# IMMUNOLOGY OF THE ORAL MUCOSA

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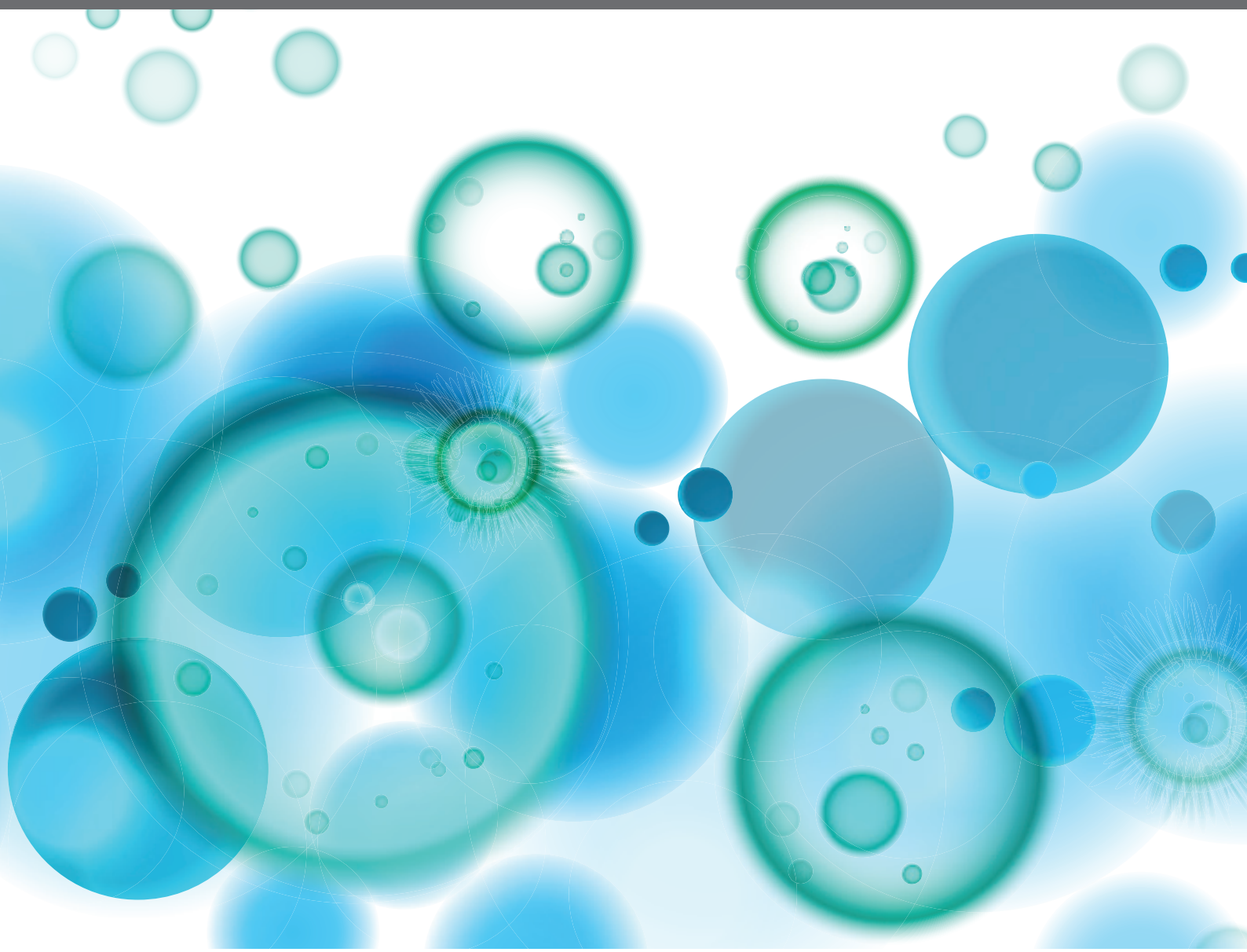

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# IMMUNOLOGY OF THE ORAL MUCOSA

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# Editorial: Immunology of the Oral Mucosa

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**Editorial on the Research Topic**

**Immunology of the Oral Mucosa**

The oral mucosa is host to over 700 species of commensal organisms (HTTP://www.homd.org) and is constantly exposed to potentially inflammatory stimuli, yet, in healthy individuals, acute inflammation is unusual. Waldeyer's ring and numerous draining lymph nodes provide inductive and effector sites for intense immune activity that maintains the integrity of the mucosal barrier. A state of immune privilege or tolerance is said to exist in the oral mucosa through the induction of T regulatory cells and the production of cytokines that support immuno-suppression of unwanted responses to innocuous antigens (1–3). Several different types of stratified epithelia are present including the lining mucosa, masticatory and specialized mucosa and range from thin non-keratinized sub-lingual and buccal mucosae to thick highly keratinized Gingiva (4) Their functions have direct effects on immune responses, such as the induction of homeostatic Th17 responses by the mechanical damage of mastication (5).

Investigations of the oral cavity range from the first observations of dental plaque by Anton van Leeuwenhoek in the 1660's to the recently published cell atlas of the oral mucosa (6) and have provided major contributions to the understanding of mucosal immune responses (7). Seminal studies emphasizing the importance of the ecological balance of the oral microbiota and the host have described dysbiosis as a driving force contributing to oral (and systemic) diseases (8).

This Research Topic has attracted an eclectic series of reviews, original research, case studies and hypotheses which are reflective of the importance and broad range of oral immunology studies and the applicability of the oral mucosa for diagnosis, intervention and prevention of disease.

Suárez et al. have compared the immune niches of the oral and gastrointestinal mucosa in terms of their anatomy, cell to cell communication, antigen handling, signaling pathways and systemic consequences in disease pathogenesis. A greater diversity of dendritic cells in the oral cavity (9), and potential synergistic interaction between TLRs and NOD receptors results in a measured response to oral bacteria (10). TLR splice variants have been observed in the buccal mucosa of the autoinflammatory condition Behçet's Disease (11) where dysregulated antigen recognition could contribute to inflammatory profiles. Suárez et al. conclude that microbial translocation contributes to systemic disease and emphasize the bidirectionality of the interface between the oral and gastrointestinal mucosa.

An intriguing paper on obstructive sleep apnea by Samiento Varón et al. suggests that children with hypertrophied tonsils have abnormally active pro-inflammatory B and T cells, where

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modulation of the microbiome allows penetration into tonsil tissues and potential breaching of the epithelial barrier and is supported by previous studies (12).

Characterization of the human oral mucosa cell atlas suggests that a stromal-neutrophil axis regulates tissue immunity (6). Metcalfe et al. have reviewed the role of polarized phagocytes in the oral mucosa, suggesting a particular heterogeneity of neutrophils in this tissue. The role of neutrophils in the pathology of the periodontal pocket was recently reviewed (13, 14) and up-regulation of neutrophil activation in inflammatory disease with oral manifestations has been described (15). Ozuna et al. have demonstrated that gram-negative bacteria associated with periodontal disease are able to *highjack* neutrophil function by upregulating azurophilic granule exocytosis as survival mechanism. The plasticity of neutrophils in oral tissues is highlighted suggesting that these cells are underestimated (16). The use of the neutrophil/lymphocyte ratio was suggested as an indicator of disease progression (17).

Zhang et al. have reviewed the role of T-regulatory cells in the context of Head and Neck cancers as well as periodontitis. Other studies suggest that a uniquely large population of FOXp3$^+$ Tregs are found in the oral mucosa (18). Tregs promote the generation of IL-17-producing Th17 cells by consuming IL-2, an important survival factor, but also a negative regulator of Th17 differentiation (19).

IL-17 is an important cytokine in the oral mucosa and has been shown to regulate host-microbe interaction (20) as well as being up-regulated in response to mastication (5). Its role in the pathology of periodontitis is well documented (21). In contrast some Th17 cells act as negative regulators of inflammation by secreting IL-10 and are thus regarded as non-pathogenic (22). In this context oral mucositis was shown to be mitigated by IL-17 receptor signalling in a clinically relevant murine model of irradiation induced mucositis which might lead to therapeutic interventions (Saul-McBeth et al.)

The use of pro-biotics is a topic of popular interest for restoring/maintaining the *normal* microbiome (23, 24). Wang et al. suggest this might be a useful adjunct in oral cancer therapy as demonstrated in a mouse model of mucositis using *Strep. salivarius* K12 to help reconstitute the dysbiotic oral microbiome.

Other materials have been suggested as potential down regulators of inflammation and Mooney et al. present a proof-of-concept study using Quercetin, a plant based polyphenolic flavonoid, to modify the oral microbiome both *in vivo* and *in vitro*. This flavonoid has been shown to have health benefits in humans and animals (25) and works through modulation of the NF-κB:A20 axis.

Molecular profiling of multiple cell types and multiple effector molecules provides large amounts of data and moves analysis of health and disease from reductionist theory to systems biology. Profiles of multiple pro-resolving lipid mediators have been investigated by Lee et al. in periodontal inflammation, suggesting a correlation between these molecules, receptor genes and the sub-gingival microbiome that have the potential to skew the periodontal microbiome. Failure to resolve inflammation was investigated by Alvarez et al. in experimental periodontitis where the T cell component was inhibited, but neutrophil and macrophage infiltration was unaffected by early administration of RvE1. Previous studies have suggested that salivary levels of lipid mediators might be used as indicators of health and disease of the periodontium (26)

Salivary flow in the oral cavity is greater than 1 liter/day and the surface epithelium is constantly in turnover. About 70% of the total numbers of lymphocytes are found in the gut (27) and the compartmentalization of the mucosal immune system has been investigated, particularly in the context of vaccination (28–30). While the systemic and mucosal immune systems are regarded as distinct there is constant communication between them in order to maintain the homeostasis of "health". Many systemic diseases present with oral manifestations and oral health has a considerable impact on systemic disease (31).

Classically IgA has been regarded as the predominant functional antibody in saliva and during the SARS-CoV-2 (Covid) pandemic it has been surprising that this antibody has been somewhat neglected (32). Most analysis of immune responses have concentrated on the serum IgG response to this mucosal infection.

Chellamuthu et al. present data on IgG antibodies in the saliva of SARS-CoV-2 infected individuals and suggest this would be an effective alternative to serum-based assays. Secretory IgA (sIgA) antibodies in individuals that have ***not*** been infected with SARS-CoV-2 have been reported (33). However, these cross-reactive antibodies may be a result of the ability of sIgA to bind virus in a non- specific manner as part of its function of virus exclusion from mucosal surfaces. While the contribution of IgA to the immune response to SARS-CoV-2 requires further investigation, a recent review elaborated a greater degree of interaction between IgG and IgA (as well as IgM and IgD) in driving sIgA responses in increasingly complex cross-talk between the mucosal and systemic systems (34).

The detection of soluble mediators of inflammation (cytokines) in serum compared with saliva was highlighted by Novak et al. Salivary cytokine profiling would be less invasive than blood sampling and might reveal more accurately, either disease activity or disease progression in individuals with oral manifestations of disease, and host and environmental influences (35).

Immunohistochemical signatures as clinical monitoring for mucosal melanoma are presented by Xu et al. This disease is difficult to diagnose with a poor prognosis and methods that improve outcomes are very important (36).

Similarly, genetic markers can provide indicators of progression, manifestations and the propensity for diseases. A case report of a family with hereditary gingival fibromatosis (HGF) presented by Gao et al. suggest that the presence of high levels of Human β defensins in the gingival tissues had a beneficial effect in preventing the thickening of the gingiva in this condition.

Qian et al. have used transcriptomic analysis to identify a new set of inflammation promoting cell subsets in chronic periodontitis. Their study shows NLRP3$^+$ macrophage involvement and expression of HLA-DR on endothelial cells and CXCL13$^+$ fibroblasts were highly associated with regulatory

profiles in Asian patients. This is consistent with recent papers on the involvement of NLRP3$^+$ cells in periodontal disease (37) and in periodontitis associated with uncontrolled type-2 diabetes (38).

A recurring theme in several submissions is the diversity and plasticity of the cells of the oral mucosa. This may reflect the observations that the oral mucosa, like fetal tissues heals quickly without scarring. The immaturity and diversity of some cell types within the oral mucosa may be the key to the unique physiology of this tissue.

Future studies specifically designed to further develop the oral mucosa and saliva as analytical tools for both oral and systemic disease are an exciting prospect both in the context of new technologies such as multi-colored flow cytometry, multiplex analysis and fast affordable genetic analysis and as potential routes for vaccinology and drug delivery (39).

## AUTHOR CONTRIBUTIONS

LB drafted the editorial and ND, PS and HK contributed to the final submitted version. All authors contributed to the article and approved the submitted version.

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# Role of Tonsillar Chronic Inflammation and Commensal Bacteria in the Pathogenesis of Pediatric OSA

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Immune responses at the boundary between the host and the world beyond are complex and mucosal tissue homeostasis relies on them. Obstructive sleep apnea (OSA) is a syndrome suffered by children with hypertrophied tonsils. We have previously demonstrated that these tonsils present a defective regulatory B cell (Breg) compartment. Here, we extend those findings by uncovering the crucial role of resident pro-inflammatory B and T cells in sustaining tonsillar hypertrophy and hyperplasia by producing TNFα and IL17, respectively, in *ex vivo* cultures. Additionally, we detected prominent levels of expression of CD1d by tonsillar stratified as well as reticular epithelium, which have not previously been reported. Furthermore, we evidenced the hypertrophy of germinal centers (GC) and the general hyperplasia of B lymphocytes within the tissue and the lumen of the crypts. Of note, such B cells resulted mainly $(IgG/IgM)^+$ cells, with some $IgA^+$ cells located marginally in the follicles. Finally, by combining bacterial culture from the tonsillar core and subsequent identification of the respective isolates, we determined the most prevalent species within the cohort of OSA patients. Although the isolated species are considered normal oropharyngeal commensals in children, we confirmed their capacity to breach the epithelial barrier. Our work sheds light on the pathological mechanism underlying OSA, highlighting the relevance taken by the host immune system when defining infection versus colonization, and opening alternatives of treatment.

**Keywords: tonsils, oral bacteria, obstructive, sleep, apnea, mucosal immunity**

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## INTRODUCTION

Human paired palatine tonsils are lymphoid epithelial tissues of the oral mucosa around the oropharynx. They are part of the Waldeyer's ring of lymphoid tissue, which also comprise the adenoids and the lingual tonsils. The palatine tonsils (tonsils, from now on) are strategically located to generate mucosal immunity as they are constantly exposed to dietary and airborne antigens (Ags). Moreover, they have evolved for direct transport of foreign material from the exterior to the lymphoid cells through deep and branched crypts and the absence of Ag-degrading digestive enzymes (1). The surfaces of the human body, including the oropharynx, are colonized by several microbes, mainly bacteria, that establish a mutualistic relationship with the host. Tonsils are predominantly B-cell organs, immunologically most active between 4 and 10 years old. Some children (also some adults, but were not included in this study) present tonsillar hyperplasia and hypertrophy for yet unknown reasons. Such enlargement is the major pathophysiological sign underlying OSA, a highly prevalent disease, recognized as a major public health burden. OSA is characterized by repeated events of partial or complete upper airway obstruction during sleep that lead to disruption of normal ventilation with all the consequences implied due to hypoxemia. An increment in pro-inflammatory cytokines have been reported in blood from OSA patients (2–4). It is assumed that the B cell hyperplasia and hypertrophy that cause OSA are the result of chronic inflammation of palatine tonsils in children.

B cells contribute to immune responses during infectious, inflammatory and autoimmune diseases. In the last two decades, we have learned that B cells are able to modulate physiological and pathological processes not only by producing antibodies (Abs) and presenting Ags but also by producing cytokines. It has been shown that B cells can occur in the form of several cytokine-secreting subsets with either pro- or anti-inflammatory functions (5). Being an important reservoir of human B cells, the tonsils serve as a platform to study such subsets. Within this framework, we have recently demonstrated that OSA tonsils rendered significantly lower percentages of interleukin 10 (IL10) producing B cells (Bregs) than tonsils excised due to recurrent tonsillitis, showing that Bregs have a more complex and interesting role in tonsillar disease than was hitherto appreciated. Moreover, such defect in Breg population correlated with an increment in the proportion of germinal center (GC) cells. This correlation reveals a role for the Breg subset in controlling GC reactions, as it is by means of GC cells that tonsils are hypertrophied (6). GCs are the microanatomical sites within secondary lymphoid organs, critical for memory B and plasma cell generation. There are also many other cellular and molecular players involved in controlling GC activity. For instance, it has been long known that the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) is a required autocrine B-cell growth factor (7).

In the present paper, we found that tonsillar mononuclear cells (TMC) from OSA tonsils effectively exhibit a pro-inflammatory cytokine profile rapidly in culture. Interestingly, under certain stimulating conditions, B lymphocytes became one of the main cell populations driving TNFα levels in culture *ex vivo*. On the other hand, tonsillar interleukin 17 (IL17) was produced primarily by $CD4^+$ T cells. At tissue level, we discovered CD1d expression by tonsillar stratified as well as reticular epithelium and corroborated the persistent hypertrophy of GC and the concomitant hyperplasia of B lymphocytes, prevalently IgG/IgM positive $(IgG/IgM)^+$. We identified a number of bacterial species in the tonsillar core of patients with tonsillar hypertrophy, considered normal oropharyngeal commensals in children. However, we confirmed their presence beyond the epithelial boundaries by fluorescence *in situ* hybridization (FISH). Thus, our observations suggest that tonsillar hypertrophy is a multifaceted condition not associated to the presence of a particular microorganism but more likely to a failure of normal immune homeostatic mechanisms caused by the local loss of the capacity to discriminate between commensals and pathogens of the host. All in all, when such discrimination is lost, commensals become pathogens. Therefore, we support the notion that OSA in children is of infectious nature, clearly not associated to a single species.

## MATERIALS AND METHODS

### Isolation of cells

Primary human mononuclear cells were isolated from tonsils obtained from patients (total n=54 taking into account all experiments, the particular number of samples per experiment were detailed in the corresponding Figure legends) undergoing tonsillectomy due to OSA. TMC were prepared as follows. Briefly, tonsils were collected in phosphate buffered saline (PBS) buffer containing 50 μg $ml^{-1}$ amphotericin B (Richet, BA, Arg). Tissues were chopped with a scalpel in IMDM medium (see below) and passed through a 70 μm-pore-size cell strainer (Falcon, Thermo Fisher, BA, Arg). TMC were purified by density gradient centrifugation with Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). The viability of primary cells, as determined by trypan blue exclusion was greater than 99% in all preparations. Informed consent was obtained from subjects before the study. The institutional ethics committee (Clinical Hospital, School of Medicine, Buenos Aires) approved the collection and use of clinical material, conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). Informed consent was obtained from all participants and/or their legal guardian/s. FACS experiments were performed with freshly isolated cells only.

### Cell culture

TMC were cultured in IMDM medium (Life Technologies, CA, USA) containing 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES), 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol (all from Invitrogen, CA, USA). Human IL2 (20 ng/ml; R&D Systems, MN, USA) and human IL4 (20 ng/ml; R&D Systems, MN, USA) were added immediately before experiments also as supplements. When indicated, human recombinant hCD40L (250 ng/ml; R&D Systems, MN, USA) and 25 μM CpG-ODN 2006 (InvivoGen, CA, USA) were used. Cells were cultured at

$1x10^6$ cells/ml either in 24-well culture plates (1ml) or 48-well culture plates (0.5ml).

## Antibodies and fluorescence-activated cell sorting (FACS)

Fluorochrome conjugated mAbs specific for human CD3 (Pacific Blue, clone UCHT1), human CD20 (FITC, clone L27 and APC H7 clone 2H7), human CD4 (PerCP, clone SK3), human IL17A (APC, clone BL168), TNFα (PE, clone IT-5H2 and BV711, clone Mab11), and respective isotype control mAbs were purchased from BD Biosciences (CA, USA) and Biolegend (CA, USA). Fixable viability dyes used were either eFluor 780 (eBioscience, CA, USA), Zombie Green (Biolegend, CA, USA) or Zombie Aqua (Biolegend, CA, USA) depending on the staining scheme. To detect intracellular cytokines in cultured cells, the latter were stimulated with PMA/Ionomycin/Brefeldin A for the last 5 hs of culture. Then, they were incubated with Cytofix/Cytoperm (BD PharMingen, CA, USA) for 20 minutes (min) in the dark and washed with Perm/Wash solution (BD PharMingen, CA, USA). Following permeabilization, the cells were stained with the respective anti-cytokine mAb. Cells were acquired using FACSAria II (BD Biosciences, CA, USA) and analyzed with FlowJo software (Treestar, OR, USA). Single stained controls were used to set compensation parameters. Fluorescence minus one and isotype-matched Ab controls were used to set analysis gates.

## Statistical analyses

The results were analyzed using GraphPad Prism 7.0 software (Graph Pad Inc, CA, USA). The normality of variable distribution was assessed by the Shapiro–Wilk test, the hypothesis of normality was accepted when $p > 0.05$. Once the hypothesis of normality was accepted, comparisons were performed using unpaired Student t test. When normality resulted rejected, a Mann-Whitney test was used. In both cases, the null hypothesis being that 2 populations do not differ.

## Bacterial cultures

Tonsillar samples were cultured on Columbia agar containing 5% sheep blood and chocolate agar (Laboratorios Britania, Argentina) at 37°C with 5% CO2 for 24 to 48 h. Anaerobes were cultured onto Brucella agar supplemented with hemin and vitamin K under anaerobic conditions. All isolates were identified by MALDI-TOF MS. The isolates were identified by the direct colony on plate extraction method as previously described (8). Mass spectra were acquired using the MALDI-TOF MS spectrometer in a linear positive mode (Microflex, Bruker Daltonics). Mass spectra were analyzed in a m/z range of 2,000 to 20,000. The MALDI Biotyper library version 3.0 and MALDI Biotyper software version 3.1 were used for bacterial identification.

## Immunofluorescence

Cryostat sections (5-10 μm thickness) of tonsils were fixed and stained with mouse anti-human CD1d (clone NOR3.2/13.17 Santa Cruz Biotechnology, CA, USA) followed by chicken anti-mouse IgG antibody AlexaFluor 488 (Thermo Fisher Scientific, BA, Arg). $(IgG/IgM)^+$ cells were detected by addition of goat anti-human IgM+IgG (H+L) $F(ab')_2$ (Jackson Immunoresearch, PA, USA) followed by donkey anti-goat IgG AlexaFluor 594 (Jackson Immunoresearch, PA, USA). $IgA^+$ cells were detected by addition of goat anti-human IgA $F(ab')_2$ (InVivoGen, CA, USA) followed by anti-goat IgG AlexaFluor 594 (Jackson Immunoresearch, PA, USA). Cell nuclei were visualized with 4,6-diamidino-2-phenylindole staining (DAPI, Thermo Fisher Scientific, BA, Arg). Finally, slides were rinsed with phosphate buffered saline, air dried, mounted in Vectashield (Vector Labs, CA, USA) and sealed with a glass coverslip. Samples were examined with a Nikon Eclipse Ti-E fluorescence microscope (Nikon instruments Inc, Tokyo, Japan). Corresponding isotype controls were always added (see **Supplementary Information**).

## Bacterial localization and immunostaining

A solution of 0.5 mg/ml of lysozyme (Sigma Aldrich, Darmstadt, Germany) in 0.1 M Tris-HCl and 0.05 M Na2EDTA was added to the cryosections, followed by incubation at 37°C for 3 h. Fixed, permeabilized tonsillar sections were then incubated in a moist chamber for 4hs at 48°C in hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl (pH 7.6), 0.01% sodium dodecyl sulfate, 30% formamide] containing either the universal probe EUB388 AlexaFluor 488 labeled probe or the negative control (nonsense) NONEUB388 AlexaFluor 546 (Thermo Fisher Scientific, BA, Arg). Stringent washing was performed by incubating the slide in washing buffer [20 mM Tris-HCl (pH 7.6), 0.01% sodium dodecyl sulfate, 112 mM NaCl] at 48°C for 15 min in a moist chamber a number of times, and subsequently rinsed with ddH2O for 5min. Finally, probed-hybridized tonsil cryosections were subjected to immunofluorescence staining as described above to determine the distribution of bacteria within the different tissue compartments. Images were acquired with a Nikon Eclipse Ti-E microscope (Nikon Instruments Inc., Tokyo, Japan) or an Olympus FV1000/IX81 confocal microscope (Olympus Corporation, Tokyo, Japan) using an oil-immersion objective (60X; numerical aperture, 1.42).

# RESULTS

## Characterization of TNFα Production by B Cells From Hypertrophied Tonsils

It is long established that B-cell–derived TNFα plays a crucial role in the development of follicular dendritic cells (FDCs) and B-cell follicles in spleen (9–11). TNFα and IL10 are cytokines with antagonistic actions. Interestingly, tonsils from OSA patients present a defective Breg compartment which correlates with higher proportion of GC B lymphocytes [BGC (6)], larger GC (12), and increased numbers of T follicular helper cells (Tfh) (13) than tonsils excised by other pathologies. Taking all into account, it would be expected that OSA tonsils exhibit a significant lymphocyte–derived TNFα compartment. We examined TNFα expression at the single cell level by FACS, upon TMC culture. A set of cultures was treated for 24 hs with IL2 and IL4 (IL2+IL4), which promote survival of all lymphocyte subsets through slight stimulation (14). Another set of cultures was supplemented for 24

hs and 48 hs with CpG and CD40L (in addition to IL2 and IL4, IL2+IL4+CD40L+CpG) to target stimulation towards B cells, aiming to assess specifically their capacity to contribute to the tonsillar pool of TNFα. Single cells were selected based on FSC-A vs FSC-H analysis (Singlets gate, **Figure 1A**). The cells that were already dead prior to permeabilization were excluded from the analysis through a fixable dead cell staining (Viable gate, **Figure 1A** and **Supplementary Figure 1**). In agreement with previous observations (14), elevated levels of cell death were linked to stimulation of tonsillar cultures (**Figures 1A, B**), dominated by a variety of terminally differentiated and highly activated B cells. In fact, we used this decay in the viability of the TMC cultures to monitor for suitable activation. In our experience, TMC culture stimulation is quite susceptible to minimal changes in experimental conditions (cellular density or FCS and activation cocktail batches, for instance). Therefore, when comparing cytokine secretion within a cohort of patients, those cultures that did not present such evolution in terms of viability, proportion of $CD3^+$ and $CD20^+$ populations and CD20 down-modulation (**Figure 1A**), were not considered. Also, $CD3^+$ cells served as an internal control for cytokine expression (**Figure 1C**). As expected, we observed that TMC from hypertrophied tonsils produced considerable amounts of TNFα when stimulated. At 24 hs post stimulation, approximately one third (29% ± SD 22%) of the cells from the IL2+IL4 stimulated cultures, expressed TNFα and that percentage reached ~40% (37%± SD 16%) in CD40L+CpG+IL2+IL4 stimulated cultures. The latter cultures were particularly affected by the surface CD20 down-modulation which takes place in response to general stimuli (15), albeit more pronounced with CD40L stimulation, as it has been extensively described previously (6, 16). Interestingly, B cells ($CD20^+$) expressing TNFα were those presenting lower levels of CD20 ($CD20^{down}$ $TNF\alpha^+$ cells, **Figure 1A**) suggesting that TNFα expression might be another functional consequence of CD20 modulation and the downstream signaling pathways triggered post internalization. These findings confirmed that the extent of CD20 down-regulation correlates with the degree of B cell activation (**Figures 1A, C**) (6, 15, 16). Despite the fact that lymphoid populations other than $CD20^+$ and $CD3^+$ cells in TMC are negligible (**Figure 1A**, non-cultured panel, double negative cells), their putative expansion, under the culture conditions used, needed to be tested as they would represent a putative contaminant of the $CD20^{down}$ cells. Tonsillar dendritic cells (tDC) identified as $CD20^-CD3^-CD11c^+$ appeared to increase their percentage within the $CD20^+CD20^{down}$ gate under stimuli. Even so, they represented 0.4%-0.7% of the latter, depending on the stimulus (**Supplementary Figure 2A**). Moreover, we could not detect any $CD11b^+$ cells among the cultured TMC (**Supplementary Figure 2B**). The proportion of innate lymphoid cells (ILC) was also assayed (**Supplementary Figure 3**) and proved to be negligible as well (detailed in the next section).

In order to compare TNFα expression by $CD20^{+/down}$ population with that of $CD3^+$ control cells within the same sample, we measured the median fluorescence intensity (MFI) for $TNF\alpha^+$ cells on both populations (**Supplementary Figure 4**). Regardless of the type and length of stimulation, average MFI for $CD3^+$ cells resulted around twice the same parameter for $CD20^{+/down}$ cells (**Figure 1D**). We next determined in which proportion $CD20^{+/down}$ and $CD3^+$ cells were contributing to the total $TNF\alpha^+$ population (**Figure 1E**). Comprehensibly, at 24 hs post stimulation with CD40L+CpG+IL2+IL4, when B cell activation peaked, $CD20^{+/down}$ cells represented the majority of tonsillar $TNF\alpha^+$ cells (52.4% ± SD 13.4% $CD20^{+/down}$ cells vs 41.7% ± SD 12.8% $CD3^+$ cells). On the other hand, at 48 hs post stimulation with CD40L+CpG+IL2+IL4, when tonsillar B cells showed strongly reduced viability, tonsillar $TNF\alpha^+$ population was dominated by $CD3^+$ cells (30.0% ± SD 11.7% $CD20^{+/down}$ cells vs 63.0% ± SD 15.9% $CD3^+$ cells). The cultures stimulated for 24 hs only with IL2+IL4 presented a tendency towards a slightly higher $CD20^{+/down}$ cells contribution to the $TNF\alpha^+$ cell pool than $CD3^+$ cells (**Figure 1F**). Importantly, the ability of tonsillar B cells to express TNFα was proved to be independent on the presence of the T cells in the TMC sample (**Supplementary Figure 5**). Finally, we also tried stimulating the TMC cultures using as a surrogate antigen F(ab′)2 goat antibodies specific for IgM and IgG (in addition to CD40L+IL2+IL4) (14), obtaining very similar results to those of CD40L+CpG+IL2+IL4 stimulation (data not shown). Kim et al. (17) have previously reported that upon culture, TMC from hypertrophied tonsils rendered higher levels of $TNF\alpha^+$ in the supernatant than TMC from children with recurrent tonsillitis. These results extend such findings, indicating that being exposed to various stimuli, B cell population can largely contribute to the TNFα pool, supporting (if not promoting) tonsillar inflammation.

## Presence of Inflammatory T Cells Co-Expressing IL17 and TNFα in Hypertrophied Tonsils

It has been suggested that development of OSA is associated with peripheral Th17/Treg imbalance and is characterized by a pro-inflammatory cytokine microenvironment (2). We have previously reported on the ability of tonsillar B cells to secrete IL17 (6). However, while B cells as a population can dominate tonsillar TNFα production, we found IL17 secretion is indeed controlled by $CD3^+$ cells at every culture condition tested, including those specifically stimulating B lymphocytes (**Figures 2A, B**). TMC cultures supplemented with IL2+IL4 as well as those supplemented with CD40L+CpG+IL2+IL4 rendered an $IL17^+$ population which comprised ~ 90% $CD3^+CD4^+IL17^+$ cells at 24 and 48 hs. We confirmed this observation by measuring cytokines on the supernatant of some of the cultures by multiplex biomarker immunoassay (Luminex). We tried 4 different conditions to specifically stimulate B cells (CpG; CD40L; anti-(IgM/IgG)+CpG; anti-(IgM/IgG)+ CD40L), all of them in addition to IL2+IL4. We also added a culture treated only with IL2+IL4, as control. IL17 concentration did not significantly varied with culture condition compared to the control, as opposed to that of TNFα, which exhibited significant increments under B cell stimulating conditions (data not shown). As expected, $CD3^+IL17^+$ cells were confirmed to be $CD3^+CD4^+IL17^+$ (Th17) cells (**Figure 2B**). Moreover, we tried to estimate the putative contribution of cells other than Th17 and B

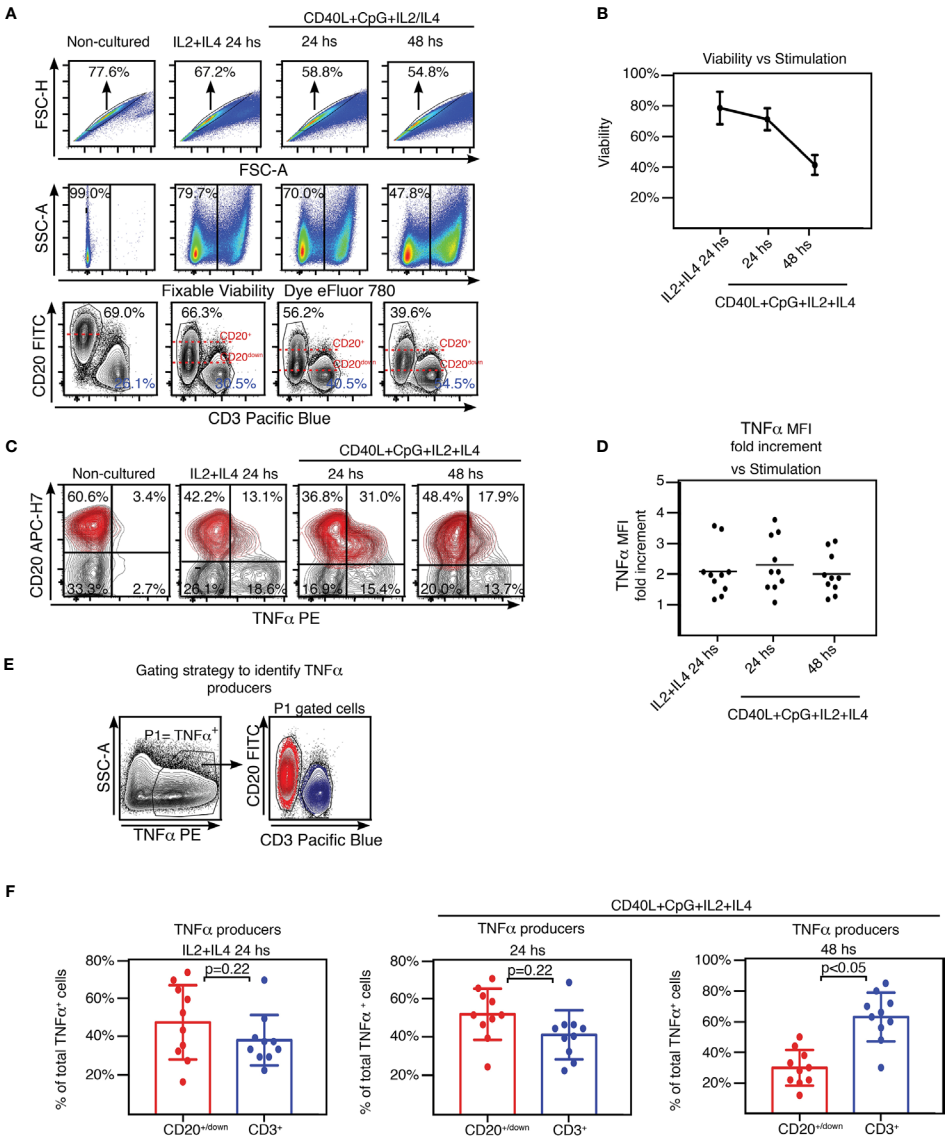


**FIGURE 1** | TNFα expression by tonsillar mononuclear cells. **(A)** Freshly isolated TMC were cultured on IL2+IL4 alone or CpG+CD40L+IL2+IL4, for the time points indicated on the top of each panel. Non-cultured cells served as control (conserved at $4^0$C). Cells were stained for surface CD20, CD3 and intracellular TNFα. Samples were subsequently analyzed by FACS. Gating strategy is partially illustrated. Singlets were gated by plotting FSC-H vs FSC-A for each sample (upper panels). Within the singlets population, dead cells were determined by a fixable viability dye. In this case, the viable gate corresponds to the eFluor $780^-$cells (middle panels). Within the viable gate, lymphoid gate was determined through SSC-A vs FSC-A (not shown). Lower panels: CD20 vs CD3 contour plots. Percentages designate frequencies of the populations indicated. Dashed red lines show the decline of expression of CD20 post stimulation. Data from one experiment representative of 10 independent experiments performed with different individuals each one, are presented. Gates were manually adjusted due to the changes experienced by the cells in culture upon each treatment. **(B)** Line graph plotting the mean percentage ± SD of the frequencies of the viable gate pooled from the 10 independent experiments performed with stimulated TMC and detailed in **(A)**. **(C)** TMC were cultured under the same conditions and stained for the same molecules as in **(A)**. Gating strategy as in **(A)** (singlets-viable-lymphoid, not shown). Contour plots show TNFα and CD20 expression by TMC. Percentages designate frequencies of populations in each quadrant. $CD20^{+/down}$ cells appear highlighted in red, based on the gating performed on the corresponding CD20 vs CD3 graph as in **(A)**, using the backgating option, to exclude any CD3 contamination. Quadrants were manually adjusted due to the changes experienced by the cells in culture upon each treatment. Data from one experiment representative of 10 independent experiments performed with different individuals each one, are presented. **(D)** Graph showing the MFI fold increment=(TNFα MFI of $CD3^+TNFα^+$/TNFα MFI of $CD20^{+/down}$ $TNFα^+$) for each independent experiment at every stimulating condition. Calculations were made following FACS analysis depicted in **Supplementary Figure 4**. **(E)** Gating strategy used to estimate B and T cell specific contribution to the $TNFα^+$ cell pool from the 10 samples analyzed as shown in **(A–C)**. P1 denotes percentage of $TNFα^+$ cell population determined by TNFα vs SSC-A contour profile under the stimulations described above. Panel on the right, representative contour plots for CD20+/down (red) and $CD3^+$ (blue) populations within the P1 gate. **(F)** Histograms presenting the mean percentage ± SD of the $CD3^+$ and $CD20^{down/+}$ cell population frequencies from 10 independent experiments within the $TNFα^+$ cell pool for each stimulating condition, calculated as shown in **(E)** *p* value was calculated through unpaired *t* test, normality was confirmed by the Shapiro-Wilk test.

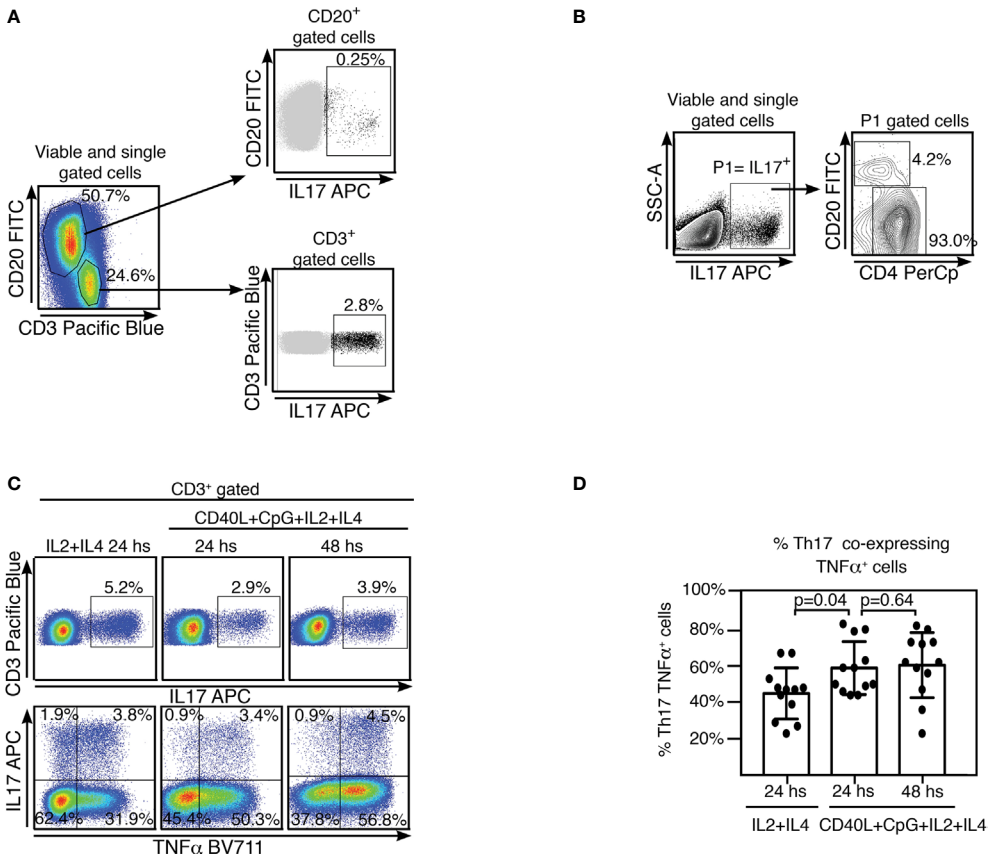


**FIGURE 2** | Tonsillar Th17 cells co-express TNFα and IL17. **(A)** Freshly isolated TMC were cultured on CpG+CD40L+IL2+IL4 for 24 hs. Samples were subsequently analyzed by FACS. Gating strategy is partially illustrated. Singlets were gated by plotting FSC-A vs FSC-H for each sample (not shown). Within the singlets population, dead cells were determined by a fixable viability dye (see Methods). Within the viable gate, lymphoid gate was determined through SSC-A vs FSC-A (not shown). Cells were stained for surface CD20, CD3 and intracellular IL17. Pseudo-color plots show CD3 and CD20 expression by TMC upon stimulation, percentages designate frequencies of populations in each gate (left panel). Upper right panel: dot plot shows IL17 and CD20 expression by TMC, $CD20^+IL17^+$ population is highlighted in black and its frequency within $CD20^+$ population is indicated as percentage. Lower right panel: dot plot shows IL17 and CD3 expression by TMC, $CD3^+IL17^+$ population is highlighted in black and its frequency within $CD3^+$ population is indicated as percentage. Data from one experiment representative of 10 independent experiments performed with different individuals each one, are presented. **(B)** Gating strategy used to estimate the populations contributing to the $IL17^+$ cell subset upon stimulating strategy as in **(A)**. Singlets were gated by plotting FSC-A vs FSC-H for each sample (not shown). Within the singlets population, dead cells were determined by a fixable viability dye (see Methods). Within the viable gate, lymphoid gate was determined through SSC-A vs FSC-A (not shown). P1 denotes $IL17^+$ cell population determined by IL17 vs SSC-A contour profile. Panel on the right, representative contour plots for $CD20^+$ and $CD4^+$ populations within the P1 gate. Percentages designate frequencies of the populations indicated relative to P1 gate. Data from one experiment representative of 6 independent experiments performed with different individuals each one, are presented **(C)** Freshly isolated TMC were cultured on IL2+IL4 alone or CpG+CD40L+IL 2+IL4, for the time points indicated on the top of each panel. Singlets were gated by plotting FSC-A vs FSC-H for each sample (not shown). Within the singlets population, dead cells were determined by a fixable viability dye (see Methods). Within the viable gate, lymphoid gate was determined through SSC-A vs FSC-A (not shown). Cells were stained for surface CD20, CD3, intracellular TNFα and IL17 and finally analyzed by FACS. Upper panels: pseudo-color plots show IL17 vs CD3 expression by TMC. They correspond to events within singlets, viable, lymphoid and $CD3^+$ gates. Percentages designate frequencies of $IL17^+$ cells within $CD3^+$. Lower panels: pseudo-color plots show TNFα and IL17 expression by TMC, gated as in the upper panels. Percentages designate frequencies of populations in each quadrant, relative to the $CD3^+$ gate. Data from one experiment representative of 12 independent experiments performed with different individuals each one, are presented. **(D)** Histograms presenting the mean percentage ± SD of the Th17 $TNFα^+$ cell population frequencies from 12 independent experiments for each stimulating condition, calculated as shown in **(C)**, top right quadrant. *p* calculated through a Mann-Whitney test as the distribution of data in the middle column was not normally distributed.

lymphocytes, to the $IL17^+$ cell population pool. ILC3 are characterized by their capacity of secreting IL17 when stimulated. ILC3 represented around 0.03% of the TMC lymphoid gate (P3, **Supplementary Figure 3**). Their negligible fraction precluded us from detecting whether the culture conditions used stimulated them to express IL17. The same applied to Natural Killer (NK) cells which collectively signified 0.2% of the TMC lymphoid gate (P3, **Supplementary Figure 3**) but only a sub-fraction would be able to express IL17.

Th17 cells have appeared to exhibit plasticity of function and often co-produce other pro-inflammatory cytokines, particularly at sites of inflammation. Hence, we investigated whether tonsillar Th17 also expressed other cytokines as TNFα and interferon γ (IFNγ). The pseudo-color dot plots shown in **Figure 2C** were

gated on $CD3^+$ cells and show CD3 vs IL17 (upper panel) and IL17 vs TNFα (lower panel) staining. We detected co-expression of TNFα in a significant fraction of the Th17 cell population upon all culture conditions tested. At 24 hs post stimulation, nearly half of the Th17 population (44% ± SD 14%) from the IL2+IL4 stimulated cultures co-expressed TNFα. This percentage significantly increased when TMC were cultured with CD40L+CpG+IL2+IL4 for 24 hs (59% ± SD 14,5%) as well as for 48 hs (60%± SD 18%) indicating a positive correlation between higher frequencies of TNFα and IL17 and TNFα co-expression (**Figure 2D**). Low levels of expression of IFNγ observed upon the stimulating conditions tested precluded us from definitive conclusions regarding co-expression of IFNγ and IL17.

We concluded that tonsillar IL17 was produced primarily by Th17, which largely coproduced TNFα. Interestingly, several studies have linked the Th17 pathway with formation of GCs in mice spleens and ectopic B cell follicles at sites of inflammation (18–20). On the whole, TMC from hypertrophied tonsils promptly exhibited a pro-inflammatory cytokine profile in culture.

## The Complex Histological Milieu of Tonsils and the Potential B Cell Contribution to Their Tissue Specific Inflammation

Like all mucosae, tonsillar immune actions (anti- and pro-inflammatory) must be tightly regulated, to balance the protection against virulent germs and the tolerance to harmless flora and innocuous Ags entering with air and food. Collectively, the results described above and others we have previously published (6, 14) are in agreement with the notion that tonsillar hypertrophy is a result of an imbalance between regulatory and effector immune functions which in turn lead to a local chronic inflammation. To this point, we have examined isolated cells. However, *in vivo*, immune processes occur in a complex tissue context which has to be comprehended. In order to recognize the histologic compartments within tonsils, we performed immune-fluorescence staining on cryosections from excised tonsils. The palatine tonsils are secondary lymphoid organs belonging to the mucosal associated lymphoid tissue (MALT), with unique histological characteristics. Unlike the lymph nodes, tonsils do not have afferent lymphatics, but present deep and branched crypts. Ag sample occurs through their distinctive tissue architecture and this is why tonsils are called lympho-epithelial organs. The tonsillar epithelium provides protection as well as serving to transport foreign material from the lumen to the lymphoid compartment. To evidence this mucosal surface, we tested the tonsillar epithelial cells' reactivity to antibodies against CD1d, since it has been reported its expression in other mucosal epithelial cells, like intestinal (21), epidermal keratinocytes (22) and vaginal epithelium (23). We confirmed CD1d expression in tonsillar epithelium. Anatomically, the surface epithelium of the palatine tonsils is an extension of the stratified squamous epithelium of the oral mucosa (**Figure 3A**, **Supplementary Figures 6, 7**). Importantly, in the crypts, the stratified epithelium becomes narrower and laxly textured [reticulated epithelium as termed by Oláh in 1978 (24)] and highly infiltrated with lymphocytes (**Figures 3B–D**). We observed CD1d expression in reticulated epithelium as well as in stratified squamous epithelium. Nevertheless, the level of that expression did not appear uniform at all levels of the multilayered squamous epithelium. The basal cells, which maintained an orientation perpendicular to the basement membrane, presented negligible immunoreactivity to anti CD1d. Conversely, CD1d was expressed strongly in the epithelial cells of the supra-basal and intermediate layers, significantly declining its expression in the apical layers. A similar pattern of CD1d staining is observed in the vaginal stratified epithelium (23). Expression was restrained mostly to the cell membrane in all cases (**Figure 3**). This epithelium was supported by a layer of connective tissue (**Figure 3A**) that separates it from the lymphoid component, and resulted negative for CD1d, with the exception of sporadic infiltrating lymphoid cells. **Figures 3B, C** show a region of crypts lined by reticulated epithelium. In fact, the crypt in **Figure 3C**, is lined by reticulated epithelium on one side and stratified squamous epithelium on the other. Two characteristics of the former are clear from the figures, and these are the desquamation of the upper cell layers and the absence or disruption of the basal layer of the epithelium. In this case, there is no boundary between the epithelium and the underlying lymphoid tissue. The cells in the intermediate layer were distorted and often separated from one another by the invading lymphoid cells, yet again CD1d exhibited high expression in this layer. The reticular epithelium trailed the curving shapes of the underlying follicles. To our knowledge, this is the first report on CD1d expression by tonsillar epithelium.

To visualize the lymphoid compartment, we stained for $(IgM/IgG)^+$ B cells (**Figure 3**). Our results confirmed previous reports (25). $(IgM/IgG)^+$ B cells (and plasma cells, distinguish by their size) appeared located not only in follicles, GC and their mantle zone, but also uniformly scattered throughout the tonsillar tissues (**Figures 3A–F**), generally associated in clusters when infiltrating reticular epithelial tissue (**Figure 3D**). $IgA^+$ B/plasma cells on the other hand, were mainly found outside the germinal centers, especially in the epithelium of crypts and rarely appear in the GCs (**Figure 3F**). Also, it can be observed the protrusion of lymphocytes and plasma cells from the sites of desquamation to the lumen (**Figures 3C, D**). IgG and IgM can be observed in its soluble form, accumulated on the apical surface of the epithelial layer (**Figures 3A, D**). It has been previously shown that nasopharyngeal epithelium widely expresses FcRn, which mediates IgG transport across the epithelial cell layer (26). On the other hand, it does not express pIgR, hence the transport of dimeric IgA is unfeasible through this kind of epithelia (27), making IgG the predominant protective isotype in tonsils. The histological findings we described above are in agreement with those reports. Moreover, FcRn and CD1d belong to the same family of proteins, the non-classical MHC class I molecules and both have been shown to display a restriction in their expression to specific tissues, namely epithelial cells from other mucosal sites (28). Finally, follicular hyperplasia was present in all samples as well as intensively active germinal centers (**Figure 4**). Such hyperplasia is attributable to proliferation of BGC, as we and others have previously shown (6, 12, 29). In light of the results shown in previous sections, such misguided

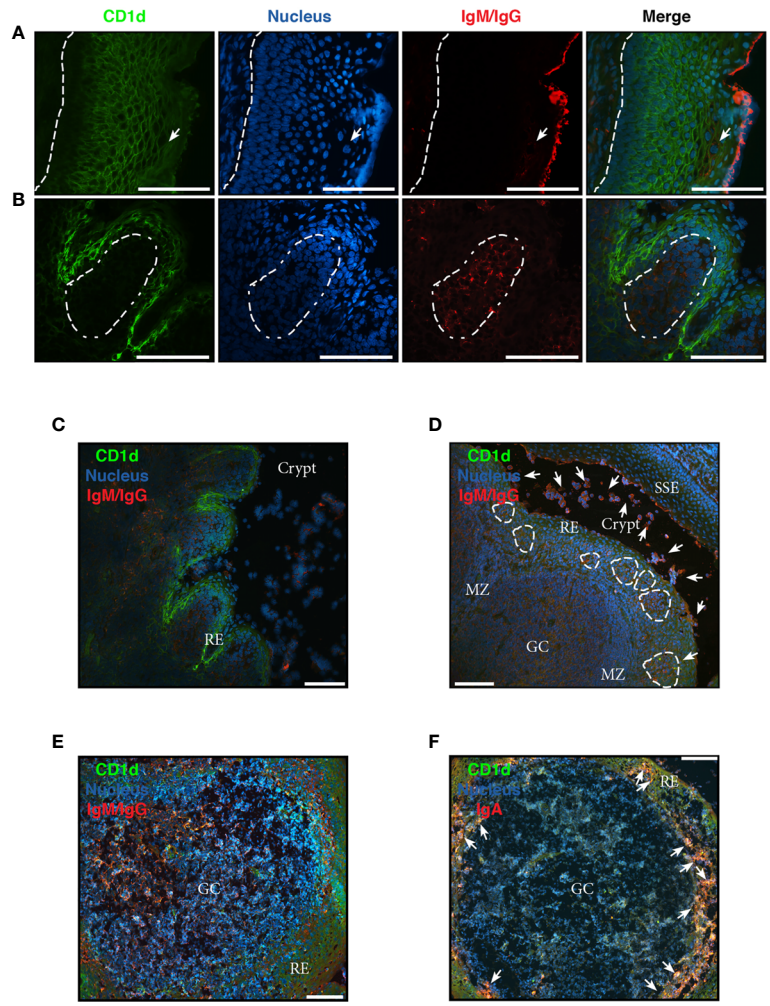


**FIGURE 3** | Analysis of chronically inflamed human tonsils by immunohistology. **(A–F)** Three-color immunofluorescent microscopy of tonsillar frozen sections. **(A)** Representative immunofluorescence staining showing stratified squamous epithelium with CD1d (green), IgG/IgM (red) and nucleus (blue). Dashed line represents the limit between the basal membrane and the epithelium. Arrow indicates $IgG^+/IgM^+$ plasma and B cells intercalated in the mesh of epithelial cells. **(B)** Representative immunofluorescence staining showing reticular epithelium with CD1d (green), IgG/IgM (red) and nucleus (blue). Dashed lines represent the limit of the follicle. **(A, B)** Scale bar, 100 µm. **(C)** A less magnified image of the section shown in **(B)** to evidence the particular characteristics of the lympho-epithelium covering the crypts. RE stands for reticular epithelium. Scale bar, 100 µm. **(D)** Representative immunofluorescence staining showing a germinal center (GC) with its mantle zone (MZ), the reticular epithelium (RE) over it highly infiltrated with lymphocytes. Clusters of B/plasma cells indicated by dashed lines. The crypt and the stratified squamous epithelium (SSE) on the other border of the crypt are also designated in the picture. CD1d (green), IgG/IgM (red) and nucleus (blue) staining. Arrows indicate some of the many $IgG^+/IgM^+$ B and plasma cells scattered all over the tissue and crypt. Scale bar, 100 µm. **(E)** Representative immunofluorescence staining showing single a germinal center (GC). CD1d (green), IgG/IgM (red) and nucleus (blue) staining. Scale bar, 100 µm. **(F)** Representative immunofluorescence staining showing a single germinal center (GC). CD1d (green), IgA (red) and nucleus (blue). Arrows indicate some of the many $IgA^+$ B and plasma cells into the mesh of the reticulated epithelium (RE). Scale bar, 100 µm. Samples were examined with a Nikon Eclipse Ti-E microscope.

B cell response can potentially fuel the local inflammatory microenvironment, extending the impairment of immune homeostatic control.

## Microbiological Aspects of Hypertrophied Tonsils

The most evident potential source of a local persistent immune response would be a local persistent infection. However, as opposed to recurrent tonsillitis, tonsillar hypertrophy has been long considered of non-infectious etiology. Actually, hypertrophied tonsils serve as non-infected control for tonsils affected by recurrent tonsillitis in a number of publications, including relevant and recent ones in which such control should be crucial (30). It has been generally considered that bacterial cultures from hypertrophied tonsils largely reflect oropharyngeal colonization. It is actually quite complex to distinguish infection versus colonization, provided that tonsils, being part of the mucosal immune system, are constantly exposed to the environment.

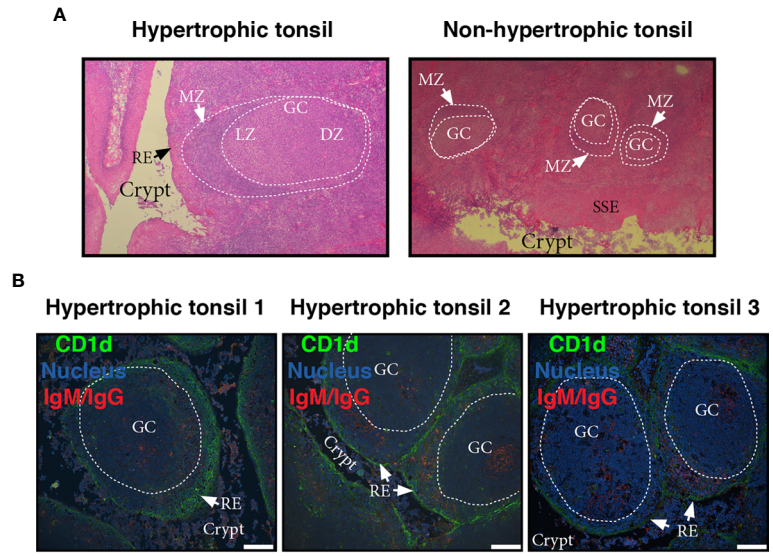


**FIGURE 4** | Microscopic features of germinal centers from hypertrophic tonsils. **(A)** Comparative light microscopy of GC from an hypertrophic tonsil (left) and a non-hypertrophic tonsil (right) which evidence the difference in size largely documented in literature. H.E, original magnification 40x. MZ of the follicles is indicated by white arrows. LZ stands for light zone and DZ stands for dark zone. RE and SSE are also denoted in the pictures. **(B)** Three colour immunofluorescence staining showing GC from tonsils excised from three different OSA patients (1, 2, 3, see also colour code). Note the lymphoid hyperplasia, the degree of infiltration of the reticular epithelium (RE) in all cases, and the crypts invaded by lymphoid cells (most B and plasma cells according to staining). Scale bar, 100 µm. Samples were examined with a Nikon Eclipse Ti-E microscope.

In order to try to discriminate between commensals and pathogens we started by naming the bacterial species that we could retrieve alive from the core of the samples upon cauterization of the surface, trying to identify abundant, relentless and viable populations from the clinical tissue. We cultured the core tonsillar tissue of 44 children undergoing tonsillectomy due to OSA, whose ages ranged from 2 to 15 years old (**Table 1**). Within our cohort of patients, the frequency of tonsillectomy varied with the age, peaking at 5-6 years old (**Figure 5A**). All samples rendered viable bacterial cultures except for one. At phylum level, the tonsil cultures were dominated by *Firmicutes* (predominantly from the genera *Streptococcus* and *Staphylococcus* in that order), *Proteobacteria* (mostly from the genera *Neisseria* and *Haemophilus*), *Bacteroidetes* (principally genera *Prevotella*), *Actinobacteria* and *Fusobacterium* (**Tables 2** and **3**) in agreement with recent data obtained by next generation sequence (NGS) (31).

**TABLE 1** | Basic demographic data of tonsils donors and number of viable bacteriological isolates produced by their samples.

| Age group (years) | N° patients [a] (%) [b] | N° bacterial isolates [c] | Co-isolated bacteria [d] |
|---|---|---|---|
| 2 -5 | 18 (41) | 58 | 3,2 ± 1,5 |
| 6 -9 | 18 (41) | 52 | 2,9 ± 1,3 |
| 10 -13 | 7 (16) | 17 | 2,4± 1,0 |
| 14 -15 | 1 (2) | 6 | 3,0 ± 1,4 |
| **Gender** | | | |
| Males | 27 (61) | 80 | 3,0 ± 1,6 |
| Females | 17 (39) | 53 | 3,1 ± 1,3 |

[a]*The values represent the number of patients in each group who donated samples after undergoing surgery due to obstructive sleep apnea (OSA). n=44 patients were analyzed, n=1 of them did not render viable bacterial cultures.*
[b]*The percentages were calculated relative to the total number of patients (n=44).*
[c]*Total number of bacterial isolates.*
[d]*Mean ±SD numbers of species co-infecting each individual in this cohort of patients per category of gender and age.*

Considering that certain microorganisms like *Streptococcus pyogenes (S. pyogenes), Streptococcus pneumoniae (S. pneumoniae), Moraxella catarrhalis (M. catarrhalis), Haemophilus influenzae (H. influenzae)* and *Staphylococcus aureus (S. aureus)* had been found either causing ear, nose and tonsil (ENT) pathology or as harmless local flora, both situations in competent hosts, we wanted to investigate their prevalence in our patients. We did not detect *M. catarrhalis* within our cohort of patients. We isolated *S. pneumoniae* from two patients. On the other hand, *S. aureus*, isolated from 45% of patients, accounted for the most frequent potentially pathogenic bacteria among the tonsils we analyzed, closely followed by *H. influenzae* (present in 27% of the patients) and *S. pyogenes* (on 23% of the samples). Bacterial isolates potentially pathogenic from the excised samples are shown in **Figure 5B**. In Argentina, vaccination against *H. influenzae* type b, *S. pneumoniae, Neisseria meningitidis (N. meningitidis)* and *Corynebacterium diphtheria*, which have been long known as commensal species in tonsils, is mandatory. We did not detect any patient carrying *Corynebacterium diphtheriae* but we found *Neisseria meningitidis* in the tonsils of a single patient and we did not serotype any isolate of *H. influenzae*.

