces variety of effects specifically in cell growth and development (tumor regulation, promotion, and angiostatins) [99]	Yes
MCP- 1 (CCL2)	Monocyte chemoattractant protein upregulated in inflammatory conditions; relational with other MCPs and MIPs to influence type and magnitude of immune response [100]	Yes
MIP- 1-α(CCL3)	Chemotactic secreted by macrophages to recruit inflammatory cells, promote wound healing, inhibition of stem cells, and maintains effector immune response; important in bone resorption [101]	Yes
MIP- 1-ß (CCL4)	Lymphocyte attractants, augmenters for T lymphocyte adhesion to VCAM- 1, induce monocyte chemotaxis, calcium mobilization, and exocytosis; immature dendritic attractants [102]	Yes
RANTES (CCL5)	CC-chemokine for migration and metastasis of human cancer cells (oral); contribute to neuronal function and metabolic disorders [103]	Yes
SDF- 1-α	Produced by fibroblasts, responsible for bone marrow progenitor cell movement and transendothelial leukocyte migration; tissue remodeling; implicated in periodontitis and neutrophil migration [104]	Yes
Growth Factors	Secreted, biologically active molecules affecting cell growth, mitosis, or differentiation [105]	
BDNF	In neurotrophins family; can induce cellular apoptosis in pro-form and cell development, differentiation, survival, and plasticity in mature form [106]	Variable
EGF	Epidermal growth factor for epithelial regeneration after physical and chemical aggression [107]	Yes
FGF- 2	Fibroblast growth factor is a neurotrophic protein associated in protection against depression, anxiety, and stress [108]	Yes
HGF	Endogenous growth substance produced by all cells of mesenchymal origin, important in regulating embryogenesis, morphogenesis, and mitogenesis; ability to repair and regenerate damaged tissues [109]	Yes
NGF-β	Nerve growth factor majorly produced by cells in oral tissues, secreted into saliva, for oral wound healing [110]	Yes
PDGF-BB	Exogenous platelet-derived GF that mediates epithelial-mesenchymal interactions; inflammation and tissue injury causes stimulation of proliferation and migration of certain cells to mucosal fibroblasts [111]	Yes
PIGF- 1	Placental GF within vascular endothelial GF family; supports growth and differentiation of trophoblasts, released by many cells upon stimulation and closely connected to placental or maternal biological activity [112]	Yes
SCF	Salivary conditioning film forms on all surfaces of mouth, acting as interface between components of the mouth (tongue, oral mucosa, enamel); plays role in lubricating oral cavity [113]	Yes
VEGF-A	Vascular endothelial GF that is crucial for maintenance of mucous membrane homeostasis and can accelerate wound healing in oral cavity; induces angiogenesis, increases microvascular permeability [114, 115]	Yes
VEGF-D	Can be secreted into saliva, regulates lymphangiogenesis; can bind to receptors on lymphatic endothelial cells and stimulate their proliferation and migration [116]	Yes

some diseases, but, conversely, there seems to be a lack of association between increased cytokines and specific symptoms. The complexity of the pathway mechanisms requires more specific research for diagnostic power, but there is definitive evidence for the importance of cytokines in understanding how diseases are progressed and how the threshold from acute infection or disease could lead to something more chronic or autoimmune.

Salivary cytokines

Pathways of inflammation are controlled by salivary cytokines and their imbalance causing an excessive response, potentially leading to tissue damage, opening possibilities of therapeutics to target these imbalanced pro-inflammatory cytokines. Looking into salivary cytokines reveals interesting relationships between pro- and anti-inflammatory cytokines, as well as other signaling factors, in systemic diseases such as HIV or tuberculosis, oral and dental diseases such as periodontitis or gingivitis, and oral and neck cancers [61]. Periodontitis is an inflammatory disease of the periodontal oral region and is initiated and progressed by pathogenic oral microbiota changes and the resulting immune response [117]. Similar to SIgA, cytokines can be produced locally from oral epithelial cells and resident immune cells [77], marking distinct pathways from blood cytokines. While there is still communication and potential cross-signaling to and from the blood, which suggests some relation between cytokines, local cytokine production in the oral cavity leads to differences in recorded cytokine levels between blood and saliva. In saliva, compared to blood, there is a greater amount of secreted cytokines in response to various diseases or pathogenic invasions. A study comparing blood vs. saliva cytokines in older adults found high correlation between plasma IFN-γ and salivary TNF-α, IL-12p70, IL- 2, and IL- 10 [77], illustrating some methods of cytokine communication/signaling.

Lactoferrin

Lactoferrin (Lf) is an antibacterial and antiviral glycosylated protein that is secreted by exocrine glands and neutrophils in sites of infection or inflammation [118]. Depending on the saturation of iron, Lf can either be regarded as apo-lactoferrin or holo-lactoferrin when devoid of iron or fully saturated with iron, respectively. However, Lf has many anti-bacterial and anti-viral properties that are independent from its ability to sequester iron ions. For example, human Lf was found to inhibit the short term growth of *P. gingivalis* and *P. intermedia* by either apo- or holo-lactoferrin suggesting that the mechanisms at play don't involve iron and the chelating ability of Lf [119]. These properties can be explained by two mechanistic pathways. The first increases the permeability of the bacterial cell membrane causing the release of cell contents and directly impeding bacterial function [120]. The second mechanism of Lf will detach external nutrients needed for bacterial survival from the bacterial wall through a process called hydrolysis [120]. Additionally, some of the antiviral properties of Lf are independent from its ability to chelate iron such as its ability to prevent the adsorption of HCMV [121]. Nonetheless, many of its functions as a key player in the innate immune system are dependent on its ability to bind to ions of irons. Lactoferrin competes with bacteria's ability to accept free floating iron needed for growth or the production of biofilm. If the affinity for iron is greater in Lf than the bacterial receptors for capturing iron then Lf will produce bacteriostatic properties [122]. Lf also plays a role in promoting an immune response. Lactoferrin can increase the activity of natural killer (NK) cells, as well as promoting phagocytosis in neutrophils and the activation of macrophages [123]. Lactoferrin can also control the production of proinflammatory cytokines, TNF-α, IL- 6, and IL- 1β, depending on the local environment [123]. Due to the antibacterial and antiviral properties of lactoferrin, many papers have found success in the oral administration of Lf to prevent the progression of an infection [124, 125].

Mucins

Mucins are another important salivary protein that gives saliva its important function in aiding the innate immune response. Multiple mucins exist in the body, but the mucins found within the oral cavity are MUC5B, MUC7, MUC19, MUC1, and MUC4 and can have various functions such as giving a gel-like consistency to saliva, antimicrobial properties, and aiding in cell signal transduction [126]. Mucins that give saliva its gel-like consistency create a physical barrier that microbes must first pass before reaching any mucosal tissue. *Streptococcus sanguinis*, *S. sobrinus*, and *S. oralis* have all shown to bind to MUC7 showing there are receptors within mucins that may interact with microbes [127]. Additionally, MUC7 was shown to have direct antifungal properties against *C. albicans* and tested at various conditions to model the variation in pH, salt, and temperature of the mouth [128]. These findings could indicate that mucins may have a role in aggregating bacteria for easy clearance by swallowing or coughing while preventing microbes from coming in contact with the epithelial wall. However, many pathogens have ways of dealing with the mucus layer alone to gain access to tissue [129]. Therefore, mucins must also serve as a vehicle for other antimicrobial proteins and effectors of the innate immune system to deal with microbes. MUC7 and MUC5B are two mucins that are heavily involved in enhancing the antimicrobial properties of saliva. For example, MUC5B has shown to interact with antibacterial and antiviral proteins to prolong their

availability within saliva by protecting these proteins from proteolytic degradation [130]. These findings show the importance of mucins to act as the delivery system in saliva.

Defensins

The family of defensins are another set of proteins in saliva with antimicrobial and antiviral properties. These small peptides are cationic with multiple cysteine residues forming three types of defensins (α, β, and γ) based on the pattern of the peptide chain and their disulfide bridges [131]. α-defensin- 1 (human neutrophil peptide 1) is stored in granules of neutrophils and secreted during neutrophilic apoptotic processes for both bactericidal properties and the regulation of inflammatory responses by other immune cells [132, 133]. Additionally, α-defensins have been found to have antiviral properties by inactivating HSV types 1 and 2, cytomegalovirus, vesicular stomatitis virus, and influenza virus A [134]. In SARS-CoV, defensins such as α-HD5 and HD6 can interfere with ACE2 and prevent 2019-nCoV from binding to the receptor making defensins very popular as a treatment option [135]. However, beta-defensins (hBDs) are the primary effectors of the innate immune response within saliva. hBDs are produced by epithelial cells in the oral cavity as a response to bacteria or inflammatory cytokines. The antimicrobial function of hBDs can be described by their unique ability to quickly kill bacteria by intercalating into the outer membrane to create holes in which contents of the cell are expelled [136]. hBD- 3 has antimicrobial function against both negative-gram and positive-gram bacteria, while hBD- 1 and hBD- 2 only have functions against negative-gram bacteria. Additionally, hBD- 1 and hBD- 3 have shown to have antifungal properties against *C. albicans in vitro* [137]. Within saliva, hBD- 1 and hBD- 2 can be found in saliva at concentrations of 150 ng/mL and have shown to synergistically work with other antimicrobial peptides [138].

Histatins

Histatins are another family of salivary peptides that primarily reside within saliva while exhibiting antimicrobial properties. Histatin- 1, histatin- 3, and histatin- 5 are the primary histatins that are found within human parotid saliva at concentrations of 2.5 μM, 1 μM, and 4 μM, respectively [139]. Histatin- 5 is produced from histatin-3 after post-translational modifications and is considered to be the most chemically active of the three. The mode of antimicrobial action for these histatins has been largely disputed on whether this peptide directly alters the cell membrane or its uptake disrupts cellular metabolism subsequently leading to cell rupture [140]. Nonetheless, this protein is capable of causing the destabilization of cell membranes, based on its electrostatic charge, leading to cell death. Antimicrobial interactions between histatins can be observed in gram-positive and gram-negative bacteria along with yeast, fungi and parasites [139]. Such an example is observed in *C. albicans* where the cell walls appear fragile after cells are treated with histatin- 5 [139]. However, it is worth noting that the rate at which histatin- 5 kills off *C. albicans* is much slower than when compared to other antimicrobial proteins. Another paper found that Histatin- 5 could be advantageously used as a therapeutic against oral candidosis in immunocompromised patients [141]. In addition to histatin's role in preventing infections, these peptides are also capable of playing a role in wound healing. Histatins, particularly histatin- 1, have been found to induce the migration of epithelial cells to seal off wounds in vitro, not just within the oral mucosa but also in skin [142]. Another report found that the use of histatin- 5 also promotes epithelial cell migration and MAPK/ERK pathways in vitro and corneal wound healing in murine models [143]. Lastly, due to histatin's role in antimicrobial and wound healing properties this protein is of great interest as a biomarker and therapeutic. One study found that the level of histatin- 5 can be used as a biomarker in the progression of childhood caries [144]. This has the potential to improve caries treatments and inhibit the progression of severe caries. Histatin- 5 has also been proposed as a promising candidate for a therapeutic gel that treats oral mucositis in patients undergoing cancer treatment with results showing improved tissue health along other benefits [145]. Histatins play an important role in maintaining the barrier of the oral cavity by preventing tissue infections while promoting a healing tissue environment.

Lysozymes

Another salivary protein with promising diagnostic use are lysozyme proteins that play a pivotal role in the defense against pathogens. Lysosomes are polypeptides of 130 amino acids and a positive charge found at concentrations that range between 0.5 and 4.0 ng/mL [146]. These proteins are produced by cells capable of undergoing phagocytosis such as macrophages and neutrophils [147, 148]. Due to the cationic structure of lysozymes, these proteins are capable of inserting themselves into negatively charged bacterial walls to form pores that disrupt the stability of the cell membrane. In addition, through a different mechanism, lysozymes can also hydrolyze the peptidoglycan cell membrane, extracellularly exposed in gram-positive bacteria, which contributes to the destabilization of the bacterial membrane leading to antimicrobial effects [149]. However, research on mechanisms of antibiotic resistant bacteria have found that some Gram-positive bacteria engulfed by phagocytic macrophages can evade autolysis through lysozymes that induce the β-lactam resistant L-form [150]. This

illustrates the complex relationship between agents of the innate oral immune system and the changing landscape of pathogens. It is also worth noting that lysozymes offer some protection against viral pathogens, such as SARS-CoV- 2, by reducing the cell entry capabilities of the virus, although the exact mechanisms are still unknown [151]. Research has also shown that lysozyme concentration in the oral cavity changes as the innate immune system becomes overloaded and, therefore, can be used as a diagnostic tool for the progression of caries, cancer, and hypertension among other diseases [152–155]. Additionally, depletion of lysozyme levels was found to have a strong correlation with common microbes, such as *Streptococcus*, *Prevotella*, *Haemophilus* and *Veillonella*, that form a dysregulated microbiota possibly caused by an exhausted and inflammatory innate immune response [156].

Defense mechanisms of the gingival barrier

Gingival crevicular fluid (GCF) in periodontal health and disease

In addition to saliva, gingival crevicular fluid (GCF) is an exudate that flows through the epithelial cells of the gingival sulcus, a concave space surrounding each tooth [157]. This fluid plays a critical role in periodontal health and disease, facilitating the transport of inflammatory mediators, immune cells, bacterial antigens, and enzymes of both host and bacterial origin. The GCF is closely tied to the subgingival biofilm, a dynamic microbial community that thrives in the anaerobic environment of the sulcus, protected from the cleaning action of saliva [158].

GCF composition and role in inflammation

The composition of GCF includes various biological markers, such as cytokines, enzymes (e.g., MMPs, collagenases), immune cells (primarily neutrophils), and inorganic ions. These components offer insights into the inflammatory status and the healing potential of the periodontium. In particular, the GCF serves as a valuable tool for assessing periodontal disease activity, as its composition reflects the tissue breakdown and immune responses within the periodontal pocket [159, 160].

GCF has emerged as a promising tool in the diagnosis of periodontal disease due to its ability to provide specific samples of biomarkers of inflammation and bone resorption directly at the site of the lesion. Among the most common biomarkers evaluated in GCF are IL- 1β, IL- 6, IL- 8, TNF-α, MMP- 8, MMP- 9, MMP- 13 and CRP, which are associated with active periodontal tissue destruction [161].

GCF, as a biopsy fluid sample, allows for more accurate diagnoses of the type and severity of periodontal disease, facilitating early detection of inflammation and potential bone destruction. Collecting GCF can help clinicians identify active inflamed sites, as well as predict disease progression by identifying early signs of periodontitis. This approach is especially valuable in the current context, where efforts are being made to optimize resources and make more rational and cost-effective treatment decisions.