In most cases we obtained co-isolates of different bacteria. Only 4 patients rendered cultures of single species, 3 of those

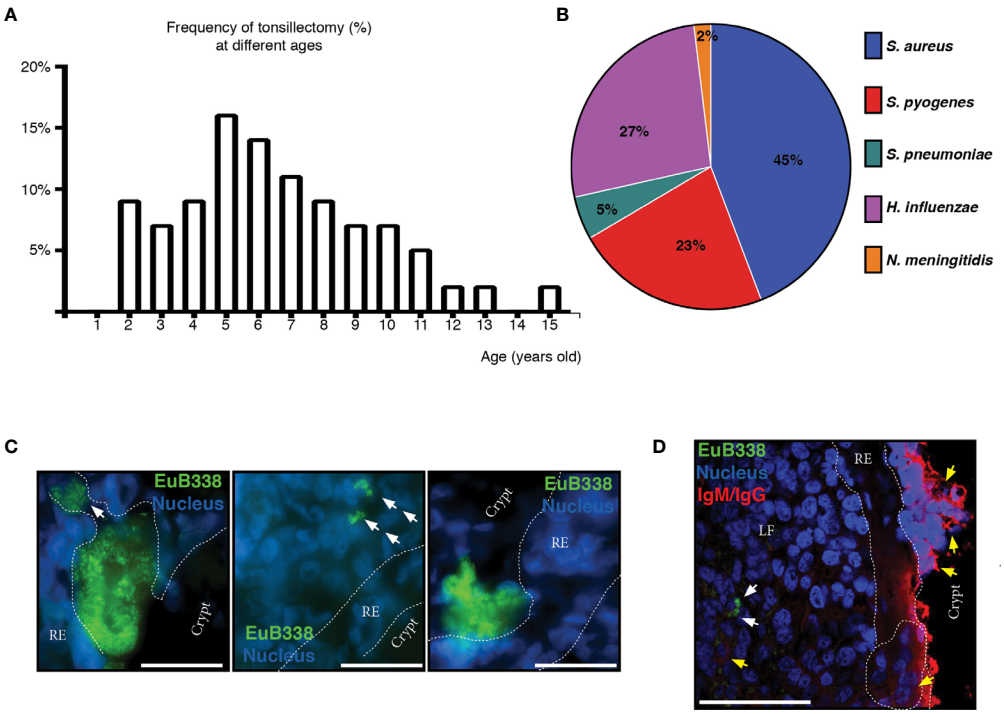


**FIGURE 5** | Microbiological aspects of hypertrophic tonsils. **(A)** Age distribution of patients who had tonsillectomy due to OSA. **(B)** The graph shows the frequency of potential pathogenic bacterial detection in tonsils. **(C)** Microphotographs of the bacterial aggregates in tonsillar frozen sections from different OSA patients evidenced by fluorescence *in situ* hybridization (FISH) with a general eubacterial probe (EUB338-Alexa 488, green). Host cells nuclei were stained with DAPI (blue). Dashed line represents the limits of the reticular epithelium (RE) on both margins. White arrows indicate the conglomerates of bacteria that have penetrated the epithelial barrier. Scale bar, 50 µm. Samples were examined with a Nikon Eclipse Ti-E microscope. **(D)** Three-color immunofluorescent confocal microscopy of tonsillar frozen sections. Bacterial aggregates in tonsillar frozen sections were evidenced as in **(C)** and are indicated with white arrows. Yellow arrows indicate B and plasma cells. Dashed line represents the limits of the reticular epithelium (RE) on both margins. Clusters of B/plasma cells are enclosed by dashed lines. LF stands for Lymphoid Follicle. Scale bar, 50 µm. Samples were examined with an Olympus FV 1000 confocal microscope.

were *Streptococcus pyogenes* group A. Co-isolates ranged from 2 to 7 species. Details of the mean of species co-isolated from each child and all isolated organisms from the samples are listed in **Tables 1–3**. As we isolated a plethora of viable bacteria considered commensal and potential pathogenic as well, we were still unable to discriminate whether the tonsillar hypertrophy is of infectious nature.

The basic distinction between a pathogen and a commensal is that the first one displays aggressive tools for invasion. Usually this represents that such pathogen is able to penetrate the epithelial barriers. Therefore, we set out to detect bacterial presence in tonsillar biopsies by FISH, which allowed us to investigate its distribution and organization *in situ*. We used a fluorescent universal eubacterial (EUB338) probe followed by immune-fluorescence staining on cryosections from excised tonsils. As shown in **Figures 5C, D**, we detected bacterial aggregates not only associated with the surface of the epithelium but also within the lymphoid compartment, having breached the reticular epithelium. Therefore, while we cannot ascertain that the microorganisms detected *in situ* as well as through culture are the initiators of the ongoing inflammatory response, we did evidence that the chronification of the process must be certainly related to bacterial spreading beyond the normal boundaries. Also, such invasion is not associated to a single species as it can be observed in different patients harboring different colonizers, which have become pathogens, given the host´ requirement of the surgery to restore homeostasis.

## DISCUSSION

Tonsils offer a useful model to study the interactions between the human mucosal immune system and the microbes that populates them at the epithelial level. Clinical material is not scarce, as tonsillectomy remains one of the most frequent pediatric surgeries carried out worldwide as a result of diagnosis with OSA. They rank among the secondary lymphatic organs, so every single developmental B cell subset is represented. In this study, initially we show that B cells as a population are able to produce TNFα at similar levels than those reached by T cell population, depending on the stimulating conditions. We have previously demonstrated B cell ability to rapidly produce other pro-inflammatory cytokines as IL6, IL8 and IL17 (6). Taken together, these results led us to conclude that B cell hyperplasia boost the pathological inflammatory processes and tissue alterations characteristic of OSA tonsils. Furthermore, we

**TABLE 2 |** Taxonomic breakdown of isolates from the core of hypertrophied tonsils at phylum level.

| Phylum | N° species | N° bacterial isolates[a] (%)[b] |
|---|---|---|
| Firmicutes | 16 | 65 (49) |
| Proteobacteria | 13 | 39 (29) |
| Bacteroidetes | 7 | 15 (11) |
| Actinobacteria | 5 | 8 (6) |
| Fusobacterium | 2 | 6 (5) |
| **Total** | 43 | 133 (100) |

[a]*The values represent the number of bacterial isolates from tonsils represented at phylum level.*
[b]*The percentages were calculated relative to the total number of isolates identified from the patient's samples.*

**TABLE 3 |** Taxonomic breakdown of viable bacterial isolates from the core of hypertrophied tonsils at genus and species level.

| Aerobic bacterial isolates | N° patients[a] (%)[b] |
|---|---|
| *Actinomyces odontolyticus* | 1 (2) |
| *Actinomyces graevenitzii* | 1 (2) |
| *Corynebacterium argentoratense* | 1 (2) |
| *Escherichia coli* | 1 (2) |
| *Gemella haemolysans* | 1 (2) |
| *Haemophilus influenzae* | 12 (27) |
| *Haemophilus parainfluenzae* | 1 (2) |
| *Neisseria elongata* | 3 (7) |
| *Neisseria flavescens* | 3 (7) |
| *Neisseria macacae* | 1 (2) |
| *Neisseria meningitidis* | 1 (2) |
| *Neisseria perflava* | 1 (2) |
| *Neisseria sicca* | 1 (2) |
| *Neisseria* spp. | 2 (5) |
| *Neisseria subflava* | 10 (23) |
| *Rothia dentocariosa* | 1 (2) |
| *Rothia mucilaginosa* | 4 (9) |
| *Staphylococcus aureus* | 20 (45) |
| *Staphylococcus intermedius* | 1 (2) |
| *Streptococcus anginosus* | 4 (9) |
| *Streptococcus constellatus* | 1 (2) |
| *Streptococcus dysgalactiae* | 2 (5) |
| *Streptococcus intermedius* | 1 (2) |
| *Streptococcus mitis* | 3 (7) |
| *Streptococcus parasanguinis* | 1 (2) |
| *Streptococcus pneumoniae* | 2 (5) |
| *Streptococcus pyogenes* | 10 (23) |
| *Streptococcus salivarius* | 9 (20) |
| *Streptococcus viridans* | 6 (14) |
| *Aggregatibacter aphrophilus* | 2 (5) |
| *Aggregatibacter segnis* | 1 (2) |
| **Anerobic bacterial isolates** | |
| *Capnocytophaga* sp. | 1 (2) |
| *Fusobacterium necrophorum* | 4 (9) |
| *Fusobacterium nucleatum* | 2 (5) |
| *Prevotella intermedia* | 5 (11) |
| *Prevotella oris* | 3 (7) |
| *Prevotella melaninogenica* | 1 (2) |
| *Prevotella nanceiensis* | 3 (7) |
| *Prevotella nigrescens* | 1 (2) |
| *Prevotella* spp. | 1 (2) |
| *Solobacterium moorei* | 1 (2) |
| *Veillonella atypica* | 2 (5) |
| *Veillonella parvula* | 1(2) |

[a]*The values represent the number of patients that rendered the isolated designated in the column of the left.*
[b]*The percentages were calculated relative to the total number of patients (n=44).*

detected a relevant proportion of $CD3^+$ cells secreting IL17, even though we did not use culture conditions capable of specifically stimulating T cells. It has been previously reported that Th17/Treg ratio measured in PBMC is positively related to the severity of OSA and serum levels of C-reactive protein (2). Interestingly, there are a number of human diseases in which B cells contribute to the pathology through the acquisition of a pro-inflammatory cytokine profile and thereby supporting T cell–mediated inflammation (18–20, 32, 33).

While others have reported on the expression of CD1d in different mucosal epithelial cells (21–23), we are the first ones to report its expression by tonsillar stratified as well as reticular epithelium. CD1d is a non-polymorphic MHC class I-like antigen-presenting molecule, restricted to lipid Ag presentation to invariant natural killer T cells (*i*NKT) cells. CD1d-binding lipid Ags from tumors as well as commensal and pathogenic microorganisms have been well characterized by many authors including ourselves (34–37). Tissue specific functions of *i*NKT cells were recently reviewed in (38). Of note, CD1d-mediated Ag presentation by epithelial/parenchimal cells can shape the outcome of immune responses by regulating *i*NKT cells function. This has been demonstrated for the case of hepatocytes, which through CD1d-lipid presentation induce *i*NKT cell tolerance in the liver, protecting from hepatic inflammation. Whereas *i*NKT cells were confirmed to be present in tonsils (39), it remains unknown their specific function at this tissue. Provided we found abundant epithelial expression of CD1d, the diversity of the tonsillar microbiota and the constant contact with environmental Ags, we suggest *i*NKT cells might be functionally as relevant at this site as they are in the gut (40).

Another non-classical MHC class I molecule which has been shown to be expressed by tonsillar epithelial cells is the FcRn. Tonsillar epithelium has been classified as a type II mucosal surface (27). In fact, such definition would fit for the stratified squamous epithelium. The specialized epithelium that covers the tonsillar crypts is only found in that location and is called either reticular or lymphoepithelium. Neither the squamous nor the reticular epithelium express pIgR, but they do express FcRn (41). Hence, the major post switch protective Ig isotype translocated to the lumen from the tonsillar crypts is IgG. Of note, IgM and IgG are the best isotypes to activate complement. This capacity makes them particularly efficient but also more injurious to the host due to the inflammation associated with such effector immune function. Through anti (IgM/IgG) staining we evidenced the hypertrophic GCs, origin of the IgG locally produced (**Figures 4**, **5**). We also stained for IgA and confirmed that $IgA^+$ B cells were mainly found outside the germinal centers. Of note, it has been recently discovered in murine intestine a network of cellular interactions that drives IgM-to-IgA class switch recombination in extrafollicular B cells, compatible with the pattern we observed (42).

When studying the pathogenesis of OSA caused by hypertrophic tonsils, host immune response is only one side of the equation. The other side of it, is the microbiota populating the tonsillar mucosal surface. A diverse pool of bacteria comprising both commensal and pathogenic organisms have been isolated from OSA tonsils. In this study we worked with a

cohort of patients whose ages ranged between 2 and 15 years old. In agreement with previous reports from patients elsewhere (43), the frequency of tonsillectomies within that age limit peaked at 5-6 years old, which is coincident with the beginning of the transition from deciduous to permanent dentition for most children. Interestingly, it has been shown that such process implies the most striking change in the salivary microbiome of all ages, when considering children from birth until 18 years old (44). Choi et al. (45) recently demonstrated that the microbiome profiles of saliva and tonsils are largely similar both, in terms of diversity and composition, in children operated due to OSA. Given the location of tonsils at the back of the oral cavity, it was somehow expected the close relation between the two microbial groups. We could therefore speculate that the important changes in the bacterial species detected when starting to acquire permanent teeth might exert extra pressure on the tonsils of some children, being the draining immune sites first handling oral Ags. Again, probably a number of factors are responsible for the loss of homeostasis, including those of the microflora but also an altered host cell immune function.

In relation to the specific bacterial species identified by culture in our samples, they are much in agreement with other studies performed either by culture or, more recently, by employing 16S rRNA gene sequencing (**Tables 2**, **3**) (31, 45–48). In our experiments, *S. aureus* (45%), *H. influenzae* (27%), *N. subflava* (23%) and S. *Pyogenes* (23%) were the most prevalent bacterial species in the core of hypertrophied tonsillar tissue within our cohort of patients. They were followed by different species of the genus *Streptococcus* and *Neisseria*. Finally, among the anaerobic prevalent isolates, we found species of *Prevotella, Veionella* and *Fusobacterium.* As we have pointed out, all these microorganisms have been isolated from around the world, from tonsils excised due to OSA and also because of recurrent tonsillitis. Clearly, there are some differences depending on the vaccination schemes operating in different countries, which might impact in differences in the prevalence within tonsils' crypts of *S. Pneumoniae* or *N. meningitidis*, for example. However, in general, there is consensus that the bacterial species we have found are normal oropharyngeal commensals in children. A point of particular interest to discuss is whether this means that such commensal can never be injurious. Noticeable, complications in the host immune system would imply the redefinition of most commensals as pathogens. On the other hand, a change in the model of life of these bacterial communities would have the same result. In this context, we identified bacterial aggregates *in situ*. Bacteria can subsist either in planktonic or biofilm states. Planktonic cells are freely motile entities persisting in a liquid environment. In contrast, most of the bacterial species we named above (*Haemophilus, Sptaphylococcus, Streptococcus*) have long been known to have the capacity of forming biofilms. In biofilms, bacteria exist as sessile aggregates encased in a self-produced complex polysaccharide matrix attached to a surface (49). The establishment of biofilms is acknowledged as a virulence factor as it allows bacteria to better resist host immune responses. While we could detect extensive aggregates attached to the surface, we did not intend to particularly identify biofilms, as we did not have samples from normal tonsils to ascertain whether biofilm presence is exclusive of pathology or not. In any case, we detect bacterial penetration through the epithelial layer to the lymphoid compartment.

The obstacle entailed by the lack of healthy pediatric tonsils to use as control group or any other comparator sample constrains the conclusions of our study to some extent. The findings described here are limited to OSA tonsils. We do not have grounds either to rule out or to extrapolate them to any other experimental model or physiological situations.

To conclude, the data presented here shed light on the contribution of the B cell compartment to the inflammatory response in OSA tonsils and underscore the importance of the interplay between the host immune system and the commensal microorganisms able to switch from asymptomatic colonization to invasive disease.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The institutional ethics committee (Clinical Hospital,School of Medicine, Buenos Aires) approved the collection, and use of clinical material conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). Informed consent was obtained from all participants and/or their legal guardian/s. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

LS and JR processed all the samples used in this study, performed most of the experiments and analyzed the data. RR performed a number of experiments. LB and PB performed cell sorting and advised on design of FACS experiments. GB and NS performed cryosections and H&E stainings. LS, JR, PF and BP critically reviewed and edited the manuscript. MA and BP provided samples. CB, CV and FM performed the microbiologic culture and identification. EA supervised and designed research, analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.648064/full#supplementary-material

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# RvE1 Impacts the Gingival Inflammatory Infiltrate by Inhibiting the T Cell Response in Experimental Periodontitis

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Periodontitis is a chronic inflammatory disease associated with the formation of dysbiotic plaque biofilms and characterized by the progressive destruction of the alveolar bone. The transition from health to disease is characterized by a shift in periodontal immune cell composition, from mostly innate (neutrophils) to adaptive (T lymphocytes) immune responses. Resolvin E1 (RvE1) is a specialized pro-resolution mediator (SPMs), produced in response to inflammation, to enhance its resolution. Previous studies have indicated the therapeutic potential of RvE1 in periodontal disease; however, the impact of RvE1 in the microbial-elicited osteoclastogenic immune response remains uncharacterized *in vivo*. In the present study, we studied the impact of RvE1 on the gingival inflammatory infiltrate formation during periodontitis in a mouse model. First, we characterized the temporal-dependent changes of the main immune cells infiltrating the gingiva by flow cytometry. Then, we evaluated the impact of early or delayed RvE1 administration on the gingival immune infiltration and cervical lymph nodes composition. We observed a consistent inhibitory outcome on T cells -particularly effector T cells- and a protective effect on regulatory T cells (Tregs). Our data further demonstrated the wide range of actions of RvE1, its preventive role in the establishment of the adaptive immune response during inflammation, and bone protective capacity.

**Keywords: T cells, Periodontal disease, inflammation, RvE1, SPMs (specialized pro-resolving mediators)**

## INTRODUCTION

Periodontitis is a chronic inflammatory disease characterized by the progressive destruction of the tooth-supporting apparatus, comprised of the periodontal ligament, radicular cement, and alveolar bone (1, 2). While the microorganisms that colonize the dental biofilm are considered as primary etiological agents, the main determinant of the disease progression is the host's immune response against the microbial challenge (3). Due to a chronic stimulation by the continuous presence of microorganisms, various types of immune cells infiltrate the periodontal tissues under physiological conditions, keeping a state of mild inflammation (4). Among these patrolling cells, neutrophils are significantly enriched and play a critical role in periodontal homeostasis while the lymphocytic compartment, particularly $CD3^+$ T cells, is the dominant population with the vast majority bearing a $CD45RO^+$ memory phenotype (4).

The transition from health to disease in periodontal tissues is characterized by a shift in immune cell composition. There is an increase in the proportion of neutrophils ($CD15^+CD16^+$ cells), which may possess a hyper-inflammatory phenotype characterized by the over-expression of reactive oxygen species and proinflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) (4, 5). Tissue-resident macrophages are expanded, and circulating monocytes are recruited to be differentiated into macrophage-like cells (6, 7). The chronic inflammatory infiltrate in periodontal tissues during disease is mostly constituted by $CD3^+$ T cells (4, 8). $CD4^+$ Th17 cells are significantly expanded by the microbial dysbiosis around the tooth, requiring local IL-6 and IL-23 production for their expansion (8, 9). Th17 cells are key drivers of the host defense against a dysbiotic microbial community, as demonstrated by the inhibition of Th17 cells accumulation in the oral mucosa and draining lymph nodes due to broad-spectrum antibiotic treatment in the periodontitis model (9). These cells are also critical players in the induction of bone loss by directly expressing RANKL and being the primary producers of IL-17, which further promotes RANKL expression by periodontal ligament fibroblasts and osteoblasts (9). These observations suggest a critical role for T cell-mediated transition from health to periodontitis and an osteoimmunological basis for destruction of alveolar bone (3). It is unclear whether this process can be reversed by restoring the inflammatory and immunological homeostasis in periodontal tissues.

Unlike the anti-inflammatory process where inflammation is blocked, resolution of inflammation is an active phenomenon regulated by specific mediators named specialized pro-resolution mediators (SPMs), a genus of endogenous lipid mediators that include separate molecular families, such as lipoxins, resolvins, protectins, and maresins (10). Resolvin E1 (RvE1, 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-EPA) is an SPM produced by converting eicosapentaenoic acid (EPA) into 18R-hydro (peroxy)-eicosapentaenoic acid (HEPA), which is metabolized by activated leukocytes into RvE1 (11). RvE1 is produced in response to the activation of inflammation, where it enhances the resolution phase by decreasing neutrophil chemotaxis and enhancing a non-phlogistic and macrophage-directed clearance of apoptotic neutrophils (12). Evidence over the years has further highlighted the wide range of actions of RvE1 on different cell types, such as inhibiting osteoclast differentiation and bone resorption capacity (13), attenuates the damage of macrophages by induced oxidative stress (14), inhibits excessive proliferation of fibroblasts after injury (15), regulates platelet activation (16), inhibits dendritic cell migration (DCs), and T cell activation (17).

RvE1 prevents and reverses the alveolar bone loss in experimental periodontitis in rats and rabbits (18–21). *In vitro* studies of RvE1's effect on neutrophils from patients with periodontitis showed that it reduced their superoxide production (18). The treatment with RvE1 enhanced the phagocytic capacity of macrophages from patients with aggressive periodontitis to a similar level to that of healthy controls, indicating the possible rescue of the phagocytic activity (16). Even though RvE1 is not intrinsically antibacterial, the prevention and treatment of periodontitis with topical RvE1 markedly modulated the oral microbial composition in a rat model of periodontitis (20). The impact of RvE1 in the microbial-elicited osteoclastogenic immune response remains uncharacterized *in vivo*. In the present study, we sought to investigate the impact of RvE1 on the gingival inflammatory infiltrate formation during periodontitis in a mouse model. We characterized the temporal-dependent changes of the main immune cells infiltrating the gingiva and the impact of RvE1 administration. We observed a consistent inhibitory outcome on T cells -particularly effector T cells- and a protective effect on regulatory T cells (Tregs). Our data further demonstrated the wide range of actions of RvE1, its preventive role in the establishment of the adaptive immune response during inflammation, and bone protective capacity.

## RESULTS

### Characterization of the Gingival Inflammatory Infiltrate During Periodontitis

To characterize the time-dependent frequency of the cellular components of the gingival inflammatory infiltrate within all $CD45^+$ cells during the periodontal disease initiation and progression, we experimentally induced periodontitis in mice by placing ligatures for 1, 3, 5, or 10 days (**Figure 1A**). As previously observed by our group (22), the alveolar bone loss was significant by day five after the placement of ligatures ($P<0.01$) and reached its peak on day 10 ($P<0.001$; **Figures 1B, C**). To analyze the gingival immune infiltrate composition, we used the clustering algorithm FlowSOM and examined the behavior of 8 extracellular markers among the $CD45^+$ gingival cells. We detected that at all time points, the three dominant populations were neutrophils ($CD45^+LY6G^{high}LY6C^{mid}CD11b^{+/-}$), macrophages ($CD45^+CD64^+CD11b^+MHCII^+$), and T cells ($CD45^+CD3^+$), which collectively represented >70% of all $CD45^+$ cells (**Supplementary Figure 1**).

We then analyzed the shift in the frequency of these three cell populations as the disease progressed. We observed a stereotyped behavior (**Figures 1D, E**), where neutrophils peaked earlier than the other cell types, reaching their higher frequency 24h after the

ligature placement (≈45%, P<0.05), suggesting an early activation of the innate immune response. Later (on day 3), macrophages reached their peak frequency (≈40%, P<0.05), indicating a transition to the adaptive immune response. T cells, which are the predominant immune cell type at the steady-state in the gingiva (P<0.05), showed a stable increase in their frequency, reaching similar levels as baseline by day 10 after the placement of ligatures (**Figures 1E, F**). When the cell numbers were analyzed, similar tendencies as frequencies were observed although the number of T cells decreased at later time points (**Figure 1G**).

## Early RvE1 Application Prevented the Progression of Bone Loss and Gingival T Cell Infiltration

To assess the impact of RvE1 on the development of the gingival inflammatory infiltrate, we administered RvE1 topically daily, starting 2h before the placement of the ligatures (**Figure 2A**). The early administration of RvE1 had a clear impact on the prevention of the alveolar bone loss; the accumulated bone loss was significantly diminished compared to non-treated animals (P<0.05; **Figures 2B, C**). Daily administration of the vehicle we used to dilute the RvE1 (Sham) did not have any impact on the alveolar bone loss (**Supplementary Figure 2**).

To effectively monitor the changes in gingival immune composition, we then used a gating strategy that emulated the cell-population clustering identified initially by the FlowSOM algorithm (**Supplementary Figure 3A**). Using this gating strategy, we were able to identify the shift of the gingival immune cells and demonstrated the spatiotemporal changes in neutrophils, macrophages, and T cells as the three major cell populations at all time points (≈60%) (**Figures 2D, E**). We did not detect any significant differences between treated and

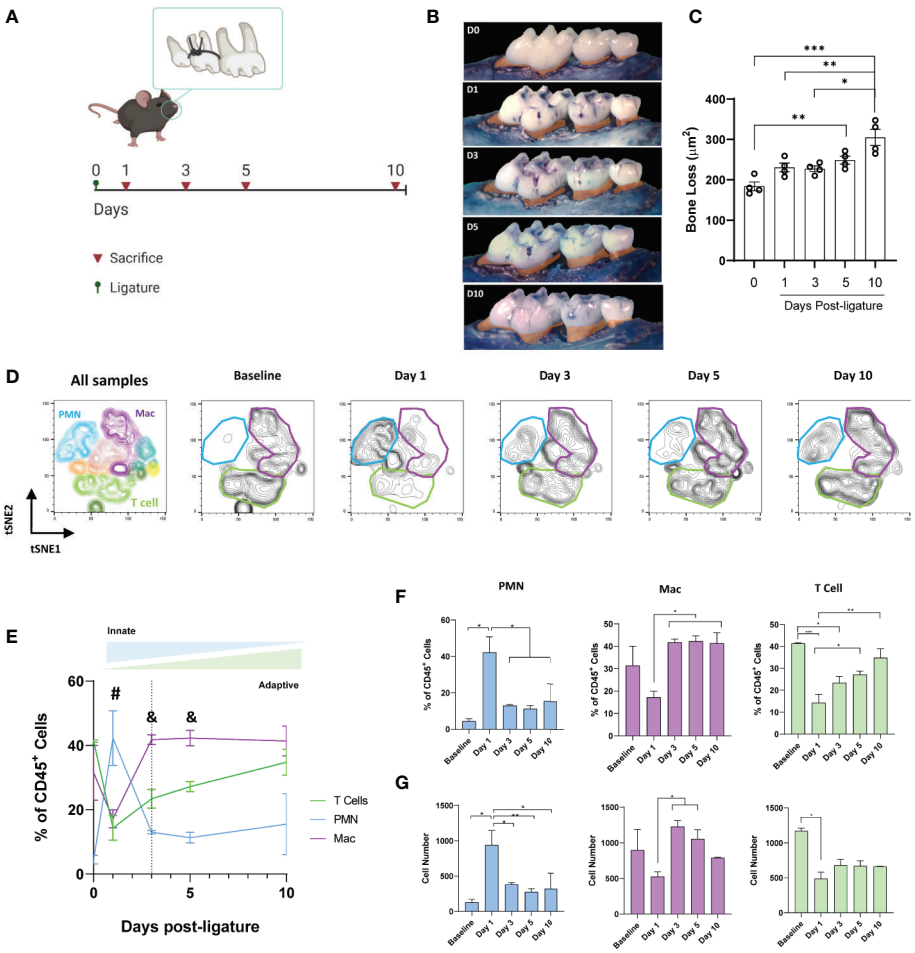


**FIGURE 1** | Gingival immune-infiltrate shift during experimental periodontitis progression. **(A)** Experimental design. **(B)** Representative palatal views of maxillary molars from animals at baseline (D0) or sacrificed at days 1, 3, 5, or 10 after ligature placement. Bone loss area is highlighted in orange. **(C)** Bone loss measurements from the cementoenamel junction to alveolar bone crest. **(D)** Identification of gingival neutrophils (PMN, $CD45^+LY6G^{high}$ $LY6C^{mid}CD11b^+$), macrophages (Mac, $CD45^+CD64^+CD11b^+MHCII^+$), and T cells ($CD45^+CD3^+$) as clustered by FlowSOM. tSNE graphs of concatenated data (4 animals per group) showing CD45+ gingival cells. **(E)** Comparison of the peak frequencies of PMN, T cells, and macrophages within $CD45^+$ gingival cells over time. Dotted line represents day 3 (highlighting transition from innate to adaptive immune response) **(F)** Percentages and **(G)** cell number of neutrophils, T cells, and macrophages in gingiva. $^{\#}$P<0.05 PMN *vs* T cell and Mac; $^{\&}$P < 0.05 Mac *vs* T cell and PMN; *P < 0.05; **P 4< 0.01; ***P < 0.001.

untreated animals in the frequency of $CD45^+$ cells among live cells in the gingiva (**Supplementary Figure 3B**). When we compared the kinetics of the frequencies of those three cell populations in non-RvE1-treated versus RvE1-treated animals, there were no significant changes in the innate immune cells (**Figures 2F, G**) while the gingival infiltration of T cells was significantly reduced on days 3 and 5 after the ligature placement in the RvE1 group ($P<0.05$; **Figure 2H**).

## Early RvE1 Application Reduced the Frequency of Effector T Cells in Cervical Lymph Nodes

To further corroborate the effect of RvE1 on the T-cell response, we analyzed the impact of early RvE1 application on $CD4^+$ T cells in cervical lymph nodes at day 10 after the placement of ligatures (**Figure 3**). Animals with periodontitis showed a reduced frequency of $CD4^+$ T cells within all cells in the lymph nodes as a direct result of increased organ size (lymphadenomegaly) compared to baseline (65% less, $P<0.001$) and RvE1-treated animals (42% less, $P<0.01$). RvE1-treated animals recovered the frequency of $CD4^+$ cells significantly compared to non-treated animals ($P<0.05$; **Figures 3A, E**). The percentage of $Foxp3^+$ cells in the $CD4^+$ gate was increased in animals with periodontitis compared to both baseline and RvE1-treated animals (both ≈70% difference, $P<0.001$; **Figures 3B, F**). Finally, we analyzed the expression of IL-17 and IFNγ on $CD4^+$ cells. We detected a significant reduction in the percentage of $CD4^+IL17^+$ cells in RvE1 group (≈75% less, $P<0.001$) comparable to healthy animals (**Figures 3C, G**). A similar tendency was observed with $CD4^+IFNy^+$ cells, although the differences were not significant (**Figures 3D, H**).

## Delayed RvE1 Application Selectively Inhibited the T-Cell Mediated Response

Since RvE1 modulated neutrophil activity as an initial step in the induction of the inflammatory process, we used a delayed RvE1 administration to measure the T cell infiltration in response to RvE1 after the neutrophils were activated. The working hypothesis in this experiment was that T cell response could be directly impacted independent of neutrophil activation during the course of inflammation. We placed the ligatures for 24 hours, allowed the neutrophils to infiltrate the gingival tissues and then started the RvE1 application. We performed a single readout at day ten after the placement of ligatures (**Figure 4A**). The delayed administration of RvE1 significantly reduced alveolar bone loss by ≈40% ($P<0.05$; **Figures 4B,C**).

Using the same gating strategy as above, we compared the frequency of dominant cell populations in the $CD45^+$ cells in gingival tissues (**Figures 4D, E**). T cell percentage was significantly reduced compared to non-RvE1-treated animals ($P<0.05$; **Figure 4F**). The percentage of DCs, which were the fourth dominant cell population among $CD45^+$ cells in this particular experiment, were significantly reduced by the RvE1 administration ($P<0.05$; **Figure 4I**). Macrophages were not significantly impacted by the delayed application of the RvE1 (**Figure 4G**) while neutrophils were significantly increased in the RvE1 group compared to both untreated ($P<0.05$) and baseline animals ($P<0.05$), representing the predominant cell population (≈50% of all $CD45^+$ cells, **Figure 4H**).

Next, we analyzed the mRNA expression of cytokines in the gingiva (**Figure 4J**). IL-17 and IL-6 were significantly reduced by the delayed RvE1 administration, reducing their expression in 78% and 65%, respectively, compared to non-treated animals ($P<0.05$; **Figures 4K, L**). In parallel, we measured the leukocyte composition in cervical lymph nodes (**Figures 5A, B**) and analyzed the percentage of T and B cells within $CD45^+$ cells to identify their proliferative changes due to the inflammatory process. We detected a significant increase of T cell frequency in animals with periodontitis and a significant reduction in animals that received the delayed application of RvE1 (≈20% less, $P<0.05$; **Figure 5C**). There was no comparable impact on B cells (**Figure 5D**).

## Delayed RvE1 Application Restored Regulatory T Cell Response in Experimental Periodontitis

Since the impact of RvE1 was profound on the T cell responses in both gingiva and cervical lymph nodes, we then tested whether this effect was primarily targeting the effector T cells, thus sparing regulatory T cells (Tregs; **Figure 6**). For this set of experiments, we used wild-type FVB mice. First, we examined the percentage of $Foxp3^+$ cells among $CD4^+CD25^+$ cells. As expected, there was an imbalance between the proliferation of effector T cells ($CD4^+CD25^+Foxp3^-$) and Tregs ($CD4^+CD25^+Foxp3^+$) during the experimental periodontitis. Frequency of Tregs among $CD4^+CD25^+$ cells were reduced ($P<0.001$). RvE1 significantly recovered the Tregs frequency ($P<0.05$; **Figure 6B**). We also analyzed the expression of IL-17 on Tregs and detected a small but significant population of $IL\text{-}17^+$Tregs in diseased animals ($P<0.0.05$), which was reduced in both percentage and number in the RvE1 group (**Figures 6A, D, E**).

# DISCUSSION

In the present study, we measured the impact of RvE1 on the formation of the gingival inflammatory infiltrate and cell composition in cervical lymph nodes in a murine model of ligature-induced periodontitis. Using different experimental designs, we demonstrated that the RvE1 inhibited the infiltration of T cells in gingival tissues and effector T cell response in cervical lymph nodes. Our assessment of the immune cell populations infiltrating the gingiva at different time points indicated that neutrophils, macrophages, and T cells were the most prevalent leukocytes during the progression of periodontitis. The early administration of RvE1 dampened the gingival infiltration of T cells and reduced the frequency of $CD4^+IL\text{-}17^+$ T cells in lymph nodes without affecting neutrophils or macrophages. When we delayed the RvE1 administration to 24h after the placement of ligatures, we observed a reduced gingival T cell infiltration associated with less gingival IL-6 and IL-17 mRNA expression and dampened $CD4^+$ proliferation in cervical lymph nodes. We also detected that RvE1 promoted the Treg response and prevented their phenotypic instability. Collectively, our data provides a new insight to the role of T cells during the

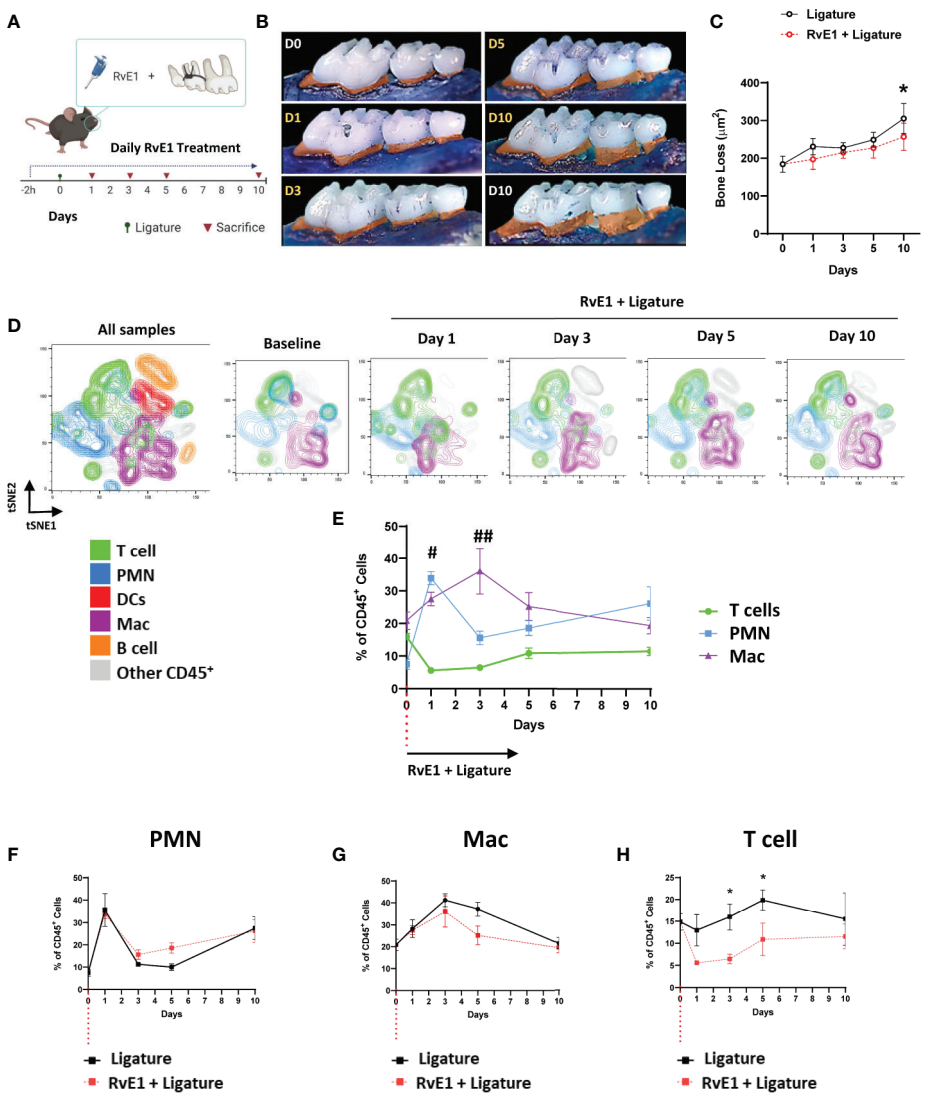


**FIGURE 2** | Temporal-dependent changes of the gingival immune-infiltrate in experimental periodontitis due to early RvE1 administration. **(A)** Experimental design. **(B)** Representative palatal views of maxillary molars from animals sacrificed at days 1, 3, 5, 10 after ligature application with or without RvE1 treatment (D1-D10 in yellow), baseline (D0) and ligature without treatment at day 10 (D10 white) as references. Bone loss area is highlighted in orange. **(C)** Bone loss in RvE1-treated and non-treated animals (n=5). **(D)** Identification of neutrophils (PMN, $CD45^+Ly6G^+$), macrophages (Mac, $CD45^+CD11b^+CD64^+$), and T cell ($CD45^+CD3^+$) subsets in tSNE graphs of concatenated data (n = 4 animals per group) within $CD45^+$ gingival cells. **(E)** Comparison of the frequencies of PMN, T cells, and macrophages within $CD45^+$ gingival cells **(F–H)** Comparison of the frequency of each cell subtype within $CD45^+$ gingival cells over time in animals with or without RvE1 treatment. $^{\#}P < 0.05$ T cells *vs* PMN and Mac; $^{\#\#}P < 0.01$ T cells *vs* PMN and Mac; $^{*}P < 0.05$.

progression of periodontitis and the impact of RvE1 administration on the periodontal immune response.

Our group has demonstrated that RvE1 prevents and regenerates the alveolar bone in various animal models of periodontitis (18–20). In these studies, end-point disease parameters were measured and kinetics of immune cell infiltrations were not assessed. Based on these studies, the mechanism of action of RvE1 seemed to be through neutrophils and macrophages and bone turnover was actively regulated by the restoration of osteoblastic/osteoclastic activities. Consistently, in a rat model of experimental periodontitis, RvE1 reduced the gingival expression of neutrophil chemoattractants such as CXCL1, CXCL2, and CCL3 (20). RvE1 effectively reduced the production of superoxide by neutrophils isolated from peripheral blood of patients with localized aggressive periodontitis, and increased the murine neutrophil phagocytosis of the periodontal pathogen *P. gingivalis* (18, 23). These functional changes were attributed to the binding of RvE1 to its cognate receptor, ERV1, expressed on neutrophils (23). RvE1 has also been shown to inhibit neutrophil infiltration in other inflammatory models, such as zymosan-induced peritonitis and dorsal air punch studies (21). Therefore, we expected to see less infiltration of neutrophils in animals with

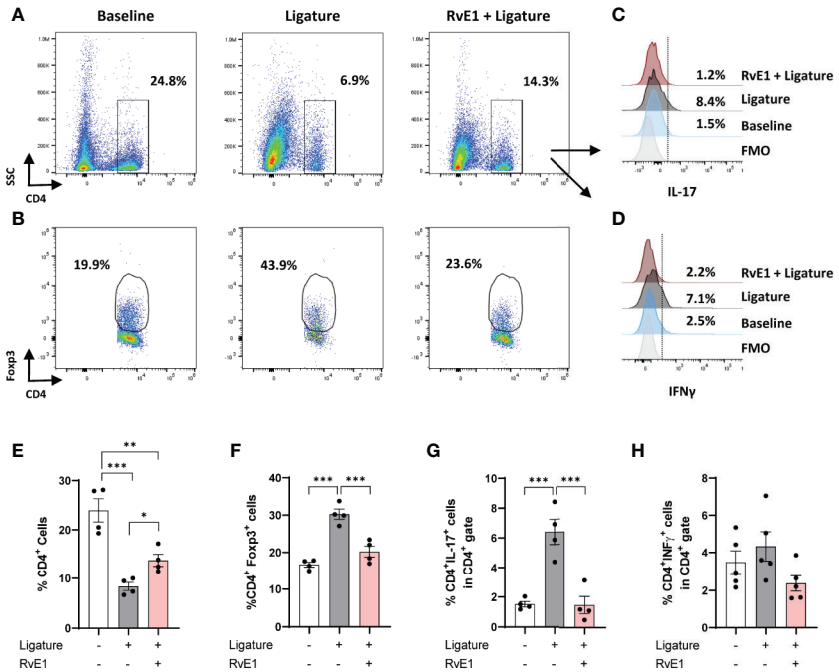


**FIGURE 3** | Impact of early RvE1 administration on $CD4^+$ cells in cervical lymph nodes. **(A, B)** Representative dot plots indicating the frequency of $CD4^+$ and $CD4^+Foxp3^+$ cells among all cells from cervical lymph nodes of animals with ligature-induced periodontitis (10 days; ligature), pre-treated with RvE1 (RvE1 + Ligature) or baseline (no ligature). **(C, D)** Representative histograms indicating the expression of IL-17 or IFNγ in $CD4^+$ cells. **(E–H)** Frequency of total CD4+ cells, $CD4^+Foxp3^+$ cells, and $CD4^+$ IL-17 or $IFN\gamma^+$ cells in cervical lymph nodes (n = 4). FMO, Fluorescence minus one. *P < 0.05, **P < 0.01, ***P < 0.001.

ligature-induced periodontitis that received early administration of RvE1. However, we did not detect such changes in the infiltration kinetics of neutrophils. Instead, we observed a significant alteration in the infiltration of T cells. CXCL1, CXCL2, and CCL3 production is dependent on the expression of Th17-related cytokines such as IL-17 and IL-23 (24, 25). Our research group previously demonstrated that at day five of experimental periodontitis begins the gingival over-expression of Th17-related cytokines, paired with increased differentiation of Th17 cells in cervical lymph nodes (22). Notably, the Th17 response commences while the alveolar bone loss becomes significant. Hence, we inferred that the reported reduced expression of IL-17-induced chemokines such as CXCL1, CXCL2, and CCL3 in experimental periodontitis was not necessarily associated with less neutrophil infiltration, but derived from a reduced Th17-response, which is associated with a decreased alveolar bone loss.

We detected a higher frequency of neutrophils among gingival $CD45^+$ cells in animals with periodontitis that received a delayed administration of RvE1. Since we allowed the neutrophil response to occur normally, by postponing the treatment until after neutrophils peaked in frequency at the gingiva, our finding indicated that RvE1 promoted the renewal of early arrived gingival neutrophils at later stages of the periodontitis progression, and limited the T cell infiltration. Thus, RvE1 prolonged the innate response, which was associated with reduced alveolar bone loss. Neutrophils were once thought to be relatively monofunctional cells, with a role comprising purely the early recruitment to the site of injury to kill and remove infectious agents. However, the diversity of their immunomodulatory functions has become increasingly apparent (26), mainly through apoptosis, which provides a powerful anti-inflammatory signal. Neutrophils exert a sustained anti-inflammatory phenotypic reprogramming of macrophages, which is reflected by the sustained reduction in the release of pro- but not anti-inflammatory cytokines from macrophages (27). Recent evidence has demonstrated the suppressive role of neutrophils during microbial infections (28). For example, neutrophils are able to produce IL-10 following interaction with LPS-stimulated Tregs by direct cell-cell contact. $IL\text{-}10^+$ neutrophils are also induced by exogenous IL-10 in a positive feedback loop. $IL\text{-}10^+$ neutrophils have been detected in patients with periodontal abscess induced by Gram-negative bacteria (29). Since we detected a downregulated expression of the proinflammatory cytokines IL-17 and IL-6 in the periodontal tissues of animals treated with RvE1, it is possible to infer that the increased infiltration of neutrophils could be associated with an anti-inflammatory response, which in turn decreases the activation of the later adaptive immune response (26). However, further studies are needed to evaluate the direct impact of RvE1 on the resolution-phase associated functions of neutrophils, such as the production of IL-10 and their interaction with effector T cells and Tregs.

Emerging evidence indicates that SPMs regulate the adaptive immunity by modulating the development of B cells and T cells

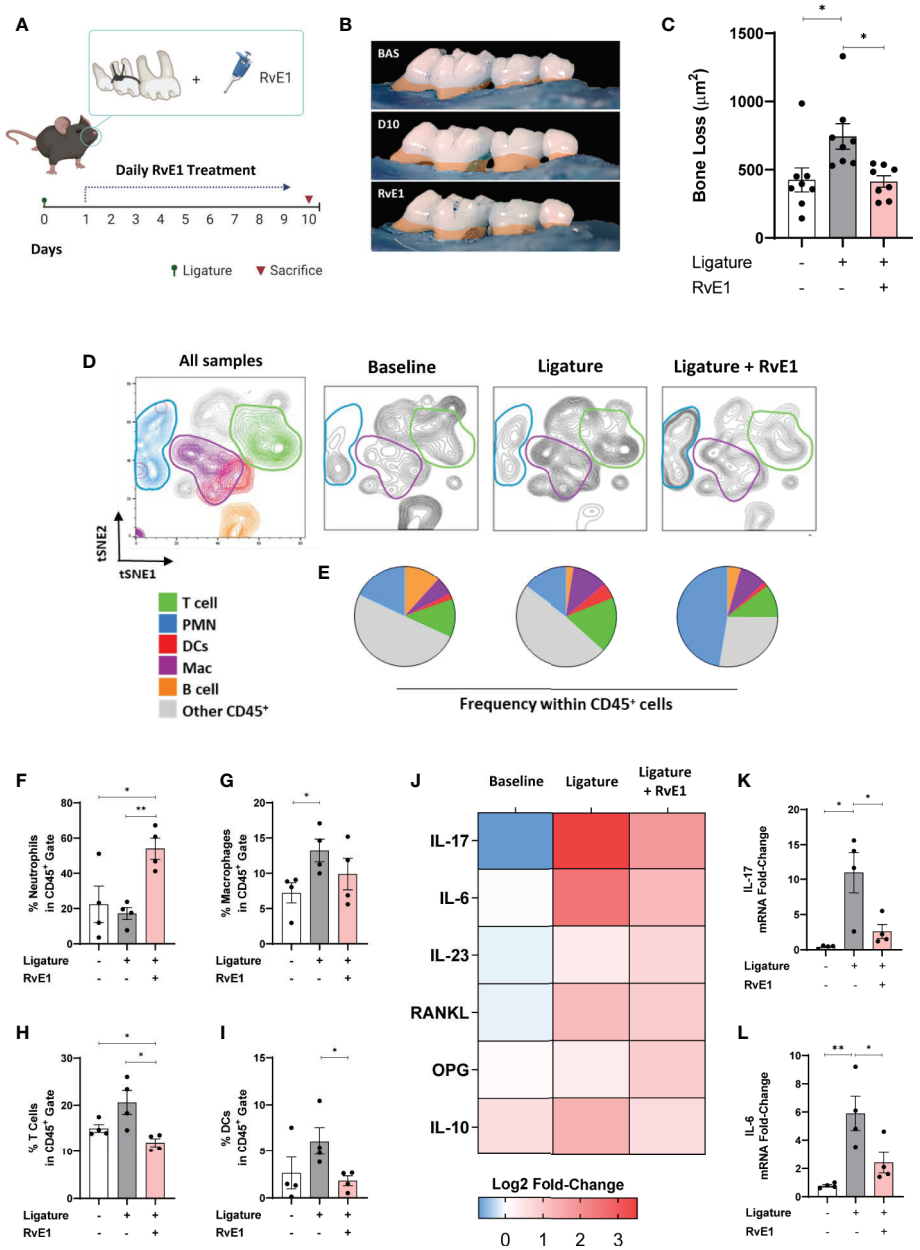


**FIGURE 4** | Impact of delayed RvE1 administration on the gingival immune-infiltrate composition. **(A)** Experimental design. **(B)** Representative palatal views of maxillary molars from animals at baseline (BAS), untreated ligature-induced periodontitis (D10), or treated with RvE1 after day 1 (RvE1). Bone loss area highlighted in orange. **(C)** Bone loss measurements from the cement-enamel junction to alveolar bone crest. **(D)** tSNE plots of concatenated data (n = 4 animals per group), and **(E)** pie charts indicating the frequency of neutrophils (PMN, $CD45^+Ly6G^+$), macrophages (Mac, $CD45^+CD11b^+CD64^+$) and T cells ($CD45^+CD3^+$), Dendritic cells (DCs, $CD45^+CD11c^+MHCII^+$), B cells ($CD45^+CD19^+MHCII^+$), and other cells within gingival $CD45^+$ cells. **(F–I)** Percentages of neutrophils, macrophages, T cells, and DCs in $CD45^+$ gate. **(J)** Heat map representing the log2 fold-change gingival expression of mRNA of cytokines. **(K, L)** Gingival mRNA expression of IL-17 and IL-6. *P < 0.05, **P < 0.01.

through direct contact or indirectly by changing the fate of antigen-presenting cells (30–32). RvE1 inhibits the inflammatory phenotype of different T cell-dependent pathologies. In an allergic airway inflammation model in mice, RvE1 reduced the expression of both IL-23 and IL-6, which are critical to sustaining the differentiation of Th17 cells (31). RvE1 inhibited the Th1/Th17 responses in corneal allograft transplantation in mice (33) and reduced the Th2-response in a murine model of asthma (34). Based on our previous description of the temporal progression of bone loss and appearance of Th17 markers in gingiva during ligature-induced periodontitis, we decided to analyze the changes in the gingival immune infiltrate due to RvE1 treatment for 10 days (22).

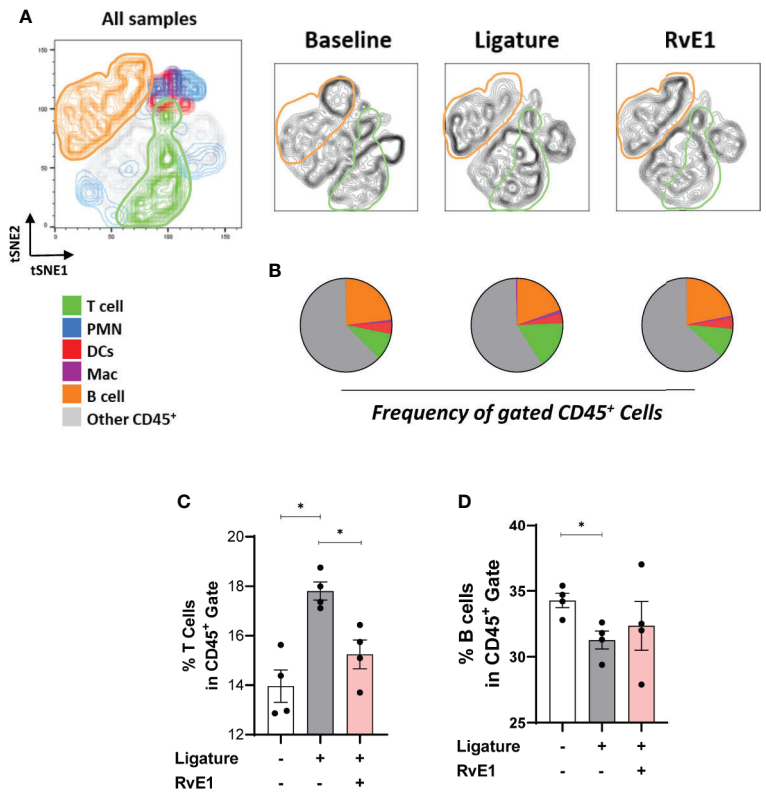


**FIGURE 5** | RvE1-induced changes in leukocyte composition in cervical lymph nodes. **(A)** tSNE plots of concatenated data (n = 4 animals per group), and **(B)** pie charts indicating the frequency neutrophils (PMN), macrophages (Mac), T cells, dendritic cells (DCs), B cells, and other cells within the $CD45^+$ cell population in cervical lymph nodes from animals at baseline, untreated ligature-induced periodontitis (Ligature), or treated with RvE1 from day 1 (RvE1). **(C, D)** Percentages of T and B cells within $CD45^+$ cells in cervical lymph nodes. *P < 0.05.

We observed that both early and delayed administration of RvE1 altered the T cell response. Remarkably, we detected a decreased gingival frequency of T cells at day 3 and 5 after the ligature placement, a period that represents the transition between innate and adaptive immune responses. Our previous study demonstrated the significant lymphadenomegaly of cervical lymph nodes due to the periodontal inflammation in mice (22). Due to the significant increase in the total cell number of cervical lymph nodes, when we measured the frequency of $CD4^+$ cells among all cell types (including non-leukocytes), the percentage decreased in animals with periodontitis compared to healthy mice. The opposite was observed when we analyzed the frequency of $CD4^+$ cells among $CD45^+$ cells, indicating $CD4^+$ T cell proliferation during periodontitis. We also detected a significant reduction of $CD4^+IL\text{-}17^+$ cells in cervical lymph nodes, indicating a decreased Th17 response. Collectively, RvE1 rescued the periodontitis-induced changes in $CD4^+$ cells. We recognize that the analysis performed is limited by the number of markers used simultaneously. A more detailed analysis is needed to characterize further which $CD4^+$ T cell phenotypes and functions are being affected by the treatment as well as other less described immune compartments.

Tregs are essential modulators of the bone-destructive immune response and promoters of inflammatory resolution and tissue regeneration (35). We previously demonstrated that Treg phenotype and functions were altered during periodontitis, acquiring pro-inflammatory functions such as the expression of IL-17 (22). Since Treg stability depends on environmental cues that are significantly modified by the effector immune response-particularly Th17, we analyzed the impact of RvE1 on this paradigm (36). As documented before, when we analyzed the frequency of $Foxp3^+$ cells among all $CD4^+$ cells in cervical lymph nodes, the percentage increased in periodontitis as a feedback mechanism in the inflammatory process. On the contrary, the frequency of $Foxp3^+$ cells within the $CD4^+CD25^+$ compartment, which only includes activated T cells, was reduced during periodontitis, suggesting an imbalance between effector and regulatory T responses (22). RvE1 recovered the frequencies of Tregs in both early and delayed application comparable to those levels in healthy animals. RvE1 reduced the small but significant population of $IL\text{-}17^+$ Tregs detected in periodontitis. Hence, RvE1 restored the Treg/effector T cells balance and protected Treg regulatory phenotype.

Our results indicated that the RvE1 impacted the adaptive immune response selectively, since it regulates the effector but not the regulatory responses. A limitation of our study was that we did not identify whether RvE1 had a direct influence on T cells, by

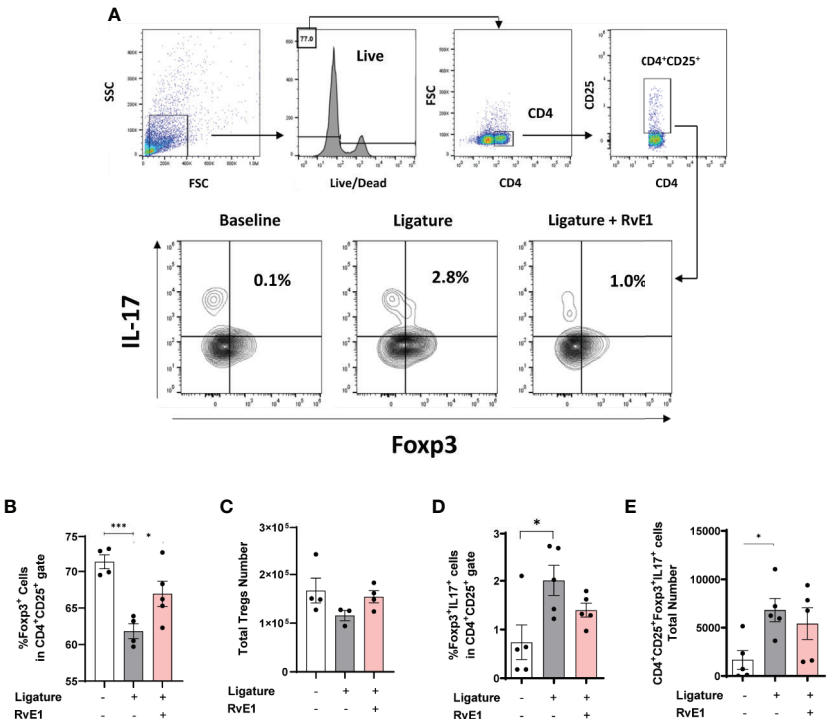


**FIGURE 6** | Impact of delayed RvE1 administration on Treg cells from cervical lymph nodes. **(A)** Representative plots indicating the gating strategy and percentage of $CD4^+CD25^+Foxp3^+IL17^+$ cells in cervical lymph nodes of animals at baseline, with ligature-induced periodontitis (Ligature), or treated with RvE1 from day 1 (RvE1). **(B)** Quantification of the percentage and **(C)** total number of Treg cells. **(D)** Quantification of the percentage and **(E)** cell number of IL-$17^+$ Treg cells. *$P < 0.05$, ***$P < 0.001$.

binding to its receptor, or an indirect impact through modulating other cell types that expressed cytokines and chemokines required for the differentiation and migration of effector T cells towards periodontal tissues. Given the amount of accumulated evidence indicating the robust effects of RvE1 on other cell types, we can infer that the outcome described on T cells is result of the net combination of functional cellular changes and the overall modulation of the inflammatory process; however, further research is needed to establish the direct role of RvE1 on $CD4^+$ T cells differentiation and functionality.

Tipping the balance between regulatory and effector functions to restore a long-term 'ceasefire' is needed to reset a dysfunctional immune response therapeutically. The use of SPMs to promote the active resolution of inflammation constitutes an important approach that takes advantage of an endogenous mechanism with a wide range of biological actions. In this study, we identified the kinetics of major immune cell types in the gingival tissues. Our work has demonstrated T cells -particularly effector T cells- as the main impacted population by early and delayed administration of RvE1 during ligature-induced periodontitis independent of neutrophil involvement. These findings align with recent studies that highlighted the critical role for T cells in the inflammatory alveolar bone destruction providing new queries to further explore the role of RvE1 in the modulation of the adaptive immune response *in vivo*.

# METHODS

## Animals

C57BL/6 and FVB wild type mice were purchased from the Jackson Laboratory (Maine, USA). 8-12-week-old male and female littermate animals were used. All animals were maintained in a pathogen-free environment, 24 ± 0.5°C, and 12:12 hours light/dark cycle at 40-70% of relative humidity. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Forsyth Institute.

## Ligature-Induced Periodontitis and RvE1 Administration

Experimental periodontitis was induced by placing 6.0-silk ligatures around the second bilateral upper jaws molars. Micro-Castroviejo forceps (Fine Science Tools), a magnification and cold-light source system, and an animal-holding structure were used to allow the maximum mouth opening of the anesthetized animal. Animals were subjected to ligatures at baseline (on day 0) of the experiment. In both early and delayed application of RvE1, the animals were treated daily with 10μL of 1μM RvE1 (Cayman chemicals) diluted in sterile phosphate-buffered saline (PBS) which was applied on the ligatures. To ensure the maximum bioactivity of RvE1, all aliquots were prepared for administration immediately before

use in amber glass vials, replacing the inner vial air with nitrogen gas, and transported at 4°C.