Diagnostic value of GCF in periodontal disease

The flow of GCF and its composition can provide valuable diagnostic information regarding periodontal disease activity. Research has demonstrated a strong correlation between protease activity (such as collagenase) and clinical parameters, like pocket depth, which are commonly used to assess the severity of periodontal disease. As a non-invasive biomarker, GCF offers a promising avenue for monitoring disease progression and evaluating the effectiveness of periodontal treatments. In conclusion, GCF is a complex fluid that plays a crucial role in periodontal immunity and disease. Its composition reflects both the microbial and host responses in the periodontal pocket, making it a valuable tool in the diagnosis and management of periodontal disease. Future research into the molecular and cellular components of GCF will likely continue to expand its clinical applications [157].

Host-microbial challenge and the gingival epithelium barrier

Tooth eruption is the unique condition in which a hard tissue perforates the soft tissues, resulting in a disruption of epithelial continuity, jeopardizing the internal environment [162]. These gingival tissues are primarily designed to protect the host against oral microbial challenges [163]. The first tissue barrier against oral microbial invasion is the epithelial lining of gingival tissues. Basically, three different epithelial structures may be distinguished: the gingival epithelium (GE), the sulcular epithelium (SE) and the junctional epithelium (JE). GE and SE are derived from oral epithelium and JE is embryologically formed by the combining of the outer cells of enamel reduced epithelium and the basal cells of the oral epithelium after tooth eruption [162–165].

The GE is derived from the oral epithelium and is classified as a keratinized stratified squamous epithelium, containing all cell layers: basal (*stratum basale* or *germinativum*), prickle (*stratum spinosum*), granular (*stratum granulosum*) and keratinized (*stratum corneum*). This structure acts as a physical and immunological barrier against microbial challenge through different mechanisms, including increased proliferation rate, cell signaling, cell differentiation, apoptosis, and tissue homeostasis [162, 163], In addition to its role as a physical barrier, GE has a key role in the pathogenesis of periodontal diseases, since it represents one of the first defense mechanisms against microbial challenge. Keratinocytes are the

major cell component of GE, but other cell types exist, including melanocytes, Langerhans, and Merkel's cells [162–164].

It was a common thought that GE acted as a physical barrier to the invasion by microorganisms. However, more recent findings suggested that the host-microbe interaction is able to induce the synthesis of cytokines, adhesion molecules, growth factors, chemokines, and matrix metalloproteinases by GE cells. Under physiological conditions, GE allows fluxes of solutes and nutrients, collection of antigens and surveillance by mucosal immune cells [166].

Biofilms can modulate the epithelial cellular immune response based on their properties and composition [166]. Some keystone pathogens and pathobionts such as *Porphyromonas gingivalis* (*P. gingivalis)* can trigger innate and adaptive immune responses and disrupt the homeostatic state. The dysbiosis of the periodontal microbiota implies changes in the relative abundance of bacterial species compared to health, leading to alterations in the host-microbe interaction that result in inflammation and periodontal tissue destruction [167]. Once the epithelial barrier integrity is compromised by the biofilm, oral microorganisms may invade periodontal tissues and trigger an inflammatory response. Since that, cell-to-cell connections in the GE may be considered as a critical part of the innate immune response to resist oral microbiome challenge [166].

GE cells are predominantly keratinocytes, where progenitor lineage resides in the basal layer. As the keratinocyte moves from the basal to superficial layers, substantial changes in cell morphology and structure are observed, including the flattening of the cell and its nucleus, which can disappear (orthokeratinized epithelium) or be entrapped into the flattened cell and keratin layer (parakeratinized epithelium) [162–164].

A great number of different types of cell-to-cell junctions are observed in GE. Besides a 4 times greater number of desmosomes than in JE, tight (TJ), adherens (AJ) and gap (GJ) junctions are seen in GE Different signaling pathways that regulate cell differentiation, proliferation, and polarization in GE are coordinated by claudin and occludin, components of the TJ, and don't exist in JE or SE [166].

This epithelial barrier can be disrupted by oral microbes in different manners, including cleavage of endothelial cells, degradation of cellular signaling molecules, and inactivation of cellular functions related to healing, regeneration, and homeostasis of periodontal tissues. *P. gingivalis* was shown to interfere with the cell-to-matrix and cell-to-cell adhesion of oral keratinocytes, reducing its adhesion to the extracellular matrix and altering its morphology and motility. When challenged with *P. gingivalis* gingipains, cultured oral keratinocytes showed proteolysis of focal contact components, such as focal adhesion kinase, catenins and adhesion signaling molecules [168] *P. gingivalis* fimbriae type II enhance bacterial adhesion and invasion of epithelial cells, degrade focal adhesion kinase, inhibit cellular migration and induce the serum release of IL- 1β and IL- 6 [169].

In addition, the modulation of epithelial cells signal transduction pathways by *P. gingivalis* can affect gene encodings. IL- 8 is down-regulated by *P. gingivalis* even in the presence of other stimulatory organisms, such as *F. nucleatum.* In health, IL- 8 forms a gradient of expression that directs neutrophils toward sites of infection, protecting the host against bacteria and neutrophil-mediated tissue degradation. Low expression of IL- 8 is important to maintain gingival health. Inhibition of IL- 8 accumulation by *P. gingivalis* can impair innate host defense at microbe-epithelium interface and the host could no longer be able to detect bacteria and direct neutrophils for their removal [169].

Immunity from gingival cells

GE cells present TLRs, transmembrane proteins that recognize molecular structures classified as *pathogen associated molecular patterns* or PAMPs. TLRs play a central role in the recognition of invading pathogens and the subsequent activation of inflammatory and immune responses. The TLR family harbors an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic domain similar to IL- 1 receptor (IL- 1R). When stimulated, TLR recruits IL- 1R-associated protein kinases, activating nuclear factor-kappa B and mitogen-activated protein kinases. The response to TLR ligands varies, suggesting different signaling pathways [170]. In the intestinal epithelium, TLR1-TLR6 and TLR10 are expressed on the cell surface for recognition of extracellular microorganisms and ligands, while TLR3 and TLR7-TLR9 are intracellularly localized in the cytosolic endosomal compartment, binding microorganisms and ligands which passed the membrane of the host cell [171]. In the GE, TLR2 is highly expressed in the basal layer, and to a lesser extent, in the more superficial cell layers [166]. A mouse GE cell line (GE1) constitutively expresses TLR4 and TLR7. The stimulation of GE1 cells with CL075 induced cytokine, chemokine and antimicrobial peptide expressions and differed from the stimulation with LPS, with higher mRNA levels of IFN-β, CXCL10, and β-defensina 14 (analogous of human BD3), lower levels of TNF, CCL5, CCL11, CCL20, CXCL2, and CX3 CL1 [172].

In addition to TLRs, GE expresses NOD1 and NOD2, which are nucleotide-binding oligomerization domain receptors that bind to peptidoglycans present in bacterial cell walls [173]. TLR 2, TLR4, NOD1 and NOD2 are expressed in normal oral epithelium and gingival tissues [166, 173, 174]. These structures are also expressed

in oral, tongue, salivary gland, pharyngeal, esophageal, intestinal, cervical, breast, lung and kidney epithelial cells, in addition to TLR3 and TLR7 [166, 173]. When challenged in vitro with bacterial components, GE cells increase the expression of TLR2, TLR4, NOD1 and NOD2, without significantly affecting cytokines secretion, although PRPGs and beta-defensin 2 were significantly upregulated [173–175]. The higher expression of TLR2 and TLR4 is associated with the release of inflammatory mediators and worsening of periodontal tissues [173, 176–179].