## Bone Loss Measurements

Bilateral maxillary bones were defleshed by dermestids for 7 days at the animal facility at the Forsyth Institute. The bone samples were then cleaned of remaining debris, treated with 12% $H_2O_2$ for 4h, and stained with methylene blue. The images were taken at 0.63×10 magnification under a dissecting microscope (Axio observer A1, ZEISS) using the AxioVision 4.8 software. The area between the alveolar bone and cementoenamel junction (CEJ) on the palatal side of each maxillary molar was measured using the Fiji software (ImageJ) as bone loss.

## Flow Cytometry

Gingival samples from the mice were placed in RPMI-1640 + 1% penicillin-streptomycin (Sigma). The tissues were then treated with 0.15 μg/mL DNase I (GIBCO) + 3.2 mg/mL Collagenase Type IV (GIBCO) (Collagenase-DNase media) in 500 μL of RPMI-1640 + 1% penicillin-streptomycin in a 6-cm culture dish, as previously reported (37). The tissue sample minced with a sterile #15 scalpel blade. All tissue fragments were collected in a 15 ml tube containing 4 mL Collagenase-DNase media, and the tubes (one per animal) were placed in a water bath at 37°C for 1h. Fifty μL of 0.5M EDTA were added during the last 5 min of incubation. The samples were spun down at 1200 rpm for 10 min and the supernatant eliminated. The fragments were mashed onto a 70-μm cell strainer. The cells were filtered by washing the cell strainer repeatedly with RPMI-1640 + 1% penicillin-streptomycin + 0.15 μg/mL DNase I. The final cell suspension was spun down at 400*g* for 10 min; the supernatant was eliminated and the cells were resuspended in 200 μL of PBS +5% fetal-bovine serum (FBS). The cells were counted and stained using the LIVE/DEAD™ Fixable Yellow Stain kit (Invitrogen) according to the manufacturer's instructions.

Then, the Fc receptors were blocked by TruStain FcX Antibody (Biolegend) according to the manufacturer's instructions. The extracellular staining was performed in PBS containing 5% FBS, using the following antibodies for 30 min at 4°C in the dark: anti-I-A/I-E (M5/114.15.2, Biolegend), -CD3 (17A2, Biolegend), -CD19 (6D5, Biolegend), -CD64 (X54-5/7.1, Biolegend), -CD11b (M1/70, Biolegend), -CD11c (N418, Biolegend), -CD45 (30-F11, Biolegend), -Ly6G (IA8, Biolegend) and -Ly6C (HK14, Biolegend). We analyzed B lymphocytes ($CD19^+MHCII^+$), T lymphocytes ($CD3^+$), macrophages ($CD11b^+$ $CD64^+$), dendritic cells ($CD11c^+$ $MHCII^+$), neutrophils ($Ly6G^+$) and other cells ($Ly6G^-Ly6C^{+/-}$) within gate $CD45^+$ (**Supplementary Figure 3**).

Single-cell suspensions were obtained from cervical lymph nodes using 70 μm cell strainers (Sigma-Aldrich) rinsed-out with PBS + 5% FBS. The extracellular staining was performed as done for gingival samples. For cytokine detection, $2\text{-}4\times10^6$ cells were incubated for 4 hours in RPMI-1640 +10% FBS + 1% penicillin-streptomycin, 1/1000 Brefeldin A (eBioscience), 50 ng/ml PMA (Sigma), and 1 μg/ml Ionomycin (Sigma). Cells were then washed with PBS and stained for Live/Dead as explained before. The intracellular staining was done using a fixation/permeabilization staining kit following the manufacturer's instructions (eBioscience) and using the following antibodies: anti-Foxp3 (MF-14, Biolegend), -CD4 (GK1.5, eBioscience), -CD25 (PC61, Biolegend) – IFNγ (XM61.2, Biolegend), and IL-17A (9B10, Biolegend).

All samples were analyzed on an Attune™ NxT acoustic focusing cytometer (Invitrogen). To set the flow cytometry compensation, the AbC™ Total Antibody Compensation Bead Kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions. The multiparametric data analysis was performed using the FlowJo software v10.7.1 (CA, USA). The data was analyzed using both a dimensionality reduction with the t-Distributed Stochastic Neighbor Embedding (tSNE) algorithm and the clustering FlowSOM algorithm. To effectively compare different samples and experiments, the following workflow was used: 1) Data clean up by applying manual gates to exclude doublets, debris, and dead cells from each sample, 2) Down sample of the $CD45^+$ gated populations of each sample to 20,000 events, 3) Concatenate samples (4 samples per experimental group), 4) Dimensionally reduce (create tSNE parameters) on the concatenated file using default settings in FlowJo, iterations 1000, perplexity 30, and learning rate (eta) 521, and 5) Analysis of every population and identifying phenotypes using FlowSOM and a later established gating strategy (**Supplementary Figures 1, 3**).

## Gingival RNA Extraction and qRT-PCR

The gingival collar around all maxillary molars, without including palatal or buccal mucosa, was carefully excised using scalpel blade (#15) under a dissecting microscope. All samples were transported in RNAlater (Life Technologies). To lyse the samples, we used glass tissue homogenizers and 300 μL of RLT lysis buffer (Qiagen), then we further homogenized the samples in QIAshredder columns (Qiagen). The RNA was obtained from the homogenate using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. The first-strand of cDNA was synthesized from 1 μg of RNA using the reverse transcription kit (SuperScript III, Invitrogen), according to the manufacturer's instructions. Quantitative real-time PCR amplification of cDNAs (50 ng) was performed using TaqMan™ Fast Advanced Master Mix (Thermo Fisher) and the respective TaqMan probe for each targeted gene. The Step One™ Real-Time PCR System (Applied Biosystems) instrument was used for quantification. All transcript levels were normalized to transcript levels of mouse18S rRNA. The data was presented as a fold-change of relative quantity using the $2^{-\Delta\Delta Ct}$ method.

## Statistical Analysis

All data sets were analyzed using Prism 8 software v8.4.2 (GraphPad). The data distribution was analyzed using the Shapiro-Wilk test. To determine differences between two experimental groups, unpaired Student-t or Mann-Whitney-U tests were performed. ANOVA or Kruskal-Wallis tests were used when more than two groups were compared, followed by

multiple comparison Tukey or Dunn post-hoc tests. P values < 0.05 were considered statistically significant.

### Original Figures

Created with BioRender.com.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Forsyth Institute.

## AUTHOR CONTRIBUTIONS

CA designed the study, carried out the experiments, analyzed the data, and drafted the manuscript. SS and HA contributed to the experiment's readout. PR analyzed bone loss. Y-CW contributed to the flow cytometry data analysis. RA contributed to the maintenance of the animal colonies and animal treatments. MG, RV, and AK conceived the study and helped to draft the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.664756/full#supplementary-material

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# Probiotic *Streptococcus salivarius* K12 Alleviates Radiation-Induced Oral Mucositis in Mice

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**Background:** Oral mucositis is the most common oral complication of cancer patients receiving radiotherapy and/or chemotherapy, leading to poor quality of life. Limitations of the current interventions on radiation-induced oral mucositis (RIOM) urge the development of novel therapeutics. Here, we evaluated the treatment outcome of probiotic *Streptococcus salivarius* K12 on RIOM mice, and oral microbiota that is associated with the progress of RIOM was further investigated.

**Methods:** An experimental RIOM mouse model was established, and *S. salivarius* K12 was applied to the mouse oral cavity daily. Histological analyses were performed to evaluate the severity of oral mucositis and the treatment outcome of *S. salivarius* K12. The oral microbiota of mice was further analyzed by 16S rRNA sequencing, microbial culture and qPCR.

**Results :** Irradiation induced conspicuous mucositis in the oral cavity of mice. *S. salivarius* K12 treatment was beneficial for the healing of RIOM, as reflected by reduced ulcer size, increased basal layer epithelial cellularity and mucosal thickness, and elevated epithelial proliferation and attenuated apoptosis. RIOM mice presented significant oral microbial dysbiosis, with an overgrowth of oral anaerobes. *S. salivarius* K12 treatment reconstituted the oral microbiota and decreased the abundance of oral anaerobes of RIOM mice. In addition, *S. salivarius* K12 treatment inhibited NI1060 in *Pasteurella* genus and downregulated the expression of nitrate reductase.

**Conclusions:** *S. salivarius* K12 treatment can alleviate RIOM and reconstituted the dysbiotic oral microbiota in mice. *S. salivarius* K12 may represent a promising adjuvant treatment to improve the quality of life of cancer patients receiving radiotherapy.

**Keywords: *Streptococcus salivarius* K12, radiotherapy, oral mucositis, dysbiosis, probiotics**

## INTRODUCTION

Oral mucositis, characterized by inflammation and mucosal damage of oral mucosa, is the most common oral complication of cancer patients receiving radiotherapy and/or chemotherapy. The incidence of oral mucositis is almost 100% in head and neck cancer patients receiving radiotherapy (1, 2). 85% of patients receiving intensive chemotherapy for hematopoietic stem cell transplant also develop oral mucositis (3). Oral mucositis induced by radiotherapy/chemotherapy can cause pain, dysphagia and malnutrition, seriously affecting the quality of life of patients and interrupting anti-cancer treatment. Till now, the management of oral mucositis is still challenging. Interventions include growth factors, antibiotics, chlorhexidine, cryotherapy, low-level laser therapy and anti-inflammatory agents, but with limited efficacy (4). At present, only keratinocyte growth factor-1 (palifermin) has been approved by the US Food and Drug Administration to mitigate oral mucositis in a very limited segment of the at-risk population (5). Hence, there is still a need for the development of novel therapeutics for the better management of oral mucositis.

The pathogenesis of mucositis induced by radiotherapy/chemotherapy has been suggested in previous studies (6, 7). Sonis et al. depicted the development of oral mucositis and intestine mucositis as a dynamic process including five stages: initiation, primary damage response, signal amplification, ulceration and healing (6). Recent studies have suggested the role of oral microbiota in the development and progression of oral mucositis (8). Microbial dysbiosis, invasion and colonization of oral mucosa were involved in the pathophysiology of oral mucositis (9, 10). Pathogens contributed to the development of oral mucositis by activating inflammatory responses through pathogen-associated molecular patterns (PAMPs), which bind to pattern recognition receptors (PRRs), subsequently activate NF-κB and induce the release of pro-inflammatory cytokines (10).

Probiotics which consist of beneficial viable bacteria and bacterial components, have shown various beneficial effects on human health, particularly *via* modulating the disease-related dysbiotic microbiota (11, 12). *Streptococcus salivarius* is a commensal bacterium in the oral cavity, and the *S. salivarius* K12 strain that was originally isolated from the oral cavity of a healthy child, has been well recognized as an oral probiotic being used for the treatment of multiple oropharyngeal pathogen-related diseases including oral candidiasis, pharyngitis, and halitosis (13–15). *S. salivarius* K12 has a regulatory effect on oral microflora (15, 16), likely due to its potent production of bacteriocin-like inhibitory substances (BLISs) including Lanibiotics salivaricin A and salivaricin B. The production of BLISs contributes to the competitiveness of *S. salivarius* K12 over pathogens and thus benefits the oropharyngeal health (17). The regulatory effects of *S. salivarius* K12 on oral microbiota imply its potential use in the treatment of RIOM. Hence, we hypothesize that radiation can alter oral microbiota and predispose the host to oral mucositis, and *S. salivarius* K12 can alleviate mucositis by modulating the oral microbiota. To validate this hypothesis, we established a RIOM mouse model, and analyzed the ecological impact of radiation on the oral microbiota. In addition, beneficial effects of *S. salivarius* K12 on the healing of oral mucositis were further evaluated.

## MATERIALS AND METHODS

### Radiation-Induced Oral Mucositis Mouse Model

Seven-week-old male BLAB/c mice were purchased and housed under specific pathogen-free conditions. All animal procedures in this study were approved by Ethics Committee of State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, China. This study conformed to the "Animal Research: Reporting of *In Vivo* Experiments" guidelines for preclinical studies.

After one week of environment acclimation, the mice were randomly divided into three groups (N=11 per group, 5 for macroscopic analyses and 6 for histological analyses and microbial analyses): irradiation-free (control), irradiation+Saline (IR+Saline) and irradiation+ *S. salivarius* K12 (IR+K12). The mice were immobilized for irradiation with chloral hydrate. The technique and set-up for head-only radiation treatment in mice were modified based on previously published studies (18). Custom-made lead shields were used for mice to limit the radiation to the heads. Mice received a high dose, single fractionated 28Gy X-ray radiation directly to their head region at rate of 3.5 Gy/min. Except for the control group, the mice received X-ray radiation. Post-irradiation mice recovered on heated pad before return to vivarium.

### Treatment With Probiotics

Probiotics solution was prepared by dissolving probiotics tablets, which contain $1\times10^{10}$ CFU of viable bacteria per tablet according to the manufacturer's instruction (NOW Foods, USA), in sterilized saline to a concentration of $1X10^{10}$ CFU/ml of *S. salivarius* K12. 100 $\mu$l probiotic solution ($1\times10^{9}$ CFU of *S. salivarius* K12 per day) was applied to the oral cavity of mouse using a micropipette from day -3 to day 8. After administration, food and water were unavailable within the next 30 min to keep the probiotics in mouth as long as possible. The mice in IR+Saline group were treated with saline as placebo. All mice were sacrificed at day 9. The microbial samples were taken before sacrifice.

### Macroscopic and Histological Analyses

Mice were sacrificed and the whole tongue was then removed from oral cavity. Excised tongues were stained with 0.05% toluidine blue to visualize the ulceration (19). Excised tongues of mice were stained with 0.05% toluidine blue for 10 min and rinsed with 10% acetic acid for 1 min to visualize the ulceration (19). Ulcers were visible as a deep blue color after staining. Then, the percentages of stained area to whole area of tongue surfaces were measured by pixels on Image J software to determine the ulcer area.

Paraffin sections were prepared for histological analyses. For staining based on hematoxylin and eosin (H&E), cell proliferation (PCNA), or apoptotic cells (TUNEL), specimens were fixed in 4% paraformaldehyde and embedded in paraffin. After deparaffinization, sections were stained with hematoxylin and eosin to confirm the histologic changes. To measure the epithelial thickness, two sites in five randomly chosen hematoxylin and eosin-stained sections (six specimens/group) were measured. The basal layer cellularity was measured by counting the absolute number of cells at basal layer at the area of interest in five randomly chosen sections (six specimens/group). To confirm the tissue-regenerative activity (cell proliferation) of damaged tissues, sections were stained with rat monoclonal anti-mouse PCNA (1:100; Abcam, Cambridge, UK). For the apoptosis assay, TUNEL (terminal deoxynucleotidyl transferase dUTP nick and labeling) staining was performed with a TUNEL kit (Beyotime, China) according to manufacturer's protocol to detect apoptotic cells. Slides were mounted with coverslips using DAPI Fluoromount-G (Southern Biotech, China). To quantify of cell proliferation (PCNA) and apoptotic activities, the absolute number of positive cells at the area of interest in five randomly chosen sections (six specimens/group) were counted.

## 16S rRNA Sequencing

Bacterial DNA was extracted from oral samples from the mice. DNA library was prepared with uniquely barcoded primer targeting the V3/4 region of the 16S rRNA as described previously (20). The library construction and sequencing data analyses were performed as previously described (21). The oral swabs from the mice were sequenced at Majorbio Co. (Shanghai, China). 338F (5'- ACTCCTACGGGAGGCAGCAG-3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3') primers were used to amplify the V3/V4 region of 16S rDNA. Barcoded 16S rDNA amplicon sequencing was performed through Illumina MiSeq platform. Sequences were trimmed using Trimmomatic (22) based on quality scores of 20, and pair-end reads were merged into longer reads by FLASH (23). Unqualified sequences, too short or contained ambiguous residues, were removed. Operational taxonomic units (OTUs) were clustered using Usearch version 7.0 (http://drive5.com/uparse/) at the 97% similarity level, and final OTUs were generated based on the clustering results. All sequencing data were uploaded to NCBI SRA database with an accession number SRP276563.

The pre-processed sequencing data was further analyzed with the following statistical methods. (1) Alpha diversity analysis was based on Shannon index. (2) PCoA (principal coordinates analysis) was used to compare the beta diversity within groups. Two non-parametric analyses for multivariate data, multivariate analysis of variance (Adonis) and analysis of similarities (ANOSIM) using distance matrices, were used to examine the community difference within groups. (3) Taxonomic annotations were assigned to each OTU's representative sequence by blasting with the oral "CORE" reference database. The relative abundances of bacterial taxa at genus levels were analyzed and compared. All analyses were performed with I-Sanger online tools (http://www.i-sanger.com/).

## Quantitative Real-Time PCR

The qPCR amplification was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). For quantification of total bacterial load, the reaction mixture (25 μl) contained SYBR® Premix Ex Tag II (Takara Bio), microbial genomic DNA (2 μl), and forward and reverse primers (10 μM each). Threshold cycle (CT) values were determined, and relative ratio of 16S and 18S was calculated based on the $2^{-\Delta\Delta CT}$ method (24). NI1060 was quantified with the protocol described previously (25). For quantification of the expression level of *napA*, microbial RNA was extracted with TriZol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of RNA into cDNA was performed with the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio). The expression levels of *napA* were measured with that of the control group as control. The sequence of the primers (16S, 18S and *napA*) used referred to the previous study (24).

## Anaerobic Bacteria Cultivation

Oral mucosa of mice was swabbed for 30 s and the swabs were then inserted into Eppendorf tubes containing 100 $\mu$L of Wilkins-Chalgren medium (Oxoid). The samples were serially diluted and plated on blood agar for two days under anaerobic conditions at 37°C. Colonies were enumerated to determine the colony-forming units (CFUs) of total cultivatable oral anaerobic bacteria (24).

## Statistical Analysis

For 16S rRNA sequencing data, statistical analyses were performed with I-Sangers online tools (http://www.i-sanger.com/) (25, 26). The differences in beta diversity (revealed by PCoA) within groups were compared; the alpha diversity data and genus-level microbial composition data were analyzed by Wilcoxon rank-sum test for two group comparison and Kruska-Wallis H test with the Dunn's test for three group comparisons. All other data were statistically analyzed by GraphPad Prism 6. Differences between groups were analyzed by one-way analysis of variance test followed by Tukey's test. A two-tailed $P<0.05$ was considered significant.

# RESULTS

## *S. salivarius* K12 Ameliorates Radiation-Induced Oral Mucositis in Mice

The body weights of mice that received irradiation decreased sharply, while *S. salivarius* K12 treatment alleviated the body weight loss (**Figure 1A**). The total body weight loss of *S. salivarius* K12 treatment group (-8.33g) was significantly less than that of irradiated mice (-12.05g) on the 9$^{th}$ day after irradiation (**Figure 1B**). In the oral cavity of irradiated mice, conspicuous mucositis, particularly on the lingual mucosa was observed as reflected by toluidine blue staining (**Figure 1C**). Topical application of *S. salivarius* K12 significantly reduced the severity of oral mucositis in irradiated mice (**Figure 1C–E**). Specifically, the relative area of mucositis including ulcers was

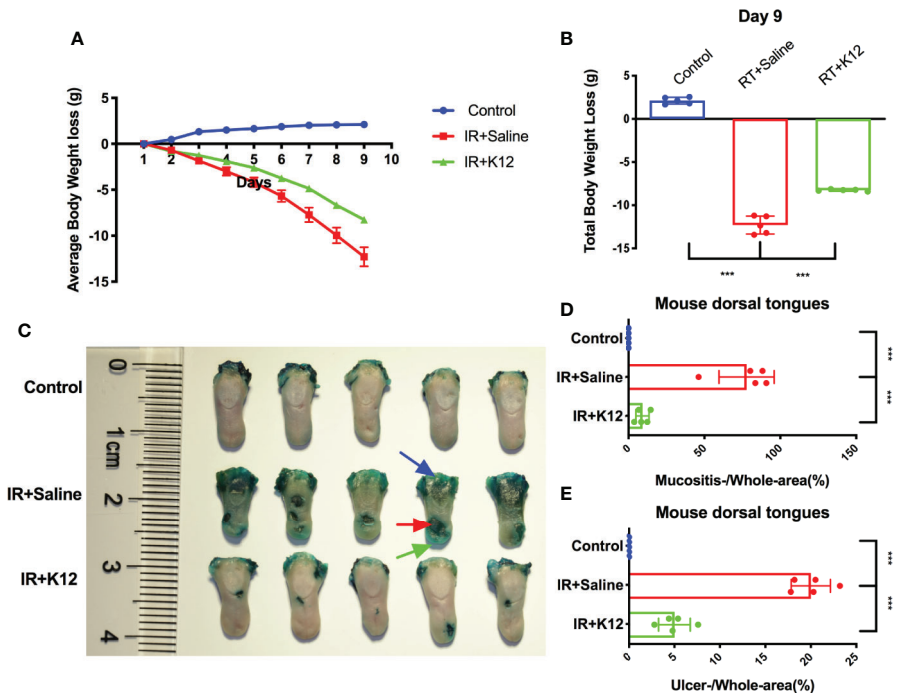


**FIGURE 1** | *Streptococcus salivarius* K12 alleviates body weight loss and reduces tongue ulcer area in RIOM mice. **(A)** Average body weight loss. **(B)** Total body weight loss. **(C)** Toluidine blue staining of harvested tongues. The area of mucositis with (red arrow) and without ulcer (green arrow) was stained blue. Blue arrow: staining at the site of incision to remove tongue. **(D)** Quantitative analyses of mucositis area (mucositis+ulcer/whole surface area). **(E)** Quantitative analyses of ulcer area (ulcer/whole surface area). Data are presented as mean ± SD. ***$P < 0.001$. N = 5 per group.

significant reduced in the IR+K12 group (9.03%) as compared to that in the IR+Saline group (77.42%) (**Figure 1D**). The relative ulcer area in tongues of the *S. salivarius* K12-treated mice (5.02%) was also significantly lower than the IR+Saline group (20.21%) (**Figure 1E**). H&E staining showed conspicuous mucosal hypoplasia and ulceration in the tongue of irradiated mice, while *S. salivarius* K12 treatment partially restored the integrity of the lingual mucosa (**Figure 2A**). Topical use of *S. salivarius* K12 also significantly increased mucosal thickness (**Figures 2B, C**) and basal layer epithelial cellularity in both ventral and dorsal tongues (**Figures 2D, E**).

Further immunohistology showed that irradiation attenuated the proliferation of basal layer epithelial cells and induced significant apoptosis in both ventral and dorsal tongue of RIOM mice as compared to the irradiation-free controls (**Figure 3**). *S. salivarius K12* treatment significantly rescued this pathology, as increased number of $PCNA^+$ proliferative cells in the baser layer epithelium (**Figures 3A–C**) and decreased number of $TUNEL^+$ apoptotic cells (**Figures 3D–F**) were observed in both dorsal and ventral tongues of the IR+K12 group compared to the IR+Saline group.

## *S. salivarius* K12 Modulates Oral Microbiota in RIOM Mice

16S rRNA sequencing data showed that the oral cavity of RIOM mice harbored a microbiota with lower alpha diversity relative to the irradiation-free controls (**Figure 4A**). Principal coordinates analysis (PCoA) based on Bray-Curtis distance showed that the oral microbiota of RIOM was distinct from that of irradiation-free controls (**Figure 4B**), indicating an altered microbial structure. Further analysis of the top 15 abundant bacterial taxa revealed genus-level differences between RIOM mice and irradiation-free controls (**Figure 4C**). The oral microbiota of RIOM mice had significantly increased abundance of *unclassified_f_Pasteurellaceae*, *Pasteurella*, *Muribacter*, *Corynebacterium* and *unclassified_O_Lactobacillales* and lower levels of *norank_f_Bacteroidales_S24-7_group*, *Rhodococcus*, *norank_c_Cyanobacteria*, *Lactobacillus*, Lachnospiraceae_NK4A136_group and *Escherichia-Shigella* as compared to the irradiation-free controls.

The oral microbiota of RIOM mice with/without *S. salivarius* K12 treatment showed no difference in alpha diversity (**Figure 4A**). PCoA based on Bray-Curtis distance revealed differential clustering of the oral microbiota among the IR+Saline, IR+K12, and irradiation-free controls, suggesting partial reconstitution of microbial structure after *S. salivarius K12* treatment (**Figure 4B**). More importantly, *S. salivarius K12* treatment altered the microbial composition of RIOM mice. An enrichment of *Pasteurella* was observed in IR+Saline group as compared to the irradiation-free controls, and *S. salivarius K12* treatment reduced the amount of *Pasteurella* in the RIOM mice (**Figure 4C**). Further species-specific qPCR analysis showed that a periodontitis-associated pathogen NI1060 in the

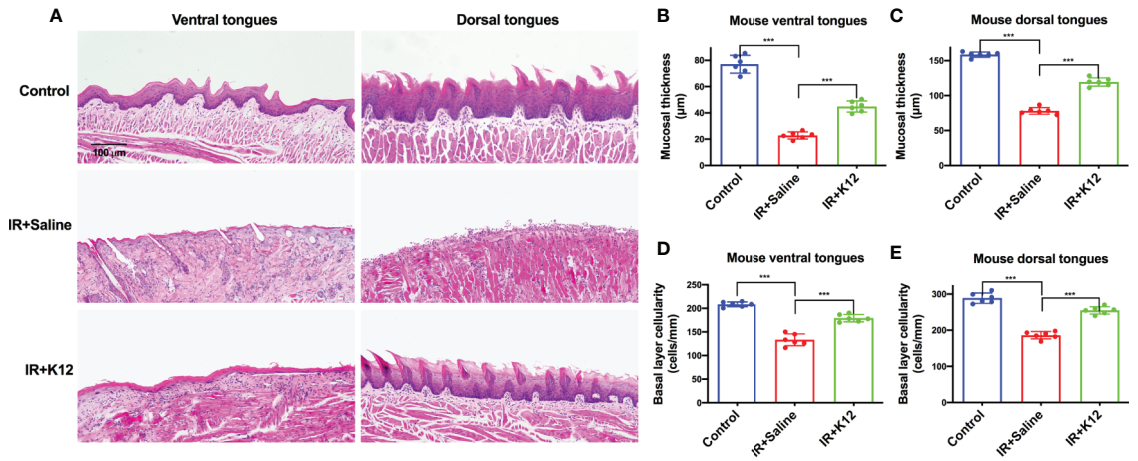


**FIGURE 2** | *Streptococcus salivarius* K12 promotes RIOM healing in mice. **(A)** Representative images of H&E staining indicating the integrity of lingual mucosa (Scale bar, 100 μm). **(B, C)** Quantitative analysis of mucosal thickness of ventral tongues and dorsal tongues, respectively. **(D, E)** Basal layer epithelial cellularity of ventral tongues and dorsal tongues, respectively. Data are presented as mean ± SD. N=6 per group. One-way ANOVA test followed by Tukey's test. ***$P$<0.001.

*Pasteurella* genus was significantly enriched in IR+Saline group, and *S. salivarius* K12 treatment significantly reduced its abundance in the oral cavity of RIOM mice (**Figure 4D**).

## *S. salivarius* K12 Suppresses the Overgrowth of Oral Anaerobes in RIOM Mice

Microbial samples from the oral swabs were further analyzed to investigate the effect of *S. salivarius* K12 treatment on the oral microbiota of RIOM mice. The oral cavity of IR+Saline mice was colonized with twice the number of bacteria as compared to the irradiation-free controls; while *S. salivarius K12* treatment significantly reduced the overall microbial load (**Figure 5A**). More importantly, the IR+Saline group presented an increased amount of cultivated anaerobic bacteria as compared to the irradiation-free controls, and *S. salivarius K12* treatment significantly reduced the amount of cultivated anaerobic bacteria in RIOM mice (**Figure 5B**). Consistently, an elevated expression of gene encoding nitrate reductase (*napA*) was observed in the oral microbiota of RIOM mice as compared to that of irradiation-free controls, and *S. salivarius* K12 treatment significantly downregulated *napA* expression (**Figure 5C**).

# DISCUSSION

Radiotherapy is one of the most commonly used treatment modalities for head and neck cancers, but with frequent complications as oral mucositis. Limited evidence has suggested the involvement of oral microbes in the development of oral mucositis in this context (8, 27, 28). In the current study, we demonstrated that radiation caused oral microbial dysbiosis of RIOM mice. More importantly, topical use of probiotic *S. salivarius K12* ameliorated oral mucositis in RIOM mice by modulating the oral microbiota, representing a promising approach to the management of RIOM.

The pathogenesis of radiation-induced oral mucositis remains undefined, and the complex interaction between microbiota and host has been suggested (9, 10, 29). Recently, increasing evidence suggests that microbiota has played an important role in the pathogenesis of mucositis (29, 30). Hu et al. analyzed the supragingival plaque of eight nasopharyngeal carcinoma (NPC) patients receiving head and neck radiotherapy and found that the relative abundance of core microorganisms changed dynamically during radiotherapy (27, 28). Zhu et al. revealed that bacterial community structure altered progressively in NPC patients during radiotherapy, accompanied with a marked increase of certain Gram-negative bacteria. Patients who eventually developed severe oral mucositis harbored a higher abundance of *Actinobacillus* during the phase of erythema-patchy mucositis (8). Reyes-Gibby et al. found that changes in the abundance of genera, including 1) *Cardiobacterium* and *Granulicatella* at the baseline; 2) *Prevotella*, *Fusobacterium* and *Streptococcus* immediately before the development of oral mucositis; and 3) *Megasphaera* and *Cardiobacterium* immediately before the development of severe oral mucositis, over the course of treatment in the patients with squamous cell carcinoma of the head and neck were associated with the onset of severe oral mucositis (31). These data suggest the involvement oral microbial dysbiosis in the development of RIOM.

*S. salivarius* K12 was first isolated from the throat of a healthy child, and is currently used as a probiotic for the treatment oral malodor, oral candidiasis, secretory otitis media and pharyngotonsillitis (14, 15, 32–35). Given the presence of oral microbial dysbiosis after irradiation, we used *S. salivarius* K12 to treat the RIOM mice. Our results showed that *S. salivarius* K12 could modulate oral microbiota, and effectively alleviated RIOM, as reflected by a significant reduction of ulceration, increased

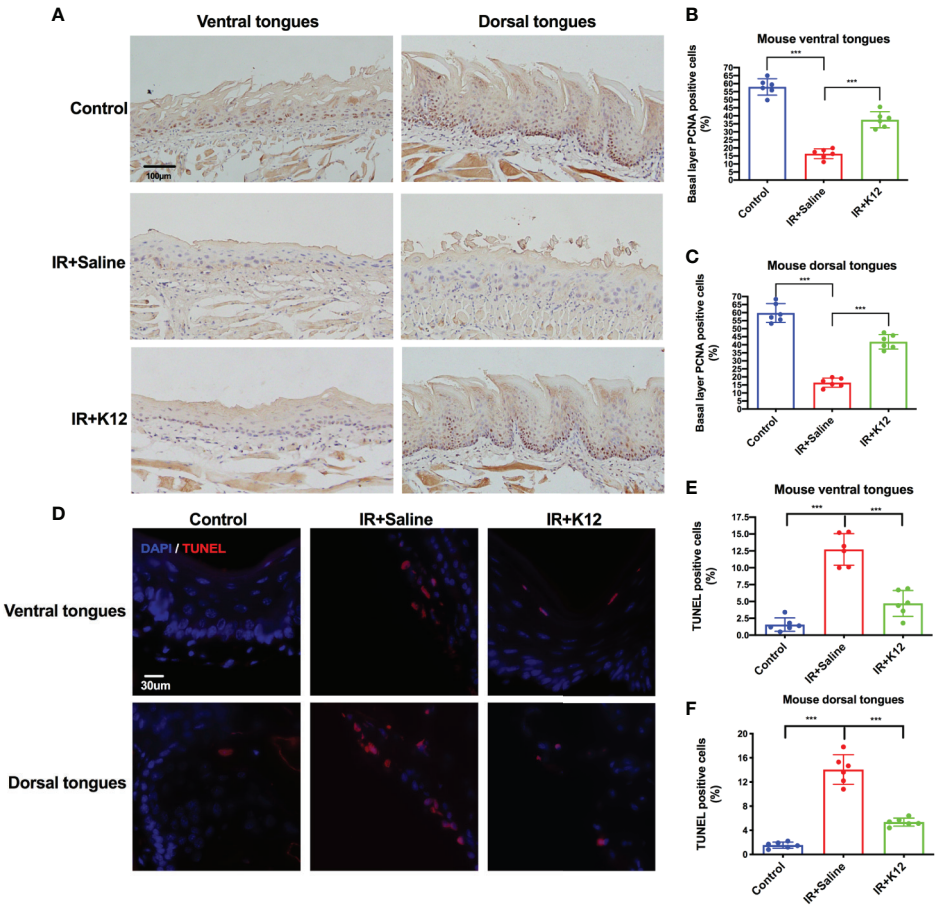


**FIGURE 3** | *Streptococcus salivarius* K12 promoted the proliferation of mouse tongue basal layer cells and reduced apoptosis of mouse tongue mucosal cells. **(A)** Representative microscopic images of mouse tongues PCNA staining (Scale bar, 100 μm). **(B)** Percentage of basal layer PCNA positive cells in mouse ventral tongues. **(C)** Percentage of basal layer PCNA positive cells in mouse dorsal tongues. **(D)** Representative microscopic images of TUNEL staining on mouse tongues (Scale bar, 30 μm). **(E)** Percentage of TUNEL-positive cells in mouse ventral tongues. **(F)** Percentage of TUNEL-positive cells in mouse dorsal tongues. Data are presented as mean ± SD. N=6 per group. One-way ANOVA test followed by Tukey's test. ***$P < 0.001$.

thickness of tongue mucosa and the density of basal cells, enhanced basal cell proliferation, and attenuated apoptosis. Although few studies have demonstrated beneficial effects of probiotics in the treatment/prevention of oral mucositis, satisfactory outcomes have also been reported by others using probiotics to treat intestinal mucositis induced by radiotherapy/chemotherapy. Kato et al. found that *Bifidobacterium bifidum* G9-1 could improve the microbial dysbiosis and thus ameliorated 5-Fluorouracil-induced intestinal mucositis in mice (11). Consistently, another study found that administration of a probiotic mixture DM#1, which includes four probiotic strains as *Bifidobacterium breve* DM8310, *Lactobacillus acidophilus* DM8302, *Lactobacillus casei* DM8121 and *S. thermophiles* DM8309, ameliorated 5-Fluorouracil-induced intestinal mucositis *via* the reestablishment of microbial homeostasis and regulation of the TLR2/TLR4 signaling pathway (36). *Lactobacillus rhamnosus* GG also shows a protective effect on the intestinal epithelium from radiation injury, possibly through the release of lipoteichoic acid that activates the radioprotective TLR2 (37).

In addition to the shift of oral microbiota after irradiation, we also observed an increased microbial load and overgrowth of anaerobic bacteria in the oral cavity of RIOM mice, along with an elevated expression of nitrate reductase gene (*napA*). Consistently, Kato et al. reported an elevated amount of anaerobic bacteria in mice with 5-fluorouracil-induced intestinal mucositis (11). Nassar et al. also reported a higher expression levels of *napA* in Gas6−/− mice, which harbored dysbiotic microbiota with expansion of anaerobic bacteria (24). NapA, as an important nitrate reductase, plays an important role in the growth of anaerobic pathogenic bacteria. By expressing nitrate reductase, the anaerobic bacteria can use electron acceptors generated as a byproduct of inflammation to support their growth by anaerobic respiration (24, 38). Lipopolysaccharides (LPS) produced by Gram-negative anaerobes could induce inflammatory responses by activating toll-like receptor 4 signaling pathway (39), which may contribute

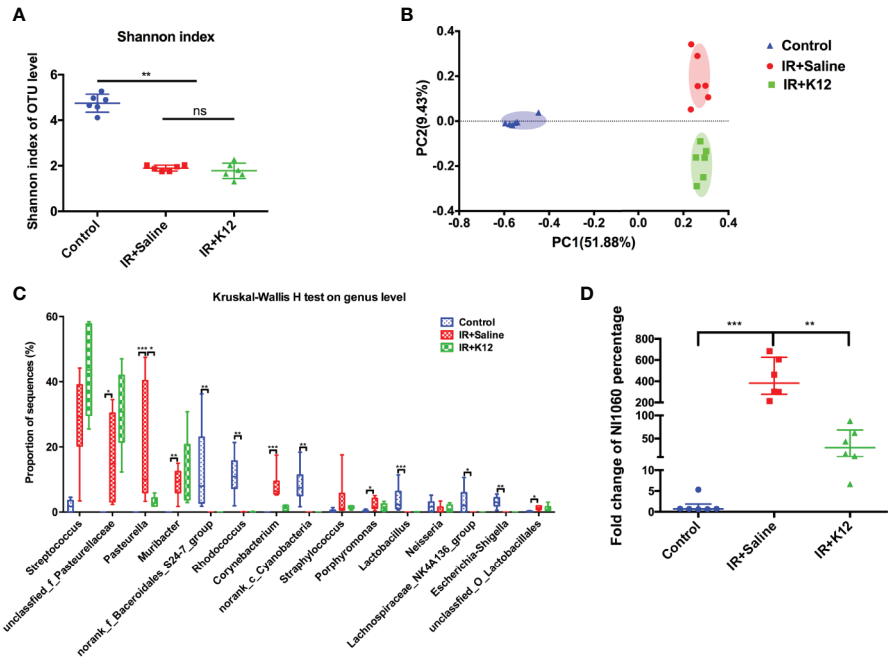


**FIGURE 4** | *Streptococcus salivarius* K12 modulates oral microbiota in RIOM mice. **(A)** The alpha diversity of oral microbiota. **(B)** Principal coordinate analysis (PCoA) of oral microbiota based on Bray-Curtis distance. **(C)** Prevalent genus with significant difference in abundance. Values are presented as median, interquartile range, minimum, and maximum. Kruska-Wallis H test with *post hoc* tests applying the Dunn's test for multiple comparisons. **(D)** qPCR quantification of NI1060 (mean ± SD). One-way ANOVA test followed by Tukey's test. N=6 per group. ns, not significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

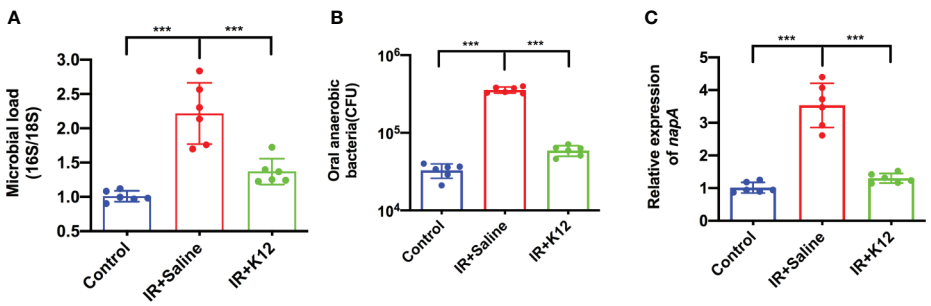


**FIGURE 5** | *Streptococcus salivarius* K12 suppresses the overgrowth of oral anaerobes in RIOM mice. **(A)** Total bacteria in oral swabs were quantified by qPCR normalized as 16S/18S rRNA. **(B)** Total cultivable oral anaerobes in oral swabs. **(C)** Relative expression levels of nitrate reductase *napA* gene of oral microbiota. Data are presented as mean ± SD. N=6 per group. One-way ANOVA test followed by Tukey's test. $^{***}P < 0.001$.

to the development of oral mucositis. In the current study, *S. salivarius* K12 treatment reduced the number of anaerobic bacteria and downregulated the expression of *napA* in RIOM mice. In addition, *S. salivarius* K12 treatment reduced the abundance of bacterial genera including *Pasteurella, Corynebacterium*, *Porphyromonas*, and *Staphylococcus*. Intriguingly, an increase of NI1060 in the *Pasteurella* genus was observed in RIOM mice. NI1060 is an inflammation-related bacterium identified in murine ligature-induce periodontitis (40). The genomic sequencing of NI1060 revealed its possible virulence genes involved in lipooligosaccharide synthesis, adhesins and bacteriotoxic proteins, which were potentially important for host adaption and induction of dysbiosis through bacterial competition and pathogenicity (41). Our recent study on periodontitis also suggests the involvement of this bacteria in periodontitis (25). The enrichment of NI1060 in the oral cavity of mice receiving radiation suggests a possible involvement of this pathogenic bacteria in the development of RIOM. Although more studies are still needed, this bacterium may provide a potential target for the treatment/prevention of RIOM.

Some cautions should be taken when interpreting data from the current study. Firstly, the RIOM mouse model was established using a single high-dose irradiation referring to a previous study (18).

Nevertheless, conventional radiotherapy for cancer patients consists of low fractionated X-ray doses lasting for weeks in most cases (42). Since single high-dose irradiation and low-dose fractionated irradiation could exert different effects on oral mucosa and oral microbiota, future studies using animal models closer to the clinical radiation regimens are warranted. Secondly, we only sampled oral microbial samples on day 9 post irradiation because overt ulceration in mouse tongue mucosa peaked 9 days after irradiation. Whether *S. salivarius* K12 can accelerate the restoration of microbial dysbiosis still needs dynamic sampling after irradiation. Thirdly, although *S. salivarius* K12 showed effectiveness in the treatment of RIOM and the inhibition of oral anaerobes in the current study, whether this probiotic strain can directly act on tissue inflammation other than competing oral anaerobes to promote the healing of RIOM still needs further investigations. It should be noted that changes in commensal bacteria took place at the early stage of radiotherapy (27, 28), suggesting that microbial alteration could possibly be an initiating factor rather than a consequence of oral mucositis. A recent systematical review including five clinical studies of 435 patients has indicated that probiotics may help reduce the incidence and mitigate the severity of cancer therapy-induced oral mucositis (43). However, as the selection and combination of probiotics, application method and target population vary among these studies, more evidence is still needed to justify the clinical application of probiotic in this scenario. In addition, the differed host responses of probiotics warrant customized probiotic interventions on patients receiving varying anti-cancer treatment modalities (44). It is also noteworthy that the use of probiotics may cause invasive infection in patients with compromised immunity (45). More work is still needed to translate the application of probiotics to the management of RIOM in the future.

In summary, our data show that probiotic *S. salivarius* K12 can modulate oral microbiota and ameliorate radiation-induced oral mucositis in a RIOM mice model. *S. salivarius* K12 as a probiotic represents a promising therapeutic against RIOM. Since probiotics have been proposed as a potential approach to the management of radiotherapy/chemotherapy-induced mucositis (46), *S. salivarius* K12 as an oral probiotic represents a promising adjuvant treatment to improve the quality of life of cancer-patients receiving radiotherapy.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, SRP276563.

## ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, China.

## AUTHOR CONTRIBUTIONS

YW, JTL, XiZ and XX contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. HZ, XuZ and JW contributed to data acquisition and interpretation, critically revised the manuscript. XJ, XP, JZ, LZ and JYL contributed to data interpretation, critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Treg: A Promising Immunotherapeutic Target in Oral Diseases

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With the pandemic of COVID-19, maintenance of oral health has increasingly become the main challenge of global health. Various common oral diseases, such as periodontitis and oral cancer, are closely associated with immune disorders in the oral mucosa. Regulatory T cells (Treg) are essential for maintaining self-tolerance and immunosuppression. During the process of periodontitis and apical periodontitis, two typical chronic immune-inflammatory diseases, Treg contributes to maintain host immune homeostasis and minimize tissue damage. In contrast, in the development of oral precancerous lesions and oral cancer, Treg is expected to be depleted or down-regulated to enhance the anti-tumor immune response. Therefore, a deeper understanding of the distribution, function, and regulatory mechanisms of Treg cells may provide a prospect for the immunotherapy of oral diseases. In this review, we summarize the distribution and multiple roles of Treg in different oral diseases and discuss the possible mechanisms involved in Treg cell regulation, hope to provide a reference for future Treg-targeted immunotherapy in the treatment of oral diseases.

**Keywords: Treg, immunotherapy, oral diseases, periodontitis, oral cancer**

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## INTRODUCTION

Regulatory T cell (Treg) was first reported to be involved in maintaining self-tolerance as early as the 1970s, but there was still a lack of specific molecular markers (1). Until 1995, Sakaguchi et al. found that the IL-2 receptor α-chain (CD25) was constitutively expressed on Treg cells, the concept of regulatory T cells was formally put forward (2). Treg, as a subset of $CD4^+$ T lymphocytes, is crucial for maintaining self-tolerance and immune homeostasis. It characteristically expresses the transcription factor forkhead box P3 (FOXP3), identified as the main regulator for Treg development and function (3).

During the emergency of COVID-19, maintenance of oral health has increasingly become the main challenge of global health due to the possibilities of increasing viral transmission (4). Oral diseases are ones of the common public health problems. Among them, periodontitis is the most important cause of adult permanent teeth loss; lip and oral cancer, as the 15th most common cancer worldwide, is closely related to the quality of human life (5). As the epitome of the whole body system, the oral cavity is affected by a variety of diseases and disorders, including apical periodontitis

and periodontitis as acute and chronic infectious diseases, autoimmune diseases such as Pemphigus Vulgaris (PV), oral cancer, and oral potentially malignant disorders (6). Increasing evidence shows that the regulation of Treg cell number and function in different diseases have opposite expectations. In autoimmune diseases, Treg cells are expected to be more stable and polyclonal and play a practical immunosuppressive role (7). On the other hand, Treg cells suppress the anti-tumor immune response, accelerate tumor proliferation and metastasis in some tumors. Therefore, Treg targeted immunotherapy is often at the forefront of anti-tumor therapy (8). However, it is worth noting that the research on the role and regulatory mechanisms of Treg cells in oral diseases is incomplete. In this review, we discuss the immunosuppressive mechanisms of $FOXP3^+$ Treg cells and summarize their distribution and function in different types of oral diseases, especially the possible mechanisms involved in the regulation of Treg distribution, proliferation, and function, to provide some new prospects that may eventually apply to clinical treatment.

## TREG BIOLOGY

Treg cells are involved in maintaining immune tolerance, accounting for 5% - 10% of $CD4^+$ T cells in the peripheral circulation (2, 9, 10). According to their different origins, the Treg population is further divided into three subgroups: thymus-derived Treg cells (tTregs), peripheral Treg cells (pTregs) and induced Treg cells (iTregs). tTregs are mainly derived from T-cell precursors stimulated by TCR signal and costimulatory molecules in the thymus; while pTregs are induced from naïve $CD4^+$ T cells that are exposed to cytokine TGF-β and IL-2 in the periphery. Besides, iTregs are induced in the TGF-β environment *in vitro* with unstable Treg phenotypes (11). The transcription factor forkhead box P3 (FOXP3) has been considered as a specific Treg molecular marker, essential for its differentiation, phenotype maintenance, and immunosuppressive function (12). FOXP3 gene mutation leads to the impairment of Treg cells development and inhibition function, resulting in human IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) disease and scurfy in rodents, respectively (13, 14). IPEX is also named X-linked autoimmunity allergic dysregulation syndrome (XLAAD). Patients with the disease will present many immunopathological symptoms within infancy, including enteropathy, diabetes, dermatitis, thyroid disease, and anemia (15). The scurfy mice are characterized by scaly and ruffled skin, spleen and lymph node enlargement, and premature death about a few weeks after birth (16, 17).

The intrinsic commitment and stable maintenance of the Treg lineage depend on the sustained high expression of FOXP3. It endows Treg with a variety of essential characteristics, including high expression of CD25 and cell surface molecules like cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), suppression of proinflammatory cytokines as IL-4 and IL-17 conversely (18). At the same time, FOXP3 can interact with ~700 target genes and multiple microRNAs to regulate the development and function of Treg collectively (19, 20).

In addition, the stability of the Treg lineage is also regulated by epigenetics. The FOXP3 locus contains several conserved noncoding enhancer sequences (CNS) that are targeted by epigenetic modifications and several transcription factors (21). Mothers against decapentaplegic homologue 3 (SMAD3) and nuclear factor of activated T (NFAT) bind to CNS1 after the activation of TGF-β signal and promote FOXP3 expression, which plays a key role in the induction of pTreg cells (22). During tTreg cell development, CpG elements within CNS2 manifest demethylation progressively. Besides, both runt-related transcription factor 1 (RUNX1) and core-binding factor subunit (CBF-β), forming a trimeric complex at the CNS2, enable the stable expression of FOXP3 (23). CNS3 also facilitates FOXP3 transcription *via* the combination of c-Rel (in the NF-κB pathway) after TCR signal activation (24). In general, Treg stability is closely related to the complex and interrelated genetic landscape shaped by FOXP3 and the higher-level epigenetic regulation involved in the induction and maintenance of FOXP3 expression.

However, the stability of Treg cells is not always immutable. It has strong adaptability in an inflammatory environment. Under the local inflammatory stimuli, dendritic-cell-derived IL-6 can induce Treg cells to transform into Th17 cells (25, 26). Th17 cells, as the representative of $CD4^+$ T cell pro-inflammatory subsets, mainly secrete pro-inflammatory cytokine interleukin IL-17 (27). Retinoid-related orphan receptor γt (ROR γt) is a unique lineage-specific transcription factor of Th17 (28). Both Th17 and Treg cells share a common key regulatory factor TGF-β, which participates in the activation of RORγt and FOXP3 (29). In the stimulation of proinflammatory cytokines such as IL-6 or IL-21, a low concentration of TGF-β induces the development of Th17 cells, correspondingly, a high concentration of TGF-β can promote the differentiation of naive $CD4^+$ T cells into Tregs and maintain immune tolerance (30). IL-6 and IL-21 also upregulate the expression of RORγt *via* inhibiting FOXP3 activity in a signal transducer and activator of transcription 3 (STAT3) dependent manner (31). In addition, pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) could down-regulate the expression of FOXP3 by binding with tumor necrosis factor receptor RII (TNFRII) and interfere with the inhibitory function of Treg cells (32). At the same time, it promotes the recruitment of protein kinase C-θ (PKC-θ) and inhibited Treg function by activation downstream Akt signal (33). Therefore, the inflammatory microenvironment may induce the instability of Treg cells, and further exacerbate inflammatory responses and tissue damage in inflammatory diseases, such as apical periodontitis.

On the contrary, tumor-infiltrating Treg cells showed quite active inhibitory phenotypes, with high expression of immune checkpoint molecules, including CTLA-4, programmed cell death 1 (PD-1), T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), lymphocyte activation gene-3 (LAG-3) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (34). Tumor cells can suppress the secretion of IL-6 in dendritic cells by the overexpression of indoleamine

2,3-dioxygenase (IDO), inhibit the reprogramming of Treg cells to Th17 cells, and further enhance the stability of Treg cells in the tumor microenvironment by silencing the expression of the Akt/mTOR pathway (35). Therefore, the enhanced stability of Treg cells in the tumor microenvironment may contribute to the inhibition of anti-tumor immunity and immune escape.

## MECHANISMS OF TREG-MEDIATED SUPPRESSION

Treg cells exert immunosuppressive function through cell-contact-independent or cell-contact-dependent mechanisms. Cell-contact-independent mechanisms mainly include secretion of inhibitory cytokines and metabolic disruption. Cell-contact-dependent mechanisms mainly include modulation of antigen-presenting cell (APC) function and mediating cytolysis or apoptosis of target cells.

### Induction of Inhibitory Cytokines

Treg cells secrete cytokines with vital immunosuppressive function, including IL-10, TGF-β, and IL-35 (36). IL-10 downregulates the expression of class II major histocompatibility complex (MHC II) and costimulatory molecules, and directly inhibits the synthesis and secretion of inflammatory factors, thus inhibiting the capacity of antigen-presenting cells (APCs) and playing an anti-inflammatory role (37). Interestingly, IL-10-producing T regulatory type 1 (Tr1) cells are also endowed with similar inhibitory functions without FOXP3 expression (38). TGF-β also affects the differentiation, development, and function of various immune cells. TGF-β inhibits APCs' function and limits cytotoxic T lymphocyte (CTL) proliferation (39). At the same time, immature $CD4^+$ T cells could be induced to Tregs by antigen stimulation in an enriched TGF-β environment *in vitro* (40). Stimulated Treg cells also exert an immunosuppressive effect in the form of cell-cell interaction with persistently expressing TGF-β at a high level on the cell surface (41). As a novel member of the IL-12 family, IL-35 is another inhibitory cytokine explicitly secreted by Treg cells, involved in the maintenance of its maximum inhibitory function. Ectopic expression of IL-35 confers regulatory activity on naive T cells in a titrable fashion, whereas recombinant IL-35 alone is sufficient to suppress T-cell proliferation (42).

### Regulation of Antigen-Presenting Cell (APC) Function

CTLA-4, constitutively expressed in Treg cells, is an inhibitory receptor associated with the T cell costimulatory molecule CD28 (43). CTLA-4 and CD28 compete for costimulatory receptors (CD80, CD86) on antigen-presenting cells, resulting in the downregulation of these two costimulatory molecules, thus inhibiting the T cell response (44, 45). Furthermore, CTLA-4 promotes the upregulation of the enzyme IDO by dendritic cells (DCs), which catalyzes the decomposition of tryptophan, an essential amino acid. The potential downstream effects lead to cell cycle arrest and more sensitivity to apoptosis of effector T cells, along with impairment of APCs function (46). Besides, lymphocyte activation gene 3 (LAG-3/CD223) is highly expressed on the surface of Treg cells, which combines with MHC class II molecules in higher affinity than CD4. It inhibits DC function and immunostimulatory capacity through the inhibitory signal pathway mediated by immunoreceptor tyrosine-based inhibition motif (ITAM) (47). Blocking LAG-3 attenuates the inhibitory effect of Treg cells, while the ectopic expression of LAG-3 endows $CD4^+$ T cells the inhibitory activity (48).

### Mediating Cytolysis or Apoptosis of Target Cells

Treg cells also cause immunosuppression by inducing target cell death *via* cell contact. Treg cells kill target effector cells, which are mediated by releasing granzymes A and B in the perforin dependent or independent manner (49–51). Additionally, Tumor-necrosis-factor-related apoptosis-inducing ligand-death receptor 5 (TRAIL-DR5) pathway has been proved to be an important component of Treg-induced cytotoxicity (52).

### Disruption of Metabolic Pathways

Another potential mechanism of Treg-mediated suppression is the metabolic blockade. Treg cells highly express the high-affinity IL-2 receptor (CD25), resulting in competitive consumption of IL-2 with effector T cells. Therefore, the effector T cells are prone to Bim-mediated apoptosis for the deprivation of the crucial metabolic and survival cytokines (10, 53). Treg cells express the ectoenzymes CD39 and CD73, which hydrolyze adenosine triphosphate (ATP) or adenosine diphosphate (ADP) to cAMP and adenosine, driving the accumulation of adenosine nucleosides and disrupting effector cell metabolism (54). Treg cells also promote the transfer of inhibitory second messenger cAMP to an effector T cell *via* cell contact-dependent gap junction and unexpectedly inhibit the immune function of effector T cell (55).

## DISTRIBUTION AND FUNCTIONS OF TREG CELLS IN ORAL DISEASES

### Treg Cells in Apical Periodontitis

Apical periodontitis is a local inflammatory immune response caused by bacterial infection in root canals, which often leads to periapical tissue damage and alveolar bone destruction (56). Thus, the balance between the host pro-inflammatory and anti-inflammatory responses supposedly determines the progression and outcome of apical periodontitis, which is regulated by different types of $CD4^+$ T helper cells, including at least Th1, Th2, Th17, and Treg cells (57). As a potential protective subset of $CD4^+$ T cells, accumulating studies have revealed that the beneficial role of Treg cells in restricting the overactivity of the periapical inflammatory response (58, 59).

The number of Treg cells was found remained at relatively low levels from days 7 to 21 (acute phase, the lesions markedly expanded in 3-dimensional directions) after induced periapical

lesions of the lower first molars in rats, and then increased significantly by day 35 (chronic phase, the lesions expanded slowly). Interestingly, the ratio of $IL17^+/Foxp3^+$ and the number of osteoclasts correlated negatively with Treg cells (60). In addition to artificially induced acute periapical lesions in animal models, some human studies on chronic periapical lesions assessed the expression of FOXP3, which was associated with the histological type of lesion, the intensity of the inflammatory infiltrate, and the thickness of the cystic epithelial lining. Chronic periapical lesions include periapical granulomas (PGs), radicular cysts (RCs), and residual radicular cysts (RRCs). Periapical granulomas are the most common type of chronic apical periodontitis. It is granulomatous tissue composed of lymphocytes, fibroblasts, and other inflammatory cells to replace the normal bone structure. With the persistence of chronic inflammation, the epithelial cells of Malassez are stimulated by cytokines and growth factors, proliferate into epithelial masses, then liquefy and necrose in the center, and gradually form into RCs (61). The RRCs are defined as radicular cysts which remain in the jaw without proper treatment after the affected tooth was extracted (62). FOXP3 expression in RRCs, RCs, and PGs increased sequentially. The number of $FOXP3^+$ cells was significantly higher in the inflammatory infiltrate grade III, followed by that in grades II and I (63).

In another study, the percentage of $Foxp3^+$ Treg cells continued to increase after pulp exposure and was negatively correlated with (sphingosine-1-phosphate receptor 1) S1P1-positive cells by day 14 after the induction of periapical lesions in rats. Upregulated S1P1 triggers a series of intracellular responses to promote the receptor activator of nuclear factor kappa B ligand (RANKL) expression, which is related to osteoclast formation during the pathogenesis of periapical bone destruction (64). Besides, S1P1 promotes inflammatory cell infiltration and inhibits the function of Treg through the Akt-mTOR pathway in the acute stage. Therefore, the complex and precise regulatory network between S1P1 signal and Treg cells better explains the process of periapical lesions.

By contrast, inhibition of Treg function with anti-GITR (a phenotypic marker of Treg cells) in mice impelled the exacerbation of severity of periapical lesions at 14 and 21 days, increased expression of pro-inflammatory cytokines and destructive tissue mediators, thus preventing the formation of the inactive/stable status. Similar results were observed in CCR4KO mice. Conversely, the expansion of Treg cells attenuates lesion progression *via* the injection of cytokine C-C motif ligand 22 (CCL22)-releasing particles in the root canal system in a CCR4-dependent manner (58). These findings suggest that Treg chemoattractant application may be a promising option in the treatment of apical periodontitis.

A recent trial is also yielding promising results that Treg cells were enriched around regenerating tissues in the root canals of dogs after regenerative endodontic treatment (RET). In vitro, stem cells from the apical papilla (SCAP) promoted the conversion of pro-inflammatory T cells to Treg cells. It may suggest that the anti-inflammatory and anti-apoptotic abilities of upregulated Treg cells promote successful tissue repair and regeneration *via* releasing more cytokines and pro-healing growth factors, which may create an appropriate immune microenvironment for tissue regeneration (59).

These findings highlight that the infiltration of Treg cells is crucial for preventing the progression of apical periodontitis and promoting tissue regeneration. Treg cell dynamics plastically regulate pathogenic Th1, Th2, or Th17 cell phenotypes to maintain normal homeostasis and restrict inflammatory reaction's overactivity (65). Therefore, promoting endogenous Treg recruitment-based therapy may provide a promising strategy for the treatment of periapical lesions and osteolytic diseases. At the same time, Treg enrichment creates an appropriate immune microenvironment for tissue regeneration, which lays a biological foundation for regenerative endodontic treatment. In the future, the researches on the effectiveness and biosafety of chemokine controlled release system and the exact role of Treg in the regeneration process will be conducive to the theoretical basis into clinical reality.

## Treg Cells in Periodontitis

Most tissue damage in periodontitis is caused by the host immune response to infection, although the accumulation of plaque microorganisms is the initiating factor (66). Therefore, controlling the host immune-inflammatory response remains a challenge for periodontitis therapeutically interventions. Different clinical studies have described Treg accumulation preferentially in infected tissues, limiting the immune response. For instance, a large number of Treg cells has been reported in middle and advanced chronic periodontitis biopsies than gingivitis (67). Moreover, other studies have shown that chemokines such as CCL17 and CCL22 are more abundant in tissues with higher inflammatory infiltration, which seems to recruit more Treg cells from inflammatory sites in a CCR4 dependent manner (68). However, some $FOXP3^+$ cells may function differently from conventional Treg cells. A small population of $IL\text{-}17A^+FOXP3^+$ cells were found in periodontitis, but not in gingivitis, suggesting the functional plasticity of Treg cells transforming into inflammatory Th17 cells in the periodontitis environment (69).