Human β-defensins

Are a group of small antimicrobial cysteine-rich peptides against Gram-positive and Gram-negative bacteria, fungi, and viruses [180]. Two subfamilies are described: α-defensins (6 subtypes) and β-defensins (4 subtypes) [180–183]. Human β-defensins are expressed in gingiva, oral mucosa, GE keratinocytes, parotid gland, saliva, buccal mucosa, tongue, and GCF [138, 184–186]. While the expression of HβD- 1 is constitutional, HβD- 2 expression is stimulated by IL- 1 and LPS, suggesting that they play an important role in innate immune defenses against oral microorganisms [138, 184]. HβD- 3 was detected in 88% of gingival biopsies obtained from healthy and chronic periodontitis patients, confined to the GE. In health, HβD- 3 was detected more frequently in the basal layer when compared to diseased subjects (53% vs. 18%), while in chronic periodontitis HβD- 3 expression occurred from the basal to spinous layer (82%). In healthy tissues from diseased subjects, HβD- 3 expression was noticed in the basal and deep spinous layers, while in pocket areas the expression was found in the superficial spinous layer. In healthy and diseased patients, HβD- 3 was expressed by GE keratinocytes, Langerhans and Merkel cells. These findings suggested the role of HβD-3 in periodontal homeostasis through its antimicrobial activity and adaptive immune responses [187].

Disruption of the GE barrier may also be the result of excessive neutrophil elastase (NE) function. Although neutrophils are primarily involved in host responses against bacterial challenges, excessive activity may lead to the destruction of human tissues. NE is positively correlated to GCF flow rate and clinical attachment loss in periodontitis patients, but its underlying mechanisms remain unclear. For that reason, Hiyoshi et al. (2022) investigated how NE induces periodontitis severity and the role of NE inhibition in periodontitis treatment in ligature-induced periodontitis in mice. A greater neutrophil recruitment and NE activity was seen in the animals along with bone loss. The administration of an NE inhibitor significantly decreased NE activity in periodontal tissues, downregulated proinflammatory cytokines, and limited bone loss. NE cleaves cell adhesion molecules desmoglein 1, occludin and E-cadherin and induced exfoliation of epithelial keratinous layer in a 3-D in vitro model, suggesting that NE induces the disruption of the gingival epithelial barrier and bacterial invasion of periodontal tissues, contributing to a worsening of periodontitis [188].

Periodontal diseases

Periodontitis is a chronic inflammatory disease characterized by microbe-host response interaction that results in loss of periodontal attachment through the activation of host-derived proteinases that enable the loss of marginal periodontal ligament fibers, apical migration of JE, and biofilm along the root surface [189]. Biofilm is required but not sufficient to induce periodontitis since it is the host inflammatory response to this microbial challenge that results in the loss of periodontal tissues [167, 190]. The initiation and progression of periodontitis depend on disruption of homeostasis by ecological changes in the microbiome induced by inflammation triggered by host responses to bacterial challenge in an attempt to contain it within the gingival sulcus, once the inflammation has initiated [189].

Severe periodontitis affects approximately 19% of people greater than 15 years of age, representing more than 1 billion cases worldwide, according to WHO. Prevalence across country income groups is similar, although the highest case numbers are seen in lower-middle-income countries and the lowest in low-income countries. It almost equally affects men and women, starting in adolescence, peaking at 55 years of age, and remaining high with aging [191].

Advanced periodontitis can result in tooth loss, severely compromising oral function and aesthetics, with a negative impact on the quality of life. In addition, severe periodontitis is associated with systemic diseases and conditions, such as cardiovascular diseases, diabetes, rheumatoid arthritis, lupus, and chronic pulmonary obstructive disease, among others. The pathogenic mechanisms underlying these associations may be explained by the direct migration of bacteria and its products to sites distant from the oral cavity via either hematogenous routes (direct mechanisms), increased blood levels of inflammatory mediators, or even mimetics (indirect mechanisms) [192].

The transition from health to disease involves profound alterations in the biofilm ecology, with the conversion of a symbiotic to a dysbiotic biofilm in a pathogenic mechanism named polymicrobial synergy and dysbiosis [167, 193]. This implies a change in the relative abundance of individual components of the microbial community compared to health, ultimately mediating destructive inflammation and bone loss [193, 194]. This could be related to microbial challenges and the activation of the innate and

adaptive immune system and its role in host-microbial interactions.

Besides the role of keystone pathogens, homeostasis can be disrupted by congenital or acquired immunodeficiencies, aging, systemic diseases such as diabetes and obesity, environmental factors, such as smoking and diet, and epigenetic modifications in response to environmental changes [167]. The accumulation of dental plaque at gingival margins results in the development of gingivitis, which is a reversible inflammatory lesion that affects the gingival connective tissues. The resolution of gingival inflammation is achieved by personal and professional mechanical plaque control. Not all cases of gingivitis progress to periodontitis, but a periodontitis lesion develops from a previous gingival inflammatory lesion. If untreated, periodontitis lesions may lead to tooth loss.

Some research has shown that oral fluids can be used to assess the level or progression of periodontitis. A study investigated the levels of cytokines (IL- 1, IL- 2, IL- 4, IL- 6, IFN-γ and TNF-α) in GCF and saliva of 40 patients with aggressive periodontitis before and after treatment compared to 40 healthy volunteers [195]. The results showed increased pocket depth in AP before treatment. The concentrations of cytokines in both GCF and saliva were significantly higher in AP than in healthy patients and decreased after treatment. There was a positive association between GCF cytokine levels and clinical periodontal parameters, as well as a satisfactory reliability of cytokines in saliva and GCF. Similarly, 16 s rDNA measuring the microbiota of saliva from aggressive and chronic periodontitis against a healthy control, found a close relationship between the abundance of *P. gingivalis* and periodontitis [196]. These two studies suggest that measuring cytokines and microbiota in saliva may be considered an easy and noninvasive method for monitoring periodontal disease activity.

Pathogenesis of periodontal disease

Classically, the pathogenesis of periodontal disease is described in four stages after initiation of plaque accumulation: initial (2–4 days after plaque formation), early (4–7 days after plaque formation), established (12–21 days after plaque formation), and advanced lesion [197]. The initial lesion is characterized by neutrophil recruitment and emigration through JE and a discrete inflammatory infiltrate localized immediately beneath the JE occupying 5–10% of connective tissue. This inflammatory infiltrate is mainly composed of monocytes/macrophages, lymphocytes, and neutrophils. This lesion is subclinical and can be observed only at the histopathological level. The only discrete clinical signs are the slight increase in GCF flow rate. *Streptococcus* are the main bacterial components of this stage and they are known to secrete a number of enzymes that increase SE and JE permeability. Lipoteichoic acid and peptidoglycans are components of the cell wall of these early colonizers which are capable of activating complement via the alternative pathway, inducing the production of C3a and C5a, anaphylatoxins that flow back to the tissues and establish a gradient from the gingival sulcus to the tissues, where they can induce the release of vasoactive amines from mast cells, increasing vascular permeability. Mast cells also release other cytokines, such as TNF-α, stimulating endothelial cells to express adhesion molecules, and recruiting neutrophils to the gingival tissues. Simultaneously, bacterial-released substances and C5a attract neutrophils to the JE. Within the gingival sulcus, neutrophils release their lysosomal contents (abortive phagocytosis) that return to the tissues and degrade connective tissue. PMNs also release neutrophil extracellular traps (NETs) during pathogen-induced cell death (NETosis), which represents one of the first lines of host defense against microbial challenge. NETs are released by both dead and live PMNs and are associated with severe tissue damage. Additionally, many proinflammatory substances, such as LPS, IL- 8, TNF-α, and *Streptococcus* M protein, induce NET formation. Other cell types are able to secrete extracellular traps, as well, especially mastocytes. These cells release mast cell extracellular traps which limit the entrance of bacteria and its vesicles into the tissues, also contributing to localized tissue destruction. PMNs also secrete other cytokines, such as IL- 1 and its antagonist receptor (IL- 1RA) and IL- 17, which induces the production of IL- 8 by SE cells. IL- 8 is a strong chemoattractant for PMNs and stimulates NET formation. IL- 17 has a protective role in periodontal disease, maintaining the PMN barrier in the gingival sulcus [198].

The early lesion is characterized by increased neutrophil emigration and initial lateral proliferation of JE cells, approximately 4–7 days after initial plaque accumulation. A larger inflammatory infiltrate is observed at the gingival connective tissue, along with vascular proliferation, dilated vessels, and an increase in vessel permeability and collagen loss. Therefore, the GCF flow rate dramatically increases. Similarly, there is an increase in the permeability of SE and JE, allowing the entrance of bacterial products into gingival tissues. At this stage, there is a shift from PMN-predominant inflammatory infiltrate to lymphocytes and macrophage prevalence. Clinically, it is possible to notice early signs of gingival inflammation. In the established stage, a marked increase in the lateral proliferation of JE and neutrophil emigration, along with a greatly increased leukocytic inflammatory infiltrate composed by 10–30% of plasma cells is observed. Clinically, there are clear signs of chronic gingival inflammation, characterized by bleeding on probing, swallowing, and redness of the gingival margin [197, 198].

Around 12–21 days after initial plaque accumulation, the lesion becomes clinically evident. At 21 days, lymphocytes are 70% of the inflammatory infiltrate and a 4-fold increase in the number of PMNs within JE occurs. PMNs and plasma cells represent less than 10% of the total inflammatory infiltrate. TNF-α and IL- 17 are secreted from mast cells and PMNs undergoing NETosis, leading to an increase in endothelial cell leukocyte adhesion molecule- 1 (ELAM- 1) and intercellular adhesion molecule- 1 (ICAM- 1), resulting in an increase in the secretion of IL- 8 by epithelial cells, attracting neutrophils to the JE and forming a barrier against bacterial invasion. Degradation of collagen is estimated to be of the order of 60–70%. A delayed-type hypersensitivity-like reaction develops, with the formation of perivascular lymphocyte-macrophages inflammatory infiltrate, consisting of 2 $CD4^+$:1 $CD8^+$ T cells, dendritic APCs, and macrophages. Activated T cells and SE cells express high levels of human leukocyte antigen HLA-DR and HAL-DQ. Langerhans cells existent in the GE uptake soluble antigens entering the tissues, presenting it to lymph nodes and activating T cells, which turn back to the tissues and, together with macrophages, control the entrance of invading microorganisms and reestablish the homeostasis with biofilm [198].

In cases in which gingivitis progresses to periodontitis, the gingival inflammation can exert pressure for the development of a dysbiotic and inflammophilic microbiota, including members that can subvert or evade the immune system. The severity of periodontal lesion may depend on host-related parameters that influence the host's immune, inflammatory, and regenerative response [167]. The advanced lesion corresponds to the advanced stage, in which the inflammatory infiltrate is mostly composed of plasma cells occupying > 50% of the gingival connective tissue volume. An apical proliferation and migration of the JE along with detachment and ulceration of the pocket epithelium are observed [197] In the presence of generalized, moderate, periodontal pockets, the ulcerated epithelium is estimated to correspond to the size of the palm (approximately 50 to 75 cm^2) [199], constituting an important risk factor for the development of systemic diseases.

The role of gingival innate and adaptive immune responses

The innate and adaptive immune responses to microbial challenges are therefore the key for destructive periodontal diseases. The first point to be highlighted is the massive accumulation of neutrophils within the JE, pocket epithelium, and gingival connective tissue [167, 190, 197]. Neutrophils are vital to maintain periodontal health and any impairment in its function, such as in cyclic neutropenia or any other condition, results in rupture of the tissue homeostasis [167]. Periodontal tissue destruction is mediated by the release of enzymes, such as matrix metalloproteinases, and other substances such as cytokines and ROS [200], activation of osteoclastic bone resorption [201], chemotaxis for IL- 17-producing $CD4^+$ Th17 cells by the production of CCL2 and CCL20 [202], activating T cells and leading to bone loss. B and T cells are the major source of membrane bound and secreted Receptor Activator of Nuclear Factor-kappa B Ligand (RANKL) involved in periodontal bone loss. Th1 has a protective role in periodontal lesions through the release of IFN and IL- 12 cytokines that promote cell-mediated immunity and inhibit osteoclastogenesis [203, 204], although in some instances Th1 cells may also activate bone loss by expressing RANKL [203]. Th1 cells predominate in stable lesions, while Th2 predominates in progressive lesions characterized by a B cell dominant inflammatory response [204].

Increased deposition of immune complexes and complement fragments in diseased gingiva suggests the role of plasma/B cell antibodies in inflammatory responses [167]. Indeed, greater deposition of immune complexes was found in gingival tissue samples of systemic lupus erythematosus (SLE) and periodontitis than in periodontitis-only patients, suggesting the role of inflammation in the pathogenesis of the two conditions [205]. In addition, B cells produce inflammatory cytokines and MMPs that can further contribute to tissue damage [204].

The attachment of JE cells to the enamel surface and to the underlying connective tissue is stronger than the cell-cell attachment, while the few desmosomes and wide intercellular spaces between JE cells allow the gingival crevicular fluid (GCF) and inflammatory and immune cells to transmigrate, protecting the body against microbial invasion [206]. In the course of periodontitis, there is a conversion of JE to pocket epithelium. Detachment of JE from the tooth surface seems to occur after the increasing degree of inflammation and transmigration of neutrophils, eventually leading to focal disintegration of JE [206]. Bacteria can also contribute to the detachment of JE and the formation of the pocket epithelium. *P. gingivalis* gingipains are capable of degrading components of cell-cell junctional complexes and cleaving intercellular adhesion molecule- 1 (CD54) on oral epithelial cells, resulting in disruption of the interaction of neutrophils and epithelial cells [207]. Apically, the pocket epithelium is continuous with a reduced JE, which proliferates apically as the pocket deepens, maintaining the integrity of the gingival sulcus [206].

Dental plaque represents a continuous challenge to the innate immune system. In healthy sites, this challenge is beneficial, while in diseased sites there is an imbalance between host defenses and bacterial challenges. Due to the specific characteristics of JE, a highly orchestrated

expression of mediators is released in the periodontal tissues, including e-selectin, ICAMs, and IL- 8, which facilitate the transit of neutrophils from the gingival connective tissue to the gingival crevice [190, 208]. Other innate immune mediators expressed in periodontal tissues are β-defensins (βD) 1, βD2, βD3 [184, 187, 190], CD14 and lipopolysaccharide-binding protein (LBP) [190]. LBP, produced by gingival epithelial cells and the liver as an acute phase response marker [190], and LBP mRNA are more highly expressed in healthy than diseased sites [209]. As previously described [190], gingival tissues express TLRs, especially TLR2 and TLR4 in the development of periodontal diseases. Il- 1β was demonstrated in periodontal tissues of germ-free mice.