On the other hand, the defect of Treg cells function is identified in many animal models to promote the progression of periodontitis. In the *A. actinomycetemcomitans* induced mice model of periodontitis, inhibiting the function of Treg cells by anti-GITR resulted in alveolar bone resorption and increased inflammatory cell infiltration, accompanied by the decrease of IL-10, TGF-β, and CTLA-4 (70). A similar phenomenon was observed in the IDO knockout mouse model along with lipopolysaccharide (LPS)-induced inflammation, as IDO affects the metabolism of Treg cells (71). In an experimental periodontitis model, the phenotype and function of Treg cells were also affected. The down-regulated Foxp3 expression and the damage of the inhibitory effect of Treg cells on osteoclast differentiation further promoted Th17-driven bone loss. The hypermethylation of CpG sites in the Foxp3 locus caused by periodontitis may be responsible for its function impairment (72). In a recent study, the possible reason for the aggravation of periodontal disease during pregnancy has been attributed to Treg

cells' shortage. The expression of Foxp3, TGF-β, CTLA-4, and CD28 in the gingiva of pregnant mice was reduced after periodontal disease induction. Simultaneously, the frequency and inhibitory ability of Treg cells in cervical lymph nodes were also down-regulated *in vitro* test, with the increase of inflammatory Th17 cells (73).

Currently, gratifying achievements have been reported in biochemical recruitment and positive regulation of Treg cells. Local or systemic administration of IL-35 also retards alveolar bone resorption in periodontitis mice *via* regulating the balance of Th17/Treg, down-regulating RANKL, and inducing osteoprotegerin (OPG) production (74). Similarly, an injectable and biomolecule-delivery of poly(L-lactic acid) (PLLA) nanofibrous spongy microspheres (NF-SMS) promote Treg enrichment, amplification, and Treg-mediated immunotherapy against bone loss in a mouse model of periodontitis *via* significantly releasing miRNA and IL-2/TGF-β (75). Exosomes from periodontal ligament stem cells, as communication mediators, are also involved in the regulation of Treg cell distribution and play an essential role in immunomodulation. Compared with normal conditions, the exosomes of periodontal ligament stem cells isolated from *Porphyromonas gingivalis* lipopolysaccharide (LPS) induced periodontitis environment transfer less miR-155-5p and increased Sirtuin-1 (SIRT1) protein into $CD4^+$ T cells, and then led to the up-regulation of Th17 and the relaxation of Tregs, thus exacerbating the inflammatory microenvironment of periodontitis (76). In addition, the ratio of Th17/Treg also inclines by oral administration of all-trans retinoic acid (ATRA) in experimental periodontitis and thus providing protection against periodontitis (77).

Therefore, these findings indicate that Th17/Treg ratio imbalance is considered a critical role in the procession of periodontitis. Treg cells suppress immunopathology to avoid extensive periodontal tissue damage. It has been proved to suppress osteoclast differentiation through cell-cell contact way by Treg cells (78). Inhibitory cytokines released by Treg cells, such as IL-10 and IL-4, are also largely involved in the inhibition of RANKL expression (79). On the contrary, Th17 induces the maturation of osteoclasts by promoting the expression of RANKL, accelerating the resorption and destruction of alveolar bone (80). Therefore, Treg cells and Th17 cells are considered the key cells to connect the immune system and bone. Existing studies have shown that periodontitis is closely related to diabetes (81), rheumatoid arthritis, cardiovascular diseases (82), and other systemic diseases (83). However, at present, most of the clinical treatment methods for periodontitis are still the basic treatment for its initiating factors. Therefore, exploring new immunotherapy for periodontitis in humans may provide potential help for the macro-control of systemic diseases. In the future, more researches are needed to understand the diversity and plasticity of Treg subsets for a more advanced and safer drug delivery system.

## Treg Cells in Head and Neck Squamous Cell Carcinoma

Immune escape is a characteristic of head and neck squamous cell carcinoma (HNSCC) (84). Treg cells might contribute to the occurrence and progression of HNSCC by suppressing antitumor immunity (85). Multiple pieces of evidence have described that the number and inhibitory activity of Treg cells is enhanced in tumors and peripheral circulation of patients with HNSCC, compared with healthy donors, along with the upregulated CD39, CD62L, CTLA-4, and FOXP3 (86–89). In addition, Treg level was proved to have a significant linear and positive correlation with tumor grades (90). Another study showed that the percentage of Treg in peripheral blood lymphocytes was also increasing correspondingly with the tumor malignant degree and lymph node metastasis. The higher the malignancy, the more activated Treg subsets (91). In the process of oral precancerous lesions to oral squamous cell carcinoma, Treg accumulation has also been widely proved, with the increase of the degree of epithelial dysplasia (92). Treg cells undoubtedly play a hostile role in the development of HNSCC. In precancerous lesions, the inflammatory response is at the peak, which is mainly maintained by Th17 cells with high levels of inflammatory cytokines, such as IL-2, IL-6, and IL-17. However, as the disease progresses, the increased level of TGF-β released by cancer cells promotes Treg differentiation, downregulates Th17 cells, further accelerating tumor progression (93).

However, there are some conflicting results about the prognostic value of Treg cells in HNSCC. Several data sustained that the high frequency of Treg cells in primary lesions and lymphogenic metastases were associated with a poor prognosis (94, 95). In contrast, other studies described that high Treg infiltration was associated with better overall survival (OS) of HNSCC (96, 97). These apparent contradictions were further explained in a recent study. Echarti et al. studied the effect of Treg cells on overall survival (OS) under different immune phenotypes and found that higher Treg cells level tended to worsen OS in "immune desert (stromal cytotoxic T cells (CTL) were ≤50 cells/$mm^2$)" and "immune excluded" tumors, but in "inflamed (intraepithelial CTL were >500 cells/$mm^2$)" tumors, high Treg cells significantly improved OS. This indicates that the prognostic value of Treg depends mostly on the inflammatory state of the tumor (98).

Another cross-sectional study showed that the amount of Treg cells increased and persisted in HNSCC patients after adjuvant chemoradiotherapy (CRT) compared with untreated or surgery-only patients and were resistant to activation-induced cell death (AICD) or cisplatin *in vitro*. These Treg cells have a stronger inhibitory function after CRT, which may be related to the upregulated latency-associated peptide (LAP), the glycoprotein A repetitions predominant (GARP), and CD39. This may be a potential driving factor for Immunotherapy resistance and relapse of HNSCC (99).

These studies suggest that Treg cells can block the effectiveness of antitumor immunity and contribute to tumor immune escape. Therefore, the reasonable strategy of depleting Treg cells or weakening their inhibitory functions should be pursued for immunotherapy (100). At present, blocking Treg-related immune checkpoint receptors (ICR) through immune checkpoint inhibitors (ICIS) has become one of the most

promising strategies for anti-cancer therapy, such as new monoclonal antibodies against CTLA-4, programmed cell death-ligand 1(PD-L1), and PD-1 (101). Although ICIS has been approved for clinical application, the compensatory mechanisms in the tumor microenvironment, such as the up-regulation of other immunosuppressive molecules, remain as potential challenges in cancer treatment (102). Therefore, the study of combined therapy strategy for ICIS targeted Treg cells may bring hope to optimize the anti-tumor immunotherapy (34).

## Treg Cells in Oral Mucosal Diseases

Oral mucosa is a vital barrier tissue to protect the oral cavity from the invasion of pathogens and foreign antigens. It was found that $FOXP3^+$ Treg cells were highly abundant in oral mucosa than in secondary lymphoid tissues and other mucosal barrier sites, and they expressed a large number of CTLA-4 and tissue retention molecule CD103. This indicates that a uniquely large number of highly active Treg cells are needed to maintain oral mucosal immune quiescence. Interestingly, Treg cells in oral mucosa were mainly dependent on the recruitment and migration of exogenous Treg cells, rather than in-situ induction (103). Abnormal numbers of Treg cells caused many types of oral mucosal diseases. For example, patients with autoimmune disease Pemphigus Vulgaris (PV) showed a decreased frequency of Treg cells, but an increased number of Th17 cells, with the reduced expression of CCL22 (104). In patients with chronic inflammatory disease aphthous ulcer, the frequency of Treg cells in peripheral blood and their inhibitory function were both down-regulated, which may be related to the decreased expression of IDO (105). In contrast, the number of Treg cells increased in precancerous lesions of oral mucosal tissues. Through the comparative study of oral epithelial precursor lesions (OEPL) and oral squamous cell carcinoma (OSCC), the expression of CD25 and FOXP3 was found to be positively correlated with the malignant degree of oral epithelial lesions (92). In the precancerous condition of oral lichen planus (OLP) and precancerous lesion actinic cheilitis (AC), $FOXP3^+$ cell infiltration increased, and $CD8^+/FOXP3^+$ cell ratio decreased, suggesting the promoting role of Treg in cancer progression (106, 107). In addition, during the progress from premalignant lesions to cancer, Th1 and Th17 phenotypes gradually inclined to Treg phenotype in spleen and lymph nodes (108). Therefore, Treg cells play an irreplaceable part in maintaining the immune homeostasis of the oral mucosal barrier. However, anti-Treg immunotherapy may contribute to slow down the progression of precancerous lesions. In addition, inducing the conversion of Treg to Th17-like phenotype may provide a potential prospect for intervention the progress of precancerous lesions.

In summary, Treg cells function like a double-edged sword, which plays a protective role in inhibiting the course of inflammatory diseases such as apical periodontitis and periodontitis (**Figure 1**), and autoimmune diseases, but accelerate the deterioration of precancerous lesions in oral mucosa and HNSCC (**Figure 2**).

# REGULATORY MECHANISMS OF TREG CELLS RECRUITMENT, PROLIFERATION, AND FUNCTION IN ORAL DISEASES

The recruitment, proliferation, and function of Treg cells are regulated by various complex regulatory networks, including cytokines, intracellular signaling pathways, epigenetic modification, and post-translational modification. These regulatory pathways affect the stability and plasticity of Treg from the cellular level to the expression of crucial genes (**Figure 3**).

## CCL22-CCR4 Axis

Many pieces of evidence suggest that the CCL22-CCR4 axis is related to the regulation of Treg cells, involving different types of oral diseases. CCL22 was originally recognized as a chemokine produced by dendritic cells and macrophages under the stimulation of bacterial components. It induces the migration of target cells by binding to its specific receptor C-C chemokine receptor type 4 (CCR4) (109). CCR4 is specifically expressed on human Treg cells in response to its chemotaxis (110). In the chronic inflammatory environment, CCL20 expression is upregulated by the proinflammatory cytokine IL-1β, which is further enhanced by the TGF-β-SMADs pathway through an enhancer upstream of the CCL20 promoter (111). NF-κB is a significant mediator of inflammation. Activated NF-κB can transactivate CCL22 expression. CCL22 also can activate NF-κB, forming a positive feedback loop (112). Interestingly, CCR4 expression is also upregulated by NF-κB activation mediated by TNF-α (113), highlighting the essential roles of NF-κB in the CCL22-CCR4 axis.

In apical periodontitis, studies have shown that CCL22 combined with CCR4 seems to be able to recruit more Treg cells into periapical lesions of mice. CCR4 depletion significantly impaired the migration ability of Tregs and increased the severity of periapical lesions, associated with the expansion of pro-inflammatory cells and tissue destruction factors. On the contrary, local administration of CCL22 in wild-type (WT) mice attenuated periapical lesions with increased Treg number, but failed in CCR4KO mice, suggesting that CCL22 promotes Treg cell migration in a CCR4 dependent manner (58). Besides, it has been reported that LPS promotes the secretion of CCL22 in macrophages by downregulating the expression of miR-34a in the apical periodontitis model of rats. The high expression of CCL22 is parallel to the frequency of $Foxp3^+$ Treg cells (114).

In periodontitis, chronic periodontitis patients showed high levels of CCL22 and CCR4 compared with healthy donors (68). It was early observed in murine and canine experimental periodontitis that the release of CCL22 particles could recruit more Treg cells to inflammatory sites, and significantly reduce the alveolar bone resorption (115). Furthermore, in experimental periodontitis, CCR4KO mice and the blockade of CCL22 in WT mice both showed impairment of Treg migration, accompanied by the expansive osteoclastogenic cytokine and increased inflammatory bone loss. Adoptive transfer of $CCR4^+$ Treg cells

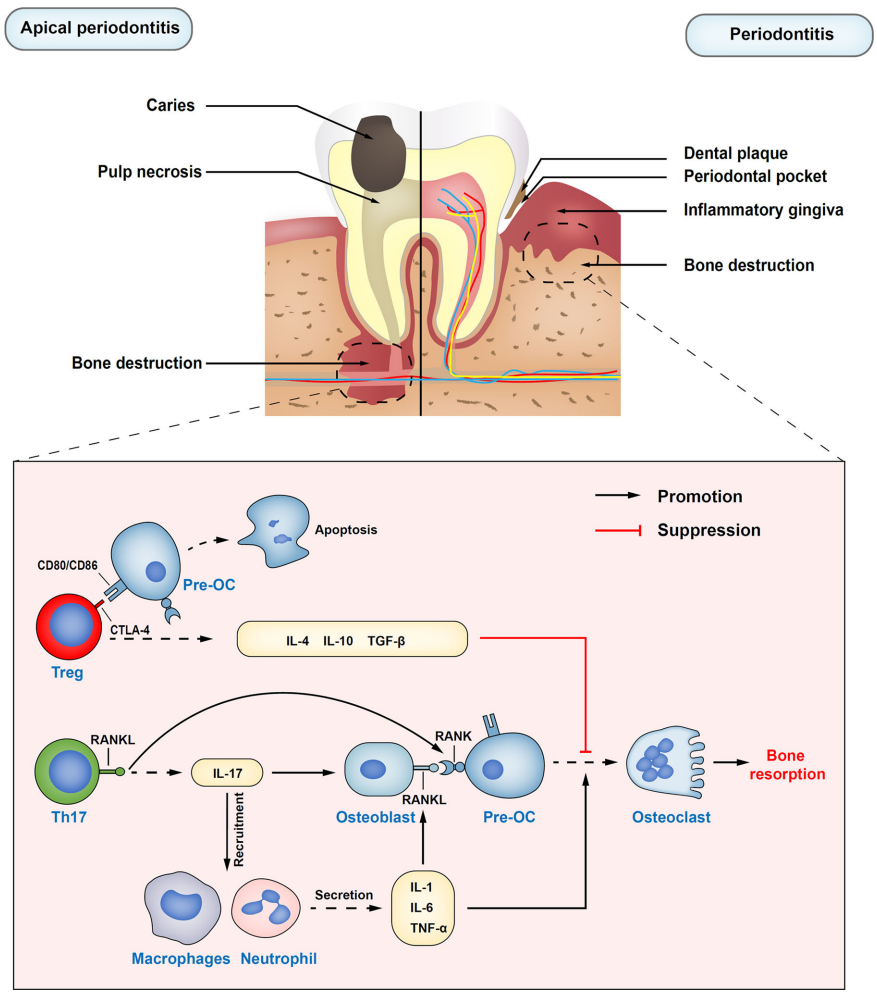


**FIGURE 1** | Immune regulatory functions of Treg cells in apical periodontitis and periodontitis. Treg cells inhibit the differentiation of osteoclast precursors into osteoclasts by secreting inhibitory cytokines, such as IL-10, IL-4, and TNF-β. Also, inhibiting receptor cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) on Treg cells directly in contact with osteoclast precursors costimulatory molecules CD80 and CD86, which can induce the production of indoleamine 2,3-dioxygenase (IDO) and induce the apoptosis of osteoclast precursors. Th17 cells upregulate osteoblasts and self-expressed receptor activator of nuclear factor kappa B ligand (RANKL) through the release of inflammatory cytokine IL-17. At the same time, IL-17 plays an important role in the mobilization and recruitment of immune cells, stimulating the release of local inflammatory factors, resulting in the expansion of osteoclasts, and the aggravation of inflammatory response. Pre-OC, osteoclast precursors; RANK, the receptor activator of nuclear factor kappa B.

to the CCR4KO mice or exogenous release CCL22 provided by poly (lactic-co-glycolic acid) (PLGA) microparticles rescued the increased disease phenotype by promoting migration of Treg cells (116).

Similar regulatory axes have also been described in oral cancer. CCL22, as an oncogene, is upregulated in oral cancer specimens to promote the migration and infiltration of Treg cells. Silencing CCL22 expression showed opposite effects. CCL22 expression in oral cancer cells was induced by IL-1β secreted by cancer-associated fibroblasts, suggesting a new therapeutic prospect by targeting the IL-1β-CCL22-CCR4 signaling axis for the treatment of oral cancer (117). Moreover, CCL22 is the target of tumor suppressor miR-34a. In cancers, CCL22 is unregulated by the suppression of miR-34a mediated by TGF-β (118).

Therefore, these findings suggest that the CCL22-CCR4 axis is involved in Treg recruitment in a variety of oral diseases, and the diverse regulation of the CCL22-CCR4 axis according to treatment goals may provide a potential immunotherapeutic target.

## The Roles of Interleukins

A variety of interleukins participate in the stability of Treg phenotype and inhibitory function through different mechanisms. Through the stable expression of FOXP3 and CD25, IL-2 is irreplaceable for the development, stability, and function of Treg cells (119). Multiple studies have shown that IL-

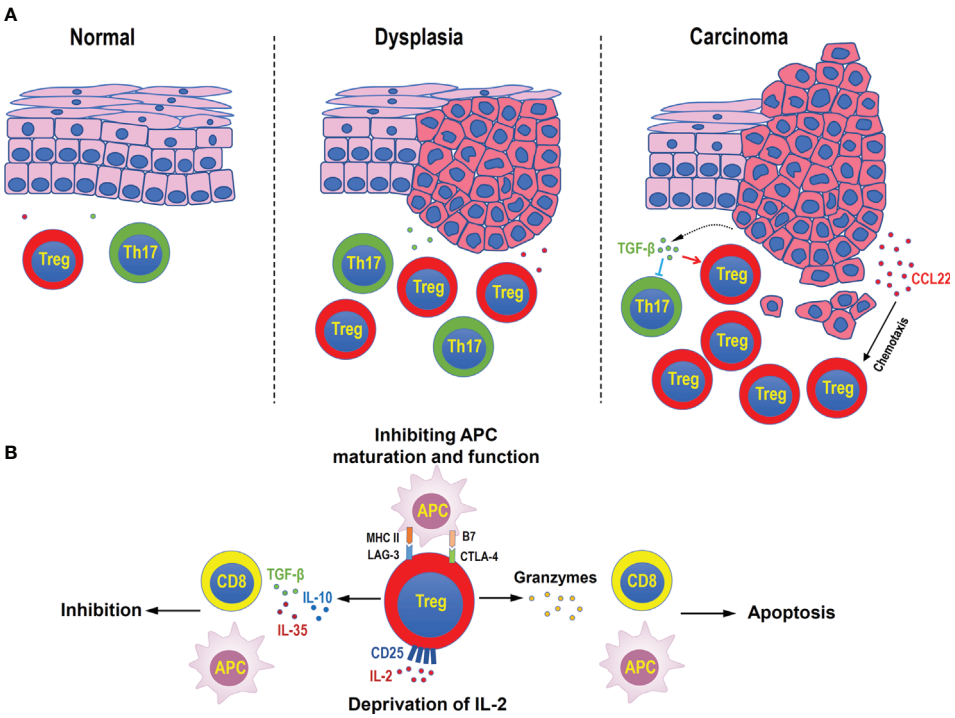


**FIGURE 2** | Distribution and functions of Treg cells in head and neck squamous cell carcinoma. **(A)** Treg cells increase during the disease progression in oral mucosal dysplasia and squamous cell carcinoma. Malignant cells can secret CCL22 to attract Treg cells, or secret TGF-β to suppress inflammatory Th17 cells. **(B)** Immune suppressive mechanisms of Treg cells in head and neck squamous cell carcinoma. Treg cells can secret inhibitory cytokines, such as TGF-β, IL-10, and IL-35, to suppress the functions of antigen-presenting cells (APC) and $CD8^+$ effector T cells, directly kill effector or APC by granzymes, consume of IL-2 by highly expressing CD25, and negatively regulate the maturation and functions of APC by immune checkpoint molecules, such as LAG-3 and CTLA4.

2 receptor signaling can mediate the phosphorylation of signal transducer and activator of transcription 5 (STAT5) by activating Janus kinases (JAKs). Furthermore, activated STAT5 binds to the FOXP3 promoter and CNS2, promoting its expression (120–122). On the contrary, when IL-2 signal transduction was deficient, FOXP3 expression stability in Treg cells was lost (123). Interestingly, FOXP3 and other transcription factors jointly inhibit the expression of IL-2 in Treg cells, making it highly reliant on IL-2 produced by activated non-Treg cells, forming feedback control on the expansion of non-Treg cells (124).

Treg cells secrete inhibitory cytokines IL-10, TGF-β, and IL-35. Among them, IL-35 can produce regenerative feedback in Treg cell response by inducing the activation and differentiation of IL-35 producing Treg cells, termed iTR35 (74). Therefore, the significant benefits of IL-35 based therapy lie in the direct inhibition of IL-35 and the synergetic amplification of iTR35 immunosuppression (125).

Furthermore, IL-33, a member of the IL-1 cytokine family, has attracted attention as an important Treg cell immunomodulator recently (126). IL-1 receptor-like 1 (ST2) is considered as the only receptor of IL-33. IL-33 could support the expansion of $ST2^+Foxp3^+$ Treg cells (127), increasing the secretion of inhibitory cytokines IL-10 and TGF-β1 in $ST2^+Foxp3^+$ Treg cells and promoting their suppressive function in head and neck squamous cell carcinoma (HNSCC). ST2 antibody made the opposite effect, which suggested that ST2 may be a potential target for immunotherapy of HNSCC in the future (128). However, IL-33 is also a pro-inflammatory cytokine. In a mouse periodontitis model, systemic administration of IL-33 exacerbated bone loss in a RANKL dependent manner (129). Therefore, given the different roles of IL-33 in different diseases, a deeper understanding of IL-33 action mode in the future will be more targeted at the IL-33-ST2 signal to treat human diseases (130).

## PI3K/Akt/mTOR Signaling Pathway

The PI3K/Akt/mTOR pathway is involved in many biological processes such as survival, proliferation, growth, apoptosis, and glucose metabolism (131). In Treg cells, the activation of PI3K/Akt/mTOR pathway by inflammatory Toll-like receptor (TLR) or T cell receptor (TCR) signals expand Treg cell amplification (132) but reduces FOXP3 expression (133). FOXP3 inhibits Akt phosphorylation and blunts PI3K/Akt/mTOR signal transduction, by which FOXP3 gives a negative feedback

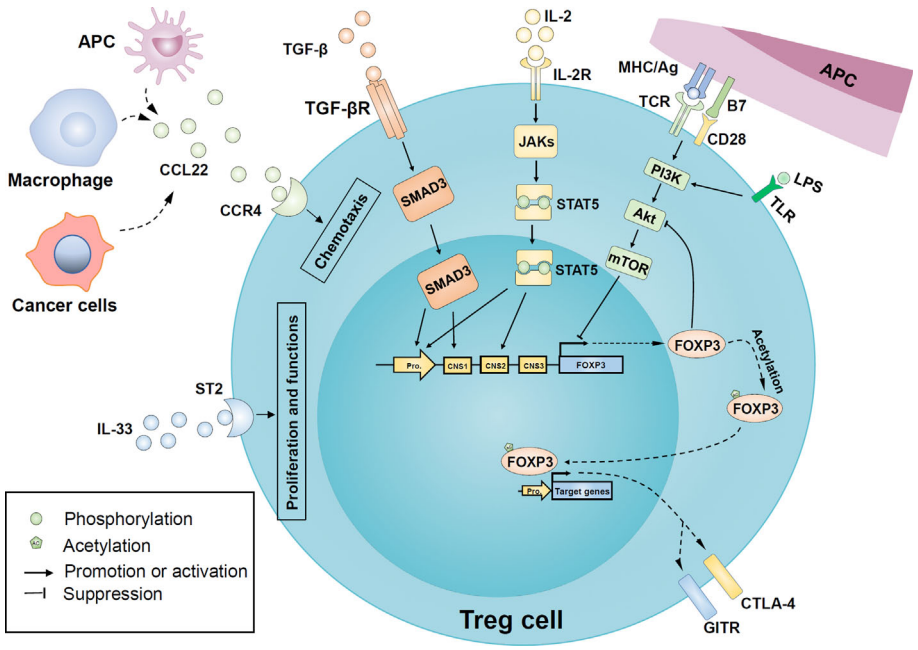


**FIGURE 3** | Regulatory mechanisms of Treg cells recruitment, proliferation, and function in oral diseases. Macrophages and antigen-presenting cells upregulate the release of cytokine C-C motif ligand 22 (CCL22) in an inflammatory environment and recruit more Treg cells to local tissues in a CC-chemokine receptor 4 (CCR4) dependent manner. Similarly, tumor cells are involved in the release of CCL22 in head and neck cancer. In addition, the binding of IL-2 and its receptor CD25 activated the JAK/STAT5 signaling pathway to induce FOXP3 expression. Transforming growth factor β (TGF-β) also has a positive effect on FOXP3 expression by activating mothers against decapentaplegic homologue 3 (SMAD3) transcription factors. Besides, IL-33 binds to the IL-1 receptor-like 1 (ST2) and further promotes the expression of FOXP3 and proliferation of Treg cells. The PI3K-Akt-mTOR pathway activated by inflammatory Toll-like receptor (TLR) or T cell receptor (TCR) signals may be involved in the FOXP3 expression inhibition and the regulation of Treg proliferation, while FOXP3 can negatively feedback on Akt activation. Post-translational modification of mature FOXP3 protein, such as acetylation, enhances both stability and activity of FOXP3. FOXP3 can endow Treg with typical characteristics, such as cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR). APC, antigen-presenting cells; CNS, conserved non-coding sequence; IL-2R, IL-2 receptor; LPS, lipopolysaccharide; mTOR, mechanistic target of rapamycin; PI3K, phosphoinositide 3-kinase; JAKs, Janus kinases; STAT5, signal transducer and activator of transcription 5; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; TGF-βR, TGFβ receptor; TLR, Toll-like receptor.

regulation and results in enhancing the suppressive function of Treg cells (134). On the contrary, phosphatase and tensin homologues (PTEN), a negative regulator of PI3K, is able to stabilize Treg cells in tumors (135). Moreover, the administration of PI3K-Akt pathway inhibitors in CT26 (a mouse colon carcinoma cell line) mouse models showed a significant therapeutic antitumor effect associated with a selective reduction in Treg cells activation and proliferation with no effect on conventional T cells. It was also demonstrated that PI3K-Akt pathway inhibitors could enhance the antitumor immune responses of the antitumor vaccine by inhibiting Treg cell proliferation (136). Interestingly, mTOR inhibition by rapamycin has been shown to support the proliferation and survival of Treg cells, which is opposed to Akt and PI3K (137–139). Specific deletion of the mTOR gene (Rictor) (140) or inhibition of mTOR activity by rapamycin promoted the induction of FOXP3 and maintained the function of Treg cells (141). On the other hand, the anti-tumor effect of PI3K, Akt, and mTOR inhibitors can directly present as the inhibition of tumor cell proliferation and angiogenesis, as well as the survival enhancement of $CD8^+$ T cells (142). Taken together, these data suggested the complicated regulatory mechanisms of the PI3K/Akt/mTOR pathway in Treg cells. Combination of multiple PI3K/Akt/mTOR pathway inhibitors, targeting different steps, may suppress both proliferation and function of Treg cells and achieve better anti-tumor effects.

## Methylation and Post-Translational Modifications

DNA methylation has long been considered as one of the important epigenetic modifications that regulate gene expression but not changing the DNA sequence (143). FOXP3 expression is also regulated by DNA methylation (144). Campos et al. evaluated DNA methylation patterns of 22 gene promoters involved in immune regulation of periapical lesions. The methylation level of the FOXP3 gene promoter was the highest in periapical granulomas and apical cysts, negatively correlated with the expression of FOXP3 mRNA. In addition, active periapical lesions showed higher levels of FOXP3 methylation than inactive periapical lesions. Therefore, the dynamic changes of

FOXP3 methylation level at different stages of periapical lesions may regulate Treg cells as a master switch, affecting the process and outcomes of periapical lesions (145).

As for cancer, thymically derived natural Treg cells were suggested as the primary type of Treg cells in tumor tissues and showed a conserved demethylated region in the first intron of the FOXP3 gene. This Treg-specific demethylated region is required for the long-term maintenance of FOXP3 expression (146, 147), which may mediate by the superfluous STAT5 and TET2 in tumor-infiltrating Treg cells (148). Therefore, Treg-specific demethylated region (TSDR) based supportive therapy may provide a novel strategy for anti-tumor immunity treatment *via* reducing intratumoral Treg cells infiltration or weakening its inhibitory function.

Besides epigenetic modification, FOXP3 function is also positively affected by a posttranslational mechanism: FOXP3 arginine methylations. The inhibition of type I protein arginine methyltransferases (PRMTs) is reported to interfere with arginine methylation of FOXP3 and damage the inhibitory function of Treg cells; while up-regulating PRMT1 could prevent Treg cells from tilting to Th1-like cell phenotype (149). PRMTs have been proved upregulated in several tumors, which indicates a poor prognosis (150). Pharmacological ablation of PRMTs is showing a promising prospect of tumor therapy by inhibiting the function of Treg cells (151). At present, the clinical research of various specific PRMTs inhibitors is in full swing and following widely interest.

In addition, other post-translational modifications are also involved in the regulation of Foxp3 functions, such as acetylation (152). Acetylation enhances both stability and activity of FOXP3 (152). Histone acetyltransferases (HTAs) and histone deacetylases (HDACs) have been widely reported to coordinate the differentiation, function, and stability of Treg cells (153). CBP and p300 are the members of the HTAs family. Their combined deletion leads to fatal autoimmunity in mice at 3 to 4 weeks (154). Interestingly, the selective deletion of p300 damages the inhibitory function of Treg cells and enhances anti-tumor immunity without autoimmune deficiency (155). By contrast, HDAC inhibitor therapy usually increases peripheral Treg cells and enhances Treg suppressive function, upregulates acetylation of FOXP3, and related markers like GITR and CTLA-4 (156).

So far, diverse regulatory mechanisms of Treg cell recruitment, proliferation, and function have been reported in oral diseases. Harnessing these mechanisms may help to treat oral diseases (**Table 1**). However, understanding these mechanisms needs to be improved.

## CONCLUDING REMARKS

Oral diseases, as one of the most common public health problems worldwide, are closely associated with immune disorders. When patients suffer from autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, oral manifestations such as chronic periodontitis, oral lupus erythematosus, and Sjogren's syndrome are also common (157, 158). However, when the body's immune function defect, whether primary or acquired, it is often accompanied by necrotizing ulcerative

**TABLE 1** | Regulatory mechanisms of Treg cells recruitment, proliferation, and function in oral diseases.

| Regulatory mechanisms | Defects or treatments | Effects on Treg cells | Oral diseases | References |
|---|---|---|---|---|
| CCL22-CCR4 axis | CCR4KO mice; Intraperitoneal injection of anti-CCL22 antibodies | Treg migration impairment | Aggravation of apical periodontitis and periodontitis | (58, 116) |
| | CCL22-releasing PLGA microparticles | Treg migration promotion | Remission of apical periodontitis and periodontitis | |
| | CCL22 gene silencing | Treg migration impairment | Impaired oral tumorigenesis | (117) |
| | CCL22 overexpression | Treg migration promotion | Promoted oral tumorigenesis | |
| IL-2-JAKs-STAT5 signaling pathway | IL-2KO mice; JAKsKO mice; STAT5a/b double KO mice | Reduction of Treg frequency | Unknown | (121) |
| | Transient activation of STAT5 in IL-2-deficient mice | Increasing Treg number | | |
| IL-35 | Intragingival injections of IL-35 | Increasing induction of iTr35 cells | Inhibition of periodontitis progress | (74) |
| IL-33 | IL-33 overexpression | Expansion of Treg population and function | Poor prognoses of HNSCC | (128) |
| | Anti-ST2 mAb | Inhibition of Treg number and function | Promotion of effector T cell proliferation | |
| PI3K/Akt/mTOR signaling | Targeting PI3K and Akt with specific inhibitors | Inhibition of Treg proliferation | Enhancement of the antitumor immune response | (136) |
| | Rapamycin (mTOR inhibitors) | Expansion of Treg | Inhibition of effector T cell function | (137) |
| FOXP3 gene methylation | Hypomethylation | Promotion FOXP3 expression; Increase of Treg infiltration | Inactive apical periodontitis; Promoted tumorigenesis | (145, 147) |
| FOXP3 arginine methylation | Targeting PRMTs | Inhibition Treg function | Enhancement of the antitumor immune response | (149) |
| FOXP3 histone acetylation | Selective deletion or pharmacologic inhibition of p300 | Inhibition Treg function | Enhancement of the antitumor immune response | (155) |

periodontitis, oral candidiasis, and the risk of tumor is significantly increased (159). Treg cells play important roles in maintaining immune homeostasis and self-tolerance in oral tissues. They play protective roles in inhibiting the course of inflammatory diseases such as apical periodontitis and periodontitis but accelerate the deterioration of precancerous lesions in oral mucosa and HNSCC. Therefore, Treg is a promising immunotherapeutic target of oral diseases. So far, the regulatory mechanisms of Treg distribution, stability, and function remain largely unclear. Further researches are required to explore these mechanisms and help to design Treg-based therapeutic strategies for the treatment of oral diseases.

## AUTHOR CONTRIBUTIONS

YZ, JG, and RJ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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# Subgingival Microbiome and Specialized Pro-Resolving Lipid Mediator Pathway Profiles Are Correlated in Periodontal Inflammation

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Failure of resolution pathways in periodontitis is reflected in levels of specialized pro-resolving lipid mediators (SPMs) and SPM pathway markers but their relationship with the subgingival microbiome is unclear. This study aimed to analyze and integrate lipid mediator level, SPM receptor gene expression and subgingival microbiome data in subjects with periodontitis vs. healthy controls. The study included 13 periodontally healthy and 15 periodontitis subjects that were evaluated prior to or after non-surgical periodontal therapy. Samples of gingival tissue and subgingival plaque were collected prior to and 8 weeks after non-surgical treatment; only once in the healthy group. Metabololipidomic analysis was performed to measure levels of SPMs and other relevant lipid mediators in gingiva. qRT-PCR assessed relative gene expression ($2^{-\Delta\Delta CT}$) of known SPM receptors. 16S rRNA sequencing evaluated the relative abundance of bacterial species in subgingival plaque. Correlations between lipid mediator levels, receptor gene expression and bacterial abundance were analyzed using the Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO) and Sparse Partial Least Squares (SPLS) methods. Profiles of lipid mediators, receptor genes and the subgingival microbiome were distinct in the three groups. The strongest correlation existed between lipid mediator profile and subgingival microbiome profile. Multiple lipid mediators and bacterial species were highly correlated (correlation coefficient ≥0.6) in different periodontal conditions. Comparing individual correlated lipid mediators and bacterial species in periodontitis before treatment to healthy controls revealed that one bacterial species, *Corynebacterium durum*, and five lipid mediators, 5(S)6(R)-DiHETE, 15(S)-HEPE, 7-HDHA, 13-HDHA and 14-HDHA, were identified in both conditions. Comparing individual correlated lipid mediators and bacterial species in periodontitis before treatment to after treatment revealed that one bacterial species, *Anaeroglobus*

*geminatus*, and four lipid mediators, 5(S)12(S)-DiHETE, RvD1, Maresin 1 and LTB4, were identified in both conditions. Four *Selenomonas* species were highly correlated with RvD1, RvE3, 5(S)12(S)-DiHETE and proinflammatory mediators in the periodontitis after treatment group. Profiles of lipid mediators, receptor gene and subgingival microbiome are associated with periodontal inflammation and correlated with each other, suggesting inflammation mediated by lipid mediators influences microbial composition in periodontitis. The role of correlated individual lipid mediators and bacterial species in periodontal inflammation have to be further studied.

**Keywords: computational biology, host microbial interaction, inflammation, metabolomics, microbiota, lipidomics, periodontitis**

## INTRODUCTION

In the United States, around 60 million adults over 30 years of age have periodontitis (42.2%) with 7.8% having severe periodontitis (1). Periodontitis is a biofilm induced chronic inflammatory disease characterized by gingival inflammation and destruction of alveolar bone. Disproportionate host responses and microbiota dysbiosis are the two major etiological factors (2). Periodontal tissue damage is primarily mediated by bacterially induced immune responses. The composition of the subgingival microbiota is associated with the inflammatory status in periodontal tissue (3). It is hypothesized that the change of local environment induced by inflammation results in the shifts of the subgingival microbiota (4, 5). Many studies have shown that the composition of the subgingival microbiota shifts following periodontal therapy performed to control inflammation (6, 7). In periodontitis, the inflamed tissues with deep periodontal pocket provide an anaerobic environment with breakdown products of tissue destruction, plasma proteins and hemoglobin as nutrients for the growth of several anaerobic gram-negative bacteria, resulting in microbiota shifts. Once inflammation is controlled, the environment is not suitable for periodontal pathogens anymore and commensal microbiota can be re-established in a homeostatic relationship with the host.

Resolution of inflammation is a proactive process induced by specialized pro-resolving lipid mediators (SPMs), including lipoxins, resolvins, protectins and maresins, that bind to specific G protein-coupled receptors on a variety of cells. In the resolution phase of inflammation, there is decreased infiltration of neutrophils, reduced levels of pro-inflammatory cytokines and lipid mediators, and increased recruitment of resolving macrophages that clear the lesion by efferocytosis (8, 9). It has been demonstrated that SPMs control inflammatory diseases, such as inflammatory bowel disease (10), diabetes (11) and periodontitis (12) in the preclinical models. Specifically, in experimental periodontitis, SPMs, such as resolvin E1, topically applied on gingiva can prevent bone loss, regenerate the lost bone, change gene expression patterns in gingiva and result in shifts of the oral microbiota (13, 14). Resolution of inflammation induced by SPMs can influence the composition of the subgingival microbiota in periodontal inflammation.

In humans, SPMs have been found in milk (15), serum, lymphoid tissue (16), saliva and gingival crevicular fluid (17, 18). The levels of SPMs and related lipid mediators in various specimens are associated with the inflammatory status of mammary glands (15), the stability of atherosclerotic plaques (19), the severity of tuberculous meningitis (20) and the disease status of periodontitis (17, 18). Recently, SPMs, SPM pathway markers and SPM corresponding receptor genes are identified in gingival tissues, the periodontal inflammation site (21). Profiles of these lipid mediators and receptor genes are associated with the severity of inflammation. However, the associations between these lipid mediators and the oral microbiome have not been explored. Since preclinical studies demonstrated that resolution of inflammation induced by SPMs is associated with shifts in the taxonomic composition of the oral microbiota (13, 14), there is a need to investigate the clinical relationship between profiles of SPM relevant lipid mediators and subgingival microbiome in periodontal inflammation to clarify how inflammatory reactions mediated by SPMs affect subgingival microbial composition in humans and vice versa.

The aim of this study was to assess correlations between levels of lipid mediators including SPMs and SPM pathway markers, expression of SPM receptor genes in human gingiva and the relative abundance of bacterial species in subgingival plaque. The results of this study indicate the potential interactions between lipid mediators and bacterial species in different periodontal inflammatory conditions.

**Abbreviations:** 5(S),6(R)-DiHETE, 5(S),6(R)-dihydroxyeicosatetraenoic acid; 5(S),12(S)-DiHETE, 5(S),12(S)-dihydroxy-*6E,8Z,11E,14Z*-eicosatetraenoic acid; 7-HDHA, 7-hydroxydocosahexaenoic acid; 12(S)-HHTrE, 12(S)-hydroxyheptadecatrienoic acid; 13-HDHA, 13-hydroxydocosahexaenoic acid; 14-HDHA, 14-hydroxydocosahexaenoic acid; 15(S)-HEPE, 15(S)-hydroxyeicosapentaenoic acid; 18-HEPE, 18-hydroxyeicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; LTB4, leukotriene B4; MaR1, maresin 1, 7(S)-MaR1, 7-epi maresin 1; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGF2a, prostaglandin F2a; RvD1, resolvin D1; RvE3, resolvin E3.

## MATERIALS AND METHODS

### Clinical Study Design

The study was conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki and

approved by the University of Texas Health Science Center at Houston (UTHealth) Committee for the Protection of Human Subjects (HSC-DB-16-0167). All participants provided written informed consent. The inclusion criteria were subjects aged ≥18 with ≥24 teeth and no history of systematic periodontal therapy within the past two years. These subjects should not have received anti-inflammatory drugs for more than one week and systemic antibiotics within three months before sample collection, did not routinely take fish oil supplements, had no presence of diabetes mellitus or any systemic condition that entails a diagnosis of "systemic disorders that have a major impact on the loss of periodontal tissues by influencing periodontal inflammation" (22), were not pregnant, and were not current users of tobacco products or nicotine replacement medication.

A comprehensive periodontal charting and examination were performed for all subjects. Probing depth (PD), level of free gingival margin (FGM), clinical attachment level (CAL) and presence of bleeding of probing (BOP) were measured at six sites per tooth. Full mouth series (FMS) radiographs were obtained prior to all treatments for all subjects to confirm alveolar bone level. The subjects in the healthy group were required to meet all inclusion criteria, as well as have all teeth with a probing depth of ≤3mm, clinical attachment loss of ≤2mm (except teeth with mid-buccal or lingual gingival recession), and radiographic bone levels ≤2mm from the cementoenamel junction (CEJ) (no radiographic bone loss due to periodontitis). The subjects in the periodontitis group were required to present with ≥8 teeth with a probing depth of ≥5mm, clinical attachment loss of ≥3mm, and radiographic bone levels >2mm from the CEJ.

## Clinical Sample Collection

Subjects in the healthy group were seen for one clinical research visit, prior to prophylaxis. During the visit, four gingival biopsy samples were obtained from interproximal sites (mesiobuccal, distobuccal, mesiolingual, or distolingual sites of each tooth) of two representative posterior teeth and subgingival plaque samples were obtained from eight interproximal sites of the same two teeth. Subjects in the periodontitis group were seen for two clinical visits; the first visit being prior to the first scaling and root planing (SRP) appointment, and the second visit eight weeks following completion of the second SRP appointment. In these two visits, gingival biopsies and subgingival plaque samples were collected from interproximal sites of the same two representative posterior teeth. The most severely affected teeth with more bone loss than other teeth were selected for gingival biopsy and plaque sample collection. All first-visit samples collected in the periodontitis subjects were from sites with deep probing depths (≥ 5mm).

Gingival biopsies were performed as previously described (21). At each site for gingival sample collection, intrasulcular incisions were made from the papilla zenith toward the base of the interproximal papillae, but not to exceed the nearest line angle of each tooth. A subsequent horizontal incision was made at the base of the interproximal papillae to connect the intrasulcular incisions. The incisions extended to the alveolar bone, and split thickness papillae were partially elevated to permit the harvesting of the interproximal gingival tissue. The gingival sample was required to include epithelium and the underlying supracrestal connective tissue. Of the four gingival tissue samples collected, the sample selected for Lipid Mediator-SPM metabololipidomic analysis was from the site with the deepest probing depth and the sample selected for real-time quantitative reverse transcription assay (qRT-PCR) was from the site with the second-deepest probing depth. The other two gingival tissue samples were stored for future use.

Prior to subgingival plaque collection, supragingival plaque was removed with a separate sterilized scaler. Each subgingival plaque sample was collected using a sterile end of a Gracey mini-curette (Hu-Friedy, Chicago, IL, USA), which was inserted into the depth of the sulcus, with a standardized 20 single strokes. The plaque sample collected from one single site was placed in a microcentrifuge tube with 150μl of Tris-EDTA (TE) buffer, using an up and down motion to ensure deposition of the plaque sample into the TE buffer. The tube was immediately placed in liquid nitrogen and then transferred and stored at -80°C. Only one of the eight plaque samples was processed for 16S rRNA sequencing where possible to avoid affecting microbiome data by pooling samples from multiple sites with different periodontal conditions. Eight plaque samples were collected because sometimes the DNA concentration of a single site could be low. For one subject, the DNA concentration of the single site was too low for PCR amplification, thus samples from two sites were combined. Generally, the selected plaque samples were from sites with the deepest probing depth matched to the sites for Lipid Mediator-SPM metabololipidomics when possible.

## Lipid Mediator-SPM Metabololipidomics for Gingival Tissue Samples

The gingival tissue samples were quantitatively analyzed for levels of SPMs, SPM pathway markers and proinflammatory mediators using Lipid Mediator-SPM metabololipidomics as described earlier (21, 23). The quantities of these lipid mediators in gingival tissue samples are presented as mean ± standard deviation ng per mg of the total protein in each sample. The lower limit of quantitation is 0.015ng.

## Expression of SPM Receptor Genes in Gingival Tissue Samples *via* qRT-PCR

Expression of SPM corresponding receptor genes was analyzed with qRT-PCR, utilizing the TaqMan Gene Expression Assays Protocol (Applied Biosystems, Foster City, CA, USA). Details of the experiment were previously described (21). The TaqMan Assay primers included *GAPDH* (assay ID: Hs99999905_m1, Applied Biosystems) as the housekeeping gene compared to *ALX (FPR2)* (assay ID: Hs00265954_m1, Applied Biosystems), *BLT1 (LTB4R)* (assay ID: Hs00175124_m1, Applied Biosystems), *ChemR23 (ERV1)* (assay ID: Hs01386063_m1, Applied Biosystems), *GPR18 (DRV2)* (assay ID: Hs00245542_m1, Applied Biosystems), *GPR32 (DRV1)* (assay ID: Hs00265986_s1, Applied Biosystems), *GPR37* (assay ID: Hs0017374_m1, Applied Biosystems),and *LGR6* (assay ID: Hs00663887_m1, Applied Biosystems), which were labeled with FAM dye. The calculated cycle threshold (Ct) values from each sample were obtained

and relative expression ($2^{-\Delta\Delta Ct}$) was calculated to determine expression of specific SPM receptor genes between groups.

## 16S rRNA Sequencing for Subgingival Plaque Samples

DNA extraction of one representative plaque sample was performed utilizing the protocol of a MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA USA) (24). Eukaryotic and prokaryotic cells from the plaque sample were lysed with garnet beads to release DNA. The DNA was isolated on a PowerSoil spin filter and subsequently washed and collected as an eluate in a separate collection tube. The DNA eluate was measured on the UV-Vis spectrophotometer (Nanodrop, Thermo-Fisher Scientific, Waltham, MA, USA) to determine the amount of DNA in the sample. Then DNA samples were processed for 16S rRNA Sequencing (LC Biosciences, Houston, TX). The 16S rRNA V3-V4 regions were amplified *via* PCR and sequenced on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). The amplification primers (forward primer (338F): 5'-ACTCCTACGGGAGGCAGCAG-3'; reverse primer (806R): 5'-GGACTACHVGGGTWTCTAAT-3') contained adapters for MiSeq sequencing and single-index barcodes that resulted in PCR products that were pooled and sequenced directly. Read pairs were de-multiplexed based on barcodes and merged. All of the samples were then processed and sequenced together, and the cleaned, merged data was imported into CLC Genomics Workbench with the Microbial Genomics module. The 16S rRNA gene sequences were allocated to specific operational taxonomic units (OTUs) at 98% identity using the Human Oral Microbiome Database (HOMD). OTUs without a match to the HOMD database were given an OTU number, and genus/species were identified by BLAST search against the bacterial 16S rRNA database (25).

## Statistical Analysis

The Lipid Mediator-SPM metabololipidomics data and qRT-PCR data were first log transformed, and Wilcoxon rank sum test was used to detect differentially expressed lipid mediators and receptor genes between two conditions except for comparison between the periodontitis prior to SRP group and the periodontitis after SRP group, where Wilcoxon signed rank test was applied. The p-values were adjusted using the False Discovery Rate (FDR) multi-test correction method. Data were considered statistically significant when $p<0.05$ (21).

For the subgingival microbiome, community diversity (alpha and beta diversity) was assessed using the Microbial Genomics Diversity module of CLC Genomics Workbench. OTUs from the abundance table were aligned using MUSCLE with a required minimum abundance of 100. Aligned OTUs were used to construct a phylogenetic tree using Maximum Likelihood Phylogeny using the Neighbor Joining method and the Jukes Cantor substitution model. Rarefication analysis was done by sub-sampling the OTU abundances in the different samples at a range of depths from 1 to 100,000; the number of different depths sampled was 20, with 100 replicates at each depth. The OTUs with relative abundance less than 0.01% were excluded. Alpha diversity measures were calculated for observed OTUs and Shannon index. Statistical significance in alpha diversity between groups was calculated with Student's t-test or paired t-test. PERMANOVA (Permutational Multivariate Analysis of Variance) analysis was used to detect significant differences in Beta diversity between groups, and comparisons were visualized using Principal Coordinate Analysis (PCoA). Diversity measures were calculated using the Bray-Curtis formula.

Differences in taxa abundance of subgingival plaque samples between the three groups were identified using DESeq2 (26). OTUs were selected through DESeq2 testing. The OTUs were color-coded according to the phyla they belong to and plotted according to their log2 fold change. The relative fold change was calculated between the three subject groups.

R random Forest package (27) was used to calculate the relative abundance of OTUs. Those OTUs with mean decrease Gini larger than 0.15 were selected for subsequent integration analysis. The lipid mediator levels, SPM receptor gene expression levels and bacterial abundance were first normalized to zero mean and unit variance, and then integrated and analyzed using Data Integration Analysis for Biomarker discovery using a Latent cOmponents (DIABLO) method implemented in R mixOmics package (28). The resulting profile correlations were visualized in DIABLO. Correlations between individual lipid mediators and bacterial species in each subject group were further analyzed using the sparse partial least squares (SPLS) method (29) implemented in the R mixOmics package. The data at the genus level were also analyzed using the same methods.

# RESULTS

## Composition of the Subgingival Microbiome

Thirteen periodontally healthy subjects and 15 periodontitis subjects before and after treatment were included in this study. All periodontitis subjects were diagnosed with generalized/localized periodontitis, Stage II or III, Grade B or C (**Supplementary Table 1**). In periodontitis subjects, the mean probing depth of sample collection sites was significantly reduced following non-surgical periodontal therapy (SRP); however, the average PD after treatment remained significantly greater than in health (**Table 1**).

Three hundred and sixty one OTUs were identified across all samples. The beta-diversity, which represents the variation in microbial composition between groups, was significantly different between the healthy group and the periodontitis prior to SRP group as well as between the healthy group and the periodontitis after SRP group ($p=<0.01,<0.01$, respectively; **Figure 1**). Significance was not seen between the periodontitis prior to SRP group and the periodontitis after SRP group ($p=0.57$), suggesting periodontal treatment did not entirely change the microbial composition to a healthy condition. The bacterial species richness (the number of OTUs) in the periodontitis prior to SRP group was significantly higher than in the healthy and periodontitis after SRP groups ($p=<0.01$, 0.03 respectively,

**TABLE 1** | Characteristics of subjects and sample collection sites.

| | Healthy (H) | Periodontitis before non-surgical therapy (P) | Periodontitis after non-surgical therapy (A) | p-value (H versus P/A) | p-value (P versus A) |
|---|---|---|---|---|---|
| **Age** | 39.31 ± 15.80 | 51.20 ± 9.99 | 51.20 ± 9.99 | 0.02/0.02 | NA |
| **Gender (Male/Female)** | 6/7 | 8/7 | 8/7 | 0.71/0.71 | NA |
| **PD (mm) of sites for metabololipidomics** | 2.85 ± 0.38 | 6.47 ± 1.13 | 5.33 ± 1.76 | <0.01/ <0.01 | 0.01 |
| **PD (mm) of sites for qRT-PCR** | 2.85 ± 0.38 | 5.87 ± 1.13 | 4.13 ± 1.51 | <0.01/0.01 | <0.01 |
| **PD (mm) of sites for 16S RNA sequencing** | 2.85 ± 0.38 | 6.47 ± 1.13 | 5.27 ± 1.69 | <0.01/ <0.01 | <0.01 |
| **CAL (mm) of sites for metabololipidomics** | 0.31 ± 0.48 | 6.00 ± 1.73 | 5.33 ± 2.19 | <0.01/ <0.01 | 0.06 |
| **CAL (mm) of sites for qRT-PCR** | 0.38 ± 0.51 | 5.33 ± 1.63 | 4.60 ± 1.76 | <0.01/ <0.01 | 0.01 |
| **CAL (mm) of sites for 16S RNA sequencing** | 0.31 ± 0.48 | 6.00 ± 1.73 | 5.27 ± 2.19 | <0.01/ <0.01 | 0.03 |
| **FGM (mm) of sites for metabololipidomics** | 2.54 ± 0.52 | 0.47 ± 1.60 | 0.00 ± 1.89 | <0.01/ <0.01 | 0.29 |
| **FGM (mm) of sites for qRT-PCR** | 2.46 ± 0.52 | 0.53 ± 1.19 | -0.47 ± 1.68 | <0.01/ <0.01 | 0.04 |
| **FGM (mm) of sites for 16S RNA sequencing** | 2.54 ± 0.52 | 0.47 ± 1.60 | 0.00 ± 1.96 | <0.01/ <0.01 | 0.29 |
| **Presence of BOP at sites for metabololipidomics** | 1/13 | 14/15 | 14/15 | <0.01/ <0.01 | 1.00 |
| **Presence of BOP at sites for qRT-PCR** | 3/13 | 14/15 | 15/15 | <0.01/ <0.01 | 0.32 |
| **Presence of BOP at sites for 16S RNA sequencing** | 1/13 | 14/15 | 14/15 | <0.01/ <0.01 | 1.00 |

*PD, probing depth; CAL, clinical attachment level; FGM, level of free gingival margin; negative value indicates gingival recession (gingival margin is below cementoenamel junction); BOP, bleeding on probing; NA, not available due to the subjects are the same in the P and A groups; p-values were calculated using Student's t-test, paired t-test, Chi-squared test or McNemar test.*

**Figure 2A**). The mean Shannon index (evenness) was the highest in the periodontitis prior to SRP group and lowest in the healthy group. There was only a significant difference in bacterial species evenness between the periodontitis prior to SRP and healthy groups (p=0.04, **Figure 2B**). These results indicate that species were the most diverse in periodontitis before treatment.

Considering the OTU species assignments, *Corynebacterium matruchotii*, *Fusobacterium nucleatum*_subsp._*animalis*, *Fusobacterium nucleatum*_subsp._*polymorphum*, *Fusobacterium nucleatum*_subsp._*vincentii*, and *Veillonella dispar*, were seen in all three groups with varying abundance (**Table 2**). The top three species that had the highest relative abundance in the healthy group were *Neisseria oralis* (10%), *Corynebacterium matruchotii* (6%), and *Actinomyces* sp._oral_taxon_169 (5%). Periodontitis sites before and after therapy were dominated by *Fusobacterium nucleatum* subsp. *vincentii* at 8% and 6%, respectively. While considering species with relative abundance ≥1%, three putative red complex periodontal pathogens, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* were present in the periodontitis prior to SRP group and *Porphyromonas gingivalis* and *Treponema denticola* were present in the periodontitis after SRP group. These species had very low abundance and were infrequently present in the healthy group.

Among two-group comparisons, there were 61 bacterial species with significant differences in relative fold change between the healthy and periodontitis prior to SRP groups, 32 bacterial species with significant differences in relative fold change between the healthy and periodontitis after SRP groups and 28 bacterial species with significant differences in relative fold change between the periodontitis prior to SRP and after SRP groups (**Figures 3A–C** and **Supplementary Table 2**). The relative amounts of *P. gingivalis*, *T. denticola*, and *T. forsythia* in both the periodontitis prior to SRP and after SRP groups were significantly higher than in the healthy group (**Figures 3A, B**), but there was no significant difference between the two periodontitis groups. Periodontal health related species, *Rothia aeria* and *Corynebacterium durum*, in the healthy group had significantly higher relative fold values than the two periodontitis groups.

## Correlations Between Lipid Mediator, SPM Receptor Gene and Subgingival Microbiome

Previously, we demonstrated that the level of six lipid mediators (5-HETE, 15-HETE, 15(S)-HEPE, 4-HDHA, 7-HDHA, 17-HDHA) and expression of D-series resolvins corresponding receptor genes (*GPR18*, *GPR32*) were significantly altered by periodontitis treatment (21). In the present study, data of lipid mediator levels, receptor gene expression and bacterial species abundance were integrated and analyzed by the DIABLO method to demonstrate profile correlations. When all samples from the three subject groups are included, the correlation coefficient between subgingival microbiome and lipid mediator profiles was 0.61, the correlation coefficient between subgingival microbiome and receptor gene profiles was 0.45 and the correlation coefficient between lipid mediator and receptor

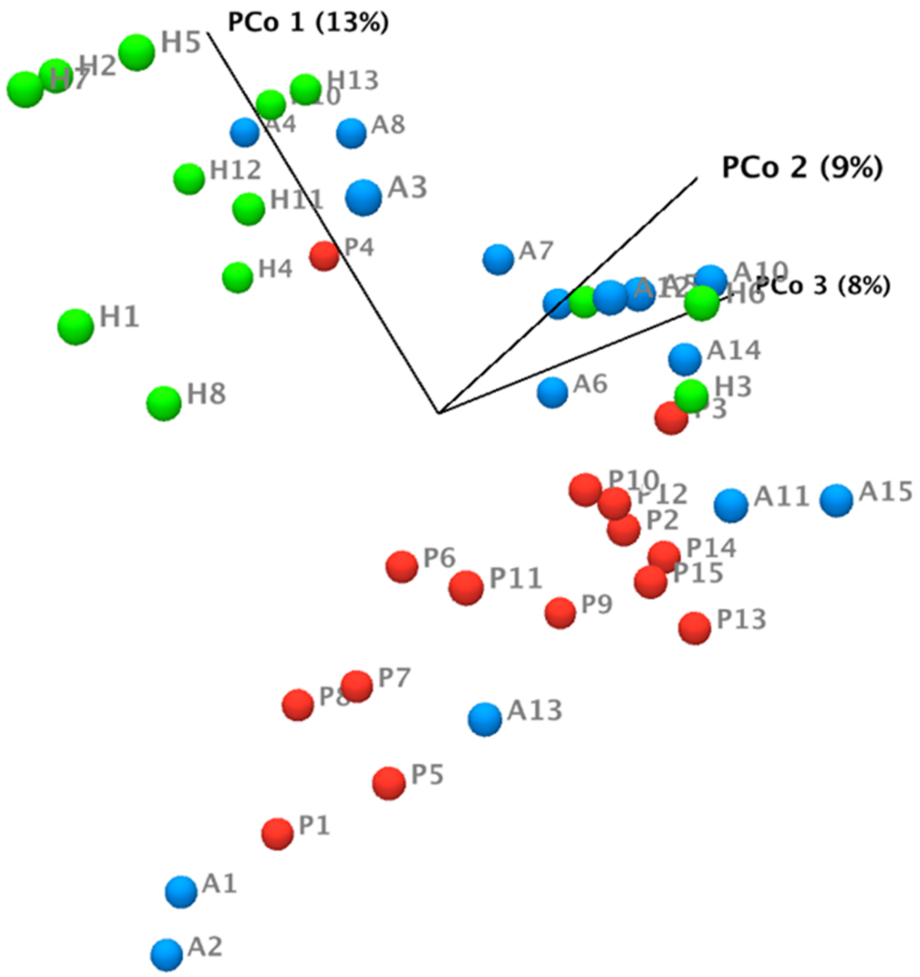


**FIGURE 1** | Bray-Curtis principal coordinate analysis (PCoA) plot for subgingival microbiome profiles. Axis one accounts for 13% of sample variance, while axis two and three account for 9% and 8% of variance, respectively. One dot represents one sample in each group. This plot demonstrates clusters of samples based on their similarity of microbial composition. These three groups display distinct microbial compositions. (H (green): healthy; P (red): periodontitis before non-surgical therapy; A (blue): periodontitis after non-surgical therapy).