Many cytokines associated with inflammation are found in GCF of periodontitis patients at higher levels than in healthy ones, including IL- 1β, TNF, and prostaglandin E2 (PGE2). In addition, soluble and membrane-bound CD14 and LBP are also present in higher concentrations in GCF from diseased than healthy sites [190].

Considering that, host responses are primarily involved in the destruction of periodontal tissues in the course of the disease. The main mechanism explaining the couple effect of osteoblasts and osteoclasts is the RANKL: Osteoprotegerin (OPG) ratio. High OPG levels prevent the RANK-RANKL interaction and binding of RANKL to osteoclast precursors, impeding bone resorption. OPG levels are regulated by bone morphogenetic proteins (BMPs) and the synthesis of RANKL is induced by pro-inflammatory cytokines, such as TNF and IL- 1, which suggests that the RANKL: OPG ratio is increased in the presence of gingival inflammation, resulting in bone loss [190]. Ultimately research demonstrates that local tissues are not affected in isolation, but rather an inflammatory process impacts systemic tissues across the human body.

Oral pathogens and their interactions with the immune system

The oral cavity hosts a wide diversity of microorganisms, including bacteria, viruses, and fungi, which coexist in balance with the host under healthy conditions [210]. However, environmental, immunological, or behavioral changes can lead to uncontrolled growth of these pathogens, triggering inflammatory processes and diseases. Among the most studied bacteria are *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Streptococcus mutans*, all of which are associated with conditions such as periodontitis and dental caries. *P. gingivalis*, for example, has the ability to modulate the host's immune response, subverting defense mechanisms to favor its own survival and contributing to chronic inflammation [211, 212].

Saliva does not have a resident microbiota, but resident oral microorganisms can be found in saliva, originating from teeth, oral mucosa and tongue, mainly. These microorganisms are essential to maintain homeostasis, since they prevent colonization by exogenous microorganisms, downregulating proinflammatory responses and converting nitrate to nitrite, contributing to antimicrobial activities, mucus production and vasodilation, which could potentially lower blood pressure. The composition of the acquired pellicle, which is formed short after the tooth enters in contact with saliva, determines the pattern of microbial colonization [213].

Based on that, since periodontitis arises from the complex interaction between subgingival microbiota composition and the host immune response, an interplay between periodontal diseases and other systemic diseases exist. For instance, in SLE patients, periodontal bacterial diversity was reduced, with an enrichment of specific periodontal pathogens dominating the microbial environment. The connectivity of *F. nucleatum* with other bacterial species and cytokines was higher in individuals with SLE compared to control subjects. *A. gerencseriae*, *C. ochracea*, and *T. forsythia* exhibited greater connectivity in both control and SLE-A individuals, whereas *S. oralis* and *P. nigrescens* showed increased connectivity in SLE-A subjects. Additionally, *S. noxia* was more prevalent in the control group, while *C. gingivalis* displayed no connectivity in control individuals. These findings suggest that subgingival bacterial species associated with SLE influence systemic host cytokine patterns, impacting overall health [214].

In addition to bacteria, viral infections also play a significant role in oral health. Herpes simplex virus type 1 (HSV- 1) is one of the most prevalent, causing recurrent lesions in the oral mucosa and impacting local immunity [215]. Epstein-Barr virus (EBV) and cytomegalovirus (CMV) can also be present in the oral cavity, especially in immunocompromised individuals, where they contribute to immune dysfunction and the worsening of periodontal diseases [216]. These viral infections can alter both innate and adaptive immune responses, promoting a microenvironment favorable for viral persistence and chronic inflammation. Furthermore, evidence suggests that the interaction between these viruses and the immune system can trigger complex mechanisms of immune modulation, favoring both the chronicity of infections and the progression of oral inflammatory condition [216].

Fungi, especially *Candida albicans*, represent another group of relevant pathogens. Although *C. albicans* is part of the commensal oral microbiota, changes in host immunity can lead to oral candidiasis. The interaction between the microbiota and the immune system is crucial in controlling *C. albicans* infections [217]. The microbiota not only protects against fungal overgrowth

Table 2 List of most common pathogens that affect the oral cavity, including the associated oral manifestations and cellular targets

Pathogen	Disease	Host Target
Bacteria		
Porphyromonas gingivalis [220]	Gingivitis, Periodontitis	Oral epithelium
Aggregatibacter actinomycetemcomitans [221]	Gingivitis, Periodontitis, Infective endocarditis, other systemic diseases	Natural habitat is oral cavity, but can enter body of the host infect many body areas, notably the endocardium
Fusobacterium nucleatum [222]	Gingivitis, Periodontitis	Oral epithelium
Prevotella Intermedia [223]	Necrotizing ulcerative gingivitis and necrotizing ulcerative periodontitis	Oral epithelium
Tannerella forsythia [224]	Gingivitis, Periodontitis	Oral epithelium
Treponema denticola [225]	Gingivitis, Periodontitis	Oral epithelium
Virus		
Herpes simplex virus (HPV) [226]	Herpetic gingivostomatitis	Oral epithelium
Epstein-Barr Virus (EBV) [227]	Periodontitis, oral inflammation, potentially Sjorgen's Syndrome	Preferential colonization of B cells, but also can infect epithelial cells
Cytomegalovirus [228, 229]	Oral mucosal ulcers, Periodontitis	Oral epithelium, endothelial cells, fibroblasts, and some lymphocyte and myeloid cells
Human Immunodeficiency Virus (HIV) [230, 231]	Linear erythematous gingivitis, necrotizing periodontitis	Tissue-resident $CD4^+$ T cells (Gut, Lymphnodes)
Fungal		
Candida albicans [232]	Pseudomembranous candidiasis, erythematous candidiasis and hyperplastic candidiasis.	Oral epithelium

but also regulates the immune response, particularly the function of cells like neutrophils and dendritic cells. Deficiency in IL- 17 is an example of how failures in the immune response increase susceptibility to fungal infections [218, 219]. New therapeutic approaches, such as the use of probiotics and immunomodulatory molecules, which can restore immune protection, are being explored [217].

Recently, advances in omics approaches, such as metagenomics, metaproteomics, and metabolomics, have allowed for a more comprehensive understanding of oral microbial communities and the endogenous and exogenous factors influencing their composition. Growing evidence supports the notion that various internal and external factors cause dysbiosis of the oral microbiota, contributing significantly to oral and systemic diseases. Current studies still focus on bacteria and *C. albicans*, with identifying the mechanisms of communication and regulation within and between the microbiome, virome, and host immune system being a challenge. Promising therapeutic strategies for oral diseases are under development, but few investigations on their effects on oral dysbiosis have produced consistent results [210].

This section details the main oral pathogens, addressing their characteristics, pathogenicity mechanisms and impact on periodontal health, summarized in Table 2.

Pathogenic bacteria

The bacterial microbiota is the main factor involved in periodontitis. Some specific pathogens are closely associated with the progression of periodontal disease.

Porphyromonas gingivalis

One of the best known and most widely studied periodontal pathogens. P. gingivalis is a gram-negative anaerobic bacterium that can invade periodontal tissues, leading to the destruction of collagen fibers and alveolar bone by inducing high levels of proinflammatory cytokines, such as IL- 1β and IL- 6 by periph-eral CD4 + T helper cells [220]. It produces enzymes such as gingipain, which interfere with the host's immune response and aid in the colonization of the periodontal environment [233].