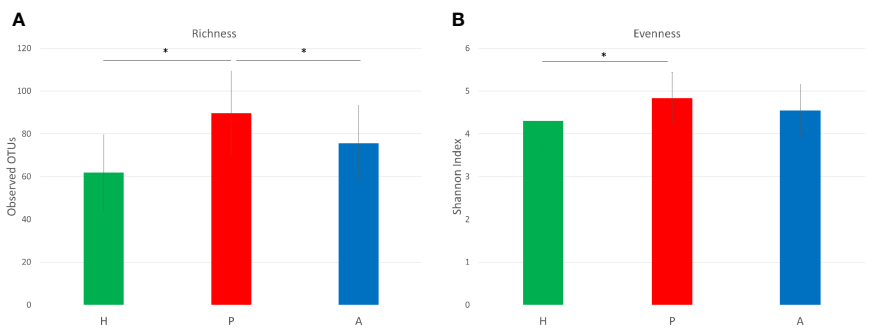


**FIGURE 2** | Alpha diversity of the subgingival microbiome. **(A)** Richness of the subgingival microbiome. Richness is represented by the number of operational taxonomic units (OTUs). **(B)** Evenness of the subgingival microbiome. Evenness is represented by Shannon index. (*$p < 0.05$; H (green): healthy; P (red): periodontitis before non-surgical therapy; A (blue): periodontitis after non-surgical therapy).

**TABLE 2** | Relative abundance of bacterial species.

| H: Healthy | | P: Periodontitis before non-surgical therapy | | A: Periodontitis after non-surgical therapy | |
|---|---|---|---|---|---|
| **Bacterial species** | **Relative abundance** | **Bacterial species** | **Relative abundance** | **Bacterial species** | **Relative abundance** |
| *Neisseria oralis* | 0.10 | *Fusobacterium nucleatum*_subsp._*vincentii* | 0.08 | *Fusobacterium nucleatum*_subsp._*vincentii* | 0.06 |
| *Corynebacterium matruchotii* | 0.06 | *Escherichia coli* | 0.06 | *Corynebacterium matruchotii* | 0.04 |
| *Actinomyces* sp._oral_taxon_169 | 0.05 | *Porphyromonas gingivalis* | 0.05 | *Fusobacterium nucleatum*_subsp._*polymorphum* | 0.04 |
| *Fusobacterium nucleatum*_subsp._*animalis* | 0.04 | *Bifidobacterium longum* | 0.05 | *Prevotella nigrescens* | 0.04 |
| *Campylobacter gracilis* | 0.04 | *Treponema denticola* | 0.03 | *Rothia dentocariosa* | 0.04 |
| *Actinomyces naeslundii* | 0.04 | *Corynebacterium matruchotii* | 0.02 | *Fusobacterium nucleatum*_subsp._*animalis* | 0.03 |
| *Actinobaculum* sp._oral_taxon_183 | 0.04 | *Fusobacterium nucleatum*_subsp._*animalis* | 0.02 | *Actinomyces* sp._oral_taxon_169 | 0.03 |
| *Fusobacterium nucleatum*_subsp._*vincentii* | 0.03 | *Fusobacterium nucleatum*_subsp._*polymorphum* | 0.02 | *Neisseria pharyngis* | 0.03 |
| *Rothia dentocariosa* | 0.03 | *Prevotella nigrescens* | 0.02 | *Porphyromonas gingivalis* | 0.02 |
| *Veillonella dispar* | 0.03 | *Veillonella dispar* | 0.02 | *Treponema denticola* | 0.02 |
| *Lautropia mirabilis* | 0.03 | *Porphyromonas endodontalis* | 0.02 | *Veillonella dispar* | 0.02 |
| *Rothia aeria* | 0.03 | *Leptotrichia buccalis* | 0.02 | *Porphyromonas endodontalis* | 0.02 |
| *Fusobacterium nucleatum*_subsp._*polymorphum* | 0.02 | *Fusobacterium* sp._oral_taxon_203 | 0.02 | *Campylobacter gracilis* | 0.02 |
| *Haemophilus parainfluenzae* | 0.02 | *Bacteroidales_[G-2]* sp._oral_taxon_274 | 0.02 | *Selenomonas noxia* | 0.02 |
| *Actinomyces* sp._oral_taxon_171 | 0.02 | *Tannerella forsythia* | 0.02 | *Actinomyces* sp._oral_taxon_170 | 0.02 |
| *Actinomyces massiliensis* | 0.02 | *Veillonella parvula* | 0.02 | *Streptococcus gordonii* | 0.02 |
| *Fusobacterium naviforme* | 0.02 | *Bacteroidaceae_[G-1]* sp._oral_taxon_272 | 0.02 | *Capnocytophaga leadbetteri* | 0.02 |
| *Corynebacterium durum* | 0.02 | *Campylobacter gracilis* | 0.01 | *Streptococcus* sp._oral_taxon_058 | 0.01 |
| *Selenomonas noxia* | 0.01 | *Streptococcus* sp._oral_taxon_058 | 0.01 | *Campylobacter rectus* | 0.01 |
| *Actinomyces* sp._oral_taxon_170 | 0.01 | *Prevotella intermedia* | 0.01 | *Neisseria oralis* | 0.01 |

*These bacterial species are listed according to their mean relative abundance ranging from high to low in each group. Only twenty species with the highest relative abundance in each group are listed. The relative abundance ranges from 0.01 to 1 (1 to 100%).*

gene profiles was 0.34. (**Figure 4A**). As the results at the species level, the correlation between subgingival microbiome and lipid mediator profiles was also the highest at the genus level. (correlation coefficient= 0.5) (**Supplementary Figure 1A**). Since the subgingival microbiome and lipid mediator profiles had the highest correlation, the subgingival microbiome-lipid mediator profile correlation was further assessed in each group and the correlation coefficients were 0.65, 0.58 and 0.43 in the healthy, periodontitis prior to SRP and periodontitis after SRP groups respectively (**Figure 4B**). The correlation patterns at the genus level in each group were also similar to those at the species level (**Supplementary Figure 1B**).

Specific bacterial species and lipid mediators were correlated (correlation coefficient ≥0.5) while including all samples of the three groups (**Figure 5**). Then, SPLS analysis was conducted to identify individual bacterial species and lipid mediators contributing to the subgingival microbiome-lipid mediator profile correlation in each group. Using a correlation coefficient cut off of ≥0.6, five bacterial species and six lipid mediators were correlated in the healthy group, five bacterial species and 11 lipid mediators were correlated in the periodontitis prior to SRP group and nine bacterial species and nine lipid mediators were correlated in the periodontitis after SRP group (**Supplementary Table 3** and **Figure 6**). These correlations indicate that the relative abundance of these bacteria species and the level of these lipid mediators are highly associated.

When comparing these correlated species in periodontitis before treatment to healthy controls, only *Corynebacterium durum*, a periodontal health related species, was identified in both conditions, but correlated with different lipids. In health, *Corynebacterium durum* was positively correlated with the leukotriene pathway marker, 5(S)6(R)-DiHETE, and one of the E-series resolvins, RvE3. In periodontitis before treatment, *Corynebacterium durum* was negatively correlated with the resolvin pathway marker, 15(S)-HEPE, and positively correlated with the proinflammatory mediator, LTB4, and SPMs, including 7 (S)-Maresin1, Maresin1 and RvD1. Both conditions had five lipid mediators, 5(S)6(R)-DiHETE, 15(S)-HEPE, 7-HDHA, 13-HDHA and 14-HDHA, correlated with different bacterial species.

When comparing these correlated species in periodontitis after treatment to healthy controls, *Treponema socranskii*, a periodontal pathogen, was identified in both conditions. This bacterial species was negatively correlated to 5(S)6(R)-DiHETE in the healthy group and positively correlated with different lipids in the periodontitis after SRP group. The E-series resolvin, RvE3, was the only lipid mediator identified in both conditions.

When comparing these correlated species in periodontitis before treatment to after treatment, a bacterial species,

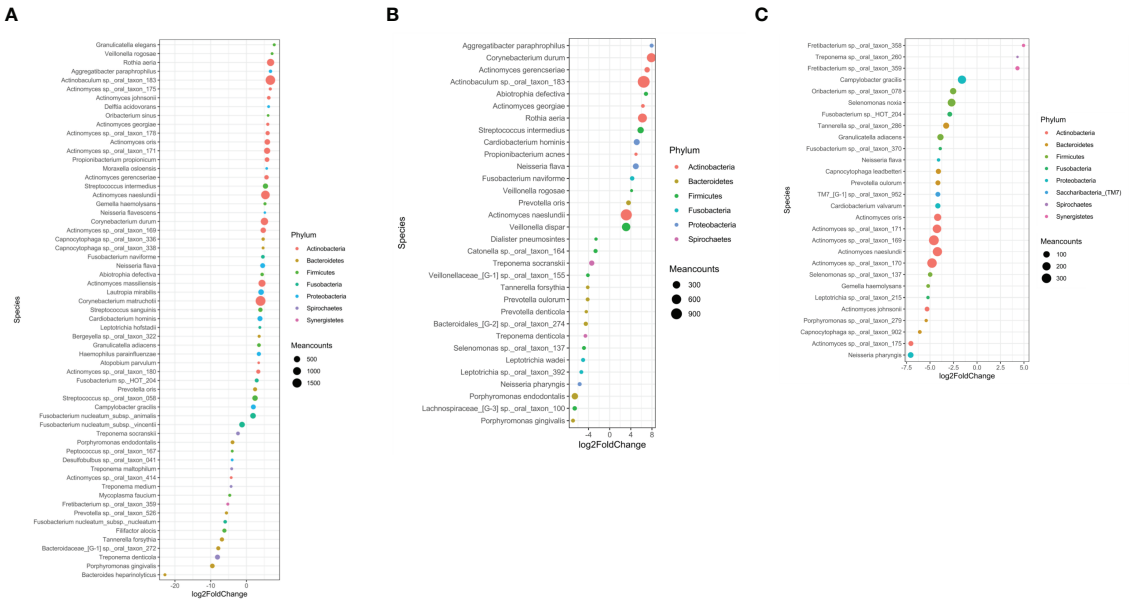


**FIGURE 3** | Differentially abundant operational taxonomic units (OTUs) in three comparisons. Each circle represents one OTU (bacterial species). The OTUs are color-coded based on the phylum to which they belong and plotted based on their log2 fold change in each comparison. The size of the circle is proportional to the mean count of each species. All differentially abundant OTUs in each comparison are listed. **(A)** Healthy (H) *vs*. Periodontitis before non-surgical therapy (P); **(B)** Healthy (H) *vs*. Periodontitis after non-surgical therapy (A); **(C)** Periodontitis before non-surgical therapy (P) *vs*. Periodontitis after non-surgical therapy (A). In these comparisons, the second group is the reference group. The fold change is calculated by dividing the bacterial abundance in the first group by the bacterial abundance in the second group (H/P, H/A, P/A respectively). These graphs demonstrate different microbial compositions between the three groups.

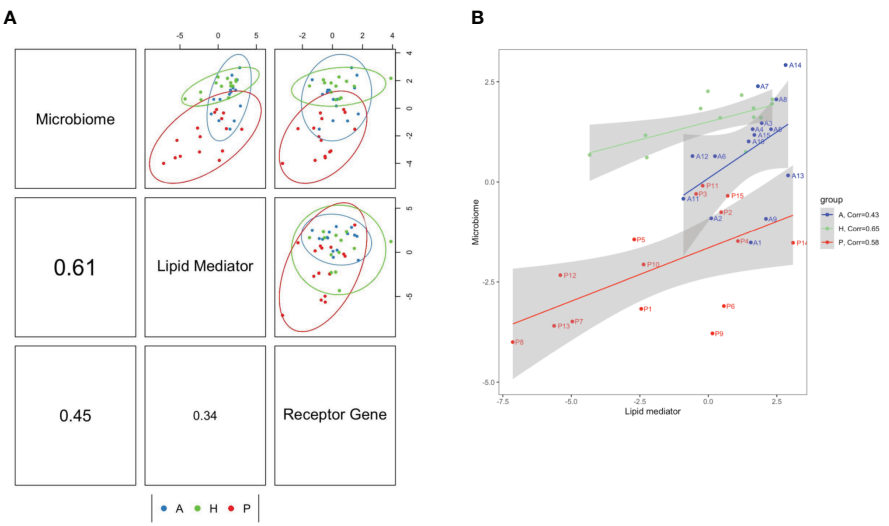


**FIGURE 4** | Correlations between lipid mediator profile, specialized pro-resolving lipid mediator (SPM) receptor gene profile and subgingival microbiome profile. **(A)** The component correlation plots represent subgingival microbiome-lipid mediator profile (upper-middle box), subgingival microbiome-receptor gene profile (upper-right box), and lipid mediator-receptor gene profile (middle-right box). The correlation coefficient of these profiles: subgingival microbiome-lipid mediator (middle-left box)= 0.61; subgingival microbiome-receptor gene (lower-left box)= 0.45; lipid mediator-receptor gene (lower-middle box)= 0.34. **(B)** The correlation plot demonstrates the subgingival microbiome-lipid mediator correlation patterns in each subject group. The correlation coefficients for the H, P and A groups are 0.65, 0.58 and 0.43, respectively. Most of the subject's subgingival microbiome-lipid mediator profiles (subjects are labeled in the plot) in periodontitis before treatment move toward the healthy patterns after treatment. (H (green): healthy; P (red): periodontitis before non-surgical therapy; A (blue): periodontitis after non-surgical therapy; one dot represents one subject in each group; numbers on axes represent relative levels of bacterial species, lipid mediators or receptor gene expression; the analyses are performed using the DIABLO method.)

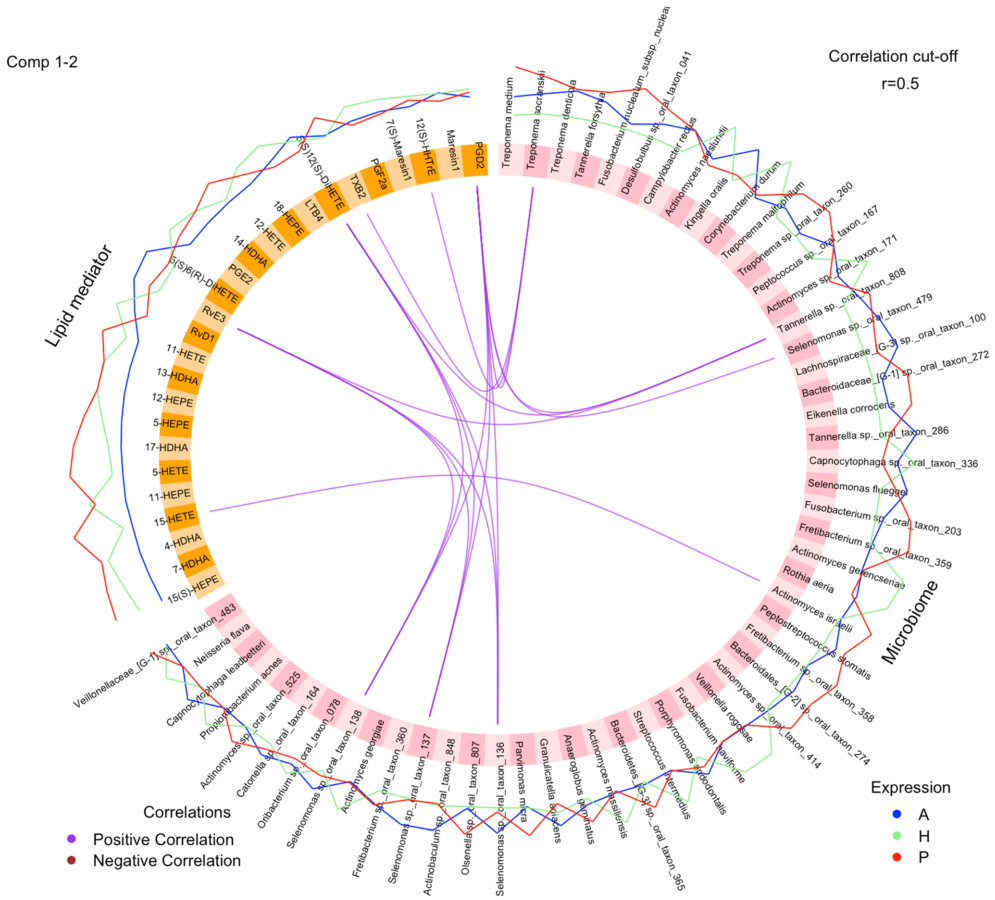


**FIGURE 5** | Correlations between individual lipid mediators and bacterial species. The circos plot shows correlations between lipid mediators (yellow) and bacterial species (pink). Three lines outside the circle represent the relative levels of lipid mediators or bacterial species (H (green): healthy; P (red): periodontitis before non-surgical therapy; A (blue): periodontitis after non-surgical therapy). Purple lines inside the circle represent positive correlations between lipid mediators and bacterial species with correlation coefficient ≥0.5. None of the bacterial species and lipid mediators has a significant negative correlation coefficient ≤-0.5. The analysis is performed using the DIABLO method.

*Anaeroglobus geminatus*, was identified in both conditions that positively correlated with different lipid mediators. Both conditions had four lipid mediators, 5(S)12(S)-DiHETE, RvD1, Maresin 1 and LTB4, correlated with different bacteria species. Among the nine bacterial species identified in the periodontitis after SRP group, four *Selenomonas* species (*Selenomonas* sp._oral_taxon_136, *Selenomonas* sp._oral_taxon_137, *Selenomonas* sp._oral_taxon_138, *Selenomonas* sp._oral_taxon_479) are highly correlated with multiple lipid mediators.

## DISCUSSION

Many studies demonstrate that shifts in the bacterial load and bacterial composition within the subgingival microbiota are correlated with periodontal conditions (30, 31). Associations between periodontal inflammation and molecular-level host responses (32, 33) or metabolite profiles (34, 35) have also been investigated. However, there is a remarkable lack of studies assessing clinical host-microbiota relationship in periodontitis. The current study showed different correlations between levels of lipid mediators, expression of SPM receptor genes and abundance of subgingival bacterial species in health vs. disease. To the best of our knowledge, this is the first study demonstrating that the molecular profiles for lipid-mediated resolution of inflammation activity are associated with microbial composition shifts in human periodontitis. The correlations between specific lipid mediators and bacterial species indicate their potential interactions, which are important for deciphering the mechanisms of resolution of inflammation in the pathogenesis of periodontitis.

In this study, most of the periodontitis subjects have severe periodontitis (Stage III, Grade C). In addition to the lack of maintenance or poor self-care, these patients may have abnormal host responses causing severe, rapid periodontal destruction and dysbiotic subgingival microbiota. Current results indicate that failure of resolution associated with the imbalanced lipid mediator profiles could be the potential mechanisms (21). This

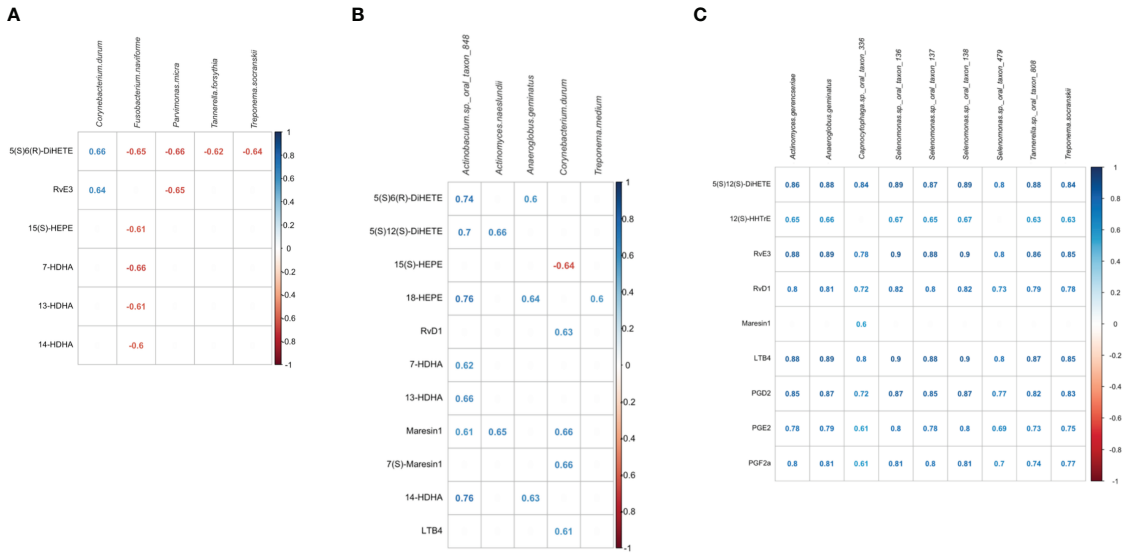


**FIGURE 6** | Correlations between bacterial species and lipid mediators in the three groups. **(A)** correlated lipid mediators and bacterial species in the healthy group. **(B)** correlated lipid mediators and bacterial species in the periodontitis before non-surgical therapy group. **(C)** correlated lipid mediators and bacterial species in the periodontitis after non-surgical therapy group. The analysis is performed using the Sparse Partial Least Squares (SPLS) method. Bacterial species are listed in alphabetical order. Lipid mediators are grouped by biosynthetic pathways; leukotriene pathway marker: 5(S),6(R)-DiHETE; lipoxygenase pathway marker: 5(S),12(S)-DiHETE; prostaglandin pathway marker: 12(S)-HHTrE; E-series resolvins and pathway markers (derived from omega-3 EPA): 15(S)-HEPE, 18-HEPE, RvE3; D-series resolvins and pathway markers (derived from omega-3 DHA or DPA): RvD1, 7-HDHA, 13-HDHA; Maresins and pathway markers (derived from omega-3 DHA or DPA): 14-HDHA, MaR1, 7(S)-MaR1; pro-inflammatory lipid mediators: LTB4, PGD2, PGE2, PGF2a. Some pathway markers are involved in multiple pathways but only one major pathway is listed. All correlated lipid mediators-bacterial species have absolute correlation coefficients ≥0.60.

study compared samples from the diseases sites of the periodontitis subjects to the healthy sites of the subjects without periodontitis because lipid mediator levels and profiles are distinct at the subject level (16, 17). However, it would be interesting to analyze samples from both diseases sites and healthy sites of the same periodontitis subjects to evaluate the subgingival microbiome-lipid mediator correlations at different local environments.

According to the profile correlation analysis, the correlation between lipid mediator profile and receptor gene profile was lower than both the subgingival microbiome-lipid mediator profile correlation and subgingival microbiome-receptor gene profile correlation. The low lipid mediator-receptor gene profile correlation supports the hypothesis that an imbalance between lipid mediator levels and receptor expression results in failure of resolution of inflammation in periodontitis (21). It is known that expression of SPM corresponding receptors is important for resolution of inflammation. Deficient expression of these receptors has been associated with increased inflammation in peritonitis (36, 37), paw edema (38), and microbial sepsis (39) in pre-clinical models. Overexpression of SPM receptors, such as ChemR23 (ERV1), was associated with immune responses favorable for inflammation resolution in a dorsal air pouch model (40).

As with the clustering in both lipid mediators and SPM receptor gene expression (21), the clusters of subgingival microbiome were associated with inflammatory conditions in gingiva (**Figure 1**). Current results of 16S rRNA sequencing show that periodontitis subjects had a higher abundance of periodontal pathogens and microbial diversity than healthy subjects. Healthy subjects also have a higher abundance of periodontal health related bacterial species. These findings were similar to published results in the literature (41–45).

The highest subgingival microbiome-lipid mediator profile correlation (**Figure 4A**) suggests that the composition of the subgingival microbiota could be significantly affected by periodontal inflammation mediated by SPMs and other relevant lipid mediators (13, 14). The correlation pattern in the periodontitis after SRP group was closer to the healthy group than the periodontitis prior to SRP group suggesting periodontal treatment moves profiles of lipid mediators and subgingival microbiota toward non-diseased profiles (**Figure 4B**). Non-surgical periodontal therapy not only improves clinical parameters but also changes molecular profiles closer to the homeostatic condition. It is possible that local debridement stimulates SPM-mediated resolution of inflammation influencing microbiota shifts in the re-establishment of the subgingival biofilm. In the correlation analysis between individual lipid mediators and bacterial species, most of the correlated lipid and bacterial species are specific for the subject group and not shared by other groups. These results also support that the subgingival microbiome and lipid mediator profiles are specifically associated with periodontal inflammatory conditions.

The correlation analysis identified some bacterial species infrequently discussed in the literature and several lipid mediators

potentially important for resolution of periodontal inflammation (**Figure 6**). A periodontal health related bacterial species, *Corynebacterium durum*, had relatively low abundance in periodontitis, but its positive correlation with several SPMs indicate that gingival tissues produce SPMs to regulate periodontal inflammation resulting in the presence of this periodontal health related bacterial species. In periodontitis before treatment, one bacterial species, *Actinobaculum* sp._oral_taxon_848 (46), had the greatest number of correlated resolution related lipid mediators, 5(S)12(S)-DiHETE, 5(S)6(R)-DiHETE, 18-HEPE, 7-HDHA, 13-HDHA, 14-HDHA, Maresin1, and higher correlations than other species. A recently identified bacterial species, *Anaeroglobus geminatus* (47), related to periodontitis and rheumatoid arthritis, was identified in periodontitis before and after treatment, but was highly correlated with different lipid mediators in the two inflammatory conditions. These bacterial species could be important for periodontal inflammation and deserve further investigation.

*Selenomonas* species were dominant in the identified bacterial species correlated with multiple lipid mediators in the periodontitis after SRP group. Although *Selenomonas* species are not frequently discussed in the literature, some studies show associations between the presence, as well as abundance, of *Selenomonas* species and periodontitis (48–50), and the fundamental role of *Selenomonas* species in the structure of subgingival biofilm (51). Tanner and coworkers found that *Selenomonas noxia* was associated with the progression of periodontitis (52). In the current study, most of the identified *Selenomonas* species had the highest relative abundance in the periodontitis after SRP group although the relative abundance was generally low (<1%, **Supplementary Table 4**). The abundance of *Selenomonas* species was highly and positively associated with levels of two SPMs, RvE3 and RvD1 in periodontitis after treatment. An *in vitro* study investigating the impact of dietary lipid supplements on the microbiota in the rumen ecosystem showed that fish oil in the diet is associated with an increased amount of *Selenomonas* species in fermenters (53) suggesting omega-3 fatty acids influence the composition of *Selenomonas* species and other bacterial species. The current findings support the association between the abundance of *Selenomonas* species and levels of lipid mediators in resolution of periodontal inflammation following treatment. Although the exact role of *Selenomonas* species in the resolution of periodontal inflammation has to be further studied, *Selenomonas* species appear to be sensitive to the resolution of inflammation after treatment and the changes of their abundance could be critically associated with the changes of periodontal inflammatory conditions.

As with *Selenomonas* species, two periodontitis pathogens, *Treponema socranskii* (50, 54, 55) and *Tannerella* sp._oral_taxon_808 (56), and an oral biofilm early colonizer species, *Actinomyces gerencseriae*, were also correlated with RvE3, RvD1 and other lipid mediators, including proinflammatory mediators, LTB4, PGD2, PGE2 and PGF2a, in periodontitis after treatment. These results indicate that the resolution phase of periodontal inflammation induced by periodontal treatment includes a combination of proresolving and proinflammatory mediators highly correlated with non-periodontitis or periodontitis associated bacterial species, explaining the specific phase of inflammation resolution between untreated periodontitis and healthy phases.

We previously reported that levels of six lipid mediators in periodontitis before treatment were significantly higher than in periodontitis after treatment (21). In this study, among the six lipid mediators, only 15(S)-HEPE, a pathway marker for E-series resolvins, was negatively correlated with *Corynebacterium durum*, and 7-HDHA, a pathway marker for D-series resolvins, was positively correlated with *Actinobaculum* sp._oral_taxon_848 in periodontitis before treatment. These two SPM pathway markers appear to be more associated with the subgingival microbiota in periodontitis than the others. Two lipids derived from arachidonic acid, 5(S),12(S)-DiHETE and 5(S)6(R)-DiHETE, were correlated with multiple bacterial species in the three subject groups. 5(S),12(S)-DiHETE is an epimer of leukotriene $B_4$ weakly chemotactic for neutrophils (57). 5(S)6(R)-DiHETE is a hydrolysis product of leukotriene $A_4$ and can bind to the leukotriene receptor (58). The identification of these two lipids in the correlation analysis suggests their relevant synthetic pathways for leukotrienes could be actively associated with changes in the subgingival microbial composition.

Resolution of inflammation induced by SPMs is critical for periodontal regeneration and healing given chronic and excessive inflammation is detrimental to homeostasis (9). Several SPMs, including RvD1, RvE3, Maresin1 and 7(S)-Maresin 1, were identified in the current correlation analysis. Resolvins are known to resolve inflammation, stimulate periodontal tissue healing and regenerate lost alveolar bone. RvE1 induces periodontal regeneration in pre-clinical periodontitis models (12–14, 59, 60), promotes regenerative properties of periodontal ligament (PDL) stem cells (61), and regulates osteoclasts as well as osteoblasts favorably for bone preservation (62–64). RvE3 demonstrates potent resolution properties by limiting neutrophil infiltration in the peritonitis model and inhibiting neutrophil chemotaxis (65–67). RvD1 inhibits osteoclast differentiation and reduces bone destruction in an arthritis preclinical model (68), inhibits production of pro-inflammatory cytokines from gingival fibroblasts (69) and enhances PDL fibroblast proliferation as well as wound closure (70). Maresins can promote periodontal tissue healing and regulate periodontal inflammation. MaR1 promotes survival and inhibits apoptosis in PDL cells (71), induces regenerative properties of PDL stem cells (61), and restores phagocytic capacity of macrophages from periodontitis patients (72). Both RvD1 and RvE3 were highly correlated with multiple bacterial species in periodontitis after treatment suggesting their important role in resolution of periodontal inflammation associated with subgingival microbiota shift. Their mean levels also increased in periodontitis after treatment as compared to periodontitis before treatment (21). According to the current findings and molecular actions described in the literature, RvD1 and RvE3 potentially could be utilized to treat periodontitis.

In general, results of the correlation analyses indicate significant associations between lipid mediator levels and subgingival bacterial abundance in periodontal inflammation. It is possible that immune responses mediated by SPMs and other lipid mediators drive the shifts of the subgingival microbiota, which can be supported by

preclinical models showing oral microbiota shifts following the local administration of SPMs (13, 14). According to *in vitro* experiments, SPMs do not have direct anti-bacterial properties (73). However, the other direct effects of these lipid mediators on bacterial growth or other activities could not be excluded (74). On the other hand, oral bacteria might affect lipid synthesis and metabolism. It is known that the metabolites from gut microbiota affects host lipid metabolism and lipid composition (75). Oral microbiota could do the same. Additionally, periodontal tissues could produce more lipid mediators to regulate excessive inflammation induced by the increased amount of bacteria in periodontitis (73). These potential mechanisms for subgingival microbiome-lipid mediator correlations have to be further studied. All of these possibilities indicate the important role of lipid mediator profiles in dysbiosis of the subgingival microbiota in periodontitis.

With some limitations, current results should be carefully interpreted. Profiles of lipid mediators and receptor genes were derived from gingival tissues. Since lipid mediators can be produced by and receptor genes can be expressed on a variety of cells, including neutrophils, macrophages, fibroblasts, osteoblasts and osteoclasts (14, 62–64, 69, 70, 76), these molecular profiles should be interpreted as a snapshot of inflammatory reactions in gingiva, but cannot fully explain cellular activities. Additionally, results of the correlation analysis could be biased by the limited number of subjects and subject variability. It is necessary to conduct *in vitro* or *in vivo* experiments to further investigate these correlations and potential interactions between bacterial species and lipid mediators.

In conclusion, profiles of lipid mediators, receptor genes and the subgingival microbiome were distinct in different periodontal inflammatory conditions. The highly correlated lipid mediator and subgingival microbiome profiles and specific correlations between individual lipid mediators and bacterial species indicate that periodontal inflammation regulated by lipid mediators drives the shifts of the subgingival microbiota. Elucidation of these correlations facilitates the understanding of resolution of inflammation in periodontitis and identification of potential biological targets for the development of novel therapies.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject ID: PRJNA726254.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UTHealth Committee for the Protection of Human Subjects. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

C-TL and TD contributed conception and design of the study. C-TL and BF collected clinical samples. C-TL, GT and BF processed clinical samples and performed experiments. C-TL, RL, LZ, GT, WZ and KM performed the data analysis. C-TL, RL, LZ, GT, WZ, KM, NA, and TD contributed to data interpretation. C-TL wrote the first draft of the manuscript. All authors wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.691216/full#supplementary-material

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# Tissue Damage in Radiation-Induced Oral Mucositis Is Mitigated by IL-17 Receptor Signaling

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Oral mucositis (OM) is a treatment-limiting adverse side effect of radiation and chemotherapy. Approximately 80% of patients undergoing radiotherapy (RT) for head and neck cancers (HNC) develop OM, representing a major unmet medical condition. Our understanding of the immunopathogenesis of OM is limited, due in part to the surprising paucity of information regarding healing mechanisms in the oral mucosa. RNAseq of oral tissue in a murine model that closely mimics human OM, showed elevated expression of IL-17 and related immune pathways in response to head and neck irradiation (HNI). Strikingly, mice lacking the IL-17 receptor (IL-17RA) exhibited markedly more severe OM. Restoration of the oral mucosa was compromised in *Il17ra*$^{-/-}$ mice and components associated with healing, including matrix metalloproteinase 3, 10 and IL-24 were diminished. IL-17 is typically associated with recruitment of neutrophils to mucosal sites following oral infections. Unexpectedly, in OM the absence of IL-17RA resulted in excessive neutrophil recruitment and immunopathology. Instead, neutrophil activation was IL-1R-driven in *Il17ra*$^{-/-}$ mice. Blockade of IL-1R and depletion of neutrophils lessened the severity of damage in these mice. Overall, we show IL-17 is protective in OM through multiple mechanisms including restoration of the damaged epithelia and control of the neutrophil response. We also present a clinically relevant murine model of human OM to improve mechanistic understanding and develop rational translational therapeutics.

**Keywords: oral mucositis (OM), inflammation, interleukin-17, oral mucosa, healing**

## INTRODUCTION

Oral mucositis (OM) is one of the most common non-hematological side effects of radiotherapy (RT) and/or chemotherapy for head and neck cancers (HNC), characterized by tissue injury of the oral mucosae (1). OM is painful and associated with nutritional deficiency, thus resulting in a large economic burden due to costs and clinical risks associated with pain management and liquid diet supplementation (2). In the 100 days following the initial development of OM, the risk of mortality

increases 3.9-fold (3). During OM the loss of oral mucosal integrity, imbalance of the oral flora and hyposalivation leads to increased susceptibility to oral infections, including oropharyngeal candidiasis (OPC) and herpes simplex virus (HSV), pronounced sensitivity to dental caries, gingivitis and periodontitis (3–5). The current therapeutic options for OM are largely ineffective and management relies on lessening symptoms, not prevention (6–9). Ultimately, severe OM can lead to increased hospitalizations and the use of feeding tubes, which may interrupt or alter cancer therapy leading to worse tumor outcomes (2, 6, 10–12).

The stages of OM in both humans and mice include initiation, primary damage with signal amplification, ulceration, followed by healing and fibrosis (2, 13). Ionizing radiation initiates OM through toxicity to basal epithelial, submucosal, and endothelial cells (3, 14). Cells with extensive DNA damage produce reactive oxygen species (ROS) that accumulate in tissue (11). Injured cells also release damage-associated molecular patterns (DAMPs) that bind receptors to initiate inflammatory signaling cascades (12). Eventually, repair and healing occurs *via* signaling from the extracellular matrix (ECM) and anti-inflammatory cytokines (2). It is understood that the progression of OM requires activation of NF-kappaB which induces transcriptional expression of pro-inflammatory mediators, such TNF-α, IL-1, and IL-6 (2, 11, 15). Sustained expression of these cytokines perpetuates tissue damage, resulting in the loss of membrane integrity and development of oral ulcers (13). While the clinical progression of OM is described, the intricate signaling networks involved in the progression and resolution have not been fully elucidated (8, 9, 11). Indeed, there is a general paucity of information regarding the immunology of the oral mucosa, highlighting a need to better understand these processes.

Interleukin-17 (IL-17) is a proinflammatory cytokine that plays diverse roles in oral health and disease. For example, IL-17 is protective against acute bone loss caused by gingivitis-related bacteria (16). However, in chronic periodontitis, IL-17 promotes inflammation-induced bone loss (17). Furthermore, due to the proliferative and inflammatory properties of IL-17, it is implicated in tumorigenesis in several cancers, including esophageal cancer (18, 19). IL-17 also supports tongue squamous cell carcinoma formation (20, 21). In other tissue compartments, the roles for IL-17 in damage and healing are not straightforward either. IL-17 protects the intestinal epithelium through maintenance of tight junctional proteins and stimulating epithelial cell proliferation (22, 23). Whereas, inhibition of IL-17 or IL-17RA leads to a weaker intestinal epithelial barrier and higher incidence of severe disease in the gut (22, 24). When considering the skin though, the proliferative capacity of IL-17 can be damaging during psoriasis, yet advantageous to tissue repair during injury, likely *via* similar effects on the epithelial layer (25–27).

Since IL-17 has distinct roles in tissue repair and maintenance it was important to determine how the cytokine functions during the severe ulceration associated with head and neck irradiation (HNI). We focused on whether IL-17RA was protective or pathogenic during oral damage and healing associated with radiation. We approached this by exposing *Il17ra*$^{-/-}$ mice to HNI-induced OM. Here, we provide evidence that IL-17RA signaling is beneficial during OM by promoting tissue regeneration and unexpectedly dampening the neutrophil response. In the absence of IL-17RA, other mediators, including IL-1, were dysregulated leading to excessive inflammation and tissue damage. Overall, while IL-17/IL-17RA are potential targets in HNC, care must be taken in establishing therapeutic strategies in patients who develop OM during radiation treatment.

## MATERIALS AND METHODS

### Mice

Mice were acquired by materials transfer agreement (MTA) with Amgen (*Il17ra*$^{-/-}$) (28). In all experiments, age- and gender-matched littermate controls or WT controls (Jax Inc.) were used. All mice were housed with food and water ad libitum under a 12-hour dark/light cycle in a specific pathogen-free facility at the University of Toledo. All animals were used in accordance with the protocol reviewed and approved by the Institutional Animal Care and Use Committee and in accordance with guidelines from the National Institutes of Health, the Animal Welfare Act, and U.S. Federal Law.

### Radiation-Induced OM

To expose mice to HNI and induce OM, mice were immobilized using an anesthesia protocol approved by the Department of Laboratory Animal Research at the University of Toledo. Mice were aligned in a custom-made Polystyrene phantom with the aid of the University of Toledo Department of Radiation Oncology staff, using the Linear Accelerator's (Linac's) field light to assure only the head received radiation. A one cm tissue-mimicking super flab bolus material was used to assure the distribution of the dose at a 99–100% level was in the entire depth intended for treatment. This assured over 99% dose to the surface of the skin and at the distal depth which was on average 1.5 cm. The energy used for this experiment was a 6 MeV electron beam and monitor units were calculated to deliver 22.5 Gy at the rate of 1,000 cGy/min in a single fraction. Based on characteristics of the 6 MeV electron profile and realizing that 50% isodose line was at the edge of the field, the mouse's head and tongue area received 80 to 85% of the nominal 22.5 Gy amounting to 18–19 Gy. The effective delivered dose throughout this manuscript should then be taken as 80 to 85% of what is presented as the nominal dose. The custom manufactured jigs at the head of the Linac were used to align the body such that only the head was exposed, and the body protected from the radiation beam. Following irradiation, animals were removed from the jig, housed in a climate and light/dark controlled environment, and allowed free access to food and water. Animals were monitored daily for changes in weight and activity.

### Macroscopic and Histopathologic Examination

Tongues were rinsed with PBS and stained with 1% toluidine blue for 2 min, followed by washing with acetic acid for 30 s to reveal ulcerative lesions. The percentage of toluidine blue-

positive areas was calculated using ImageJ software and % damage quantified by the area of toluidine blue positive area/surface area of whole tongue ∗ 100. Tissues were formalin-fixed, paraffin-embedded, and sectioned at a thickness of 5 μm. Ulcer size, mucosal thickness, and cellular infiltrate were measured in H&E-stained tissue using a Bio-Tek Cytation 5 automated microscope (Bio-Tek) and equipped with image-capturing software by The University of Toledo Advanced Microscopy & Imaging Center (Toledo, OH). Investigators analyzing staining of tongues were blinded to treatment and mouse cohort.

## Immunohistochemistry

Tissues were formalin-fixed, paraffin-embedded, and sectioned at 5 μm. Slides were dehydrated with xylene and ethanol gradient, and antigen retrieval and blocking performed. Sections were further labeled with MPO (R&D Systems, Minneapolis, MN) or ki67 (Cell Signaling Technology, Danvers, MA). Secondary biotinylated antibody was applied, and slides incubated at room temperature for 1 h. Signals were detected using Sigma Fast tablets to make the DAB solution (Sigma Aldrich, St. Louis, MO) and the reaction stopped by placing slides in TBS. For IL-1α, MMP9, and TIMP2 IHC was performed on paraffin sections using avidin-biotin-peroxidase complex (streptavidin–biotin labeled method) with the Cell and Tissue staining kit (R&D Systems, Minneapolis, MN). The manufacturer's protocol was followed. The antibodies for IL-1α, MMP9, and TIMP2 were purchased from R&D Systems, Inc. (Minneapolis, MN).

## ELISA

Fresh or frozen (−80°C) tongue tissue was submerged in cold sterile saline and homogenized by GentleMACS Dissociator (Miltenyi Biotec) in lysis buffer containing: 50 mM potassium phosphate buffer pH 6.0 and 0.5% HTAB. Samples were lysed further by 3× freeze thaw at 37°and centrifuged at 10,000×*g* 4° for 15 min. Supernatants were collected and protein concentration determined by BCA (Thermo Fisher Scientific). Following protein determination, IL-1β was semi-quantitatively measured by enzyme-linked immunosorbent assay (R&D Systems) according to manufacturer's protocol. Assay was performed in biological triplicate in technical duplicate.

## Complete Blood Count

EDTA anti-coagulated blood samples from cardiac puncture were used to obtain a complete blood count with an insight V5 Hematology Analyser (Woodley Equipment, Bolton, Lancashire).

## Real-Time Reverse Transcription-PCR

Total RNA was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO) and RNA (1 μg) reverse-transcribed by High-Capacity cDNA RT kit (Thermo Fisher Scientific, Waltham, MA) at 25° for 10 min, 37°C for 120 min, followed by 85° for 5 min. Quantitative PCR was performed using PowerUp SYBR green Master Mix and a Quant Studio 3 detection system (Applied Biosystems, Waltham, MA), as specified by the manufacturer. The crossing point was defined as the maximum of the second derivative from the fluorescence curve. For quantification, we report relative mRNA expression of specific genes using the $2^{-\Delta CT}$ method and used GAPDH housekeeping gene for normalization. Primers that were made in house are shown in **Supplementary Table 1**, otherwise primers were obtained from QuantiTect (QIAGEN, Germantown, MD). Assays were performed in biological triplicate in technical triplicate.

## RNA Sequence Analysis

RNA-seq libraries (strand-specific, single end) were generated from total tongue RNA by using a NEBNext Ultra II Directional RNA Library Prep kit (New England BioLabs, Ipswich, MA). Fifty nucleotides of the sequence were determined from one end of each cDNA fragment using the HiSeq platform (Illumina). Sequencing reads were aligned to the UCSC (University of California, Santa Cruz) mouse reference genome (mm10, GRCm38.75) using HISAT (29), and alignment files were used to generate read counts for each gene. Statistical analysis of differential gene expression was performed using the DEseq package from Bioconductor (30). A gene was considered differentially expressed if the FDR value for differential expression was <0.01. The RNA-seq analysis was performed in biological triplicate.

Enrichment and pathway analyses of the differentially expressed genes were performed using the Upstream Regulator Analytic and the Diseases and Function analytic from the Ingenuity Pathway Analysis software (Ingenuity Systems; http://www.ingenuity.com). This software assesses the overlap between experimentally derived gene lists and an extensively curated database of target genes for each of several hundred known regulatory proteins and pathways. It then uses the statistical significance of the overlap and the direction of the differential gene expression to make predictions about activation or repression of pathways.

## Antibody Treatment in OM Model

Doses of anti-IL-1R (anti-Ly6G (BioXcell), and anti-G-CSF (R&D Systems) neutralizing antibodies were based on previous studies (31–33). Isotype control antibodies dose and administration schedules are included (**Supplementary Table 2**). Mice were treated i.p. on days 0, 2, 4, 6, and 8 of the experiment with antibodies directed against IL-1R (300 μg/mouse or IgG isotype control (BioXcell) (300 μg/mouse), in age- and sex-matched controls. For the anti-Ly6G (150 μg/mouse) and anti-G-CSF (10 μg/mouse) mice were treated i.p. with both antibodies and controls (R&D Systems) on d7, followed by daily treatments of G-CSF on d8, 9, and 10 of the experiment. Mice were treated with α-IL-17A or isotype control (BioXcell) at a 150 μg/mouse on days 8 and 10 post irradiation. On day 11 following HNI, tongues were harvested and assessed for tongue damage by toluidine blue+ staining.

## Flow Cytometry

Tongue tissue was mechanically homogenized in RPMI 1640 media then incubated at 37°C for 42 min on a GentleMACS Dissociator (Miltenyi Biotec) using tissue dissociation kits (Miltentyi Biotec). Resulting solution was passed through a cell

strainer for single-cell suspensions. After brief centrifugation, cells were reconstituted with PBS supplemented with 2% FBS and 2 mM EDTA. Some $2 \times 10^6$/ml viable cells were obtained by staining with Trypan blue and counting on a hemocytometer. For analysis of immune cells, an initial incubation of CD16/CD32 Fc Block (BD Biosciences) was followed by staining with the following antibodies, all from BioLegend (San Diego, CA): CD11b-PerCP/Cyanine5.5 (M1/70), GR-1-APC (RB6-8C5), F4/80-APC/Cyanine7 (BM8), and I-A/I-E-BV510 (M5/114.15.2).

For analysis of apoptosis, separate cell suspensions were reconstituted in Annexin V Binding Buffer followed by incubation with Annexin V-PE/Cy7 antibody and Propidium Iodide (Biolegend, San Diego, CA), for 10 min before flow cytometric analysis. Flow cytometry was performed on an LSRFortessa (BD Bioscience, San Jose, CA) and analyzed with FlowJo (BD Bioscience, San Jose, CA). For flow analysis lymphocyte and myeloid populations were gated out, and the resulting epithelial cell populations were further analyzed (**Supplementary Figure 3**).

## Data Analysis

Data was analyzed on Prism (Graphpad V8.4.3). ELISA data is presented as one-way ANOVA with Tukey's *post hoc* analysis. Flow cytometry was analyzed by day with Student's *t* test and is presented as the mean ± SEM. Tongue damage data is presented as geometric mean ± SEM and analyzed by ANOVA with Tukey's *post hoc* analysis or Student's *t* test for analysis between two groups. PMN quantification was analyzed by ANOVA with Tukey's *post hoc* analysis. Gene expression data is presented as mean ± SEM analyzed by Kruskal–Wallace test with Dunn's multiple comparisons or ANOVA with Tukey's *post hoc* analysis. Normality was evaluated *via* Shapiro–Wilk tests. Each symbol represents one mouse unless indicated. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

# RESULTS

## HNI Induces an IL-17-Related Transcriptional Profile During OM

Sensitivity to OM is dependent on many factors including treatment type, dose rate, total dose, and volume irradiated. Most patients with HNC are treated with external beam radiotherapy using a relatively newer modality known as Intensity Modulation Radiotherapy (IMRT). This can only be implemented using complex treatment planning algorithms that can account for behavior of primary and scatter radiation in areas of heterogeneity (34, 35). In addition, these treatment planning systems contain optimization routines for inverse planning to achieve optimal dose to the target volume while sparing normal tissue. All of these newer features have been instrumental in reducing the severity of OM in recent years, but we still consider this as a major side effect of radiation for HNC patients that needs to be dealt with (35). In order to model OM, we used a single-dose of radiation targeting the head and neck regions of mice using a clinical linear accelerator capable of IMRT delivery. To define the stages of OM through healing, mice were irradiated and tongue tissue harvested daily for 13 days (**Figure 1A**). Irradiated mice presented with symptoms typically associated with human OM, including damage and overt lesions on the tongue. A dose of 22.5 Gy caused damage to the oral mucosa that was evident on Day 8 (~4% of tissue toluidine blue+), peaked on Day 11 (~23% of tissue, with complete healing by Day 15 (0% of tissue), which is similar to the stages of OM in humans (8, 36, 37) (**Supplementary Figure 1A**). In order to understand the immune components responsible for signal amplification and ulceration in the tongue tissue during OM, we performed RNA sequencing (RNA-Seq) analysis of mRNA from tongue tissue of non-irradiated sham mice and mice exposed to 22.5 Gy, harvested on Day 11 during peak damage (n = 3). The sequencing profiles showed that 988 genes exhibited a change in expression (FDR <0.01) in at least one of the HNI versus sham comparisons (**Supplementary Tables 3, 4**). Next, we used Ingenuity Pathway Analysis (IPA) to identify pathways activated during OM. Processes involved in epithelial cell survival and proliferation were enriched during peak damage. The most differentially expressed genes were also involved in immune functions such as global recruitment of immune cells ($p=2x10^{-18}$), movement of polymorphonuclear cells (PMNs) ($p = 4 \times 10^{-11}$), and development of Th17 cells ($p = 4 \times 10^{-6}$) (**Figure 1B**).

Further analysis of genes differentially expressed in mice with OM showed many immune components related to the IL-17 signaling pathway were activated after HNI, with stronger induction in the distal portion of the tongue where damage predominated (**Figure 1C** and **Supplementary Figure 1B**). These targets included chemokines, cytokines, signaling molecules, AMPs, and proteases that are regulated by IL-17RA in the context of other diseases (38). Since IL-17-related genes were induced during peak damage, we next determined the expression kinetics of both *Il17a* and *Il23a* throughout the stages of OM. After HNI, *Il23a* expression peaked on Day 7 followed by a rapid loss of expression by Day 9. Induction of *Il23a* was followed by *Il17a* expression, which was detected after Day 9 and increased by Day 11, the time point at which WT mice presented with severe ulcerative damage. The levels of *Il17a* dropped by Day 12 as healing commenced (**Figure 2A**). Taken together these data indicate that the IL-23/IL-17 pathway is activated following HNI, and IL-23 expression preceding IL-17 is predicted, as it is understood to be upstream of IL-17 production.

## IL-17RA Signaling Protects Against Ulcerative Damage and Epithelial Loss Following HNI

In order to determine if IL-17RA is protective or pathogenic during the most severe phase of OM, WT and $Il17ra^{-/-}$ mice were subjected to HNI and damage assessed. Larger ulcerative lesions were detected in $Il17ra^{-/-}$ mice (~11% of tongue) on Day 11 compared to WT mice (~5% of tongue) (**Figures 2B, D**). In addition to the increased surface area of damage in the $Il17ra^{-/-}$ mice (**Figure 2E**), lesions were deeper (1.8 mm *vs.* 0.6 mm) and the adjacent tissues were thinner (0.5 mm *vs.* 1.7 mm) than those in WT mice during peak damage (Day 11) (**Figure 2F**). Next, we

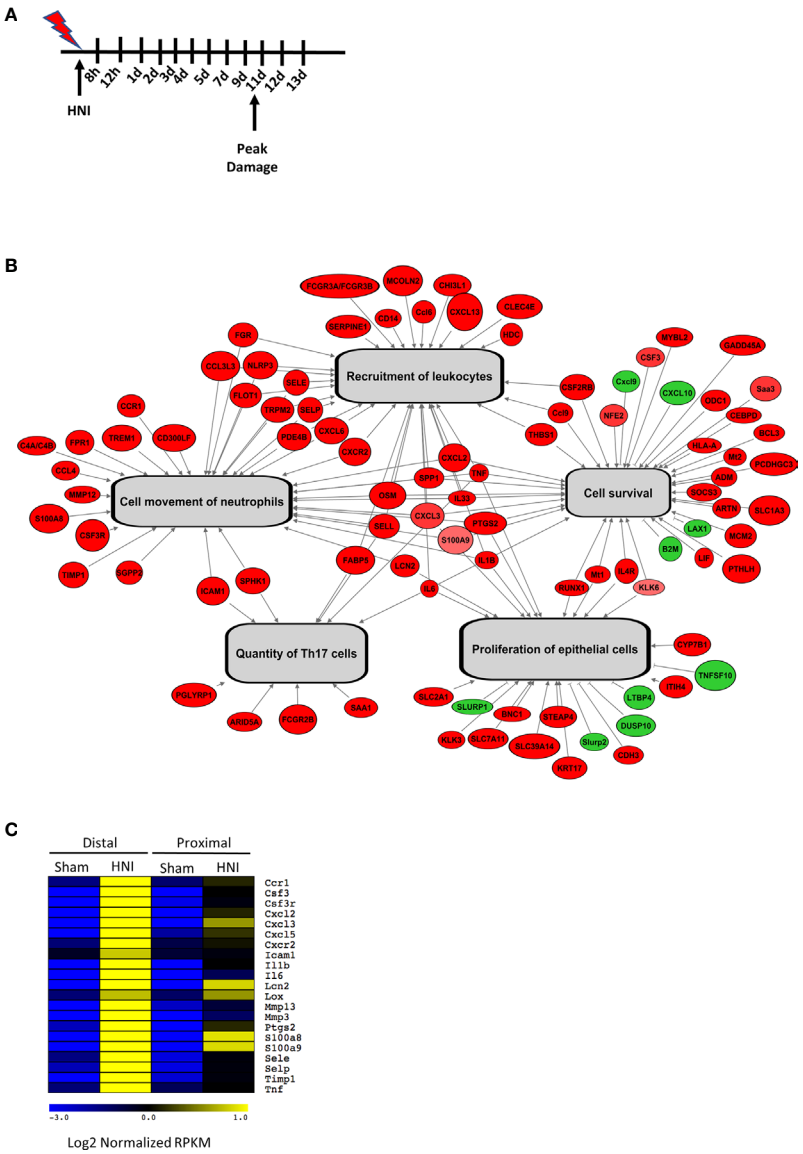


**FIGURE 1** | Targeted HNI induces OM in mice. **(A)** Model of HNI-induced OM. Mice received no radiation (sham) or HNI on D0, and tongues were harvested at various time points. **(B)** Schematic analysis of select biological functions predicted to be activated in response to HNI. Genes colored in red have HNI-induced expression and genes labeled in green have HNI-repressed expression. Gray arrows depict biological functions that are predicted by our analysis to be activated. **(C)** Comparison of IL-17RA regulated genes between sham and irradiated mice in the distal portion of the tongue.

treated mice with α-IL-17A or mouse IgG1 isotype control to determine if therapeutic blockade of IL-17 rendered mice more susceptible to OM. There were no differences in damage between mice treated with α-IL17A or *Il17ra*$^{-/-}$ mice (**Figure 2C**). Based on these findings, IL-17A/IL-17RA appear protective in HNI-induced damage to the oral mucosa.

## IL-17RA Promotes Cell Survival and Proliferation During OM

To elucidate the mechanism of IL-17RA-mediated protection, we next determined the role of IL-17 signaling in epithelial repair of the oral mucosa after radiation. Epithelial proliferation contributes to the rate of healing during OM, which is partly dependent on epidermal growth factor (EGF) and keratinocyte growth factor (KGF) (8). While expression of *Egf* was comparable between *Il17ra*$^{-/-}$ and WT mice regardless of radiation exposure, markers for early differentiated epithelial cells, keratin-15 (*Krt15*) and keratin-16 (*Krt16*), were reduced in *Il17ra*$^{-/-}$ mice, as was *Kgf* (**Figure 3A**), signifying a lack of re-epithelialization (39). Similarly, expression of *Cdc3a*, which is essential for cytokinesis, was reduced in *Il17ra*$^{-/-}$ mice. Furthermore, Ki67 staining in tongue tissue revealed fewer dividing cells in the basal stem cell layer of irradiated *Il17ra*$^{-/-}$ mice (two cells/field of view) compared to WT mice (40 cells/

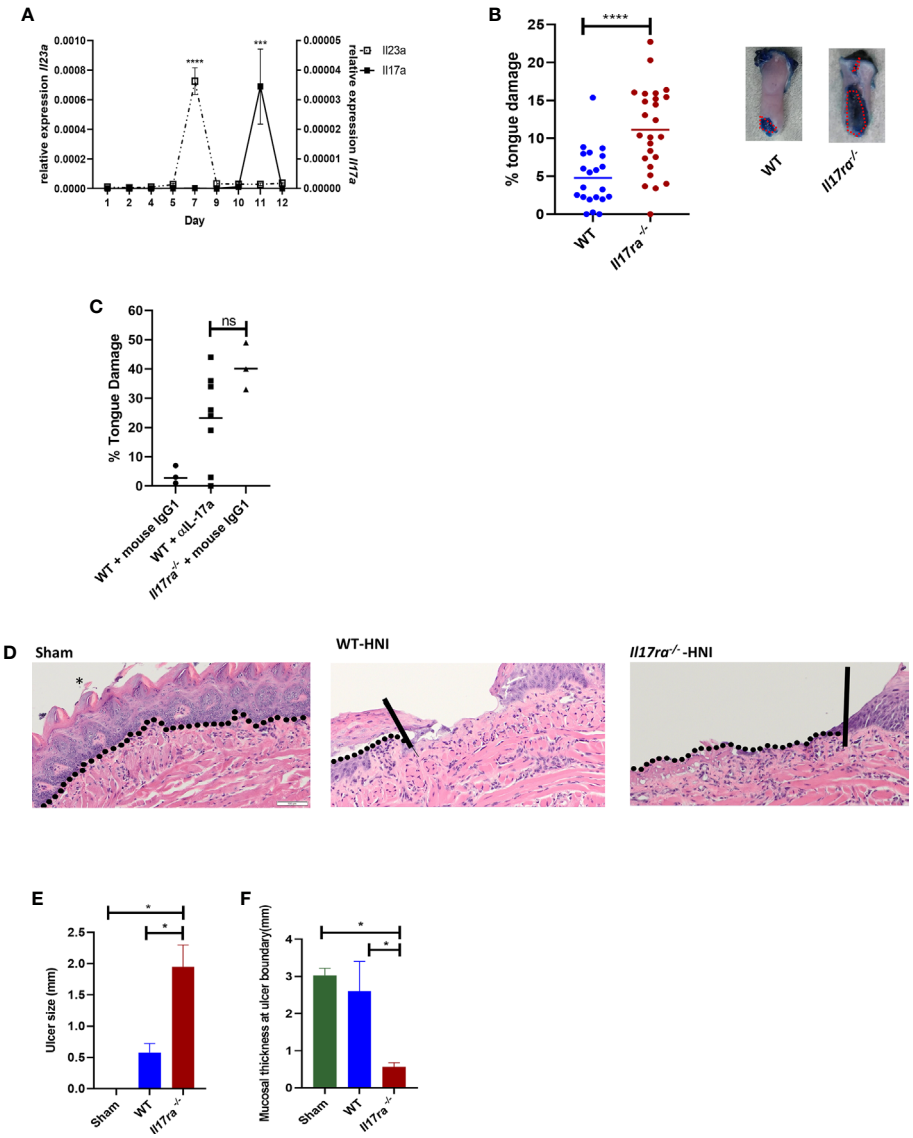


**FIGURE 2** | IL-17 is induced and is protective following HNI **(A)** Expression kinetics of *Il23* and *Il17a* following HNI assessed by qPCR. Results pooled from at least three experiments analyzed by ANOVA with Tukey's *post hoc*. **(B)** Quantification of toluidine blue staining by determining the surface area of the tongue positive for blue staining compared to the total surface area of tongue on each mouse on day 11 post radiation. The staining on the ventral side of the tongue was not included in the quantification since this included the excision site. Red dotted lines represent the surface area of a lesion as determined for all tongues. Data pooled from at least three experiments. Analyzed by Student's *t* test. **(C)** Mice were treated on days 8 and 10 with α-IL-17A or mouse IgG1 (150 μg) and damage quantified on day 11 (n = at least three per group). Control animals received appropriate isotype controls (**Supplementary Table 2**). Tongue damage analyzed by ANOVA with Tukey's. **(D)** Hematoxylin–eosin staining of mouse tongues D11 following radiation. Asterisk denotes papillae. The vertical lines in the images of tongues highlight the ulcer boundary and the dotted lines represent the epithelial-stromal boundary. **(E, F)** Quantification of lingual ulcer size and mucosal thickness (N= at least 5 per group). Data represents as least three pooled experiments. Analyzed by ANOVA with Tukey's *post hoc*. Scale bars: 50 μM. Data shown as means ± SEM, (*P < 0.05, ***P < 0.001, ****P < 0.0001. NS is not significant.

field of view) (**Figure 3B**). Also, the number of Ki67+ cells in irradiated WT mice was not significantly different compared to WT sham controls (59 cells/field of view). Consistently, transcript levels of components normally expressed during the healing process (*Mmp3, Mmp10, Sprr2*b and *Il24*) were highly induced in WT mice during OM, but were all reduced in *Il17ra*$^{-/-}$ mice (**Figures 3A**, **5A**, **Supplementary Figure 2**). IL-24 production in the suprabasal region of the tongue tissue was undetectable in *Il17ra*$^{-/-}$ mice compared to WT mice (**Supplementary Figure 2A**). In addition, a major cytotoxic effect of RT is activation of apoptosis and necrosis in the proliferating basal cell layer. Relatedly, *Il17ra*$^{-/-}$ mice had more necrotic and early/late apoptotic oral mucosal cells on Days 9 through 11 compared to WT mice (**Figure 3C**). In all, suppression of these contributors to the healing process in the

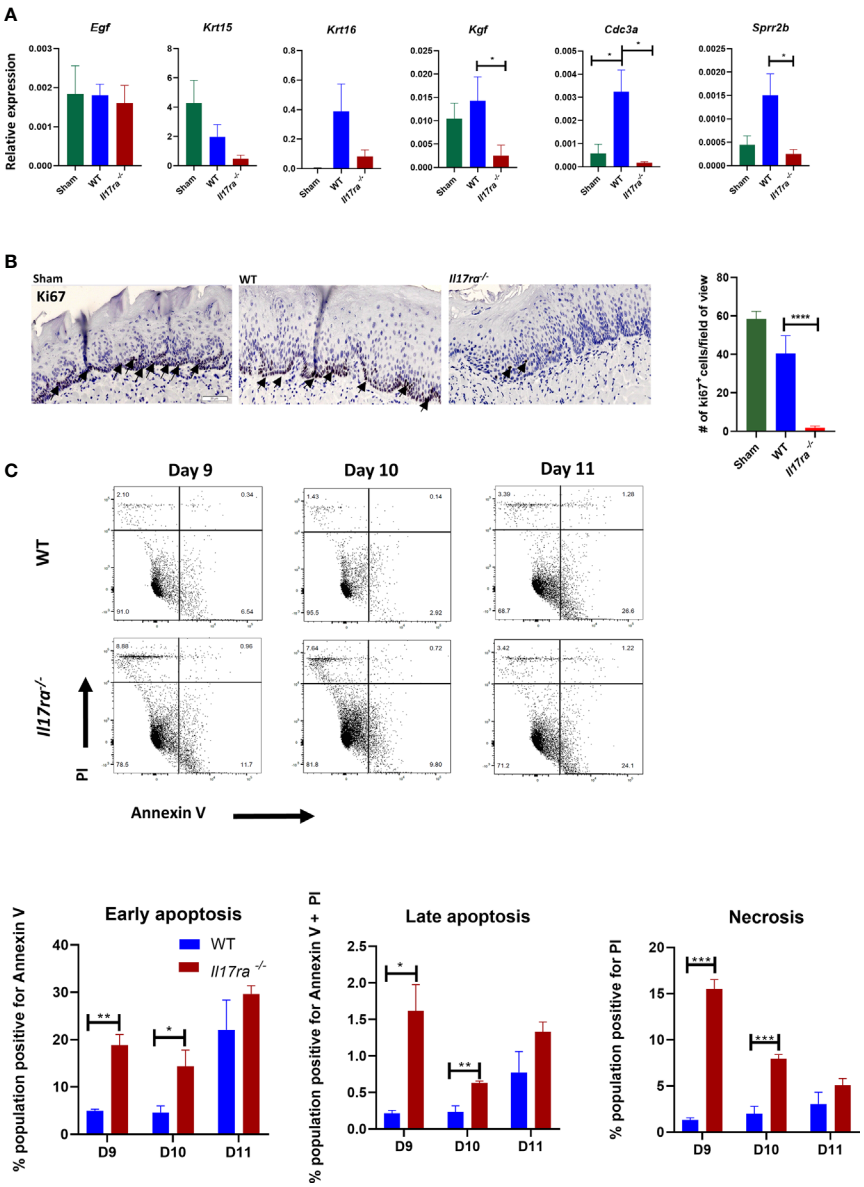


**FIGURE 3** | In the absence of IL-17RA there is enhanced apoptosis and reduced proliferation **(A)** Expression differences relative to GAPDH of genes related to healing. N = at least five mice/group. *Kgf* and *Cdc3a* analyzed by Kruskal–Wallace with Dunn's multiple comparisons test, *Sprr2b* analyzed by ANOVA with Tukey's. Data pooled from at least three experiments **(B)** Proliferation marker Ki-67 staining and quantification in the dorsal portion of the tongue obtained on day 11 following radiation. Scale bar, 100 μM. Arrows represent positive Ki67 staining. Analysis of staining by ANOVA with Tukey's. **(C)** Apoptotic and necrotic cells analyzed by flow cytometric analysis after Annexin V and PI staining. Data representative of two independent experiments and analyzed by Student's *t* test between days. Data shown as means ± SEM, (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

absence of IL-17RA further substantiates the importance of the receptor in protection and restoration of the oral mucosa during HNI-induced OM.

## Enhanced Neutrophil Response in IL-17RA-Deficient Mice

In most disease contexts IL-17 does not act on granulocytes directly, but rather promotes neutrophil recruitment through the induction of chemokines including CXCL1, CXCL2 and CXCL5 and growth factors such as G-CSF in target non-hematopoietic cells. In the oral cavity specifically, mice lacking IL-17RA in the suprabasal oral mucosae (*Il17ra*$^{fl/fl}$-K13Cre$^{+}$) have diminished neutrophil levels, contributing to their high susceptibility to oral candidiasis (40). We set out to determine if mice lacking IL-17RA had the same neutrophil defects during OM caused by RT.

In patients receiving RT or chemotherapy, the early stages of OM are characterized by neutropenia, and a lack of circulating neutrophils can be a readout for OM severity (41–43).

Neutrophils detected in the oral lesion later during the ulceration phase are generally thought to cause damage. The number of neutrophils also increases as microbes gain access to the submucosa, due to loss of barrier integrity (43, 44). By day 11 post-HNI, WT mice had levels of circulating neutrophils comparable to sham mice (**Figure 4A**), suggesting that irradiated mice were not neutropenic at this point. In contrast, the levels of blood neutrophils were elevated in *Il17ra*$^{-/-}$ mice. Next, we assessed if neutrophils were recruited to the tongue. Compared to sham, WT and *Il17ra*$^{-/-}$ mice had comparable elevated levels of neutrophils in the whole tongue, indicating irradiation induces a neutrophil influx into the tissue at later stages of OM, and this recruitment was surprisingly independent of IL-17RA signaling (**Figure 4B**). We then determined if neutrophils/polymorphonucler cells (PMNs) were localizing to ulcers in the absence of IL-17RA (**Figure 4C**). Unexpectedly again, *Il17ra*$^{-/-}$ mice showed significantly more PMNs migrated into the damaged lesions compared to WT mice. While expression of *Cxcl1* and *Cxcl2* were not affected (**Figure 4D**), there was a notable increase in *Csf3* (encodes for G-CSF) in *Il17ra*$^{-/-}$ mice compared to WT mice on Day 11 of OM.

Since neutrophil recruitment is normally compromised in mice deficient in IL-17RA we next asked if the neutrophils in the lesion were functionally competent in *Il17ra*$^{-/-}$ mice. We measured myeloperoxidase (MPO) levels in tongue, a parameter also commonly used to assess the severity of OM (8, 45, 46). The oral mucosa of irradiated *Il17ra*$^{-/-}$ mice had greater MPO production compared to irradiated WT mice on Day 11 aligning with the increased presence of neutrophils in *Il17ra*$^{-/-}$ mice (**Figure 4E**).

Neutrophils also activate MMPs through production of elastase (47, 48), so we reasoned that elevated neutrophil-mediated inflammation observed in the *Il17ra*$^{-/-}$ mice could be due to dysregulated MMPs and could impact the process of wound healing (49, 50). The increased levels of *Mmp10* and *Mmp3* in the WT tissue during OM were diminished in the *Il17ra*$^{-/-}$ tissue (**Figure 5A**). In contrast, *Mmp9*, and *Mmp12* transcripts were elevated, as well as MMP9 protein in the *Il17ra*$^{-/-}$ mice compared to sham and irradiated WT mice (**Figures 5A, B**). The skewed expression of MMPs was associated with a decrease in expression of MMP inhibitors, *Timp1*, *Timp2*, *Timp3*, and *Timp4*, and lower TIMP2 production in the *Il17ra*$^{-/-}$ mice (**Figures 5C, D**). Collectively, these data indicate that during OM, IL-17RA is not necessary for neutrophil recruitment as it is in other disease settings, nor is IL-17 signaling involved in promoting neutrophil function. Moreover, in the absence of IL-17RA there is a disruption in the balance of inflammatory mediators leading to aggravated inflammation. This further substantiates that IL-17RA signaling is required for dampening the inflammatory response during HNI-induced OM.

## IL-1 Contributes to Neutrophil Accumulation in the Absence of IL-17RA

Since neutrophils were still recruited into the OM lesion even in the absence of IL-17RA, we next determined what inflammatory mediators were responsible for the enhanced neutrophil response in the *Il17ra*$^{-/-}$ mice. In WT mice both *Il1a* and *Il1b* are induced post-HNI (**Supplementray Figure 2C**) In the *Il17ra*$^{-/-}$ mice there were elevated transcript and protein levels of both IL-1α and IL-1β on Day 11 (**Figures 6A, B**). Since both IL-1α and IL-1β are implicated in the pathology of OM, and IL-1α at least partially regulates neutrophils in other oral diseases (33, 51–53), we asked if blocking IL-1R in WT and *Il17ra*$^{-/-}$ mice would account for the increased damage when IL-17RA is lacking. Following α-IL-1R Ab administration, OM damage was decreased in WT (~1.8-fold reduction) and *Il17ra*$^{-/-}$ mice (~1.3-fold reduction) compared to mice administered hamster IgG isotype control Abs (**Figure 6C**). Of note, the mice that received isotype control Abs had 3-fold higher damage compared to radiated mice that did not receive control Abs. This may be due to species specificity, antibody concentration or the dosing schedule since mice that received mouse IgG1 control Ab at a lower dose and less frequently did not present with increased tongue damage (**Figure 2C** and **Supplementary Table 2**). Even though mice treated with hamster IgG control antibody showed increased damage in the oral mucosa after radiation it was not related to elevated neutrophil numbers within the area of ulceration (**Figure 6D**). Yet, in irradiated *Il17ra*$^{-/-}$ mice the lessening of overt damage when IL-1R was blocked correlated with a decrease in neutrophils in the areas of ulceration (**Figure 6D**). This substantiates that when IL-17RA signaling is missing uncontrolled production of IL-1 is at least partially responsible for the increased damage and enhanced neutrophil response, and that α-IL-1R is a viable treatment option to explore in OM.

## Neutrophil Blockade Accounts for the Excess Damage in the Absence of IL-17RA

To further establish the pathogenic consequence of an elevated neutrophil response in the absence of IL-17RA we used a combination α-Ly6G and α-G-CSF or rat IgG2a and goat IgG isotype controls in WT and *Il17ra*$^{-/-}$ mice (33). As in the IL-1R blockade study (**Figure 6C**), rat IgG2a and goat IgG administration resulted in increased damage compared to irradiated untreated mice, and again this higher damage was not related to an increased neutrophil influx (**Figure 7B**). Yet, α-Ly6G/G-CSF treatment resulted in the depletion of the excess neutrophils in *Il17ra*$^{-/-}$ mice (**Figure 7B**). This was associated with a reduction in tongue damage of around 2.5-fold (**Figure 7A**) and lower MPO activity (**Figure 7C**). The OM damage in WT mice was unaffected though following neutrophil depletion compared to mice that received isotype control Abs using this particular method and dosing schedule. Collectively these data suggest that IL-17RA is critical for modulating the neutrophil response during HNI, and in the absence of IL-17RA, IL-1-mediated neutrophil accumulation exacerbates inflammation and is detrimental to healing (**Figure 7D**).

# DISCUSSION

While robust models exist to study damage in the skin, highly tractable systems to study injury of the oral mucosa have been lacking. Infections that breech the mucosal barrier in the oral

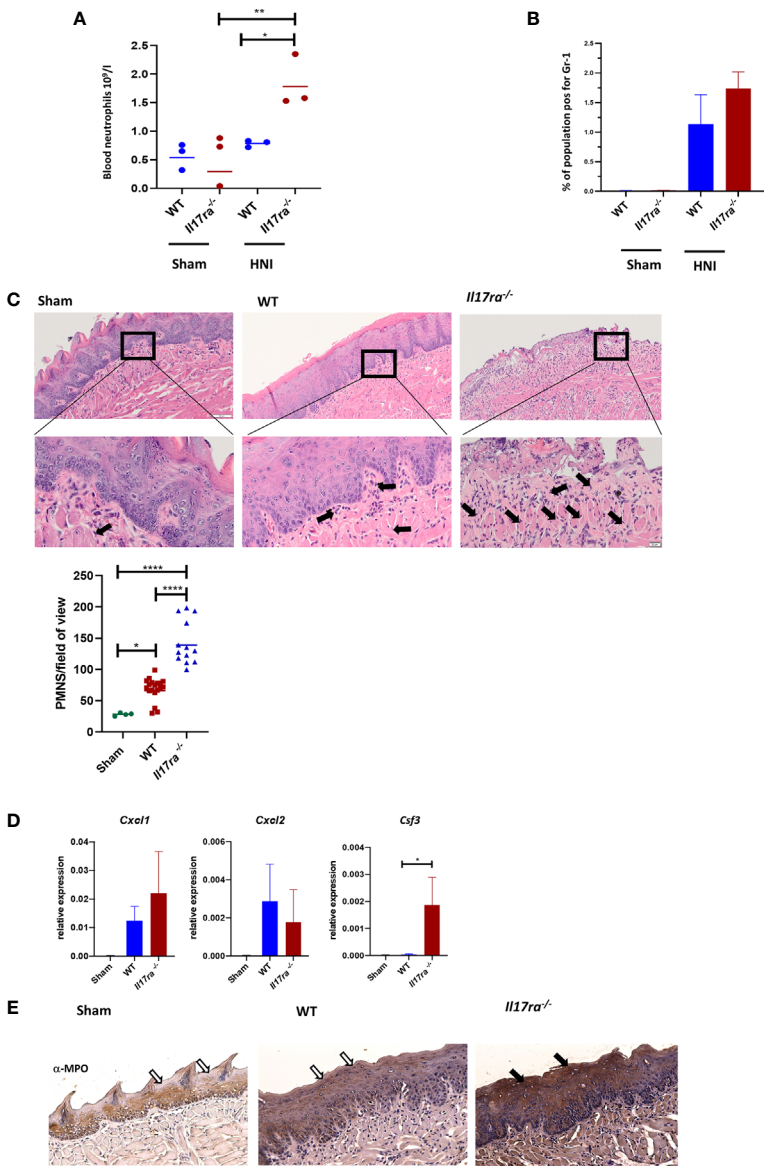


**FIGURE 4** | Neutrophil recruitment to the tongue tissue is heightened in the absence of IL-17RA. **(A)** Cardiac puncture was performed to harvest blood from non-irradiated and irradiated mice and number of circulating neutrophils measured on D11. Data is representative of two independent experiments and analyzed by ANOVA with Tukey's. **(B)** Tongues harvested on day 11 and neutrophil (Gr1+) populations analyzed by flow cytometric analysis and ANOVA with Tukey's. Data representative of two independent experiments **(C)** Staining by hematoxylin and eosin of sectioned tongues and quantification of neutrophils on day 11 following HNI. Arrows represent positive neutrophil staining in distal tongue at ulcer boundary (N = at least three tongues per group). Scale bar 100 μM (top row) and 20 μM (bottom row). Neutrophil quantification analyzed by ANOVA with Tukey's *post hoc*. **(D)** Expression of neutrophil chemokines relative to GAPDH on D11 (N = 3–5 per group). *Cxcl1* analyzed by ANOVA with Tukey's *post hoc*, *Cxcl2* and *Csf3* analyzed by Kruskal–Wallace and Dunn's multiple comparisons. Data pooled from at least three experiments. **(E)** Representative MPO-staining of ulcer boundary counterstained with hematoxylin. The filled arrow indicates positive MPO staining, while the unfilled arrows show the equivalent areas with absent MPO. Scale bar,100 μM. Data shown as means ± SEM, (*P <0.05, **P <0.01,****P <0.0001).

cavity require healing and restoration of the epithelium, but the injury is not as easily followed to the healing phase (54). To understand the immune components involved in repair processes in the oral mucosa, a model with a strong readout of damage (i.e. severe ulceration) that heals with predictable kinetics is required. We used a single dose of radiation that allowed for the development of OM that could be assessed visually and histologically, but also allowed procedural ease when using a clinical linear accelerator.

In general the pathogenesis of OM is similar between mice and humans (8). In patients, OM lesions can be detected on most keratinized tissues in the mouth that are exposed to radiation, including the tongue, buccal mucosa and the soft palate. Interestingly, overt damage was localized to the distal portion

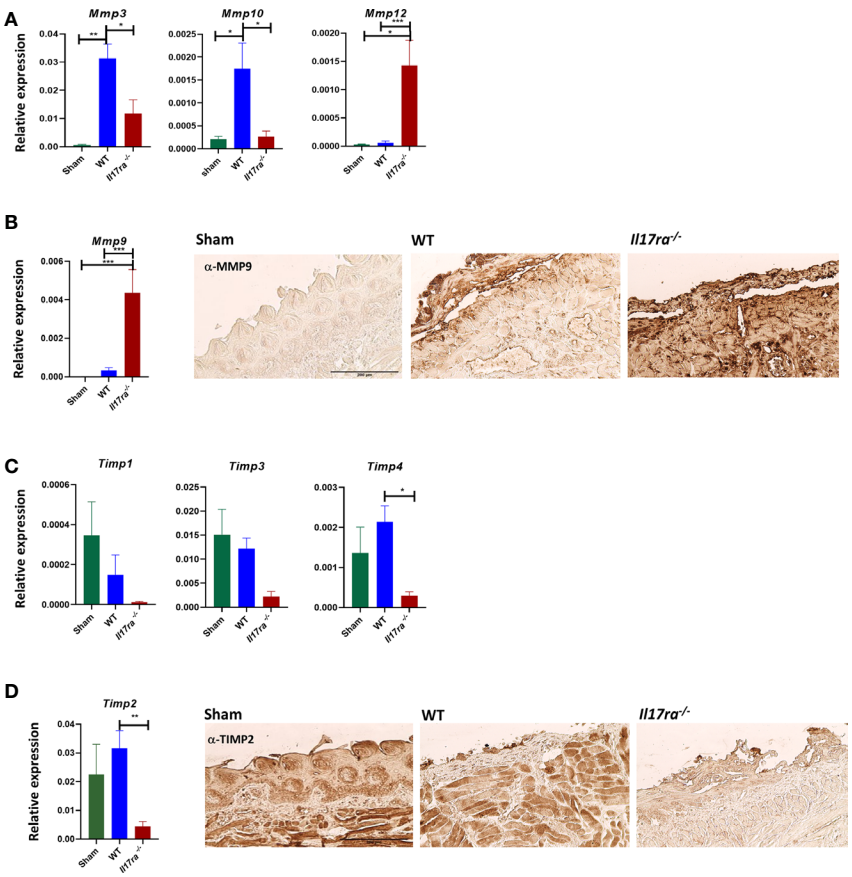


**FIGURE 5** | The absence of IL-17RA is associated with dysregulated proteases and inhibitors. **(A, C)** Gene expression relative to GAPDH of proteases and inhibitors on D11 (N = 3–5 per group). N = at least five per group. *Mmp3* and *Mmp10*, and *Timp1,2,3,4* analyzed by ANOVA with Tukey's *post hoc*, *Mmp12* analyzed by Kruskal–Wallace and Dunn's multiple comparison test. Data pooled from at least 3 experiments. **(B, D)** IHC for MMP9 and TIMP2 from the ulcer boundary zone in the distal tongue. Scale bar, 200 μM. Data shown as means ± SEM, (*P < 0.05, **P < 0.01, ***P < 0.001).

of the tongue in all mice (**Supplementary Figure 1B**). Also, IL-17RA-related gene targets were more strongly activated where the lesions predominated in WT tongue, and we postulate this is the region of the tissue that requires IL-17 to heal properly (**Supplementary Figure 1B**). The location of damaged tissue in the mouse could be an artifact of the way the mice are arranged in the apparatus of the linear accelerator or how the head and neck region is being exposed to radiation. In addition, the architecture of the mucosal surfaces in the oral cavity do vary between mice and humans, evidenced by distinct keratinization and cellular distribution patterns, which may explain the distribution of lesions in mice (55). We also have to consider the distribution of the cytokine receptors throughout the supra- and sub-basal epithelial layers of the oral mucosa. While IL-17RA is expressed ubiquitously, it is the expression of IL-17RA in the supra-basal epithelia that is required for protection from OPC (56), whereas mice deficient in IL-17RA specifically in the superficial epithelia (*Il17RA*$^{fl/fl}$-K13$^{Cre}$) are as susceptible to OPC as IL-17RA knockout mice. Therefore, follow-up studies using tissue-specific knockout mice are justified and will start to dissect these relationships.

IL-17 is highly expressed in ulcerated areas in a WT mouse and the overall effect of IL-17RA-signaling is beneficial during OM since *Il17ra*$^{-/-}$ mice showed considerably more damage. This is not the role attributed to the cytokine in other forms of recurring oral ulcers associated with leukocyte adhesion deficiency type 1 (LAD1), where both IL-23 and IL-17 are pathogenic (57). Now we establish that IL-17RA is necessary post-irradiation for maintaining the epithelial barrier through modulation of pro-apoptotic factors, while promoting effector molecules involved in cell survival and proliferation. This is similar to how IL-17RA helps maintain barrier integrity in the gastrointestinal tract (23). Furthermore, a striking phenotype of the *Il17ra*$^{-/-}$ mice was an unexpected increase in neutrophils found at the site of ulceration compared to WT mice 11 days post-irradiation. Normally IL-17 promotes an influx of neutrophils that is either protective or pathogenic depending on disease context through induction of neutrophil chemokines CXCL1,2 and 5 and growth factors such as G-CSF (58). In this way, mice deficient in IL-17 signaling components (*Il17a*$^{-/-}$, *Il17ra*$^{-/-}$, *Act1*$^{-/-}$ etc.) are particularly sensitive to infections in which prevention is dependent on the action of neutrophils, such as oral candidiasis (59). Inversely, these

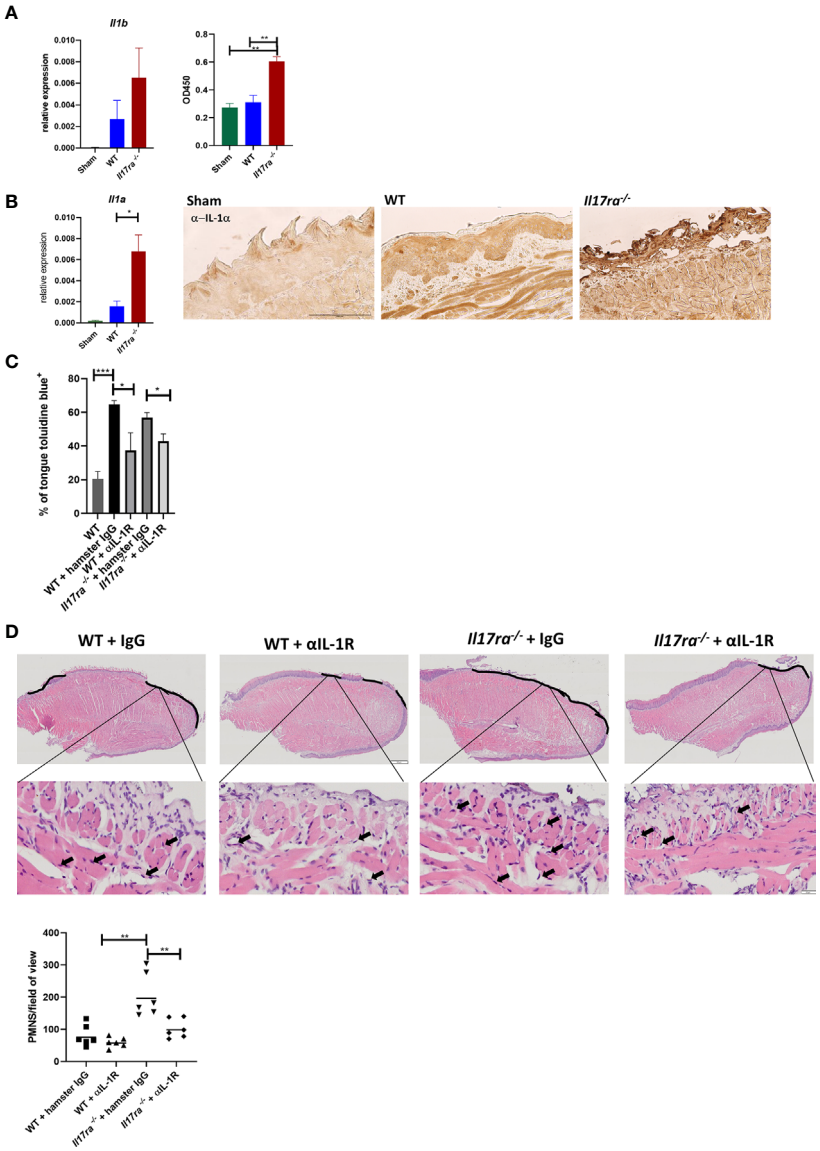


**FIGURE 6** | Neutralization of IL-1R alleviates damage in the absence of IL-17RA. **(A)** Gene expression and circulating levels of IL-1β both analyzed by ANOVA with Tukey's. For gene expression data pooled from at least three experiments. Protein expression represents one experiment. **(B)** Gene expression and IHC of IL-1α pooled from at least three independent experiments. Scale bar, 100 μM. Analyzed by ANOVA with Tukey's. **(C)** Mice were treated i.p. with αIL-1R at 300 μg on days 0, 2, 4, 6, and 8 (n = at least four). Control animals received appropriate isotype controls (**Supplementary Table 2**). Data represents one experiment. Tongues were harvested and damage quantified by toluidine blue+ staining on D11. Quantification of damage analyzed by ANOVA with Tukey's *post hoc*. **(D)** Staining by hematoxylin and eosin of sectioned tongues and quantification of neutrophils on day 11 following HNI in the ulcer boundary area. Images captured at 100 μM and zoomed in at 50 μM. Data analyzed by ANOVA with Tukey's *post hoc*. Data shown as means ± SEM, (*P < 0.05, **P < 0.01, ***P < 0.001).

same knockout mice are at least partially protected from autoimmune disease through a dampened neutrophil response (60). Yet, during HNI-induced OM, expression of neutrophil chemokines was not diminished in *Il17ra*$^{-/-}$ mice. In fact, *Csf3*, which encodes for G-CSF, was significantly enhanced in the absence of IL-17RA. Concomitantly, IL-1α and IL-1β were overproduced in *Il17ra*$^{-/-}$ mice compared to WT. The dysregulated IL-1 levels in the absence of IL-17RA appear partially responsible for the elevated PMNs since blocking of IL-1R led to a reduction, but not a complete loss, of neutrophilic cells in the lesion and lessening of OM-mediated damage. The interplay between IL-1 and IL-17 in OM does align with the role of these cytokines in other oral diseases including OPC, during which IL-1α in part controls the neutrophil response (33).

Prolonged exposure to neutrophils can lead to enhanced tissue damage. This is because neutrophils secrete a repertoire of proteases and enzymes such as MMPs, elastase, Cathepsin G, and MPO that damage tissue (61, 62). MPO is abundantly

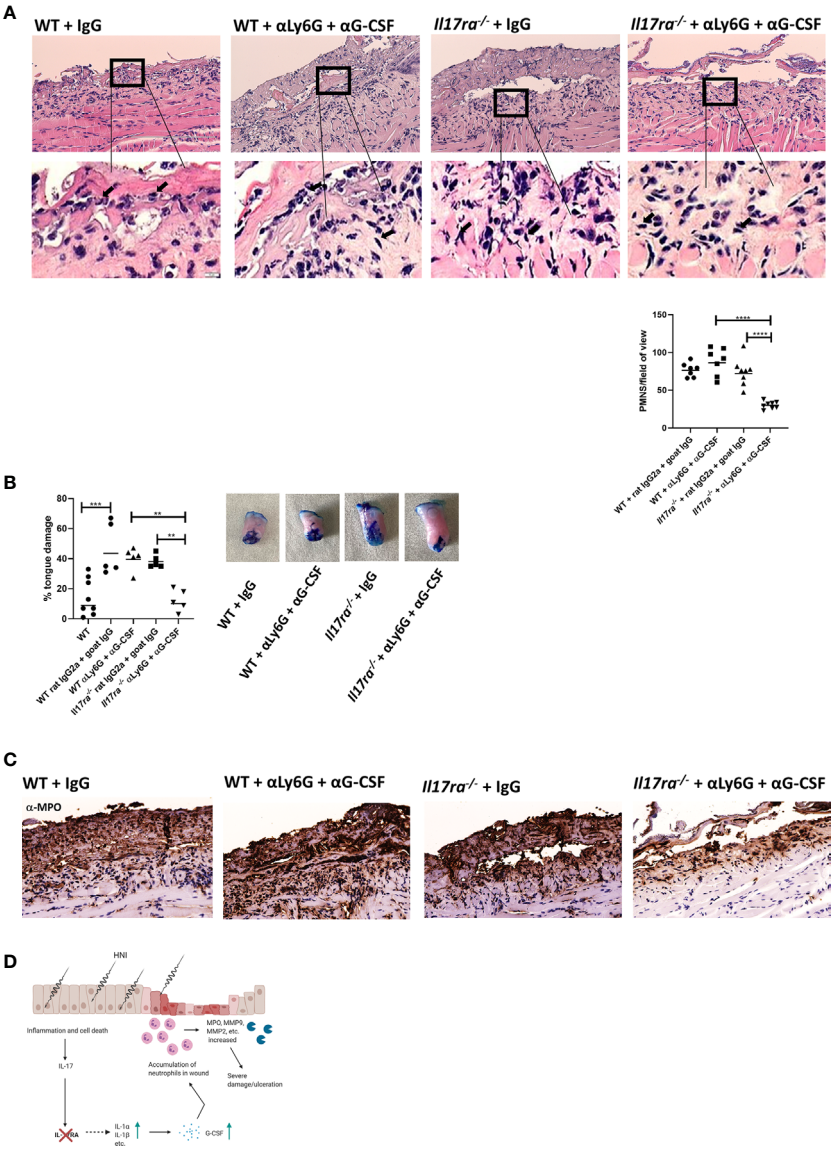


**FIGURE 7** | Neutralization of neutrophils reduces damage in the absence of IL-17RA. Mice were treated i.p. with αLy6G at 150 μg and αG-CSF at 10 μg (n = 5/group) on day 7 following radiation. On days 8–10 mice were treated with 10 μg of αG-CSF. Control animals received appropriate isotype controls (**Supplementary Table 2**). Data is representative of two independent experiments. **(A)** Staining by hematoxylin and eosin of sectioned tongues and quantification of neutrophils on day 11 following HNI. Images captured at 200 and 10 μM. Data analyzed by ANOVA with Tukey's *post hoc*. **(B)** Tongues were harvested and damage quantified by toluidine blue+ staining on D11. Data analyzed by ANOVA with Tukey's. **(C)** Representative MPO-staining of ulcer boundary counterstained with hematoxylin. **(D)** Proposed mechanism of IL-17RA-mediated protection and healing of OM. Radiation induces inflammation and apoptosis, increasing IL-23 expression. IL-23, in turn activates IL-17RA-mediated inflammation. In the absence of IL-17RA, IL-23, along with IL-1a synergizes to induce excess neutrophil chemokines like G-CSF resulting in accumulation of neutrophils in the damaged area. Increased neutrophils release proteases that further damage the wound and contribute to delayed wound healing. Image created using BioRender with permission. Data shown as means ± SEM, (**P < 0.01, ***P < 0.001, ****P < 0.0001).

expressed in neutrophil granules and is associated with severe OM lesions (45, 63) and delayed wound healing (64). Neutrophils also produce various MMPs, such as MMP-9, which was detected at elevated levels in *Il17ra*$^{-/-}$ mice, and is associated with tissue destruction during periodontal disease and OM (65, 66). While the action of MMPs triggered by DNA damage associated with ionizing radiation contributes to the ulceration of the oral mucosae, MMPs are also required to breakdown the ECM for healing. Therefore, regulation of this system is critical (67, 68). The pathogenic nature of excess PMNs in *Il17ra*$^{-/-}$ mice was substantiated by the efficacy of α-Ly6G/-G-CSF treatment that greatly reduced neutrophils in the lesion and led to a reduction in damage. Although, this therapy did not result in a reduction of PMNs or damage in WT mice. We speculate this could be due to the treatment schedule used, and modifications in dosing may result in the blocking of neutrophils

in WT mice and therapeutic efficacy. Indeed, antibody-based depletion of neutrophils can be complicated (69). Another possibility is since expression of *Csf3* was markedly increased in *Il17ra*$^{-/-}$ mice, targeting G-CSF was particularly successful in these mice. Future studies to determine the potential of neutrophil blocking treatment in IL-17RA sufficient mice are warranted.

It is also important to consider that although oral ulcers in WT and *Il17ra*$^{-/-}$ mice started to heal at the same time, peak damage was more severe in mice deficient in IL-17RA (**Supplementary Figure 1** and data not shown). This has important implications for patients that develop OM, since often it is the severity of disease that dictates termination or alteration of cancer therapies (2). It is critical to consider how the function of IL-17 in OM could also complicate targeting the Th17/IL-17 pathway to treat the associated malignancies. Because of the tumorigenic properties of IL-17 in the head and neck, it follows that the blockade of these cytokines in combination with other therapeutics might be efficacious in treating malignancies (70). However, our findings would indicate that anti-IL-17 therapy exacerbates OM symptoms, complicating the therapeutic benefit of this strategy to fight head-neck cancers.

Our finding that treatment with α-IL-17A antibody rendered mice as susceptible to OM as *Il17ra*$^{-/-}$ mice, may have implications for individuals receiving therapies that block Th17/IL-17 related immune components. Indeed, a rare case of lichenoid mucositis has been reported with secukinumab (α-IL-17A monoclonal antibody) use (71). As the administration of anti-IL-17-related treatments increase, our study indicates that these patients should be monitored for development of mucositis. Susceptibility to severe OM is partly dependent on patient factors such as smoking and overall oral health at the start of treatment, while the genetic predispositions related to the incidence of OM are less understood. Genome-wide association studies have identified genomic loci pathways related to RT-induced OM, yet no associations with the Th17 cells or IL-17 have been identified (72). Further genomic studies considering other forms of cancer therapies may elucidate a role for IL-17 and other inflammatory pathways in OM and lead to better personalized treatment regiments (73).

In conclusion, we provide evidence that IL-17RA is important for protection during HNI-induced OM (**Figure 7C**). We propose that IL-17RA is critical for preventing excessive inflammation during the ulceration phase of OM. In the absence of IL-17RA other cytokines and chemokines that are in abundance amplify immune cell migration into the oral mucosae creating an environment of excessive inflammation and damage in the mucosal layer. Further, without IL-17RA a reduced proliferative capacity of the epithelium, along with increased apoptosis, leads to a breakdown in the maintenance of the mucosal layer. IL-17RA is necessary to prevent severe damage and promote healing after ionizing radiation, and this important contribution has implications for cancer therapies related to the Th17 pathway.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject PRJNA720631, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA720631.

## ETHICS STATEMENT

The animal study was reviewed and approved by the University of Toledo IACUC.

## AUTHOR CONTRIBUTIONS

Conceptualization, Funding Acquisition and Projec Administration, HC. RNA-Seq computational analysis, VB, ACS, CM. FACS analysis, DL. Head-neck irradiation, EP and AL. Histology, AS, JS-M and JD. Experimental Support, JS-M, JD, JK, DL, EA, AW, GM, PB, and SC. Experimental Design, HC and JS-M. Paper writing, JS-M, EP and HC. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.687627/full#supplementary-material

**Supplementary Figure 1 |** WT mice exposed to radiation develop predictable OM stages **(A)**. WT mice exposed to 22.5 Gy develop OM damage that peaks on Day 11 and is cleared by day 15. Data analyzed by ANOVA with Tukey's and represents at least 3 experiments **(B)**. Solid lines depict the boundaries for considering what portion of the dorsal (top) tongue a lesion was found: proximal (near the excision-site), middle, or distal (tip of the tongue). Dashed line indicates where the tissue was portioned for RNA-seq analysis. Lesion location distribution in **(C)** WT mice (n=51) and **(D)** *Il17ra* -/- mice (n=20). Data shown as means +/- SEM, (*P<0.05, **P<0.01,****P<0.0001).

**Supplementary Figure 2 |** Changes in expression of IL-24 after exposure to HNI. Expression of *Il24* on D11 following radiation. Data pooled from at least 3 experiments and analyzed by ANOVA with Tukey's. Representative IHC images of IL-24 protein expression. Unfilled arrows represent negative staining while filled arrows represent positive staining. **(C)** Expression kinetics of *Il1a* and *Il1b* following HNI assessed by qPCR. Results pooled from at least 3 experiments. Data shown as means +/- SEM, (*P<0.05, **P<0.01,****P<0.0001).

**Supplementary Figure 3 |** Gating strategy. **(A)** Gating strategy for neutrophils. **(B)** Gating strategy for necrosis, early apoptosis, and late apoptosis.

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REVIEW
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# Oral Versus Gastrointestinal Mucosal Immune Niches in Homeostasis and Allostasis

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Different body systems (epidermis, respiratory tract, cornea, oral cavity, and gastrointestinal tract) are in continuous direct contact with innocuous and/or potentially harmful external agents, exhibiting dynamic and highly selective interaction throughout the epithelia, which function as both a physical and chemical protective barrier. Resident immune cells in the epithelia are constantly challenged and must distinguish among antigens that must be either tolerated or those to which a response must be mounted for. When such a decision begins to take place in lymphoid foci and/or mucosa-associated lymphoid tissues, the epithelia network of immune surveillance actively dominates both oral and gastrointestinal compartments, which are thought to operate in the same immune continuum. However, anatomical variations clearly differentiate immune processes in both the mouth and gastrointestinal tract that demonstrate a wide array of independent immune responses. From single vs. multiple epithelia cell layers, widespread cell-to-cell junction types, microbial-associated recognition receptors, dendritic cell function as well as related signaling, the objective of this review is to specifically contrast the current knowledge of oral versus gut immune niches in the context of epithelia/lymphoid foci/MALT local immunity and systemic output. Related differences in 1) anatomy 2) cell-to-cell communication 3) antigen capture/processing/presentation 4) signaling in regulatory vs. proinflammatory responses and 5) systemic output consequences and its relations to disease pathogenesis are discussed.

**Keywords: mouth mucosa, intestinal mucosa, gastrointestinal tract, immune system, homeostasis, symbiosis, dysbiosis, allostasis**

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## INTRODUCTION

The digestive system is a portal of entry for microorganisms we live and have evolved to coexist within a delicate symbiotic and homeostatic balance that is required for several constitutive processes in the body. Of particular importance, the digestive system is fully covered by mucosal membranes in charge of food breakdown, nutrient absorption, and waste evacuation. As such, the immune system in humans has gone through a long process of self-directed learning of "supervised tolerance" to achieve an ideal mutualistic state with microbial guests. In doing so, the digestive

system has specialized its functions by adapting its anatomical features and compartmentalizing immunity with a wide array of different microbial communities along the whole digestive tract. The overall objective of this review is to contrast the current knowledge of oral versus gut immune niches in the context of mucosal immunology and systemic output within the wide spectrum of the digestive system.

## ANATOMY OF EPITHELIA-MALT IN ORAL AND GI TRACTS

The idea of a bidirectional axis between the mouth and the gastrointestinal tract (GI) is not recent. In fact, possible relationships between the occurrence of pathologies of infectious origin in the oral cavity and inflammatory bowel disorders have been explored for decades (1). Both the oral tract and the GI tract deal with the external environment because of their vital epithelial barrier function in the digestive system, and such function depends on the interrelation between the host and the microorganisms to regulate either homeostatic balance or pathological instability. Despite such similarities, there are certain anatomical peculiarities that allow the understanding of important differences in oral epithelia vs. intestinal epithelium responses to the environment (**Figure 1**).

### Gut Epithelium Barrier

The intestinal epithelium is the second most extensive physical barrier in the human body, just second to the skin (2). As a barrier, its function is to selectively allow the absorption of nutrients, prevent pathogen invasion, prevent loss of water and electrolytes, and allow the exit of waste (3). In addition, the intestinal epithelium is currently recognized as the central axis of mucosal immunity as it is estimated that the gut houses up to 70% of the

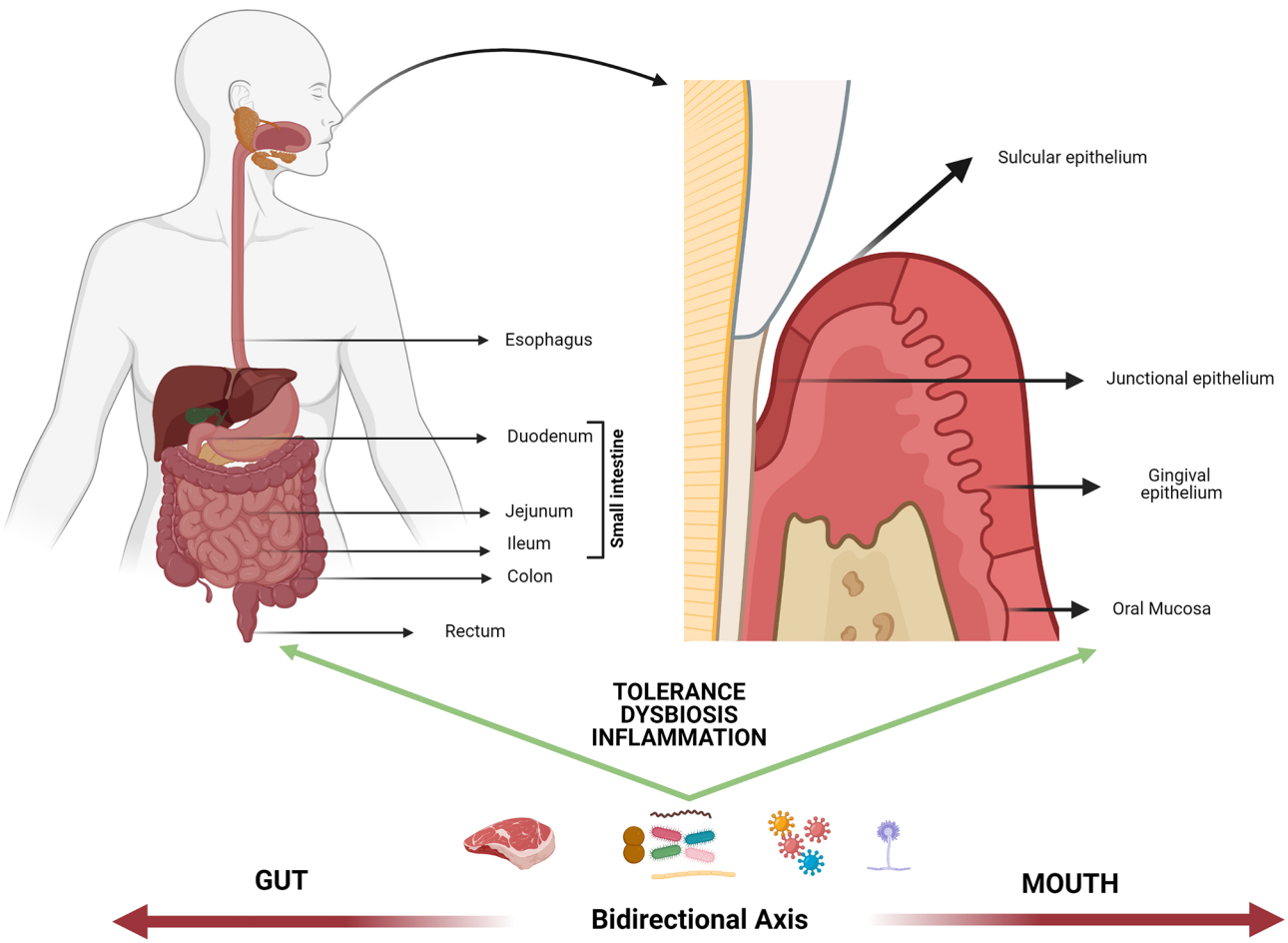


**FIGURE 1** | Mouth-gut axis. The plausible interrelation between the oral cavity and the rest of the gastrointestinal system could be mainly mediated by the response to potentially harmful self and/or foreign antigens (i.e., food, allergens, microbiome, trauma), extending beyond the control of tolerance mechanisms. Such mechanisms operate differently based on epithelia diversity of the mouth-gut axis and the direct interaction between the oral/intestinal microbiota and the host immune response, nonetheless they are thought to operate in the same immune continuum. The dysbiosis of microbial communities at both levels as well as the atopobiosis of microorganisms from the oral cavity to the intestine is seen today as a determining factor in the course of multiple inflammatory diseases.

body's lymphocyte population, making it the largest immune organ in humans (4). The structural organization of the intestine makes the immune response at the epithelial level different from immunity at the systemic level; such responses are grouped under the name of mucosal immunity. One of the most important characteristics of mucosal immunity is the presence of an inherent lymphoid tissue known as mucosa-associated lymphoid tissue (MALT), which in turn is covered by follicle-associated epithelium (FAE) (5). Although MALT are not unique structures of the intestine, as they are also found in the nasopharynx (NALT) and bronchial tissues (BALT) (6), intestine MALT are fundamental in responding to external antigens.

The intestinal epithelium is composed of IECs, which form highly organized structures (villi) and among these, other specific cells such as Paneth cells, Goblet cells, tuft cells, enteroendocrine cells, and M cells are embedded within. Paneth cells protect stem cells by means of antimicrobial peptides (AMP) release (e.g., alpha-defensins, lysozyme C, phospholipases, lectin type C and 3-gamma regenerative islet derivatives -RegIII-) (7). Goblet cells secrete mucins that lubricate and protect the intestinal surface of epithelial cells and also participate in antigen presentation, together with M cells (8). Tuft cells are in charge of chemosensing and enteroendocrine cells are hormone-secreting cells (9) (**Figure 2**).

The M or microfold cells are strategically located close to FAE areas. M cells are named for their irregular morphology in which the basement plasma membrane invaginates forming folds/pockets where these immunocompetent cells are housed (5). The epithelium of FAE, compared to the IECs, overexpresses more than 2 times multiple genes related to trafficking through the membrane, host defense and transcriptional regulation, especially ubiquitin D, tumor necrosis factor receptor superfamily 12a, and transmembrane 4 superfamily 4. This specific marker expression pattern differentiates M cells phenotype from IECs and is associated with their specific functions (10). M cells make up 10% of FAE cells and are specialized in taking antigens. M cell markers such as CCL9, Sgne-1, GP2, β1-integrin, PrP, dectin-1, claudin-4, and CD155 have been reported; these usually function as receptors for the different sensing particles (5, 11). M cells depend on RANKL for their differentiation, which is selectively expressed in subepithelial stromal cells in the domes of Peyer's patches (12). The high interaction of M cells with luminal antigens is due to a reduction in electrostatic repulsion that increases their

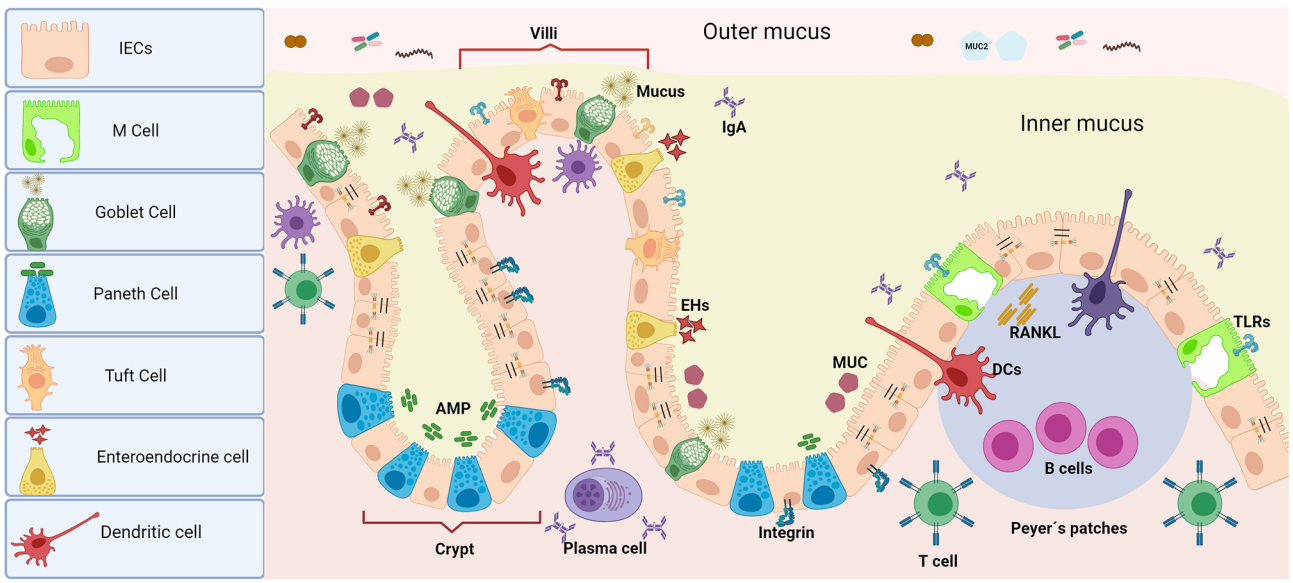


**FIGURE 2** | Intestinal epithelium barrier. Unlike oral tissues, the presence of MALT is one of the most important characteristics of mucosal immunity at the intestinal epithelia. The presence of multiple cell types with diverse functions assembled in the intestinal epithelium makes it a highly efficient chemical and physical barrier. In addition to secreting antimicrobial peptides, IECs or absorptive enterocytes control antigen persistence on the surface by epithelial shedding. Paneth cells (which are secretory by nature) are the main source of antimicrobial peptides, which gives them a decisive role in the maintenance of gut-microbiome homeostasis as well as procuring highly efficient turnover of the epithelium. Goblet cells (which are modified epithelial cells) secrete mucins in charge of lubrication and mucus protection, as well as present antigens to CD103+ DCs, complementing the function of M cells. Together, both Goblet and M cells carry out immunosurveillance and transport antigens from the intestinal lumen (from macromolecules to micromolecules) to either activate tolerance or induce an immune response along with specific resident DCs in Peyer's patches. Tuft cells mainly act as chemosensors, but they are also recognized as immune effector cells with immunomodulatory potential as they have the ability to produce cytokines (including IL25) and play an important role in the expansion of innate lymphoid cells (ILC2). Finally, enteroendocrine cells are responsible for the secretion of enterohormones which are in charge of the body's response to food as well as modulating physiological events inside and outside of the intestine (i.e., glucose tolerance modulation). The function of the barrier against microbial invasion is completed with a close control of the paracellular permeability given by intercellular junctions of different types, whose structural protein complexes are decisive in their function.

interaction given through the fucose residues of a glycoprotein coat in M cells (11). M cells also appear to be able to discriminate bacterial antigens and maintain the integrity of the barrier function of the intestinal epithelium through TLR2 (13).

In addition to the presence of multiple phenotypically and functionally different epithelial cells at the level of the intestinal barrier, protection against microbial invasion is also prevented by a close control of paracellular permeability provided by intercellular junctions of different types. Tight junctions, adherent junctions, desmosomes, and gap junctions are lateral structures involved in cell-to-cell adhesion and intracellular signaling (14). The permeability of the epithelium varies throughout the intestinal tract and the composition and abundance of different components of the tight junctions is decisive for their function. Tight junctions are composed of different transmembrane multiprotein complexes that are located in the apical part of the IECs; preventing the passage of large molecules and lipids while allowing the diffusion of ions, water and small compounds through their interaction with actin from the cytoskeleton; aided by adherent junctions. This is known as the apical junction complex. Desmosomes and gap junctions are located below the apical junction complex, and their function is to mediate intercellular adhesion and communication between adjacent epithelial cells (15). Tight junctions function is controlled by signaling molecules such as protein kinase C, mitogen-activated protein kinases, myosin light chain kinase, and Rho GTPases. Intestinal bacteria or food can differentially activate signaling pathways by changing the expression and distribution of tight junctions proteins, thus regulating the function of the intestinal barrier (16). The inter-relationship between the tight junctions and the underlying connective tissue in the lamina propria occurs through the integrins of the basolateral membrane of the epithelium that are attached to the extracellular matrix (14).

The function of the tight junctions is also complemented by that of the adherent functions, which express the proteins E-cadherin, N-cadherin, A-catenin and B-catenin. Such proteins mediate the migration speed of crypts to villus, suppression of proliferation, induction of apoptosis in crypts as well as the rate of differentiation of absorptive cells (17). TLR2 can also mediate intestinal epithelial resistance through redistribution of tight junctions proteins such as zonula occludens-1 (ZO-1) and occludins, thus altering the interaction with microorganisms and metabolites (18). Due to the location of the tight junctions (at the junction between the apical and basolateral plasma membranes), they can also regulate epithelial polarity through paracellular and transcellular pathways that allow the movement of substances to and from the lumen with concomitant energy savings (15). The function of cells and their intercellular junctions is also complemented through the production of an intestinal microclimate, which consists of an undisturbed water layer, the glycocalyx, as well as the mucus layer in which mucins play a fundamental role.

Mucus is mostly secreted by Goblet cells, viscous in consistency and enriched in mucin glycoproteins that form large polymer networks. Mucus secretion is regulated by the host upon detection of intestinal microorganisms or their metabolites (e.g., short chain fatty acids (SCFA) or Th2 cytokines) (19). Goblet cells also participate as luminal antigen presenting cells to CD103+ dendritic cells (DCs), which promote the development of regulatory T cells (Tregs) (20). The organization of mucus is different in all tracts of the intestine. Mucus in the large intestine presents as an external thick layer with abundant bacteria which is not very adherent and an internal "sterile" layer. This mechanism helps trapping bacteria, thereby increasing exposure to defensins and lysozymes (21) as well as separating microorganisms from the epithelium while conferring protection from digestive enzymes. The microbiota residing in the outer gut layers can promote the growth of pathogenic strains but also stimulate biochemical pathways that preserve the structure and function of the intestine. For example, Mucin 2 (MUC2) specifically protects the epithelium from inflammation and thus from multiple disease exacerbation (3). Microorganisms in the outer mucus layer use MUC2 as an energy source, which in turn can lead to mucin-degrading bacteria expanding in the microbiome, thus increasing the degradation of internal mucus (22). MUC2 as well as other components of mucus (CLCA1, FCGBP, AGR2, ZG16, and TFF3) are secreted by Goblet cells that are considered not only as gate-keeping cells but also antigen presenting cells (APCs). MUC2 is a gel-forming mucin, but there are other transmembrane like MUC3, MUC12, and MUC17 that form the glycocalyx (glycolipid and glycoprotein network); they help a two-way communication between structural cells and cells of the immune system, as exemplified in the secretion of IL10. If bacteria can reach the inner mucus layer, they would encounter the compound glycocalyx before reaching tight junctions (23).

The lamina propria underlying the intricate epithelial barrier houses multiple cells, not only DCs but also gut-associated lymphoid tissue (GALT), which includes Peyer's patches, lymphocytes, and intraepithelial lymphocytes (24). Thus, the lamina propria participates not only in innate but also in acquired immunity through multiple effector responses (secretion of cytokines, IgA, chemokines, proteases, and hormones) mediated by the enteric nervous system, which also regulates intestinal propulsive motility (21).

The stomach and its mucosal layer are also part of the gastrointestinal system; however, unlike the intestine, the gastric mucosa plays a limited immune function as there is no associate-MALT tissues. Current evidence suggests that the gastric mucosa immunity functions in a layer-by-layer progressive mode through innate and adaptive immunity, while maintaining microbiome balance and homeostasis. When pathogens invade the gastric mucosa, both epithelial cells and innate immune cells begin to defend *via* innate immunity; immune cells are rather recruited *via* chemokine signaling and tissue infiltration (25).

## Oral Epithelial Barrier

Similar to the GI tract, the oral tract is a microenvironment in constant contact with pathogens of all kinds. As in the GI tract,

the oral mucosa is a barrier with the external environment and must sense any stimuli to ensure balance of the system (26). The oral mucosa serves as a physical barrier (due to the structural cells and their intercellular junctions in charge of paracellular permeability) as well as chemical barrier, but unlike the intestinal epithelium, oral epithelia are multilayered. Epithelia at the periodontal level include the oral mucosa, the oral epithelium (gingiva), the sulcular epithelium and the junctional epithelium, all of them with different characteristics that are decisive in their function both as a barrier and in their response to the interaction with the oral microbiome (**Figure 3**).

The junctional epithelium, despite being considered stratified epithelia, has just 2 functionally recognized layers: one basal and one suprabasal (27). Junctional epithelium is the tissue with the highest turnover of all oral epithelia (4-6 days) but with very limited differentiation. Junctional epithelium contains specific proteins and differentiation markers such as odontogenic ameloblast associated protein, follicular dendritic cell secreted protein, and cytokeratin 19 (a consistent marker of junctional epithelium) (28), in addition to filaggrin and transglutaminase (29). Lysosomal bodies can be found in large numbers in junctional epithelial cells (27). Unlike the oral epithelium, the sulcular epithelium does not contain a granular stratum but contains a greater number of layers with more differentiated cells than the junctional epithelium with slower proliferation (6-12 days) (29).

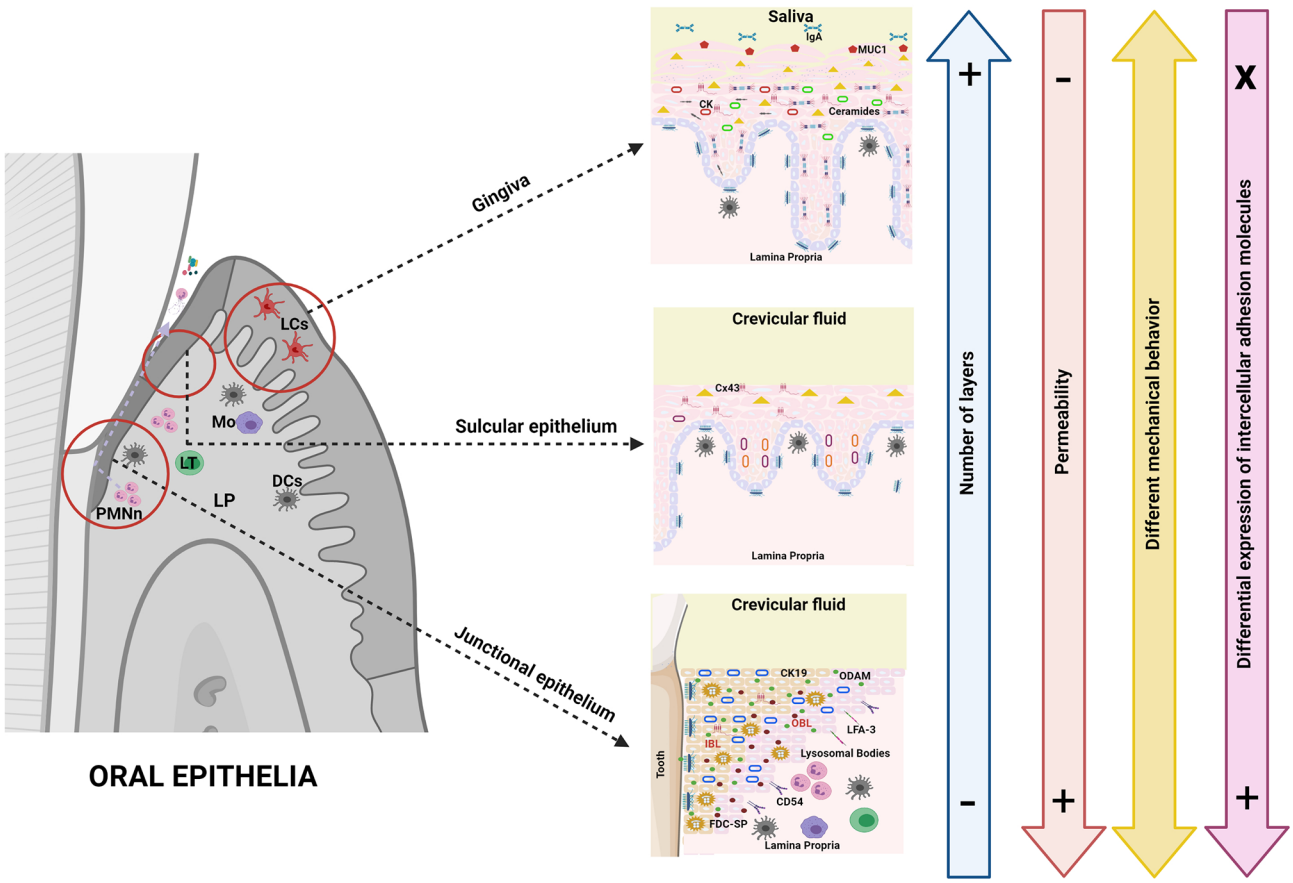


**FIGURE 3** | Oral epithelial barriers. Oral epithelia, unlike intestinal epithelia, are multilayered barriers which per se makes antigenic response highly diverse. This fact constitutes an important part of immune tolerance since it could prevent the activation of the response by the simple fact that antigens are not easily detected. The cells that conform the epithelial barriers in the oral cavity are of a similar nature to each other, thus the variations rather occur in complexity, level of differentiation and number of layers, as well as in the expression of conformational proteins of the intercellular junctions (all of the above determining greater or lesser permeability). In the oral cavity, chemosensor, hormone or mucus-producing cells are absent; rather, epithelial cells in oral barriers have different expression patterns of cytokeratins (even in non-keratinized tissues) that are important determinants of the mechanical behavior together with intercellular junctions. The cytokeratin/junction complex can lead to protein expression in the presence of inflammation, ergo modulating the response to different antigens. However, the role of tight junctions in the oral epithelia seems to be less decisive than in the GI tract. The expression of intracellular lipids (ceramides) is also different in oral epithelia; for example, cells of the junctional epithelium show a high production of AMP as well as expression of adhesion molecules that allow the transmigration of immune defense cells in the subgingival environment. Another major difference with the GI epithelia is the absence of MALT which is partly replaced in its function by the oral lymphoid foci. In fact, dendritic cells cannot sense antigens by extending their dendrites through cell junctions in multilayer oral epithelia. Finally, as in the GI tract, the expression of pattern recognition receptors (i.e., TLRs) in oral epithelial cells is highly dynamic, as such expression is inducible and differential against different microorganisms and/or antigens.