Tannerella forsythia

Also an anaerobic gram-negative bacterium, it is frequently found in individuals with severe periodontitis. It plays an important role in periodontal inflammation, causing destruction of periodontal tissue and interacting with other bacteria to form a pathogenic biofilm [224]. More recently, *Tannerella forsythia*, like *Porphyromonas gingivalis*, was found to be associated with an increased risk of esophageal cancer [234]. Although the exact mechanisms are unknown, it is thought *Tannerella forsythia* induces an inflammatory response characterized by IL- 1 and TNF-α, which can help the development of tumors through COX- 2. However, more research is required to know exactly how *Tannerella forsythia* contributes to tumor progression.

Treponema denticola

An anaerobic spirochete bacterium that is associated with aggressive periodontal diseases. *T. denticola* is

capable of forming biofilms and producing proteases that disintegrate gingival tissue [225].

Fusobacterium nucleatum

This gram-negative, anaerobic bacterium is known for its ability to interact with other bacterial species to form complex biofilms and contribute to the chronic inflammation associated with periodontitis [222]. Additionally, this bacteria has been implicated in some cancers. A study on the gene expression profile of human tumor tissues found that the level of *Fusobacterium nucleatum* was associated with a suppressive immune tumor environment characterized by $CD8^+$ T cell depletion and enrichment of $FoxP3^+$ regulatory T cells [235].

Aggregatibacter actinomycetemcomitans

A facultatively anaerobic gram-negative bacterium, often associated with aggressive periodontitis, especially in young people. It produces leukotoxins that induce leukocyte apoptosis and help the bacteria escape the immune response. Its presence is linked to rapid bone and periodontal attachment destruction [236].

Prevotella intermedia

A strict anaerobic gram-negative bacterium, adapted to the subgingival environment. Commonly associated with necrotizing ulcerative gingivitis (NUG) and necrotizing ulcerative periodontitis (NUP), severe inflammatory conditions. It produces proteolytic enzymes that degrade the extracellular matrix of healthy cells and human IgG, facilitating the destruction of periodontal tissue while evading some immune responses [223, 237].

Pathogenic viruses

Although periodontal diseases are predominantly bacterial, viruses also play an important role, especially in situations where immune function is compromised, such as in autoimmune diseases.

Herpes simplex virus (HSV)

Although HSV is most often associated with oral lesions, it can also aggravate periodontal inflammatory conditions, especially in immunocompromised patients. HSV type 1 is commonly found in lesions on the gingiva and oral mucosa, causing primary herpetic gingivostomatitis, recurrent ulcers, and exacerbation of periodontal inflammation. HSV type 2, although more commonly associated with genital infections, can be found in the oral cavity and has been implicated in the pathogenesis of oral ulcers and increased severity of periodontitis in immunosuppressed individuals [226].

Cytomegalovirus (CMV)

It is frequently present in patients with severe periodontal diseases. CMV may participate in the development of periodontitis by causing macrophages and T cells to release osteoclast-inducing IL- 1β and TNF-α. Also, gingival fibroblasts infected with cytomegalovirus exhibit diminished production of collagens I and III and enhanced generation of MMPss 1 and 2. However, although biologically plausible, the extent to which cytomegalovirus participates in the destruction of the human periodontium is still a matter of research [228].

Epstein-Barr virus (EBV)

Also detected in inflamed periodontal tissues, EBV can contribute to the chronic inflammatory response and is involved in increasing the virulence of other bacterial periodontal infections. Persistent EBV facilitates viral evasion and exacerbates the infection. Increased viral activity leads to heightened local immune and inflammatory responses, worsening the inflammatory condition, promoting B cell and plasma cell infiltration, and advancing epithelial tissue degradation. EBV-induced impairment of host defenses results in bacterial superinfection, culminating in destructive periodontitis [238].

Human immunodeficiency virus (HIV)

HIV is not directly a periodontal pathogen, but its presence profoundly alters the immune response, making individuals more susceptible to periodontal infections. Patients with HIV have a higher prevalence of severe periodontal diseases, such as linear erythematous gingivitis and necrotizing periodontitis, characterized by intense inflammation and rapid tissue destruction. Immunosuppression caused by HIV favors the proliferation of opportunistic pathogens, such as *C. albicans* and P. *intermedia* [230].

HIV- 1 infection acts as a modifying factor in periodontal diseases, and is frequently associated with the occurrence of acute periodontal diseases and exacerbation of preexisting chronic periodontitis. The great bacterial diversity and complexity in the oral microbiota of HIV- 1-infected individuals seems to be related to the chronic periodontitis progression and severity [231].

Antiretroviral therapy can modify the oral microbiota and affect the progression of periodontal disease.

Pathogenic fungi

Although fungi are not as prominent as bacteria, they do play a role in the development of oral diseases, especially in immunocompromised patients.

Candida albicans

The most common fungus found in the oral cavity, especially in individuals with compromised immune systems.

C. albicans can cause oral candidiasis, an infection that can result in painful lesions in the oral mucosa and contribute to an imbalanced microbiota, favoring pathogenic bacterial colonization [239, 240].

The impact of oral tissues and fluids in oral-systemic diseases

Oral mucosal tissues and biofluids impact on oral-systemic diseases influencing negatively when in dysbiosis. It explores the relationship between oral health and systemic conditions such as diabetes https://pubmed.ncbi.nlm.nih.gov/33426512/, cardiovascular disease https://www.amjmed.com/article/S0002-9343(23)00755-6/fulltext, oral cancer https://pubmed.ncbi.nlm.nih.gov/33155101/, and lupus https://pubmed.ncbi.nlm.nih.gov/31781106/.

Diabetes

Diabetes is a major risk factor for periodontitis [241]. In diabetes, the increased expression of TLR2 or TLR4 or both results in the release in proinflammatory cytokines, such as TNF-α and IL- 6 by adipocytes [242], which could in severe inflammatory tissue destruction as seen in subjects with periodontitis and uncontrolled diabetes [243]. In gingival tissue samples collected from subjects with periodontitis or diabetes or periodontitis and diabetes or healthy, the expression of TLR2 and TLR4 were quantified. The results showed that chronic periodontitis or diabetes had no significant effect on TLR2 expression in the oral epithelium, but an increased expression was seen at SE and a changed pattern of expression was noticed in GE. Chronic periodontitis decreased the expression of TLR in GE. Higher percentages of TLR2 and TLR4 were found in the connective tissue under SE. Chronic periodontitis and diabetes subjects showed higher percentage of TLR2 and TL4 compared with healthy and diabetics patients [244].

Cardiovascular diseases

The growing interest in salivary biomarkers for diagnosing cardiovascular diseases (CVDs) has been driven by the potential for non-invasive, convenient, and rapid point-of-care testing. Several biomarkers found in saliva have shown promise in identifying acute myocardial infarction and other cardiovascular conditions. Key salivary biomarkers for CVD include myoglobin, cardiac troponin I, creatine phosphokinase MB, MPO, brain natriuretic peptide, exosomal microRNAs (miRNAs), C-reactive protein (CRP), matrix metalloproteinases (MMP- 8, MMP- 9), and tissue inhibitor of metalloproteinase- 1 [245].

Some studies have shown that salivary C-reactive Protein (CRP) levels correlate with serum CRP levels, suggesting that it could be a useful non-invasive marker for systemic inflammation in CVD. Increased salivary CRP may reflect systemic inflammation, a key player in the pathogenesis of cardiovascular diseases, including atherosclerosis and plaque rupture. CRP is a well-established marker of inflammation and is used to assess cardiovascular risk. It is produced by the liver in response to inflammatory cytokines, and elevated levels are associated with increased risk of atherosclerosis, myocardial infarction, and other cardiovascular events [246–248].

Oral cancer

Oral cancer, a significant global health concern, has prompted extensive research into utilizing salivary proteomics for early detection and improved diagnostics. Saliva, in its intimate contact with the oral cavity, harbors a plethora of proteins and peptides that can reflect the physiological state of the oral tissues, including the presence of malignant or premalignant lesions.

Advances in mass spectrometry-based proteomics have enabled the identification and quantification of thousands of salivary proteins, opening up new avenues for biomarker discovery. Several studies have reported panels of salivary proteins that show differential expression in oral cancer patients compared to healthy controls [249–253]. These potential biomarkers include proteins involved in inflammation, immune response, cell signaling, and tissue remodeling. Notably, some of these proteins have been detected in saliva even before the clinical manifestation of oral cancer, highlighting the potential of salivary proteomics for early diagnosis. In addition to proteomics, other detection methods and types of markers have been proposed as tools for cancer diagnosis such as salivary miRNAs [254–256].

However, the path to clinical implementation of salivary biomarkers for oral cancer is not without challenges. The heterogeneous nature of saliva, inter-individual variability, and the influence of confounding factors such as oral hygiene and diet can complicate the identification of robust and reliable biomarkers [257–259]. Ongoing research is focused on addressing these challenges through the development of standardized saliva collection and processing protocols, advanced proteomic technologies, and sophisticated data analysis algorithms [260, 261].

Despite these challenges, the potential of salivary proteomics for oral cancer detection and monitoring remains promising. With continued research and technological advancements, salivary biomarkers could become a valuable tool for early diagnosis, risk assessment, and personalized treatment of oral cancer, ultimately improving patient outcomes and survival rates.

Lupus

Oral bacteria play a crucial role in modulating immune responses and contributing to dysbiosis, which can

promote systemic inflammation. The oral cavity, particularly niches such as the gingival sulcus and fluid, is a site where pathogens may induce local and systemic inflammation, contributing to various chronic diseases like type 2 diabetes, rheumatoid arthritis, cardiovascular diseases, systemic lupus erythematosus (SLE) and others [262, 263].

Recent studies have highlighted the role of oral pathogens in exacerbating chronic inflammatory conditions, including SLE. Elevated levels of inflammatory cytokines, such as IL- 1, IL- 6, IFN-γ, and TNF-α, have been observed in lupus patients compared to healthy controls, with some differences in cytokine profiles between lupus patients and those with periodontal disease [264, 265].

Research by our team has shown that lupus patients, particularly those with active disease, exhibit an increased regulation of pro-inflammatory cytokines. Additionally, the oral microbiota of lupus patients is dysbiotic, with significant elevations in *T. denticola* and *T. forsythia*, which are associated with higher systemic inflammatory cytokine levels [214]. Some findings suggest that oral pathogens can influence disease activity, and the immune response in SLE may be exacerbated by oral infections, particularly in those with concurrent periodontal disease [205].

Interestingly, salivary biomarkers are emerging as non-invasive indicators for the early detection and monitoring of SLE. Studies show that salivary IgA levels, as well as specific cytokines, correlate with disease activity and can differentiate SLE patients from healthy individuals. Furthermore, salivary cytokines could be valuable tools for early diagnosis, monitoring disease progression, and assessing treatment efficacy [266]. However, further research is necessary to fully understand the pathogenic mechanisms linking the oral microbiome to SLE and other autoimmune diseases, and to validate salivary biomarkers as reliable diagnostic tools for SLE [267].

Future directions

Salivary biomarkers have a promise on how we envision precision medicine. Deeper exploration of salivary biomarkers holds the potential to revolutionize diagnostics and therapeutics. Identifying specific salivary signatures associated with various diseases could enable early detection, personalized treatment plans, and monitoring of therapeutic responses. Therapeutic modulation of salivary immunity is still underexplored. Investigating interventions that can modulate salivary immune responses opens new avenues for disease management. This could include targeted therapies to enhance beneficial immune components, suppress harmful inflammation, or restore a balanced oral microbiome.

Longitudinal studies for disease progression would provide better validation of salivary markers in health and disease longitudinal studies are crucial to understand the long-term impact of salivary immune responses on disease progression. Tracking changes in salivary biomarkers over time can reveal valuable insights into disease trajectories and inform preventive strategies. By integrating salivary and systemic data we will better understand how and why saliva is representative of general health in addition to the oral health metrics. Integrating salivary data with other systemic health information can provide a more comprehensive understanding of disease mechanisms and individual health profiles. This could lead to the development of integrated diagnostic and treatment approaches.

The salivary microbiome has incredible potential in personalized medicine. Further research into the salivary microbiome and its interactions with the host immune system can unlock opportunities for personalized medicine. Tailoring interventions based on an individual's salivary microbiome profile could improve treatment efficacy and minimize adverse effects.

Conclusions

This comprehensive review highlights the critical role of saliva in oral and systemic health. Saliva, with its diverse cellular components, proteins, cytokines, and immunoglobulins, acts as a dynamic and multifaceted barrier against pathogens. Understanding the intricate immune mechanisms within saliva is essential for unraveling the complex interplay between oral and systemic health. Future research in this field holds immense promise for developing innovative diagnostic tools, personalized therapeutic interventions, and preventive strategies to improve overall health and well-being.

Abbreviations

AJ	Adherens junction
APCs	Antigen-presenting cells
BAFF	B-cell activating factor
BMI	Body-mass index
BMP	Bone morphogenetic protein
citH3	Citrullinated histone H3
CPM	Count per million
CVD	Cardiovascular disease
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
ELAM	Epithelial leukocyte adhesion molecule
ELISA	Enzyme-linked immunosorbent assay
E-selectin	Endothelial-selectin
FC	Fold-change
FDR	False-discovery rate
GCF	Gingival crevicular fluid
GE	Gingival epithelium
GO	Gene Ontology
GWAS	Genome-wide association studies
HbA1c	Hemoglobin A1C
hBD	Beta-defensin
HCMV	Human Cytomegalovirus
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

IL- 1R	Interleukin-1 receptor
IP- 10	Interferon gamma-induced protein 10
JE	Junctional Epithelium
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBP	Lipopolysaccharide-binding protein
LCs	Langerhans cells
Lf	Lactoferrin
LLOQ	Lower limit of quantification
LRR	Leucine-rich repeat
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
MUC	Mucin
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain
OECs	Oral epithelial cells
OPG	Osteoprotegerin
PAMPs	Pathogen-associated molecular patterns
PCA	Principal component analysis
PMA	Phorbol 12-myristate 13-acetate
PPRs	Pattern-recognition receptors
P-Selectin	Platelet-selectin
QC	Quality control
RANKL	Receptor Activator of Nuclear Factor-kappa B Ligand
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RvE1	Resolvin E1
SC	Secretory component
SE	Sulcular epithelium
sICAM- 1	Soluble intercellular adhesion molecule-1
SIgA	Secretory Immunoglobulin A
SPM	Specialized pro-resolving lipid mediator
ST2	Suppression of tumorigenicity 2
T2D	Type 2 diabetes
TJs	Tight junctions
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
TMM	Trimmed mean of M-values
TSLP	Thymic Stromal lymphopoietin

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Declarations

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Competing interests
The authors declare no competing interests.

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