Keratinized epithelia (e.g., gingiva and hard palate) have, in addition to keratinocytes, a cornified envelope made up of different types of keratins. Such keratins are composed of extracellular proteins that provide mechanical resistance as well intracellular lipids (ceramides) that regulate permeability (30). In the stratum corneum, there are evenly spaced lamellae and within those spaces there are other disorganized lamellae and electrodense material (membrane lining granules), while in non-keratinized barriers there is a single broad lamella at the periphery of cells. Cholesterol is the main lipid in all epithelia, and ceramides are also part of nonkeratinized epithelia, although in lesser amounts. The buccal stratum corneum is very similar to the stratum corneum of the epidermis in its lipid content and its permeability, but in the gingiva, there is a small portion of phospholipids, much more abundant in non-keratinized tissues and also characterized by low amounts of ceramide 2 as well as abundance of glucosylceramides. In the stratum corneum of the gingiva, the predominant lipids are triglycerides, phospholipids, and glycolipids and their proportions differ with respect to the stratum corneum of the epidermis (31). Therefore, keratins are very important to determine the mechanical behavior of cells and help together with intercellular junctions as support for the barrier in different layers of epithelia (32, 33).

Regarding the underlying layers in the gingiva, the basal layer is composed of mitotic cells that are constantly renewed, which in turn repair the integrity of the barrier (34); whereas in the junctional epithelium, the apical and the basal lamina continues with the basement membrane. Basement membranes are specialized extracellular matrices including collagen types IV and VII, laminin, heparan sulfate proteoglycan, fibronectin, nidogen (entactin), and the proteoglycan perlecan that interpose between connective tissues and the epithelium, endothelium, muscle fibers, and the nervous system. These, in addition to compartmentalizing, participate in selective permeability and molecular screening, cell polarization, migration, adhesion, and healing (27).

As in the IECs, keratinocytes of the oral gingival epithelium are held together by tight junctions and adherent junctions while their attachment to the basement membrane is held by hemidesmosomes (26) these junctions confer paracellular permeability to the oral mucosa, by differential expression of proteins in a similar way to the intestine. There, both desmosomes and adherent junctions function as anchoring/adhesion structures and the expression of conformational proteins varies according to the type of epithelium. For example, there is a high expression of desmoglein 1 and 2 in the cell-cell contacts in the superficial layers in gingival/sulcular epithelium, but not in junctional epithelium. E-Cadherin, a main transmembrane protein of the adherent junctions can be found in the spinous layer of the oral and in the sulcular epithelium but not in the junctional epithelium (35). Thus, it seems that the junctional epithelium has few desmosomes with desmoglein 3 as the main component where adherent junctions are absent.

Connexin 43 (the main protein associated with gap junctions) is related to the activation of calcium hemichannels permeable to small molecules such as metabolites, ions, and intracellular signaling molecules (e.g., ATP, ADP, cAMP, amino acids, small peptides, glucose, inositol triphosphate, cyclic nucleotides, and oligonucleotides) (36). Connexin 43 can be found in the spinous layer of the oral and sulcular epithelium, as well as in some parts of the junctional epithelium, whereas claudin 1 and occludins can be found in the superficial layers of the gingiva. In both oral and sulcular (sulcus/pocket) epithelium, the expression of E-cadherin and involucrin (a key marker of differentiation in stratified epithelia) is decreased in the presence of inflammation, which is associated with marked alterations of actin filaments, indicating advanced damage to the epithelium structure and loss of barrier function (35). As such, all of these proteins support the barrier/antibacterial function properties at the oral/sulcular levels, but not at the level of the junctional epithelium (very few gap junctions can be found in the JE). Nonetheless, the cells of the junctional epithelium are excellent producers of defensins that can be stored in the wide intercellular spaces (37).

Paracellular permeability is based more on membrane coating granules (MCGs) than on tight junctions in stratified oral epithelia (38). In cultures of TR146 cells (a continued cell line of human buccal epithelial origin) have shown tight junctions that are not formed and zone occludens that are not seen (39). However, it has been hypothesized that the differential expression of the tight junctions repertoire in the oral cavity may be involved in the scarring process in this tissue. In a murine model, the expression of claudin 1 and occludin was analyzed and it was concluded that there are genes differentially expressed in oral tissues that can contribute to the mechanisms that lead to the expression of scar phenotypes in response to injuries (40). Keratin also connects to actin through adherent junctions and desmosomes forming a mechanical unit, while adherent junctions act as mechanosensors in the transduction of mechanical forces between the plasma membrane and the cytoskeleton of actomyosin. Desmosomes and intermediate filaments provide mechanical stability to maintain the architecture of these tissues constantly exposed to the action of mechanical forces.

As a chemical barrier saliva (given its high content of IgA, mucins, and antimicrobial peptides) plays a very important role, because of the lining of the oral mucosa. In the mouth, the main mucin is MUC1, which is found in the superficial layer of the oral epithelium and will bind to salivary proteins to form salivary films (41). Noteworthy, it has been determined that MUC1, like MUC4, can also play a role in signal transduction in the immune system. For example, MUC1 through its binding to the B-catenin of the adherent junctions and MUC4 (intramembrane ligand for a receptor tyrosine kinase -ErbB2/HER2/Neu-) activate phosphorylation and initiate corresponding signaling, acting as an important sensor mechanism in response to invasion or damage of the epithelium (42). In the oral mucosa, the membrane-anchored mucin film (MAM) is a mixed film of salivary and epithelial components, and the epithelial cells underlying this protein layer have ridges/folds in the cell membrane called microplicae (MPL), typical of the epithelial cells that are covered by mucus (e.g., esophageal mucosa). MPL

vary according to the epithelium of the oral cavity in its morphology: straight, parallel, curved and branched MPL, and honeycomb. Non-keratinized epithelial cells have surfaces with parallel or branching MPL, while keratinized epithelial cells have honeycomb appearing MPL. The exact function of MPL in the oral mucosa is not known but it is thought that they provide mechanical support, maintain the mucus layer, increase the contact surface of the cell with the external environment. This in turn maximizes the absorbance of metabolic products through the external membrane and with the glycocalyx, protects from the entry of bacteria, forming the barrier complex of the oral mucosa with highly glycosylated proteins active in bacterial aggregation. They have also been linked to drug absorption (43).

At the structural level, it is also important to report the expression of intercellular adhesion molecules (ICAM) at the junctional epithelium level. For example, the expression of the carcino-embryonic Ag-related cell adhesion molecule 1 (CEACAM1 mediates cell cohesion) is high in junctional epithelium, which is important during antigen recognition since CEACAM1 functions as a surface receptor for different bacteria, interacting with infiltrating PMNs or T cells and leading to structural alterations to facilitate the immune response (44). Similarly, the expression of intercellular adhesion molecule-1 (ICAM-1 or CD54) and lymphocyte function antigen-3 (LFA-3) is high in junctional epithelium, which supports its important role in inflammatory reactions at this level (27). In sum, the modulation of epithelial barriers at the periodontal level is widely related (although it is not the only factor) to its interaction with microorganisms, which could contribute to the initiation and progression of diseases in the oral tract.

# EPITHELIA/MALT INTERACTIONS AND THE FATE OF THE IMMUNE RESPONSE IN THE UNDERLYING LAMINA PROPRIA

## Effector Responses in Intestinal and Oral Mucosa

MALT were described more than 40 years ago as organized clusters of B cells and large tissue lymphoid aggregates in which traffic originates to the effector sites in the mucosa (45). MALT has the main function of protecting the host from pathogens but without breaking the integrity of the barrier. For this reason, it is crucial that its activation occurs against antigens that are potentially dangerous while tolerance is exhibited against non-pathogens. MALT must also be activated against dietary antigens and those whose breakdown is related to the occurrence of inflammatory problems.

With the exception of plaque-induced gingivitis, overt inflammation of the oral mucosa is not common due to the highly efficient control mechanisms such as the epithelia which is in constant contact with "invaders" of all kinds (microbial, food, allergens, trauma). Conversely, specific sites of induction of immune responses in the intestine are abundant as they are found embedded in the epithelium, such as the Peyer's patches, mesenteric, and colon lymphoid nodes (46), housing B and M cells (also present in the nasopharynx) (47) which sense/pass antigens on to professional APCs (follicular DCs, macrophage, and B cells) and additional effector sites, like the intraepithelial lymphocytes in the villi over the lamina propria (48). In fact, there is no MALT in the periodontal tissues, so it is thought that the role of DCs is crucial to process antigens, maturate, migrate to the basal lamina, and present to T cells in structures called oral lymphoid foci. Therefore, the intestinal epithelium has clearly differentiated response-inducing sites while these are absent or at least not characterized in the oral epithelia (49). It is considered that these responses for the oral epithelium rather take place in oropharynx and NALT (50).

Intranasal immunization experiments have led to the understanding that the oral cavity also connects to the cranial and nasal-associated lymphoid tissues (CONALT), which have been proposed to be part of the cervical lymph nodes (CLNs) (51), and play an important role in the induction of immune responses (52). CONALT include the facial lymphoid nodules, parotid, and submaxillary gland (superficial cervical lymphoid nodules), as well as the deep cervical lymph nodules lying dorsal to the brachial plexus in the neck musculature. The lymphoid tissue of Waldeyer's pharyngeal ring, including the adenoids (the unpaired nasopharyngeal tonsil) and the paired palatine tonsils are also part of the CLNs/CONALT in humans. CONALT help the spread of activated lymphocytes so local responses can be activated. This is facilitated by the expression of homing and addressin receptors that mediate the binding of lymphocytes to high endothelial venules of CONALT, whose expression is differential according to the migration site. For example, peripheral node addressin and L-selectin mediate lymphocyte trafficking to CONALT, addressin cell adhesion molecule-1 (MAdCAM-1) and $\alpha4\beta7$ to cervical nodules and VCAM-1 mediate trafficking to lymphoid nodules in parotid gland. Other authors have called this Cranial-oral and nasal-associated lymph nodes CONALNs, or just cervical lymph nodules given the absence of sensor M cells or organized lymphoid structures and the fundamental role of regional lymphoid nodules in the induction of immune responses (53). In sum, the induction of mucosal immunity at distant sites by intranasal immunization proves the existence of a common mucosal immune system (52).

Oral lymphoid foci are expressed under inflammatory pathological conditions in periodontal tissues, and are foci of T, B, and DCs infiltration. They contain Langerhans cells (LCs), $T\gamma\delta$ cells in the epithelium and double-positive maturing CD1a + CD83 + LCs in the lamina propria just under the basement lamina, which could suggest the local presentation of antigens at this level, in addition to the participation of the CONALNs in tolerance induction (49, 54).

## Receptor Engagement in Oral and GI Tracts

Innate immune sensing represents the most ancient form of self/non-self-discrimination (55). For this, evolution has developed a wide array of cellular receptors that recognize more diverse viral,

bacterial, fungal, and protozoan surface components, as well as endogenous molecules arising from either constitutive host cell functions or tissue damage coming from trauma or inflammation (56). These innate immune receptors are quite abundant in host cells with many pleiotropic associated pathways, so dissecting each specific agonist/receptor-mediated responses in both the oral and gut tracts is out of focus for this review. Instead, the most important and general pathogen substrate/to/receptor mechanisms in the context of oral and GI innate immunity are reviewed.

## Toll-Like Receptors (TLRs)

TLRs are a family of transmembrane proteins that have a primary role in pathogen recognition and innate immunity initiation (57). TLRs receptors bind to several microbial components or end-products known as microbial-associated molecular patterns (MAMPs), which include peptidoglycans, lipoteichoic acid, flagellin, double-stranded viral RNA, unmethylated bacterial DNA and lipopolysaccharide (LPS), among others. Currently, 10 human TLRs have been identified, including extracellular as well as intracellular receptors (58). After binding and recognition, TLRs are able to trigger an array of signaling pathways that ultimately activate downstream molecules such as nuclear factor kB (NFkB) and interferon regulatory factor 3 (IRF3) (59), which in turn mediate the expression of several proinflammatory cytokines as demonstrated in several tissues. TLRs are highly involved in responding to inflammatory processes in the presence or absence of infections and are thought to be a critical component of the innate immune response. Their flexibility in recognizing epitopes is facilitated by both physical/structural features, as well as interactions with additional innate immune receptors, soluble molecules, and subcellular trafficking mechanisms (56). In addition to TLRs expression in cells of the immune system such as macrophages and DCs, TLRs are also present in non-immune cells such as epithelial cells, keratinocytes and oral/GI mucosae, making TLRs remarkably important (60).

Oral epithelium acts as the first barrier against invaders of the oral cavity. As such, epithelial sentinel function is of key importance in maintaining tolerance/homeostasis or initiating an immune response. mRNA of all 10 TLRs has been detected in oral epithelial cells, but the actual expression and cellular localization of TLR proteins varies per anatomical location and is inducible under different situations (58). For example, oral epithelial cells do not show increased production of proinflammatory cytokines in response to bacterial components (61) but are rather capable of upregulating TLR and NOD receptors (62). These mechanisms suggest epithelial cells are capable of 1) preventing tissue destruction caused by excessive inflammatory reactions by means of tolerance (63) and 2) initiating and orchestrating an appropriate immune reaction when required. As such, TLRs contribute to the homeostatic relationship between bacteria and the host symbiosis, as previously shown in an *in vivo* model (64).

As the GI tract is heavily colonized by trillions of microorganisms, it is imperative to maintain structural/functional homeostasis during nutrient absorption, commensal/pathogen control and immune regulation crosstalk. As such, TLRs have important antimicrobial functions in preventing excessive responses towards commensal microorganisms as well as activating a strong immune response when appropriate (65). TLR1–TLR9 are expressed in different cell types in the gut, including IECs, immune cells and non-immune parenchymal/stromal cells (66, 67). In particular, human IECs have shown mechanistic capabilities of tolerance and immune regulation *in vitro* similar to oral epithelial cells (68). TLRs are also key regulators of oral tolerance/sensitization in the GI tract. In addition to the commensals and pathogens residing in the gut, food products can often be contaminated by bacteria and/or fungi, so it is plausible that contaminating organisms can also shape oral tolerance to contaminated foods *via* TLRs (69).

TLR signaling is facilitated by the intracellular Toll/IL 1 receptor (TIR) domain (sharing structural homology with the IL1 receptor). The domain itself contains TIR-containing cytoplasmic adaptor molecules (Myeloid differentiation antigen 88 -MyD88-, TRIF) that initiate signaling after dimerization by recruiting additional signaling molecules that ultimately activate the expression of numerous immune response genes through activation of AP-1, NFkB and other transcription factors. MyD88 is an example of a cytoplasmic adaptor protein that drives NFkB translocation into the nucleus and consequently the production of proinflammatory molecules such as prostaglandin E2, leukotriene A4, tumor necrosis factor-alpha (TNFα), IL1β and several chemokines (CXCL8/IL-8, CCL2, CCL3 and CCL5) (56, 70).

## NOD-Like Receptors (NLR) and the Inflammasome

Leucine-rich repeat-containing receptors (NLRs) consist of about 20 related family members of cytosolic receptors that mostly recognize intracellular ligands. NLRs can be classified into molecules that contain either a CARD (Caspase recruitment domains, or caspase activation and recruitment domain) or a Pyrin motif (71). Nucleotide-Binding Oligomerization Domain Receptors (NOD) CARD proteins mediate NFκB activation, whereas Pyrin molecules (e.g., NALP3) regulate IL1β and IL18 production. NOD receptors recognize peptidoglycan in gram positive bacteria and muramyl dipeptides in gram negative bacteria (58). These receptors are also thought to play a role as antibacterial factors.

Oral epithelial cells express NOD *in vivo* and *in vitro*, playing an essential role in mucosal innate immunity (63). NOD expression is linked with B-defensin production in epithelial cells, which makes them ideal "antibacterial" receptors. In fact, it has been shown that TLR/NOD synergism in oral epithelial cells leads to antimicrobial peptide production instead of proinflammatory cytokine production, reinforcing the theory of "measured" responses to oral bacteria (72, 73).

NLRs also encompass a large number of innate immune sensors and receptors in the GI tract. Similar to oral epithelial cells, several NLRs have been found to restrain, rather than activate, immune signaling and subsequent proinflammatory cytokine production. Noteworthy, while some NLR family members, such as NOD1 and NOD2, can cause activation of

these pathways in response to stimuli in the gut, select NLRs, such as NLRX1, NLRC3, and NLRP12, act as negative regulators of inflammatory pathways, adding to the complexity of NLR biology (74).

NLRs binding and downstream signaling can elicit an inflammatory reaction by means of cytokines, chemokines and antimicrobial peptides production. Some products can be proinflammatory (IL6, IL8, TNFα) while others can display immunoregulatory or antimicrobial properties (interferon gamma, IFNγ and human β-defensin-1, hBD-1). Importantly, one major function of NLR proteins involves the modulation of inflammatory signaling pathways, including NFκB and MAPK (58). Also, another well-defined function of some NLRs (NLRP1, NLRP3, NLRC4, and NLRP6) is the activation of a multi-protein complex, known as the inflammasome.

The inflammasome is a large multiprotein complex that recognizes a wide array of microbial, stress and danger signals, triggering the maturation of proinflammatory cytokines, including IL1β and IL18 and promoting innate immunity (75). NLRs regulate caspase-1 activation as the initial step of inflammasome formation. Even though caspases are cysteine proteases that are mostly known to regulate cellular death *via* apoptosis, they also facilitate proinflammatory cellular processes. As such, caspases can be categorized as either proinflammatory or proapoptotic (76, 77).

### Protease-Activated Receptors (PAR)

PAR receptors are a family of four G protein-coupled receptors that sense proteases. Proteases, or proteolytic enzymes, are majorly involved in all signal transduction events in both homeostasis and disease, as they can be derived from circulation (coagulation factors), inflammation (mast cells/neutrophils) or from other multiple sources (epithelial cells, neurons, bacteria, fungi); as such, proteases can act at the cellular surface level by generating or destroying receptor agonists as well as activating/inactivating surface receptors, thereby making the PAR system of vital contribution for a "backup" signal transduction events (78). Much is known about PARs functions, however understanding the specific role of proteases in physiology and pathophysiology remains largely unknown.

PAR family members PAR1 and PAR2 are highly expressed in the oral periodontium. These receptors participate in periodontal tissue metabolism by regulating inflammation and repair processes through activation of endogenous factors and/or bacterial enzymes (79). Interestingly, the keystone pathogen *Porphyromonas gingivalis* (*P. gingivalis*) is known to produce gingipain enzymes which specifically cleave PARs as part of its immune evasion mechanisms (80). Also, the abnormal activation of PAR2, also known as coagulation factor II (thrombin) receptor-like 1 (F2RL1) drives several pathophysiologic processes in the oral cavity, including oral cancers, as PAR2 promotes oral squamous cell carcinoma growth/progression *in vitro* (81).

PARs are highly expressed in the GI tract while PAR2 is the best studied. The most probable reason for the high expression of these receptors is the fact that the GI tract is continuously exposed to a wide variety of housekeeping or bacterial proteases, like digestive enzymes or proteinases, respectively. PARs play important roles in enterocyte function, intestinal ion transport, GI motility and exocrine secretion, and act as effectors of intestinal inflammatory disorders (82). Like most GPCRs, PARs couple to multiple signaling pathways and can thereby regulate many cellular functions such as cellular proliferation, migration, secretion, adhesion, and transcription.

## Antigen Capture/Presentation

DCs are considered to be the most important actors of antigen presentation at the cellular level in both the oral and GI barriers. DCs must not only attain defense against the invasion of pathogens but must also limit the immune response even after the encounter with the antigen. Therefore, the benefits that can be obtained from the encounter with commensals or from the intake of nutrients are not altered by an uncontrolled activation of the host immune system (49). DCs are specialized APCs which control a spectrum of innate and adaptive responses (83). DCs are a heterogeneous population of cells, distinguishable by surface, intracellular phenotypic markers, immunological function, and anatomic distribution (84). However, DCs have recently been defined as cells of the hematopoietic system on their own (85). Dcs are found throughout the lymphatic system but also in non-lymphoid tissues such as skin and mucosal surfaces (85), which are the most common sites of entrance for microbial pathogens (84).

DCs have been classified into five main cell types: conventional DCs type 1 and 2, monocyte-derived DCs, LCs and plasmacytoid DCs. The establishment of transcriptomic similarities between mouse and human DCs types lead to the creation of this classification (86). Each subtype resides in a different part of the body and has specific roles in the immune response (85). In most tissues, DCs are present in an immunologically immature state as immune sentinels poised to respond (87). These DCs lack the necessary accessory signals for T cell activation. Nevertheless, they are well equipped with pattern-recognition receptors (PRRs) both on and within many immune cells in the peripheral tissues of both GI and oral tracts (88). DCs capture and process antigens to form MHC–peptide complexes *via* endocytic pathways such as phagocytosis or macropinocytosis (85). DCs are also in constant movement throughout their life cycle (85), because their ability to migrate throughout the body is a critical aspect for the initiation of immunity (87). DCs also play an essential role for the discrimination of antigens, leading to autoimmune disease (89).

DCs are widely distributed residents in the oral epithelium. Myeloid DCs and LCs are the most common phenotypes, expressing CD1a and CD207 (LCs specific lectin Langerin) similarly to their intestinal counterparts. However, oral and GI DCs differ in the expression of costimulatory molecules B7.1 (CD80) and B7.2 (CD86) as well as other myeloid markers such as CD11b (49). The amount and distribution of DCs varies in the oral mucosa depending on the site, with less presence in the gingiva and the sublingual region than in other areas of the mouth (90). Oral DCs express significantly more MHC class I and II and CD40, as well as FcγRIII/CD16 and FcγRI/CD64

receptors. They express a high affinity receptor for IgE (FcεRI) even in non-atopic individuals, indicating high allogeneic stimulation. Therefore, DCs contribute beyond responses to infections in the oral cavity, such as response to allergens, for example (91). Interestingly, it is possible to find alterations in the amounts of DCs of different types in lichen planus (92) and oral carcinomas. Immature DCs (CD1a+) and LCs (CD207+) are significantly decreased in oral submucous fibrosis and oral squamous cell carcinoma, when compared to a normal oral epithelium. Additionally, an increase in plasmacytoid DCs (CD303+) was reported in oral squamous cell carcinoma, indicating possible relationship with the development of these pathologies (93).

Antigen-presenting DCs in the intestinal mucosa mostly remain in an immature state in the presence of commensals but encounter with new, potentially pathogenic antigen can initiate an active immune response. DCs in GI mucosal tissues are somehow instructed by IECs to suppress inflammation and promote tolerance, but DCs that are not "tolerated" are recruited to from Peyer's patches and circulating blood to the insult site, consequently initiating an inflammatory response. In doing so, the differential response of IECs to both commensals and pathogens controls DCs-mediated responses, while activated T and B cells in Peyer's patches are marked to return to the intestine given DCs ability to promote on-site antigen presentation through upregulation of integrin 47 and CCR9 (94). Additionally, these Peyer's patches DCs promote IgA secretion by inducing B cell differentiation (95). Such mechanism has not been reported in the oral cavity although it has been assumed that absence of increased DCs recruitment in the oral cavity could prevent over-activation at the level of the oral mucosa (49). Remarkably, *in vitro* co-culture experiments with oral epithelial cells and DCs in the presence of gram positive and gram-negative bacteria does not lead to DCs maturation, as evidenced by low MHC II, CD80 and CD86 expression with very little IL12 and TNFα when compared to non-activated DCs. Similarly, DCs in co-culture were not able to stimulate allogeneic naive CD4+ to produce INFγ and TNFα, when placed under these conditions or in the presence of Th1, anti-CD3 and anti-CD28 cells. This is thought to be one of the strategies of oral epithelial cells to avoid hyper reaction of the immune system against resident bacteria, through the inherent ability of this epithelial barrier to suppress immune responses (96). The other strategy is related to DCs allowing T cell clones to acquire memory by persisting long-term periods, tolerating self-antigens, or responding rapidly upon re-exposure to noxious antigens (83).

Macrophages are also critical antigen-presenting cells with important roles in both tissue homeostasis and inflammation of the oral and gastrointestinal tracts, as phenotypic M1 (proinflammatory pathogen-killing) and M2 (cell proliferation/tissue repair) polarization in resident macrophages can be found in the gum/gut axis (97). For example, M1 has been associated to chronic periodontitis whereas M2 is associated to health/gingivitis stages (98); nonetheless M2 has also been associated to chronic infections (99). Similarly, the GI tract is one of the most macrophage-dense organs. In a surveillance state, colonic macrophages exhibit a M2-like phenotype supported by CD206 +/CD163+ phenotype, IL-10 production, promoting epithelial cell proliferation/regeneration and promotion of Tregs proliferation; whereas several mouse IBS studies show that M1 proinflammatory macrophages dominate the large intestine during experimental colitis (100). In this regard, oral and gut are remarkably similar, however, there are clear imbalances in M1/M2 ratio responses as these are heavily influenced by the immune microenvironment.

## Adaptive Immunity in Oral and Intestinal Immune Niches

Microbial processing and clearance are essential for appropriate immune response and efficient disease resolution. DCs take up pathogens and/or antigens by means of receptor mediated phagocytosis and process these to be presented to other cells of the immune system. Phagocytosis provides an opportunity for DCs to sense the nature of the engulfed "invader" so efficient intracellular routing to specific compartments tailors an appropriate immune response (101). Lysosomal degradation then takes place through multiple mechanisms including the endocytic/phagocytic pathway (102), macroautophagy (103), microautophagy (104) and chaperone-mediated autophagy (105). Interestingly, such pathways can be subjected to bacterial exploitation (106).

Some differences between oral and gut can be described in the context of antigen presentation and immune consequence. For example, the oral mucosa is considered to be very tolerogenic in spite of continuous exposure to a wide array of antigens (commensal or pathogenic) or mechanical trauma (107), as evidenced by the fact that severe inflammatory responses in the oral cavity are relatively rare (49, 108). Furthermore, a distinctive arrange of tolerogenic Dcs compartmentalized in different locations in the oral epithelium that induce regulatory CD4 + T cells has been described, making the oral tract an ideal site for inducing tolerance (109). In the case of food allergies are mostly mediated by a type 2 immune reaction to dietary antigens processed by gut APCs (primarily different types of DCs) leading to the initial priming of T cells and the production of food specific IgE antibodies. Thus, DCs (along with monocytes and macrophages) dictate gut tolerance to allergy by shaping the T cell and subsequent B cell antibody response (110).

How DCs know where to go specifically continues to be a mystery, and to date this is still under investigation (87). Upon activation, DCs switch their behavior from endocytosis towards migration (85). Throughout this journey migrating DCs must adapt their motility skills to reach their target destination, including the capacity to traverse a wide variety of tissue types and across many anatomic barriers, recognize and adhere to specific microvascular endothelial cells, sense and follow chemoattractant signals, and interact with lymphocytes and other immune cells to allow the exchange of critical information regarding the antigens-presenting process (84, 85).

DCs undergo cytoskeletal changes to optimize DCs motility that results in fast migration (85). Expression of the G-protein

coupled receptor CCR7 located on the surface of DCs enhances their migratory capacity towards the lymphatic tissues (87). CCL19 and CCL21 are chemokines expressed by peripheral lymphatic endothelial cells that guide DCs to downstream lymph nodes (84). The interaction between the chemokine CCL21 and its receptor CCR7 is crucial for the migration of activated DCs (85). Most chemoattractant signals result in integrin activation, which causes firm adhesion and arrest of the cells leaving the blood vessels *via* diapedesis (85). A variety of cytokines and chemokines are released in response to bacterial products. Granulocyte-macrophage colony-stimulating factor (GM-CSF), TNFα, and IL1 have been described to promote DCs movement and maturation (87). Many molecular processes take place in DCs migration but the most important factor for DCs arrival at the lymph node is the chemokine-mediated guidance (85).

Once into the lymphoid tissues, DCs may complete their maturation, release chemokines that attract B and T lymphocytes, and maintain the viability of recirculating T cells (87). In the words of Ralph Steinman -the discovery of DCs- the maturation process of DCs is a critical link between innate and adaptive T cell-dependent immunity (111). Innate immunity includes rapid reactions to infection and is not specific to a particular pathogen (83). Adaptive immunity is acquired more slowly (days to weeks) and includes highly specific responses for antigens that are sustained long-term to develop improved function upon re-exposure to the antigen (83). Nonetheless, adaptive immune responses can be both immunogenic or tolerogenic (112).

Maturating DCs both activate and expand T helper-cells (Th), controlling many T cell responses (87). These Th cells then exert both inflammatory and regulatory responses (112). For example, DCs induce different types of CD4+ T cells such as Th1, Th2, or Th17 to produce powerful cytokines, which increase resistance to infection (83). The balance between subsets of T cell effector populations has been determined to be important in the quiescence as well as the progressive stages of inflammatory diseases. IL17 is a pro-inflammatory cytokine secreted by CD4+ Th17 cells that stimulates the recruitment of neutrophils and monocytes into inflamed areas (83). The role of Th17 cells is to maintain mucosal barriers and contribute to bacterial clearance at mucosal surfaces (88). In terms of regulation of the inflammatory response in the oral epithelium, there is ample evidence demonstrating the key role that Th17/Treg balance can play in the pathology of oral diseases, such as periodontitis (as in the proportion of Th17 and its products in gingival tissues can explain the severity of periodontitis) (113).

The role of the dysbiotic changes that guide Th17 subpopulations differentiation at the local level also has an impact at the systemic level, that could support the relationship between inflammatory periodontitis and other systemic diseases and could aid in diagnostic/prognostic testing (114). Recent studies have shown an association between IL17 and many inflammatory and autoimmune diseases such as systemic lupus erythematosus, sclerosis multiple, asthma and inflammatory bowel disease (IBD) (112). Alternatively, tolerogenic DCs can eliminate or block T cells, resulting in resolution of ongoing immune responses and prevention of autoimmune responses, which despite having been reported as widely prevalent in oral mucosa, seem to be related to non-pathologic responses and even represent non-recognized physiological functions as the immune removal of debris (115, 116). Alternatively, tolerogenic DCs can eliminate or block T cells, resulting in resolution of ongoing immune responses and prevention of autoimmunity (115).

The regulation of inflammation at all levels relies to a large extent on the cells that express the FOXP3 marker, which acts as a master regulator of the inflammation regulation pathways and the development of Tregs. FOXP3+ cells are generated in the thymus or induced in peripheral tissues, so their functions are different. Accordingly, thymic cells will be selected by their own antigens to control systemic immunity while peripheral cells are induced by antigens in the tissue and are related to local suppression (117). Current evidence suggests that Tregs in oral mucosa reach sites by migration and not by induction *in situ* (118). In addition, a high presence of FOXP3+ cells (Tregs) can be phenotypically different from those of lymphoid nodules or spleen, based on the differential expression of CD103+, CTLA-4, CD44+ and Neuropilin-1. The oral mucosa presents large amounts of different phenotypes of CD4+ cells with relatively known functions, unlike the intestinal mucosa where CD8+ cells predominate (119). Therefore, it could be speculated that the CD4+FOXP3+CD25+ phenotype in the mouth leads to protection against possible autoimmune reactions or against commensal organisms (120). In fact, it has been described in mice that these FOXP3 cells in oral mucosa are mainly in the lamina propria which enables such capability (119).

The process of inducing tolerance in the gut is not entirely clear but sensing of antigens by the immune system is thought to be involved. Immune responses vary if the antigen is captured from the lumen, through the gap junctions of goblet cells, versus if antigens are captured and presented by various APCs in the lamina propria (121–123). Most intestinal Tregs cells are Tr1, Th3 and CD4+CD25 + and are believed to interact with DCs while TrE CD8+ cells recognize antigens presented by epithelial cells (124). This DCs-T cell interaction is crucial in the induction of tolerance, so if a naive T cell recognizes an antigen presented by an immature dendritic then it will not differentiate into an effector cell but rather into a Tregs. In addition to gap junctions, MUC2 of Goblet cells promotes tolerogenic properties in DCs that induce pTregs, including TGFβ production and retinaldehyde dehydrogenase expression. Glycans associated with MUC2 determine anti-inflammatory properties in DCs by assembling a galectin-3-Dectin-1-FcγRIIB complex that activates β-catenin that in turn inhibits the activation of NFkB by altering the expression of pro-inflammatory (not tolerogenic) cytokines (125).

Under inflammatory conditions, Tregs that conventionally produce IL10, TGFβ and IL35, can also produce pro-inflammatory cytokines such as INFγ and IL17A (based on their expression of RORγt) (126). It is not very clear whether they retain their suppressive capacity or contribute to

inflammation, as this depends on environmental signals, local presence of proinflammatory cytokines, and metabolites. Such phenomenon makes targeting Tregs therapeutically difficult (127). Tregs can also upregulate the expression of pro-inflammatory cytokines (IL-17A and IL-22) dependent on IL-2 stimulation in a murine model of infection by candida albicans (128), although this is still controversial (129). Furthermore, short chain fatty acids correlate with an increase in the frequency of Foxp3 +, IL-17A +, and Foxp3 + IL-17A + double positive (Treg17) in lingual tissue and oral draining lymph nodes after depletion of resident bacteria by the antibiotics use, highlighting the role of the resident microflora during mucosal infections (130).

In addition to the regulation of inflammation by the induction of Tregs, DCs (like most cells) release extracellular vesicles that are an important part of immune regulatory mechanisms, participating not only in antigen presentation but also in cell-to-cell communication (131). Extracellular vesicles are small nanometric molecules that are divided into three groups according to their biogenesis: apoptotic bodies, microvesicles, and exosomes (132). All cells, including DCs can use this mechanism to carry out exchanges of proteins, lipids, nucleic acids, signaling molecules, and modulating responses (133). For example, during an infection with adherent-invasive *Escherichia Coli* (associated with Crohn's disease), exosomes secreted by infected cells can have an impact on the innate immune responses of surrounding cells to infection at the intestinal level, which can in turn be amplified by the same exosomes and contribute to associated diseases. In fact, exosomes have been suggested as diagnostic markers and therapeutic targets to both diagnose and treat intestinal inflammation (134). Interestingly, exosomes isolated from infected DCs have the ability to reprogram immune cells responsible for experimental alveolar bone loss *in vivo* (135).

## SYSTEMIC OUTPUT AND ITS RELATIONS TO PATHOLOGY

### Microbiome and Symbiosis

Microbiome is an ecological community of commensal, symbiotic, and pathogenic microorganisms that share our body space and have been determinants of health and disease (136). The composition of the microbiome varies across body places and among individuals (137). Compiled data from the MetaHit and the Human Microbiome Project identified 2,172 species isolated from humans, classified into 12 different phyla, from which 386 are strictly anaerobic and therefore will usually be found in mucosal regions such as the oral cavity and the GI tract (138). The collection of microorganisms including bacteria, archaea and eukarya that live in the GI tract is named the "gut microbiota" and has coevolved with the host to form a mutually beneficial relationship (138). The gut microbiota is the largest microbiome in our body with more than 50 different phyla and 500 bacterial species (139). However, a healthy human gut microbiota is dominated by three primary phyla: *Firmicutes* (30-50%), *Bacteroidetes* (20-40%), and *Actinobacteria* (1-10%) (139). Strict anaerobes such as *Bacteroides, Eubacterium, Bifidobacterium, Fusobacterium, Atopobium*, and *Peptostreptococcus* are major constituents of the gut microbiota, whereas facultative anaerobes including *Lactobacilli*, *Enterococci*, *Streptococci*, and *Enterobacteriaceae* are present in less proportion (139). The composition of the microbiota varies along the gut. *Bacteroidetes* and *Actinobacteria* represent more than 90% of bacterial phyla in the colon but only 50% in the small intestine, which contains around 40% *Firmicutes* species (139). The majority of gut bacteria are non-pathogenic and cohabit with the enterocytes in microenvironments within the intestine reaching a healthy homeostatic equilibrium known as symbiosis (140, 141). The gut microbiota offers several benefits to the host through a range of physiological functions including nutrient and drug metabolism, maintenance of structural integrity of the gut mucosal barrier, immunomodulation, and protection against pathogens (139).

On the other hand, the oral cavity harboring more than 770 prokaryotic species (according to the expanded Human Oral Microbiome Database) houses the second largest and diverse microbiome (142). Different habitats in the mouth (e.g., teeth, gingival sulcus, tongue, cheek, and hard and soft palate) form a diverse ecological system that allows the growth of different microbial communities, including bacteria, fungi and viruses creating the "oral microbiota" (143). Bacteria are the most common microorganisms found in the oral microbiome. Although several bacterial phyla have been described to date, the oral cavity is dominated by *Firmicutes* (36.7%), *Bacteroidetes* (17.1%), *Proteobacteria* (17.1%), *Actinobacteria* (11.6%), *Spirochaetes* (7.9%), and *Fusobacteria* (5.2%) (142). Under normal conditions, these sets of microorganisms live harmonically in communities structurally and functionally organized called biofilms. These bacteria cohabit in the oral biofilms with synergistic and antagonistic interactions that contribute to ecological stability (144). Salivary components are the major nutritional source for microbial growth. IgA, lactoferrin, lactoperoxidase, lysozyme, statherin, and histatins are required for the development of a balanced microbiome. Saliva has antimicrobial properties due to presence of certain components including nitrite and hypothiocyanite that contribute to the state of equilibrium of the microbiome (144).

Homeostasis of both oral and gut microbiomes is characterized by diverse and dynamic microbial communities. However, the oral microbiome is more diverse and dynamic as compared to the gut microbiome (145, 146). The biodiversity of this ecosystem improves its ability to resist environmental disturbances, and dynamic microbiomes are more selective of colonizing bacterial members (146). The microbiota influences the induction, training, and function of the mucosal immune system. Conversely, the immune system has evolved to allow the maintenance of a symbiotic relationship of the host with this complex ecosystem (139, 147). The immune system is directed to reinforce the immunity barrier and thereby their own containment (147). One of the most important strategies used by the host to maintain this homeostatic relationship with the

oral and gut microbiota is to minimize contact between microorganisms and the epithelial cells surface, limiting tissue inflammation and microbial translocation (147). In the GI tract, house of the largest density of commensals, several mechanisms benefit a tolerogenic immune response. In the small intestine, this objective is achieved by the action of PRRs, antimicrobial peptides, IgA, CD103+ DCs and regulatory T cells as well as cytokines including IL10, IL33, and TGFβ. In the large intestine a thick continuous mucus layer favors this goal. These structural and immunological defense mechanisms have been termed as the "mucosa firewall" (148). The active sampling of commensal, pathogens and antigens is mediated by three types of immunosensory cells (i.e., enterocytes, M cells, and intestinal DCs) (149). The gut microbiota interacts with the host cells through a well-regulated immune system that involves TLRs and NLRs. The capacity of immune cells to discriminate commensal from pathogenic bacteria is mediated partially by these receptors (149). It has been shown that intestinal epithelial integrity can be enhanced through the activation of TLRs, which results in proliferation, restitution, and protection of IECs against apoptosis. Commensal bacteria could also induce the expression of NOD2, which plays an important role in regulating the intestinal mucosal homeostasis and suppressing colonization with pathogenic bacteria (149). Commensals also control pathogenic flora through the competition for nutrients and production of antimicrobial peptides that affect the survival and virulence of pathogens (147). As a result of the interactions between the microbiome and the immune system, microbial attacks are effectively controlled by the host response, thereby maintaining a symbiotic state. Although all these processes in oral and GI mucosa induce a protective response that prevents the host from developing diseases in most cases, there is potential for these mechanisms to be disturbed either by overgrowth of microorganisms or by changes in the local host response as a result a dysbiotic state (150). Lastly, evidence from studies in germ-free animals has shown the important role of commensal bacteria on intestinal homeostasis. Germ-free animals are more predisposed to infections, have reduced vascularity and slower renewal of epithelial cells. Anatomically, such animals present longer intestinal villi, associated with crypt atrophy and smaller Peyer's patches with fewer intraepithelial lymphocytes. In addition, the mucosa and muscle wall thickness were decreased in these mice (139). Gut microbiota promotes the preservation of the integrity of the intestinal epithelial barrier and the promotion of epithelial repair after injury (139).

## Dysbiosis and Anatomic Alterations

Dysbiosis is defined as a condition in which the normal microbiome population structure is disturbed (150). The ability of a microbe within the microbiota to cause or exacerbate diseases varies. Most microbes can shift from one relationship to another based on the state of activation of the host, infection, and localization (147). Diseases, some aspects of the lifestyle (e.g., smoking, diet, oral hygiene), genetic variations, antibiotic treatments, the activity of salivary proteins, salivary flow rates, innate/adaptive immune factors, as well as pathogens have been associated with dysbiosis (137, 144). Disruption of the balance of the bacterial ecosystems in the human microbiome has been associated with a plethora of inflammatory diseases and infections including some GI disorders and periodontitis (143).

Periodontitis is a common disease in the oral cavity. The pathogenesis and development of periodontitis involves a complex gene-environment interaction model rather than the earlier proposed model caused by one or two single factors (151). There is no doubt that periodontitis is initiated by bacteria. However, the triad of microorganisms commonly named as "red complex" (*P. gingivalis-, Treponema denticola -T. denticola-*, and *Tannerella forsythia -T. forsythia-*), are not the only ones involved in the etiology (152). Evolving advances in the periodontal research field have indicated dysbiotic microbial community may be responsible for eliciting progressive inflammation and alveolar bone loss (152). Indeed, the host inflammatory response and other modifying and predisposing factors determine the clinical outcome of periodontitis (151). It is now well established that environmental factors (e.g., smoking, diet, and stress), immunoregulatory defects associated with polymorphisms or mutations, systemic diseases, aging, epigenetic modifications, and the presence of keystone pathogens may disrupt the ecosystem transforming a symbiotic microbiota into a dysbiotic one and promote the initiation of periodontitis in a susceptible host (146, 151, 153). The transition from periodontal health to disease is associated with a remarkable shift in the composition of subgingival communities (154). A symbiotic community is composed mainly of facultative bacterial genera including *Streptococci* and *Actinomyces* while a dysbiotic community is composed of anaerobic genera from the phyla *Firmicutes, Proteobacteria, Spirochaetes* and *Bacteroidetes* (155). Abusleme et al. have reported that the change occurred in community structure rather than shifts in membership. That means that most taxa seen in periodontitis were commonly seen in health although in a small number and in low proportion. Conversely, most health-associated taxa were commonly seen in periodontitis. Therefore, the ecological shifts from symbiosis to dysbiosis are a consequence of the increment of dominant species, rather than the disappearance of health-associated species. It was also reported that periodontitis is associated with enhanced uniformity thus contrasts with other disorders of the GI, in which dysbiosis is associated with a decrease in bacterial diversity (155).

In early stages of gingivitis, neutrophils are the first cells to arrive chemotactically at the inflammatory infiltrate through intercellular spaces in the junctional epithelium (156). The presence of pathogens into the subgingival crevice or pocket is followed by chemotactic cytokines secretion by epithelial cells, making the neutrophils release proteolytic enzymes disrupting the epithelial barrier. This gingival epithelial disruption allows pathogens and their products to infiltrate into the lamina propria, triggering the release of pro-inflammatory cytokines and inducing tissue breakdown and bone loss. When antigens enter the lamina propria, they can be taken up by DCs thus activating TNFα, INFγ and IL-13, which increase inflammation.

In addition, the contribution of periodontopathogens and their virulence factors to barrier disruption have been studied (156). The gram negative, black pigmented bacterium *P. gingivalis* is the most widely studied periodontal pathogen; and is considered as a keystone in the development of periodontitis (152). A variety of virulence factors including LPS, fimbriae, hemagglutinins and gingipains contribute to the pathogenicity of this microorganism (156). Katz et al. have demonstrated that *P. gingivalis* disrupted the epithelial integrity and is able to invade the connective tissue by degrading epithelial cell-cell junctions complexes, thereby allowing the spread of the bacterium (157). Likewise, *T. denticola* degraded the ZO-1 protein, disrupting the epithelial barrier and infiltrating the epithelial layers (156). *A. actinomicetemcomitans* decreases connexin-43 levels and E-cadherin expression in gingival epithelial cells (156). Taken together, these results suggest that beneficial bacteria and their products maintain the gingival epithelial barrier by improving tight junction-related gene expression and by developing a beneficial microenvironment that reduces the viability of barrier-disrupted pathogens (156).

On the other hand, GI permeability regulation is mediated by anti-inflammatory cytokines (microbiome-guided expression), while failure in the permeability of the barrier during GI inflammatory diseases can be attributed to the alteration in the expression of proinflammatory cytokines (158). Gut dysbiosis and other factors may trigger epithelial barrier dysfunction which contributes to the increased intestinal permeability, thus creating a "leaky gut" (159, 160). Other alterations of intestinal protection mechanisms including reduced secretory Ig A and generalized decreases in immunity could further affect the intestinal barrier. This leaky gut allows the entrance of bacteria and their products (MAMPs) from the gut lumen into the host. Once inside the intestinal immune system, these MAMPs interact with innate sensors such as TLRs of intestinal cells and intracellular NLRs, triggering an inflammatory response characterized by increased production of IL1, IL6, IL8 and TNFα (160). As a consequence, cytokine imbalance perpetuates intestinal inflammation, which may trigger an array of autoimmune diseases (143, 161). Moreover, leaky gut allows certain commensal bacteria in gut microbiota to escape the lumen of the gut inducing inflammation and causing systemic tissue damage once translocated into the peripheral circulation. Gut bacteria can be translocated to the liver *via* portal vein. Into the liver, these PAMPs activate hepatocytes and Kupfer cells through TLRs and NLRs such as innate immune responses including cytokine production, leading to hepatic injury (162) (**Figure 4**).

## Systemic Disease Output (Oral vs. GI and Systemic Connections)

The oral and gut microbiomes are anatomically connected as they colonize mucosal surfaces in the digestive system. As a result, mechanical and/or biochemical alterations can affect both in a bidirectional way (145, 150). In fact, oral and gut microbiota

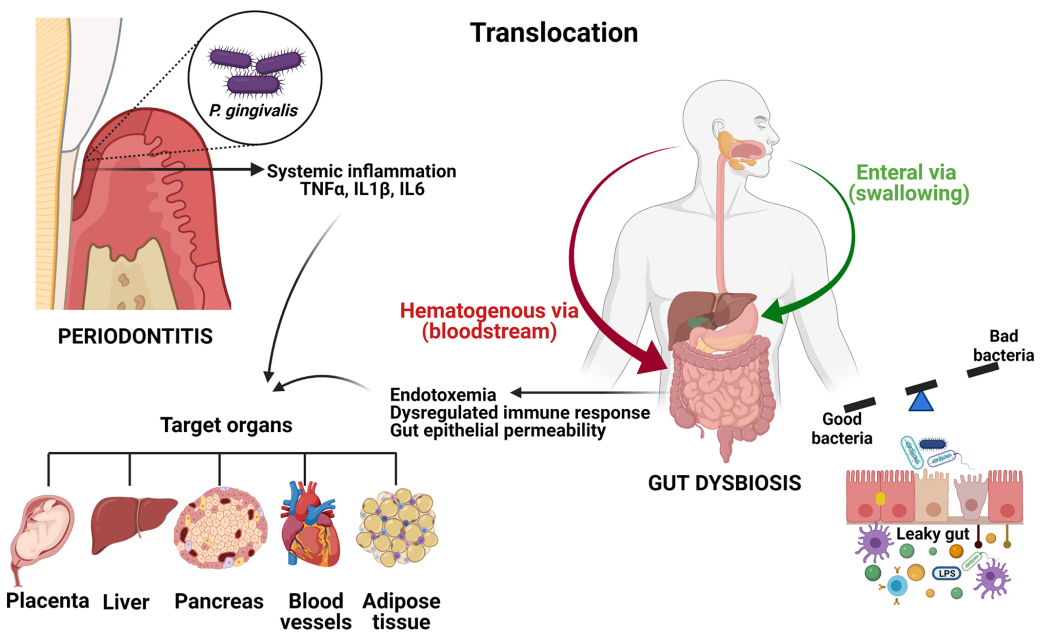


**FIGURE 4** | Oral and GI systemic connections. Oral bacteria can translocate to the digestive tract enterally or parenterally. In periodontitis, locally pro-inflammatory mediators including TNFα, IL1β and IL6 can enter the systemic circulation and induce a low-grade systemic inflammation. Disruption of the gut epithelial barrier induced by dysbiosis results in increased gut epithelial permeability, thus creating a leaky gut. This leakage generates endotoxemia, systemic inflammation and a dysregulated immune response. Since some diseases such as type 2 diabetes, non-alcoholic fatty liver disease, obesity and atherosclerosis have been associated with periodontitis, as well as with gut dysbiosis, it is reasonable to assume that swallowing oral bacteria *via* saliva could represent a possible mechanism linking periodontitis and systemic diseases.The figures were created with BioRender.com.

may be two of the most important microbiomes affecting overall human health (145).

Although the GI tract is highly effective in preventing colonization of foreign microbes, certain oral bacteria can spread from the oral cavity and be translocated to the digestive tract through both parenteral (hematogenous) and enteral routes (163). Segata et al, reported that an estimated of $1x10^{11}$ bacterial cells per day flow from the mouth to the stomach, which means that the oral microbiome might considerably contribute to distal digestive tract populations (164). Species of *Bacteroides, Eubacterium, Streptococcus, Prevotella*, and *Veillonella*, among others have been detected in both the oral cavity and stool in more than 45% of subjects in the Human Microbiome Project (164). In addition, saliva influences the microbial growth of the habitats above the stomach because saliva is a buffer and controls the pH, while its high mucin content allows for nutrient availability. Patients with intestinal diseases commonly exhibit abnormal enrichment of common oral bacteria in the luminal contents and the gut mucosal tissues. In fact, bacteria exclusive to the oral cavity, such as *Campylobacter, Porphyromonas, Prevotella, Fusobacterium* and *Actinomyces* can be found in the gut of patients with gastrointestinal diseases (143).

There is ample scientific evidence demonstrating the translocation of periodontal pathogens from the local periodontium to end-organ targets is facilitated by hematogenous systemic dissemination (157). In fact, epidemiological data suggests that periodontitis is associated with an increased risk of a plethora of diseases including type 2 diabetes, atherosclerotic vascular diseases, adverse pregnancy outcomes, obesity, rheumatoid arthritis, and non-alcoholic fatty liver disease (154, 165). Patients with severe periodontitis can swallow $1x10^{12}$-$10^{13}$ *P. gingivalis* each day (162). Theoretically, if oral bacteria can resist the pH of the stomach, they may potentially reside and colonize the GI tract. *P. gingivalis* is resistant to acid gastric, which permits migration to the colon with subsequent local effects (163). Several studies have focused on investigating if *P. gingivalis* can experimentally modulate the gut microbiome. Studies in mice have shown that *P. gingivalis* can disrupt the gut epithelial integrity through reduced expression of tight junction proteins as well as modifying the microbial composition, both *via* outgrowing in the gut and disseminating *via* endotoxemia (162, 165). Arimatsu et al. reported that oral administration of *P. gingivalis* strain W83 ($1x10^9$ CFU/ml twice a week for five weeks) in mice induced a change of bacterial composition in the ileum, accompanied by increases in the levels of plasma endotoxin, insulin resistance, and systemic inflammation. Reduction in the mRNA expression of the tight junction proteins ZO-1 in the ileum was also reported. Authors hypothesized that the endotoxemia likely was not derived from *P. gingivalis* but could be related to disturbances of the gut microbiota caused by swallowed bacteria leading to metabolic disorders (165). Similarly, Nakajima et al. have reported that a single administration of $1x10^9$ CFU/ml *of P. gingivalis* (strain W83) in mice induced dysbiosis of the gut microbiota, with increased *Bacteroidetes*, decreased *Firmicutes*, and increased serum endotoxin levels. The gene expressions of tight junction protein-1 and occludin (involved in intestinal permeability) were also downregulated. Higher amounts of bacterial DNA on the liver of infected mice were also reported. It is important to highlight that changes in intestinal microbiota preceded systemic inflammation, supporting the idea that alterations of the gut microbiota composition by swallowed periodontopathic bacteria may be a causal mechanism linking periodontitis and systemic diseases (162). However, it is worth noting that these experiments used human oral bacteria, which are exogenous to the mouse microbiome, hence limiting direct extrapolations to human pathogenesis. In sum, the periodontal microbiome affecting the gut microbiome requires much more clinical evidence while experimental results provide a new paradigm to understand the role of periodontopathic bacteria in the gut microbial composition and the plausible development of systemic diseases (165).

*Fusobacterium nucleatum (F. nucleatum)* is one of the most abundant microorganisms in the oral cavity, in both healthy and diseased individuals. *F. nucleatum* is a pathobiont that outgrows during oral dysbiosis and may act as a bridging organism, allowing for other keystone bacteria to bind *via* adhesins, thus playing a key role in periodontitis. Compelling evidence shows that *F. nucleatum* is found in the colonic mucosa of patients with IBD and colorectal cancer (143). These findings were followed by studies showing that *F. nucletaum* may play a role in colorectal cancer development, metastasis and disease outcome (166). Adhesion to the gut epithelium is mediated through surface proteins including FadA, Fap2, and RadD. Mechanisms by which *F. nucleatum* is involved in tumor progression include the creation of a proinflammatory microenvironment and TLR4-activated signaling to NFkB. Additionally, *F. nucleatum* may induce immune suppression of intestinal mucosa by affecting the function of immune cells including macrophages, T cells, and natural killer cells. The ability of *F. nucleatum* to induce resistance to chemotherapy in colorectal cancer is mediated *via* TLR4/NFkB pathway-induced autophagy. Since *F. nucleatum* is involved with the development of colorectal cancer, metastasis, and treatment outcome, strategies to selectively target *F. nucleatum* should be further explored in the future (166).

There is growing evidence that intestinal flora plays a key role in human physiology and dysbiosis of the gut microbiota is associated with the pathogenesis of several diseases within and outside the gut (145). Intestinal disorders comprise IBD, irritable bowel syndrome (IBS) and coeliac disease (CD), whereas extraintestinal disorders include asthma, allergy, cardiovascular disease, metabolic syndrome, and obesity. IBD is an idiopathic condition that causes chronic inflammation of the digestive tract and includes Crohn's disease and ulcerative colitis (143). The dramatic increases in the prevalence of IBD in recent years have made it one of the most studied imbalances between microbes and the immune system. Host genetics are involved in the IBD onset, with genetic factors being more critical for the development of Crohn's disease than ulcerative colitis. However, environmental factors are unequivocally involved (167). The contribution of the gut microbiota together with a dysregulated immune response is the current accepted disease hypothesis. Several studies have shown that gut dysbiosis in patients with IBD is characterized by a decrease in the bacterial diversity, temporal stability, and cluster separately when compared to healthy

controls. Results from animal models in colitis require the presence of intestinal bacteria to initiate inflammation, and an increased mucosal bacterial load is observed in IBD patients. These studies have also identified polymorphisms in genes involved in bacterial recognition and clearance. Noteworthy, inconsistent results have been found in some microbial compositional comparisons. Nevertheless, these studies have generally identified reductions in components of the *Firmicutes* phyla with concurrent increases in *Bacteroidetes* and facultative anaerobes such as *Enterobacteriaceae* (167).

IBS is a common disorder of gut-brain interaction worldwide (168). The gut-brain axis describes the bidirectional interaction between the emotional and cognitive areas of the CNS and the peripheral function of the GI tract (168, 169). Recently, the "brain-gut" axis is referred to as the "brain-gut-microbiota" axis (168). Although the etiology is not fully understood, changes in gut microbiota have been characterized in the different subtypes of disease compared to healthy subjects (170). The distribution of subtypes include IBS-diarrhea, IBS-constipation, and mixed IBS (168). The presence of a specific microbial signature characterizing these diseases is still unknown. However, decreased microbial diversity and the presence of *Clostridiales* species (methane-producing bacteria) associated with severe symptoms have been described (169). In general, epidemiological data suggest that there is a relative abundance of proinflammatory bacterial species including *Enterobacteriaceae* with decreased *Lactobacilli* and *Bifidobacterium*. When IBS patients are compared to healthy individuals there is an increase in *Firmicutes* to *Bacteroidetes* ratio and increased levels of *Streptococci* and *Ruminococcus* species are observed (168). In addition, genetics, diet, GI infections and psychological factors have been also proposed as potential risk factors for the development of IBS (169).

CD is a chronic intestinal inflammatory disorder due to an aberrant immune response to the ingestion of gluten proteins in susceptible subjects (171). Although the etiology is multifactorial this disorder is strongly associated with the expression of the leukocyte antigen DQ2 (170). There is evidence that a notable reduction in gram-positive bacterial populations, as well as increase of total gram-negative bacteria is characteristic of the active phase of the disease, which can contribute to loss of gluten tolerance. These results confirm that structural changes in the composition of the gut microbiota are associated with CD. In addition, lower levels of IgA-coated bacteria have been detected in CD patients compared to healthy controls. These results support the hypothesis that there is an intestinal barrier defect in CD patients, which fails to stabilize the gut microbiota and allows the entry of harmful antigens and pathogens, all triggering an inflammatory response (171).

Similarly to periodontitis, no specific pathogen seems to cause IBD, IBS, and CD (161). Periodontitis and these GI disorders share in common a dysregulated host immune response due to disruption by microbial communities or by changes in the local host immune response (154, 167). Consequently, the failure to control self-directed immune responses triggered by environmental or microbiota-derived antigens in susceptible individuals results in tissue damage.

## CONCLUSIONS

Epithelial tissues are crucial in establishing tolerance in multiple human body systems in direct contact with the external environment including the skin, the respiratory system and more crucially with the digestive tract (oral and GI compartments). However, beyond the ability to maintain a "non-responsive" state, epithelia are in charge of both having a symbiotic relationship with the microbiome and maintaining health, as well as inducing the activation of inflammatory processes to control aggressions. Therefore, epithelial-mediated inflammation *via* "sentinel receptors" (TLRs, NLRs, etc.) can be associated with clinical signs of multiple diseases in the presence of the host immune response deregulation and/or microbial dysbiosis. Although the final objective of establishing tolerance in both oral and GI epithelia is ultimately similar, the anatomical considerations here presented as well as the compartmentalization of the immune response make each pathway widely different, albeit traditionally considered part of the same continuum.

The initial host microbiome interaction with the oral epithelia is relatively unknown, while the initial antigen sensing process deciding the fate of the response is better documented in the gastrointestinal epithelia. However, it is clear that the initial antigen sensing in the oral cavity must be a highly regulated process, as this could be hypothesized from the low incidence of oral inflammatory epithelial diseases in humans (with the notable exception of periodontitis). In addition, there is also a gap in the knowledge of how different foods could alter the inflammatory processes and impact the response of the host in metabolic activities at buccal level, as well as what would be the influence of the oral microbiome in the process.

Although clear differences in epithelial function have been presented here, the authors conclusion is that undeniably, in light of the evidence, there is a strong relationship between oral microbiota translocation to the GI tract with a subsequent systemic disease occurrence triggered by the alteration in intestinal permeability. Therefore, such relationship between inflammatory oral diseases mediated by translocating microorganisms is becoming more and more relevant in overall systemic health and makes the oral microbiota a future therapeutic target for the prevention and treatment of multiple systemic diseases.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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# Prognostic Value of an Immunohistochemical Signature in Patients With Head and Neck Mucosal Melanoma

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**Purpose:** We aimed to develop a prognostic immunohistochemistry (IHC) signature for patients with head and neck mucosal melanoma (MMHN).

**Methods:** In total, 190 patients with nonmetastatic MMHN with complete clinical and pathological data before treatment were included in our retrospective study.

**Results:** We extracted five IHC markers associated with overall survival (OS) and then constructed a signature in the training set (n=116) with the least absolute shrinkage and selection operator (LASSO) regression model. The validation set (n=74) was further built to confirm the prognostic significance of this classifier. We then divided patients into high- and low-risk groups according to the IHC score. In the training set, the 5-year OS rate was 22.0% (95% confidence interval [CI]: 11.2%- 43.2%) for the high-risk group and 54.1% (95% CI: 41.8%-69.9%) for the low-risk group (P<0.001), and in the validation set, the 5-year OS rate was 38.1% (95% CI: 17.9%-81.1%) for the high-risk group and 43.1% (95% CI: 30.0%-61.9%) for the low-risk group (P=0.26). Multivariable analysis revealed that IHC score, T stage, and primary tumor site were independent variables for predicting OS (all P<0.05). We developed a nomogram incorporating clinicopathological risk factors (primary site and T stage) and the IHC score to predict 3-, 5-, and 10-year OS.

**Conclusions:** A nomogram was generated and confirmed to be of clinical value. Our IHC classifier integrating five IHC markers could help clinicians make decisions and determine optimal treatments for patients with MMHN.

**Keywords: head and neck mucosal melanoma, immunohistochemistry, nomogram, prognosis, signature**

## INTRODUCTION

Mucosal melanoma (MM) is rare in Caucasians, accounting for approximately 1.3% of all melanomas (1, 2), while it is the second most common subtype in China, accounting for 20%-25% of all melanomas (3, 4). The head and neck region has the highest incidence of MM, accounting for 55% of all cases (1). In China, MM of the head and neck (MMHN) represents approximately 1.7% of all head and neck malignancies, of which 94.56% occur in the mucosa. In China, MMHN mainly occurs in the nasal sinus and oral cavity. SEER database statistics (5) show that 72% of malignant melanomas occur in the nasal sinus, 19% occur in the oral cavity, and 9% occur in other areas, including the nasopharynx, oropharynx and larynx. MMHN is a malignant tumor with a poorer prognosis than other subtypes (3, 4, 6). According to the National Comprehensive Cancer Network (NCCN) guidelines (2019), surgical resection is preferentially recommended for localized MMHN (7). Immunotherapy and targeted therapy have recently changed the treatment landscape for MMHN (8, 9). However, these systemic therapies for MMHN have been evaluated only in small studies (10–13) or retrospective case studies (14–16). The gold standard for the diagnosis of MMHN is histopathological examination. Melanoma cells have a variety of phenotypes, including spindle, plasmacytoid, epithelial, etc. (17, 18). Tumor cells are arranged in lamellar, nest, and globoid structures at the microscopic level, and tumors with mixed cell phenotypes are more malignant than those with a similar phenotype throughout (17). Cells with cytoplasm rich in pigment granules make the disease easy to diagnose. However, approximately 13% ~ 25% of tumors lack melanin particles and need to be distinguished from other cancers, lymphomas, and sarcomas. Immunohistochemistry (IHC), an inexpensive and easy-to-use approach, is the most widely applied pathological technique in determining the expression of tumor-associated proteins. IHC analysis is often applied to distinguish different classifications of MMHN. Common immunohistochemical indicators include S-100, HMB-45 and Melan-A. It has been reported that many IHC-based markers can predict the prognosis of MMHN, but none have made it into clinical practice (19, 20). In addition, prognostic models incorporating multiple

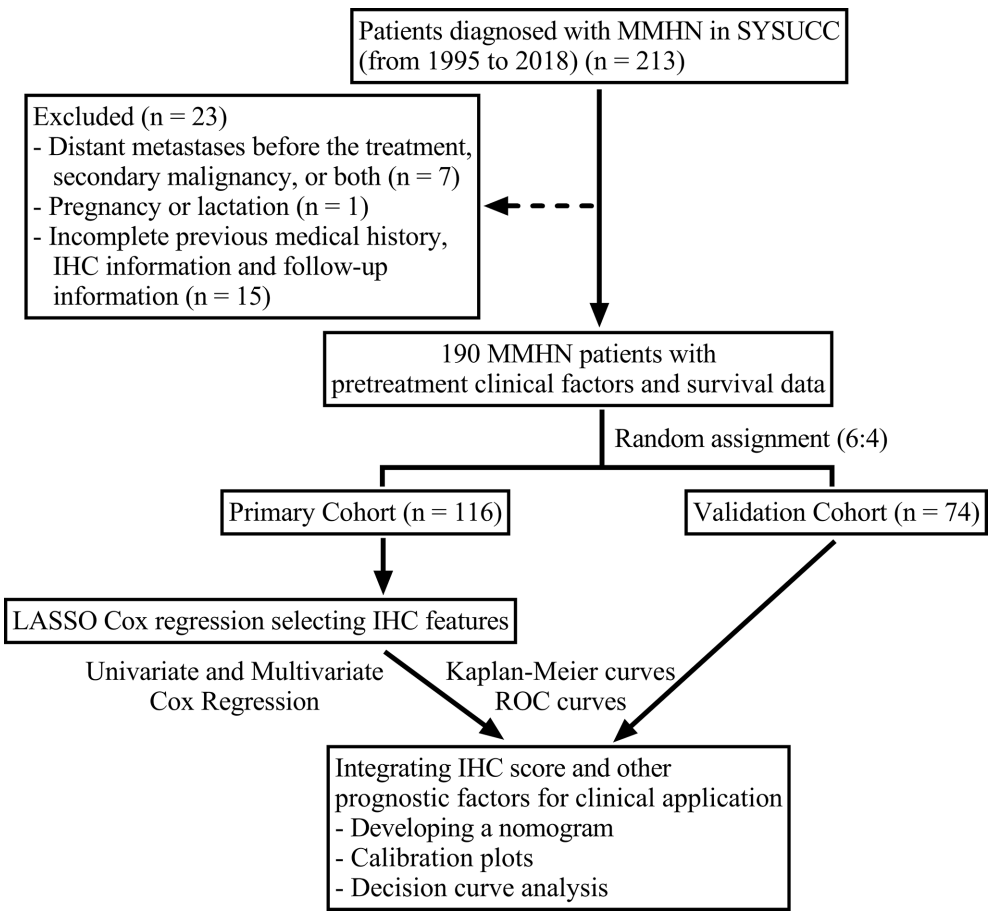


**FIGURE 1** | Flow chart of the study design. MMHN, mucosal melanoma of the head and neck; SYSUCC, Sun Yat-Sen University Cancer Center; IHC, immunohistochemistry; ROC, receiver operating characteristic.

**TABLE 1** | Baseline characteristics between the primary cohort and validation cohort.

| | Training set | | Validation set | |
|---|---|---|---|---|
| | **Low-risk patients (n = 75)** | **High-risk patients (n = 41)** | **Low-risk patients (n = 62)** | **High-risk patients (n = 12)** |
| Gender | | | | |
| Male | 51 (68.00%) | 26 (63.41%) | 36 (58.06%) | 9 (75.00%) |
| Female | 24 (32.00%) | 15 (36.59%) | 26 (41.94%) | 3 (25.00%) |
| Age (year-old) | | | | |
| <= 60 | 45 (60.00%) | 27 (65.85%) | 39 (62.9%) | 7 (58.33%) |
| > 60 | 30 (40.00%) | 14 (34.15%) | 23 (37.1%) | 5 (41.67%) |
| Tumor site | | | | |
| Others | 8 (10.67%) | 8 (19.51%) | 16 (25.81%) | 0 |
| Nasal cavity | 45 (60.00%) | 18 (43.90%) | 32 (51.61%) | 6 (50.00%) |
| Paranasal sinus | 3 (4.00%) | 4 (9.76%) | 3 (4.84%) | 0 |
| Oral cavity | 19 (25.33%) | 11 (26.83%) | 11 (17.74%) | 6 (50.00%) |
| T stage | | | | |
| T3 | 47 (62.67%) | 15 (36.59%) | 28 (45.16%) | 6 (50.00%) |
| T4 | 28 (37.33%) | 26 (63.41%) | 34 (54.84%) | 6 (50.00%) |
| N stage | | | | |
| N0 | 58 (77.33%) | 27 (65.85%) | 44 (70.97%) | 6 (50.00%) |
| N1 | 17 (22.67%) | 14 (34.15%) | 18 (29.03%) | 6 (50.00%) |

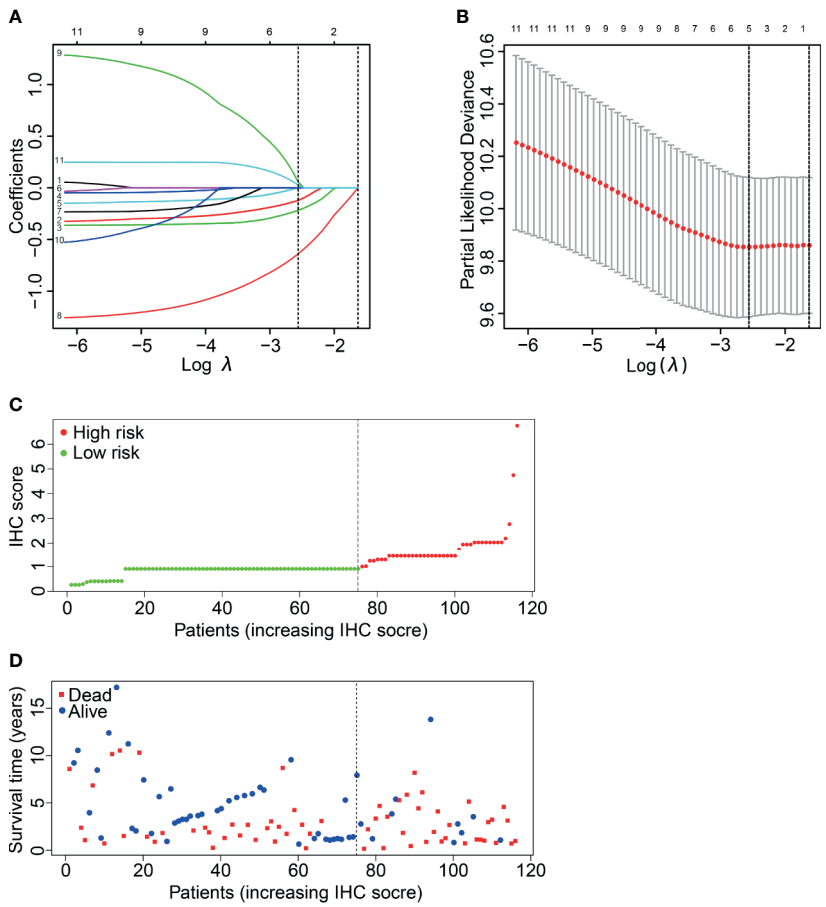


**FIGURE 2** | IHC feature selection using the LASSO cox regression model. **(A)** A LASSO coefficient profile plot of the IHC features was produced against the logλ sequence. **(B)** Ten-fold cross-validation for IHC features selection in the LASSO Cox model. The dotted vertical lines represented the λ values with minimal deviance (left) and with the largest λ value within one standard deviation of the minimal deviance (right). We then selected the coefficients of the model with the minimal deviance, which included 5 IHC features. **(C, D)** The IHC score classified MMHN patients into low-risk and high-risk groups.

biomarkers help clinicians make treatment decisions and develop optimal treatment combinations to reduce disease mortality.

The objective of this study was to develop and validate an IHC-based classifier with a least absolute shrinkage and selection operator (LASSO) Cox regression model and to develop a prognostic nomogram for MMHN patients based on IHC biomarkers and clinicopathological factors.

## MATERIALS AND METHODS

A total of 190 previously untreated, nonmetastatic MMHN patients diagnosed at Sun Yat-Sen University Cancer Center between 1995 and 2018 were retrospectively included in the study. Patients were randomly assigned to the discovery cohort (116 cases) or validation cohort (74 cases) at a ratio of 6:4. The tumor-node-metastasis (TNM) stage of the patients was reassessed according to the American Type Tissue Culture Collection (AJCC) 8th edition guidelines (21). The study workflow of patient eligibility was displayed in **Figure 1**. The exclusion criteria were as follows: (1) evidence of distant metastasis before treatment, secondary malignancy, or both; (2) pregnancy or lactation; and (3) incomplete previous medical history, IHC information and follow-up information. The ethics committee of Sun Yat-Sen University Cancer Center approved our study protocol.

### Development and Validation of an Immunohistochemical Predictor

The LASSO Cox regression method was used to shrink the regression coefficients of the features and select the best combination of survival outcome predictors. Prognostic characteristics with nonzero coefficients were enrolled in the model. Each prognostic score was calculated according to a combination of these features. We developed a multimarker classifier to predict overall survival (OS) in patients with MMHN. The LASSO Cox regression model was applied by the "glmnet" package. We applied the survival R package to calculate the risk score, regarded as IHC score. (https://CRAN.R-project.org/package=survivalAnalysis). MMHN patients were divided into high- and low-risk groups based on the median value of the IHC score.

### Statistical Analysis

The chi-square test for categorical variables was used to assess differences between two groups. The survival time of patients separated into different risk groups according to the IHC signature score was estimated by Kaplan-Meier survival analysis with the log-rank test. We applied the 'pROC' package to perform receiver operating characteristic (ROC) analysis to investigate the prognostic properties of IHC features. The independent prognostic variables were analyzed by univariable and multivariable Cox regression analyses.

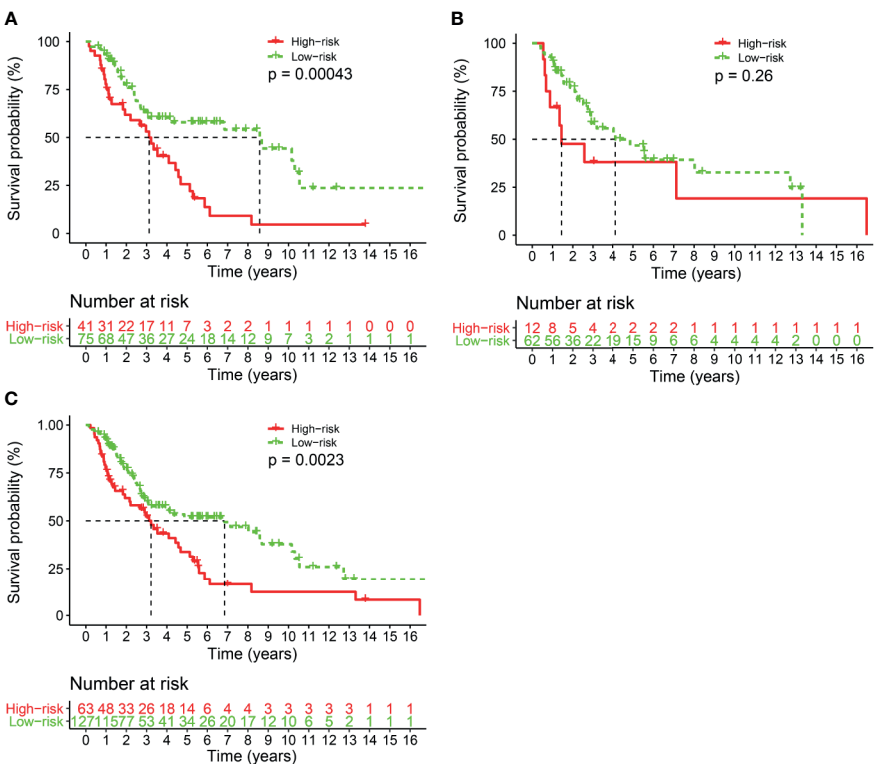


**FIGURE 3** | The comparison of overall survival between the high- and low-risk groups stratified by IHC score. **(A)** The Discovery cohort, **(B)** the validation cohort, and **(C)** the total cohort.

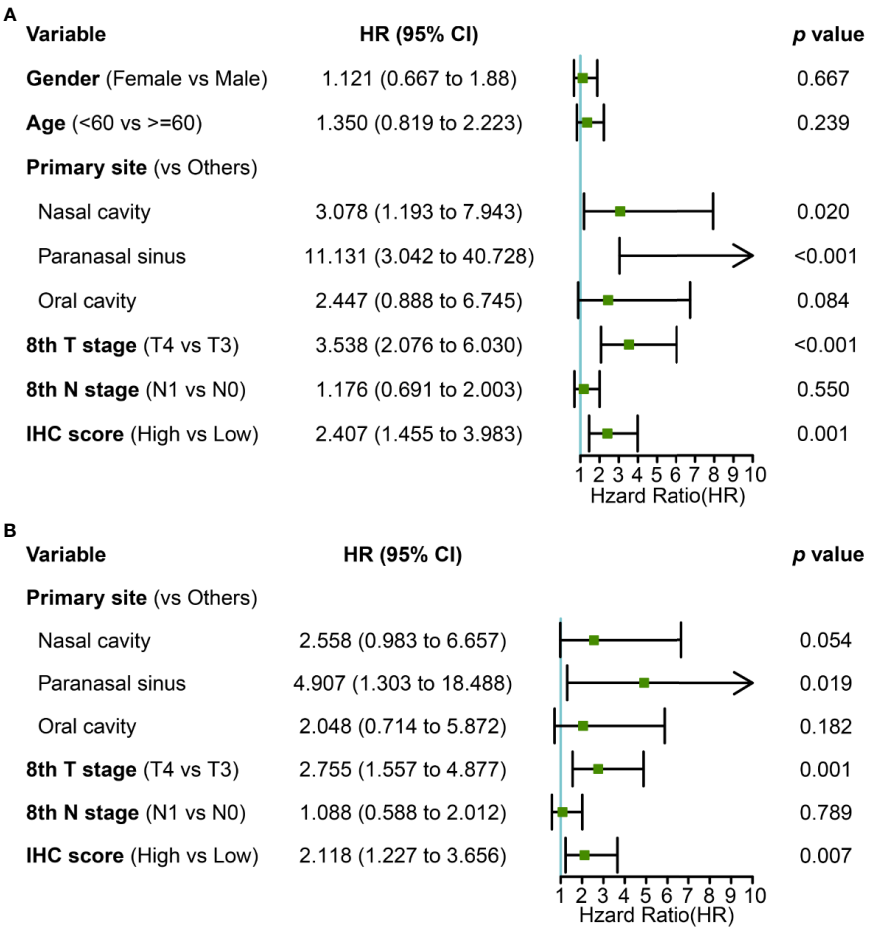


**FIGURE 4** | **(A)** univariate and **(B)** multivariate Cox regression with clinical information and IHC score for OS.

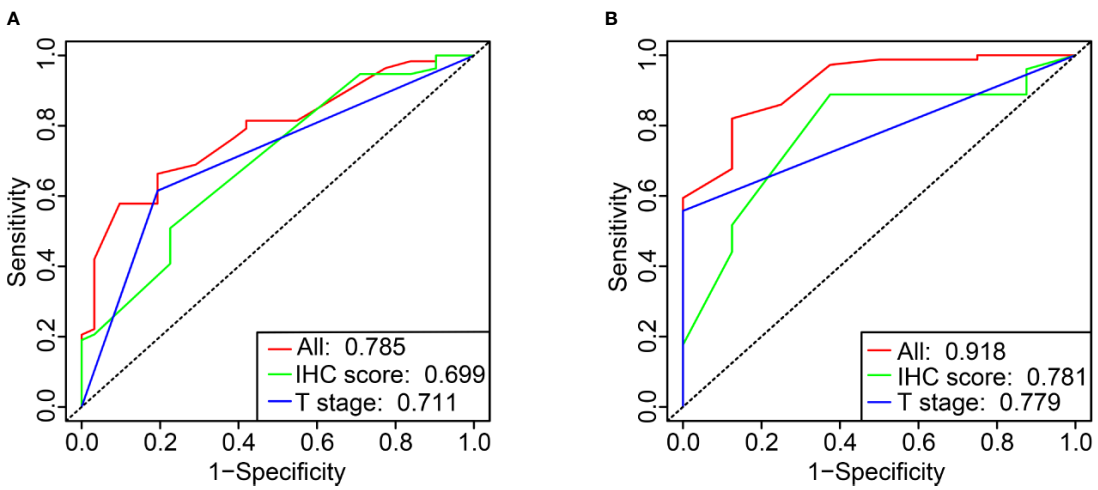


**FIGURE 5** | Time-dependent ROC curve comparing the prognostic value of IHC score in 5 and 10 years with that of T stage **(A, B)**.

A nomogram was further constructed to predict the OS probability by accounting for the Cox regression coefficients. Calibration plots were drawn on the basis of the regression analysis results. Decision curve analysis (DCA) was performed to estimate the clinical utility. All statistical analyses were carried out with R software (version 4.0.3).

# RESULTS

## Patient Characteristics

For the whole cohort, the median age was 56 (range, 19–87) years old, with a male to female ratio of 1.79:1. The primary tumor sites were as follows: The nasal cavity in 101 (53.2%) patients, the paranasal sinus in 10 (5.3%) patients, the oral cavity in 47 (24.7%) patients, and other sites such as the nasopharynx, oropharynx, eyelids, and larynx in 32 (16.8%) patients. The median follow-up time was 31 months (range, 2–206 months), during which there were 102 deaths. The baseline characteristics between the primary cohort and validation cohort are listed in **Table 1**.

## Immunohistochemical Signature Development

Eleven IHC features (S100, HMB45, Melan-AC, Ki67, Vim, SOX-10, CD56, CK, Syn, CgA1, NSE) were reduced to five prognostic markers (HMB45, Melan-AC, CK, Syn, NSE) in the training set *via* the LASSO Cox regression model (**Figures 2A, B**). Based on the median IHC score calculated with the R survival package, we divided patients of the discovery cohort into low-risk and high-risk groups. The distribution plot of IHC score showed that the risk of mortality increased with increasing IHC

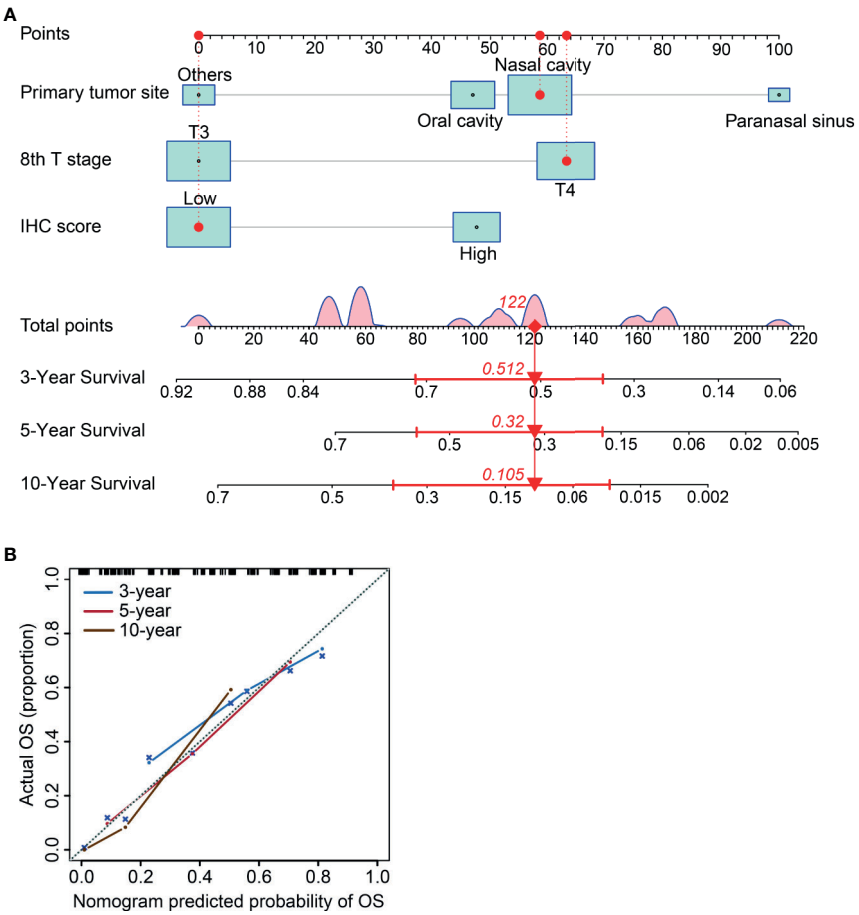


**FIGURE 6** | Establishing and validating a nomogram. **(A)** A constructed nomogram for prognostic prediction of a patient with MMHN. The patient had a local tumor in nasal cavity, T4 stage, and low IHC score. Density plot of total points shows their distribution. For category variables, their distributions are reflected by the size of the box (to view boxes of IHC score, the smaller one represents high score and the bigger one represents low). The importance of each variable was ranked according to the standard deviation along nomogram scales. To make use of the nomogram, the black dots of a single patient are located on each of the variable axis. Red dots and lines are drawn up to determine the points received by each variable; the sum (122) of these points is located on the Total Points axis, and a line is drawn downward to the survival axes to determine the probability of 3-year (51.2%), 5- year (32.0%) and 10-year (10.5%) overall survival for MMHN. **(B)** The 3-, 5-, and 10-year OS of calibration curves were used to assess the predictive ability of the nomogram. Dashed lines along the 45-degree line represented that the predicted probabilities are equal to the actual probabilities.

score (**Figures 2C, D**). The high-risk group (41 patients, 35.3%) included patients with an IHC score of 0.9 or higher, while the low-risk group (75 patients, 64.7%) included patients with an IHC score below 0.9. Patients in the low-risk group had a better 5-year OS rate [54.1% (95% confidence interval (CI): 41.8%-69.9%)] than those in the high-risk group [22.0% (95% CI: 11.2%- 43.2%); P<0.001; **Figure 3A**].

## Validation of the Signature

We also conducted the same analysis in the validation set (n=74). Patients were divided into a low-risk group (62 patients, 83.8%) and a high-risk group (12 patients, 16.2%) according to the IHC score. The 5-year OS rate was 43.1% (95% CI: 30.0%-61.9%) in the low-risk group and 38.1% (95% CI: 0.18–0.8117.9%-81.1%; P= 0.26) in the high-risk group (**Figure 3B**). There was a statistically significant difference between the two risk groups in the whole cohort (P<0.001, **Figure 3C**).

## Prediction Accuracy of the IHC Classifier

Primary tumor site, T stage, and IHC score were significant prognostic variables in the univariable analysis (**Figure 4A**). Primary tumor site, T stage, and IHC score remained independent predictors in multivariate analysis (**Figure 4B**). In addition, receiver operating characteristic (ROC) curve analysis of 5- and 10-year OS showed that the area under the receiver operating characteristic curve (AUROC) values for the IHC score was 0.699 and 0.781, respectively, which was similar to that of T stage (P= 0.866 and 0.979). Furthermore, the combination of IHC score and T stage was superior in predicting 5- and 10-year OS than T stage alone (5-year OS, P = 0.013; 10-year OS, P = 0.005). Thus, IHC score could contribute to predicting the 5- and 10-year OS (**Figures 5A, B**).

## Nomogram Building and Clinical Utility Assessment

We established a nomogram incorporating IHC score, T stage, and primary tumor site and its prognostic efficacy (**Figure 6A**). The nomogram displays an example of a given patient to predict survival probability. The total score was depended on each scores calculated by the nomogram; the total risk point for most patients in this study was from 0 and 220. Calibration curves showed high consistency between the Kaplan-Meier estimates and those from our nomogram in both data sets (**Figure 6B**). Finally, the clinical value of the nomogram was evaluated by DCA. The nomogram has promising clinical value because the range of the 3-, 5-, and 10-year threshold probabilities for OS indicate that the nomogram provides a better net benefit than all or no treatment (**Figures 7A–C**).

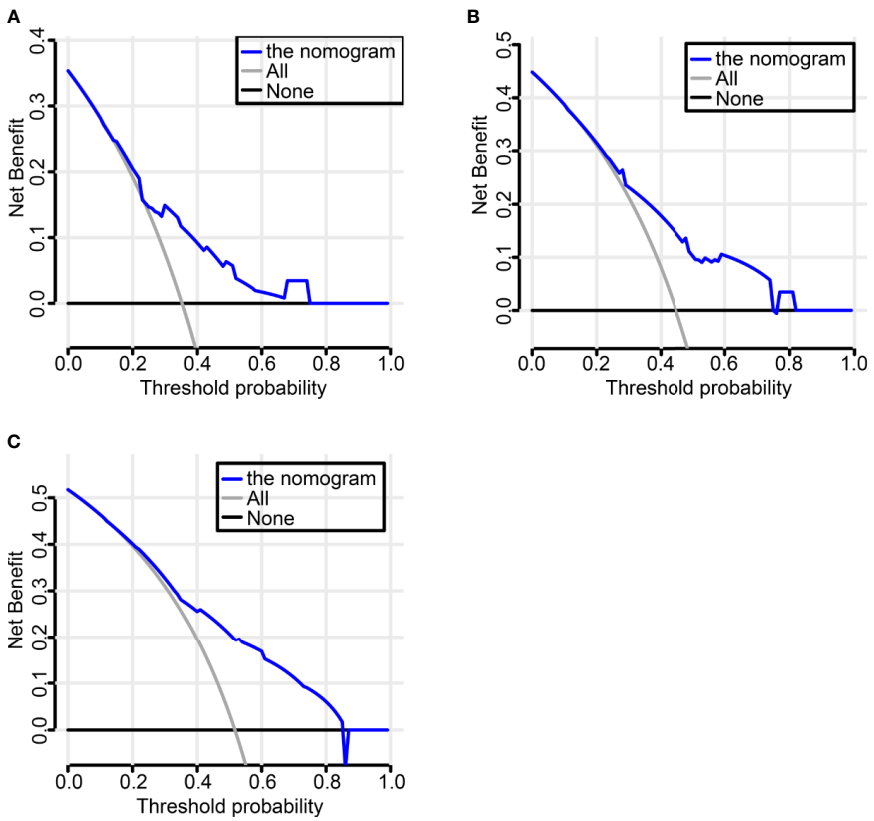


**FIGURE 7** | Decision Curve Analysis (DCA) to evaluate clinical utility of the nomogram. The y-axis measures the net benefit. The dashed line represents the nomogram. Using the IHC-based nomogram provides a better net benefit than all or no treatment **(A–C)**.

## DISCUSSION

Current treatment modalities do not provide clinically useful molecular prognostic biomarkers. We developed a nomogram for predicting 3-, 5-, and 10-year OS in patients with MMHN by combining useful immunohistochemical biomarkers and clinical features; this model can help clinicians make treatment decisions. A previous study reported the function of the biomarkers found in our study. The expression of Melan-AC is highly specific in differentiating lymph nodes from metastatic melanoma (22), whereas the prognostic function of Melan-AC remains unknown. Syn is abnormally regulated in many cancers and plays an important role in the proliferation and progression of tumor cells (23). Syn is a typical pathological amyloid protein observed in melanoma, whose expression is negatively correlated with the melanin content (24). However, the prognostic function of syn remains controversial. It has been reported that increased cytokeratin (CK) expression is connected with highly malignant melanoma (25). Our results showed that CK is expressed in many advanced unresectable metastatic melanomas. However, the prognostic role of CK in patients with MMHN remains controversial. HMB-45 plays an important role in diagnosing amelanotic melanoma (26). It could also be a good marker for diagnosing primary oral and nasal MM (27). The expression of HMB-45 appears to be highly specific for diagnosis in MM (28), but the prognostic function of HMB-45 has not been reported. NSE may be a potential target for lymphoma therapy and a prognostic marker for lymphoma (29). Patients with high expression of NSE for lymphoma had worse clinical outcomes than those with low expression of NSE. However, the tumor-promoting mechanism of NSE and the prognostic role of MMHN remain unclear. The expression of NSE could be helpful for disease assessment and for the early identification of distant metastases in patients with melanoma (30).

To our knowledge, this is the first nomogram constructed to predict the OS probability of patients with MMHN. In this study, we successfully integrated multiple immunohistochemical markers into a single signature *via* a LASSO Cox regression model, and this signature had significantly higher prognostic accuracy than a single immunohistochemical marker alone. With this model, the 3-, 5-, and 10-year OS of individual patients with MMHN can be predicted accurately. For example, a patient with T3 stage disease (0 points), paranasal sinus MM (100 points), and a low IC score (0 points) would have a total score of 100 points, yielding an estimated 3-year OS rate of 62%.

The main limitation of our study is that the nomogram is based on retrospective data from a single cancer center. Further multicenter, prospective clinical trials are needed to verify our results.

## CONCLUSIONS

We developed and validated a nomogram incorporating clinicopathological characteristics and IHC features that can precisely predict the prognosis of patients with MMHN and help clinicians develop an optimal treatment strategy.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The ethics committee of Sun Yat-Sen University Cancer Center. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Study conceptualization (LC and L-XL). Data curation and investigation (Q-QX, LC, and L-XL). Funding acquisition (LC and L-XL). Data analysis, interpretation and visualization (Q-QX, Q-JL, and C-LH). Quality control of data and algorithms (M-YC, M-FZ, and S-HY). Manuscript writing (Q-QX, Q-JL, and C-LH). Manuscript reviewing and approving (Q-QX, Q-JL, LC, and L-XL). All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The Hunger Games: *Aggregatibacter actinomycetemcomitans* Exploits Human Neutrophils As an Epinephrine Source for Survival

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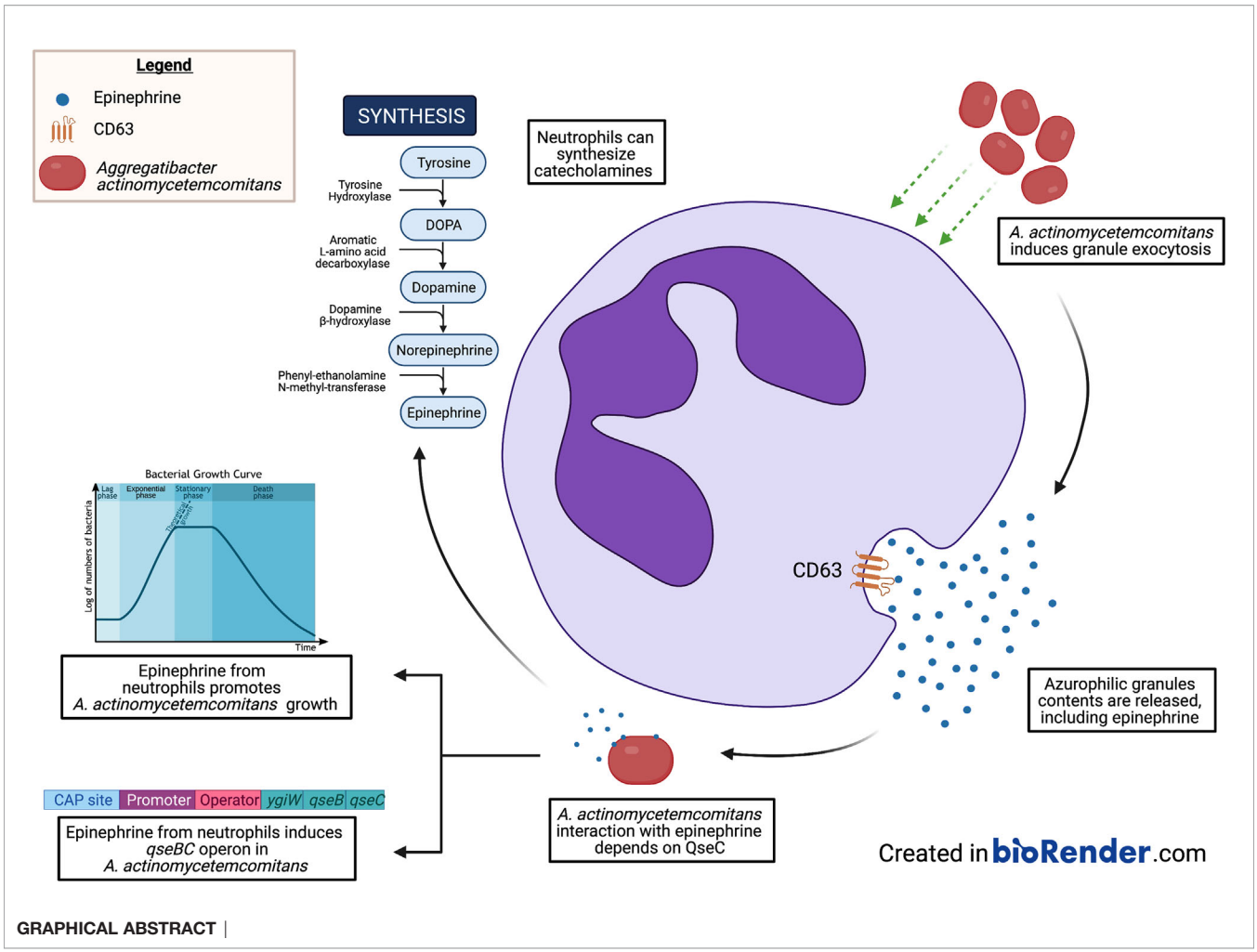
*Aggregatibacter actinomycetemcomitans* is a gram-negative facultative anaerobe and an opportunistic oral pathogen, strongly associated with periodontitis and other inflammatory diseases. Periodontitis is a chronic inflammation of the periodontium resulting from the inflammatory response of the host towards the dysbiotic microbial community present at the gingival crevice. Previously, our group identified catecholamines and iron as the signals that activate the QseBC two-component system in *A. actinomycetemcomitans*, necessary for the organism to acquire iron as a nutrient to survive in the anaerobic environment. However, the source of catecholamines has not been identified. It has been reported that mouse neutrophils can release catecholamines. In periodontitis, large infiltration of neutrophils is found at the subgingival pocket; hence, we wanted to test the hypothesis that *A. actinomycetemcomitans* exploits human neutrophils as a source for catecholamines. In the present study, we showed that human neutrophils synthesize, store, and release epinephrine, one of the three main types of catecholamines. Human neutrophil challenge with *A. actinomycetemcomitans* induced exocytosis of neutrophil granule subtypes: secretory vesicles, specific granules, gelatinase granules, and azurophilic granules. In addition, by selectively inhibiting granule exocytosis, we present the first evidence that epinephrine is stored in azurophilic granules. Using QseC mutants, we showed that the periplasmic domain of the QseC sensor kinase is required for the interaction between *A. actinomycetemcomitans* and epinephrine. Finally, epinephrine-containing supernatants collected from human neutrophils promoted *A. actinomycetemcomitans* growth and induced the expression of the *qseBC* operon under anaerobic conditions. Based on our findings, we propose that *A. actinomycetemcomitans* promotes azurophilic granule exocytosis by neutrophils as an epinephrine source to promote bacterial survival.

**Keywords: *Aggregatibacter actinomycetemcomitans*, human neutrophils, catecholamines, epinephrine, granule exocytosis, QseBC system, periodontitis**




**GRAPHICAL ABSTRACT** |

## INTRODUCTION

In the oral cavity, when host microbe homeostasis is broken, bacterial communities accumulate at the sub-gingival pocket and form biofilms that lead to oral diseases such as periodontitis (1). Periodontitis consists of a chronic inflammation of the periodontium caused by the inflammatory response of the host to plaque biofilm. The disease is characterized by the progressive deepening of the sulcus and loss of attachment between the bone and gingival tissue leading to bone loss. Recurring inflammation of the periodontium has been associated with the initiation, exacerbation, and pathogenesis of a number of other inflammatory diseases (2). *Aggregatibacter actinomycetemcomitans* (*Aa*) is a non-motile gram-negative facultative anaerobe of the *Pasteurellaceae* family (3–5). *A. actinomycetemcomitans* has been strongly associated with periodontitis and other diseases such as rheumatoid arthritis, cardiovascular diseases, atherosclerosis, urinary tract infections, and brain abscesses (6–10). *A. actinomycetemcomitans* contributes to tissue inflammation, destruction, and bone resorption by expressing a number of virulence factors such as cytolethal disentin toxin, leukotoxin A of the RTX family of bacterial toxins, and collagenase (11–14).

In the subgingival pocket, *Aa* must compete for nutrients, such as iron, in order to survive. As part of the host immune response, iron is kept unavailable to bacteria by being sequestered by catecholamines (i.e., epinephrine, norepinephrine, and dopamine) or leukocyte-produced molecules like lactoferrin and transferrin (15, 16). However, some bacteria have evolved to subvert this mechanism by producing iron scavenging molecules, known as siderophores (17–19) or using host-derived catecholamines (15). Catecholamines have been implicated as causative or contributory agents of periodontitis (20–22). Interestingly, *Aa* does not produce siderophores (23), but expresses the QseBC (quorum sensing in *Escherichia coli*) two-component system (TCS) that is activated in the presence of both catecholamines and iron (6). The *qseBC* operon encodes the genes for *qseC*, a sensor molecule, and *qseB*, the response regulator (24). Novak et al. (25) demonstrated the requirement of QseC for *Aa* biofilm growth and virulence. Furthermore, Weigel et al. (6) subsequently showed that *Aa* growth in a chemically defined media (CDM) supplemented with both epinephrine or norepinephrine and ferrous ($Fe^{2+}$) or ferric ($Fe^{3+}$) iron increased *qseBC* promoter activity and bacterial growth, indicating that both catecholamines and iron are required for the activation of QseBC. Additionally, microarray analysis showed that

activation of QseBC induced the expression of genes associated with anaerobic metabolism and respiration and reduced expression of genes involved in iron uptake and transport. However, the source of catecholamines remained to be determined. In periodontitis, neutrophils are found in large numbers at the gingival crevice (26). Murine neutrophils have been shown to release epinephrine or norepinephrine when stimulated with LPS (27). Furthermore, increased levels of the enzymes (tyrosine hydroxylase and dopamine β-hydroxylase) required for the synthesis of catecholamines and their inactivation (catecholamine-O-methyltransferase and monoamine oxidase) enzymes have been observed in murine phagocytes and human lymphocytes (27, 28). Nonetheless, the presence of catecholamines in the host environment has also been shown to promote bacterial iron uptake and growth in media limited conditions (15, 21, 22). Host-derived catecholamines have a higher affinity to iron than antimicrobials like lactoferrin and transferrin (15, 29), serving as excellent pseudo-siderophores (30) to bacteria. Therefore, we proposed that human neutrophils serve as a catecholamine source for *Aa* to sequester iron, leading to QseBC activation and bacterial growth.

In this study, we demonstrate that human neutrophils have significant levels of tyrosine hydroxylase, monoamine oxidase A, and catecholamine-O-methyltransferase, validating that cells participate in catecholamine metabolism. In addition, we present evidence that human neutrophils store epinephrine in the azurophilic granules and that interaction with *Aa* induces exocytosis of azurophilic granules and release of epinephrine. Furthermore, we show that treatment with latrunculin A followed by fMLF stimulation, which is known to induce mobilization and content release of azurophilic granules, induces epinephrine release in human neutrophils. The interaction of released epinephrine with *Aa* activates QseBC and induces *Aa* growth under anaerobic conditions. These results suggest that neutrophils may serve as an epinephrine source for *Aa*, to facilitate growth and prime anaerobic metabolism in the subgingival pocket. The findings presented in this article help shed light into the crosstalk that exits between bacteria and the host endocrine system by providing an example of the role that stress hormones play in periodontal disease and potentially other chronic inflammatory diseases.

# METHODS

## Human Neutrophil Isolation and Purification

Recruitment of human donors, blood draws, and use of required materials were done in agreement with guidelines approved by the Institutional Review Board of the University of Louisville. Human neutrophils were isolated from whole blood of healthy donors using plasma-Percoll gradients as previously described (31). When necessary, neutrophils were further purified to obtain >99% pure population. Purification was carried by negative magnetic selection using the Easy Sep Human neutrophil isolation kit (Stemcell Technologies, Vancouver, BC, Canada). Cell purity was assessed by simultaneously staining with FITC-conjugated anti-CD66b (clone G10F5; BioLegend, San Diego, CA, USA) and APC-conjugated anti-CD16 (clone CB16; eBioscience, San Diego, CA, USA) antibodies and determining the percentage of $CD66b^{+}CD16^{+}$ cells using a BD Celesta flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo for analysis (FlowJo, LLC, Ashland, OR, USA). Both pure (>90%–95%) and highly pure (>99%) neutrophils were cultured in RPMI-1640 medium without phenol red (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 5% human serum (Atlanta Biologicals, Flowery Branch, GA, USA).

## Bacteria Strains and Media

In this study, we make use of *A. actinomycetemcomitans* 652 serotype c strain, a low leukotoxic, afimbriated, smooth colony-morphotype variant. Considered an opportunistic pathogen, this strain has been isolated from healthy and in large numbers in periodontitis-positive individuals (32, 33). At sublytic concentrations of leukotoxin A, there is an increase in calcium levels and neutrophil activation leading to oxidative burst and degranulation (34). *A. actinomycetemcomitans* strains (**Supplementary Table 1**) were propagated in brain–heart infusion (BHI, Difco, BD Biosciences, Franklin Lakes, NJ, USA) broth supplemented with bacitracin (50 μg/ml) and vancomycin (50 μg/ml), unless indicated otherwise. *A. actinomycetemcomitans* was grown at 37°C under microaerophilic conditions in a closed tube. The *Aa* mutant strains (all in *Aa* 652 background, see **Supplementary Table 1**) used for epinephrine interaction experiments were previously constructed by Juarez-Rodríguez et al. (24): *Aa ΔqseC* (non-polar *qseC* gene deletion mutant, spectinomycin 50 μg/ml), *Aa qseCΔp* (QseC sensor protein with an in-frame deletion of the periplasmic sensor domain, spectinomycin 50 μg/ml), and *Aa ΔqseC-comp* (non-polar *qseC* gene deletion mutant complemented with a single genomic copy of the *qseC* gene, spectinomycin 50 μg/ml). The *Aa* strain expressing the plasmid pDJR29, which contains the *lacZ* gene controlled by the qseBC promoter (35), was used in β-galactosidase assays and was grown under anaerobic conditions in CDM (**Supplementary Table 2**) (36), supplemented with kanamycin (25 μg/ml). All reagents used in anaerobic experiments were oxygen-depleted.

*Filifactor alocis (F. alocis)* ATCC 38596 was cultured in BHI broth supplemented with 5 mg/ml yeast extract, L-cysteine (0.05%), and arginine (0.05%) for 7 days anaerobically at 37°C as previously described (36–38).

## *A. actinomycetemcomitans* Challenge, Epinephrine Detection, and Neutrophil Viability

Human neutrophils ($3 \times 10^6$ cells/ml) were challenged in suspension with *Aa* at a multiplicity of infection (MOI) (39) of 50 at 37°C in a shaking water bath for 2 h, 4 h, 8 h, and 24 h, unless indicated otherwise. As a positive control for epinephrine release, neutrophils were treated with latrunculin A (1 μM, Sigma-Aldrich, St. Louis, MO, USA) for 30 min, followed by stimulation with N-formylmethionyl-leucyl-phenylalanine (fMLF, 300 nM, Sigma-Aldrich, St. Louis, MO, USA) for 5 min at 37°C in a shaking water bath. At the end of incubation,

samples were centrifuged at 6,000 × *g* for 30 s and supernatants were collected and supplemented with 100× Halt Protease and Phosphatase inhibitor single-use cocktail (1:10 dilution; Thermo Fisher Scientific, Waltham, MA, USA). The pelleted cells were lysed with ice-cold 1× lysis buffer (10 µl per $1 \times 10^6$ of cells; [20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.5% [vol/vol] Nonidet P-40, 20 mM NaF, 20 mM $NaVO_3$, 1 mM EDTA, 1 mM EGTA, 5 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM diisopropylfluorophosphate [DFP], 21 µg/ml aprotinin, and 5 µg/ml leupeptin]). Lysates were centrifuged and cell lysate was supplemented with 100× Halt Protease and Phosphatase inhibitor single-use cocktail (1:10 dilution; Thermo Fisher Scientific, Waltham, MA, USA). Epinephrine was measured in supernatants and cell lysate with a commercially available Adrenaline/Epinephrine ELISA kit (Cat. No. E4359, BioVision Inc., Milpitas, CA, USA).

Neutrophil viability when challenged with *Aa* was determined by Trypan Blue exclusion and cytospin microscopy imaging. For Trypan Blue exclusion, neutrophils were diluted 1:20 in Trypan blue at 0 h, 4 h, 8 h, and 24 h, and live cells were counted using a hemacytometer. For cytospin images, cells were centrifuged at 6,000 × *g* for 30 s and washed twice with RPMI-1640 (no phenol red, Sigma-Aldrich, St. Louis, MO, USA) to remove bacteria. Neutrophils were resuspended at $1 \times 10^5$ cells in 200 µl of RPMI-1640 and 5 µl of human serum was added (Atlanta Biologicals, Flowery Branch, GA, USA). The cell suspension was loaded into the funnel chamber that is assembled on the cytocentrifuge clip, with slide and filter. Cytocentrifuge clip was centrifuged for 5 min at 800 rpm (Shandon Cytospin 3, Thermo Fisher Scientific, Waltham, MA, USA). The microscope slide was removed from the cytocentrifuge clip and fixed and stained using the Hema 3 Protocol staining kit (Thermo Fisher Scientific, Waltham, MA, USA).

## Catecholamine Metabolism Enzyme-Linked Immunosorbent Assay (ELISA)

Cell lysates collected from neutrophils challenged with *Aa* at 2 h, 4 h, 8 h, and 24 h were tested for the presence of enzymes involved in catecholamine metabolism. The levels of tyrosine hydroxylase (Cat. No. NBP3-06920, Novus Biologicals, CO, USA), dopamine β-hydroxylase (Cat. No. NBP2-67945, Novus Biologicals, CO, USA), catechol-o-methyltransferase (Cat. No. OKBB00966, Aviva Systems Biology Corp., San Diego, CA, USA), and monoamine oxidase-A (Cat. No. OKEH02825, Aviva Systems Biology Corp., San Diego, CA, USA) were measured following the indicated protocols supplied by the kit manufacturer.

## Neutrophil Granule Exocytosis and Exocytosis Inhibition

Neutrophils ($4 \times 10^6$ cells/ml) were challenged with fMLF (300 nM, 5 min), latrunculin A (1 µM, 30 min) + fMLF (300 nM, 5 min), or *Aa* at various time points from 5 min to 24 h at 37°C in a shaking water bath. The exocytosis of secretory vesicles, specific granules, and azurophilic granules was determined by measuring the plasma membrane increase of granule markers using fluorescein isothiocyanate (FITC)-conjugated anti-CD63 (for azurophilic granules, Ancell 215-040, Stillwater, MN, USA), FITC-conjugated anti-CD66b (for specific granules, Biolegend 305104, San Diego, CA, USA), and phycoerythrin (PE)-conjugated anti-CD35 (for secretory vesicles, Biolegend 333406, San Diego, CA, USA) by a FACSCalibur flow cytometer as previously described (31, 40). Following antibody incubation, cells were washed with 0.5% sodium azide (S2002, Sigma, St. Louis, MO, USA) in FTA buffer (211248 BD, Franklin Lakes, NJ, USA) and fixed with 1% paraformaldehyde (PX0055-3, EMD, Darmstadt, Germany). Gelatinase granule exocytosis was determined by measuring the release of matrix metallopeptidase 9 (MMP-9) by ELISA (Cat. No. ab100610, Abcam, Cambridge, MA, USA).

TAT-SNAP23 and TAT-Syntaxin 4 were used to inhibit neutrophil granule exocytosis as previously described by Uriarte et al. (31) and McLeish et al. (40). Briefly, neutrophils ($4 \times 10^6$ cells/ml) were pretreated with TAT-Syntaxin 4 (0.8 µg/ml), TAT-SNAP23 (0.8 µg/ml), or TAT-control (1 µg/ml) for 15 min, followed by challenge with *Aa* for 15 min, 2 h, 4 h, and 8 h at 37°C in a shaking water bath. Granule exocytosis was measured by increased plasma membrane expression of CD35 (secretory vesicles), CD66b (specific granules), and CD63 (azurophilic granules) by flow cytometry as described in a previous paragraph. In addition, cell supernatants were collected to measure release of secretory vesicle content by ELISA for albumin (Cat. No. EHALB, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), gelatinase content by ELISA for MMP-9, specific granule content by ELISA for lactoferrin (Cat. No. ab108882, Abcam, Cambridge, MA, USA), and azurophilic granule content by ELISA for myeloperoxidase (Cat. No. ab119605, Abcam, Cambridge, MA, USA).

## Recombinant Epinephrine Interaction With *A. actinomycetemcomitans*

Epinephrine interaction with *Aa*: Increasing colony-forming units (CFUs) of *Aa* ($0.0–4.0 \times 10^8$ CFUs) were incubated with 250 pg/ml of recombinant epinephrine (BioVision Inc., Milpitas, CA, USA) for 15 min at 37°C in a shaking water bath. As a control, wells with epinephrine alone were run in parallel. Samples were centrifuged at 6,000 × *g* for 5 min, and supernatants were collected. Immediately after, epinephrine was measured from supernatants with a commercially available Adrenaline/Epinephrine ELISA kit (Cat. No. E4359, BioVision Inc., Milpitas, CA, USA). For analysis, interpolated values were obtained from recombinant epinephrine standard curve, following manufacturer instructions. The amount of epinephrine associated with *Aa* was determined by subtracting the interpolated values of bacteria-containing wells from the values obtained for control wells.

Epinephrine interaction with *Aa qseC* mutant strains: Mutant strains of *Aa* (*ΔqseC, qseC/Δp*, and *ΔqseC-comp*) were incubated at $3.0 \times 10^7$ CFUs with increasing concentrations (0–500 pg/ml) of recombinant epinephrine (BioVision Inc., Milpitas, CA, USA) for 15 min at 37°C in a shaking water bath. Wells containing increasing concentrations of recombinant epinephrine serve as

control. Afterwards, samples were centrifuged as above, and supernatants were collected. Immediately after, epinephrine was measured and analyzed as described above. The amount of epinephrine associated with *Aa* was determined by subtracting the interpolated values of *Aa*-containing wells from the values of epinephrine control.

### *A. actinomycetemcomitans* Growth Kinetics

*A. actinomycetemcomitans* wild type was inoculated into BHI broth supplemented with bacitracin (50 μg/ml) and vancomycin (50 μg/ml) and grown under microaerophilic conditions at 37°C to an optical density at 600 nm ($OD_{600}$) of 0.3–0.4. Cells were subcultured in fresh BHI broth at a 1:30 dilution and grown as described above to an $OD_{600}$ of 0.5–0.6. Cells were washed with CDM (**Supplementary Table 2**) and inoculated into CDM supplemented as above at a 1:30 dilution. Cultures were supplemented individually or with a combination of 100 μM $FeCl_2$, 50 μM epinephrine, supernatant, or cell lysate collected from neutrophils stimulated with latrunculin A and fMLF (final epinephrine concentration of 30 pg/ml). The $OD_{600}$ was measured at various time points using a Bio-Rad SmartSpec Plus UV-vis spectrophotometer (Bio-Rad, Hercules, CA, USA).

### β-galactosidase Assay

Quantitative evaluation of β-galactosidase activity was determined using permeabilized cells incubated with o-nitrophenyl-b-D-galactopyranoside (ONPG) substrate (Sigma, St Louis, MO, USA) as previously described (41). Briefly, a primary culture of *Aa* (pDJR29) was grown at 37°C under microaerophilic conditions in a closed tube overnight in BHI broth. The culture was subcultured at a 1:30 dilution in BHI broth and grown as described in the previous paragraph for 24 h. Subsequently, the secondary overnight culture ($OD_{600}$ of 0.3–0.4) was diluted 1:30 into CDM and grown in an anaerobic chamber for 24 h at 37°C. An aliquot of 0.1 ml was then used to determine the $OD_{600}$ of the culture at 24 h and triplicate aliquots of 0.1 ml were used to measure β-galactosidase activity.

### Statistical Analysis

Unless otherwise noted, statistical experimental conditions and time points were analyzed by a one-way ANOVA, followed by post-hoc Tukey's multi-comparison test using GraphPad Prism Software (GraphPad PRISM v9, San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

## RESULTS

### *A. actinomycetemcomitans* Challenge Induces Epinephrine Release and Promotes *De Novo* Catecholamine Synthesis in Human Neutrophils

It has been previously shown that mouse neutrophils stimulated with LPS can release catecholamines (Flier 2007). However, there is as of yet no clear evidence of human neutrophils' ability to release catecholamines. To determine if human neutrophils release epinephrine, cells were stimulated with fMLF, latrunculin A + fMLF, LPS, or IL-8. Supernatants and cell lysates were collected, and epinephrine levels were determined by ELISA. Unlike what was described in murine neutrophils (27), LPS stimulation did not induce epinephrine release by human neutrophils (**Figure 1A**). Similarly, stimulation of neutrophils with fMLF or IL-8 failed to induce epinephrine release (**Figure 1A**). Interestingly, treatment with latrunculin A followed by fMLF induced significant release of epinephrine compared to fMLF alone and basal (**Figure 1A**). Significant levels of epinephrine were detected in the cell lysates but there was no significant difference between experimental conditions (**Figure 1B**). Afterward, we examined the ability of *Aa* to induce epinephrine release by human neutrophils. Cells were challenged with *Aa* at a MOI of 50 in suspension for 2 h, 4 h, 8 h, and 24 h. Epinephrine levels were measured from supernatants and cell lysates by ELISA. **Figure 1C** shows that *Aa* challenge induced significant release of epinephrine compared to basal, with maximum levels reached at 4 h. Epinephrine levels in cell lysates of human neutrophils treated with latrunculin A + fMLF had significantly reduced levels of epinephrine compared to basal (**Figure 1D**); this correlates the significant increase release of epinephrine observed in supernatants (**Figure 1C**). Similarly, low epinephrine levels in neutrophil cell lysates were detected after challenged with *Aa* for 2 h and 4 h, which could be related to the release observed in the supernatants (**Figures 1C, D**). Interestingly, epinephrine release at 2 h was similar to basal (**Figure 1C**). A potential explanation for this is that the released epinephrine could be interacting in some way with *Aa*. We repeated this experiment with highly purified (>99%) human neutrophils (**Supplementary Figure 1**) and obtained similar results.

To confirm that the release of epinephrine is not caused by a cytotoxic effect of *Aa* on neutrophils, we evaluated the morphology of human neutrophils with cytospin imaging and viability with trypan blue exclusion. Human neutrophil morphology was not altered with *Aa* challenge (**Supplementary Figures 2A, B**) and challenge with *Aa* extended the lifespan of neutrophils (**Supplementary Figure 2C**) up to 24 h. Taken together, our results demonstrate that *Aa* stimulates epinephrine release in human neutrophils, depleting storage reservoirs. In addition, the difference in released (**Figure 1C**) versus stored epinephrine at 2 h (**Figure 1D**) suggests that epinephrine may be interacting with *Aa*. The increase of epinephrine levels in cell lysates at later time points of 8 h and 24 h (**Figure 1D**) suggests that human neutrophils are capable of *de novo* catecholamine synthesis.

Next, we examined the levels of enzymes involved in catecholamine metabolism. Levels of enzymes involved in catecholamine synthesis—tyrosine hydroxylase and dopamine β-hydroxylase—and levels of enzymes involved in catecholamine inactivation—catechol-o-methyltransferase and monoamine oxidase-A—were measured in cell lysates of human neutrophils exposed to *Aa* at 2 h, 4 h, 8 h, and 24 h by ELISA. In the absence of *Aa*, tyrosine hydroxylase levels are significantly low, but after *Aa* challenge, levels increase significantly with time compared to

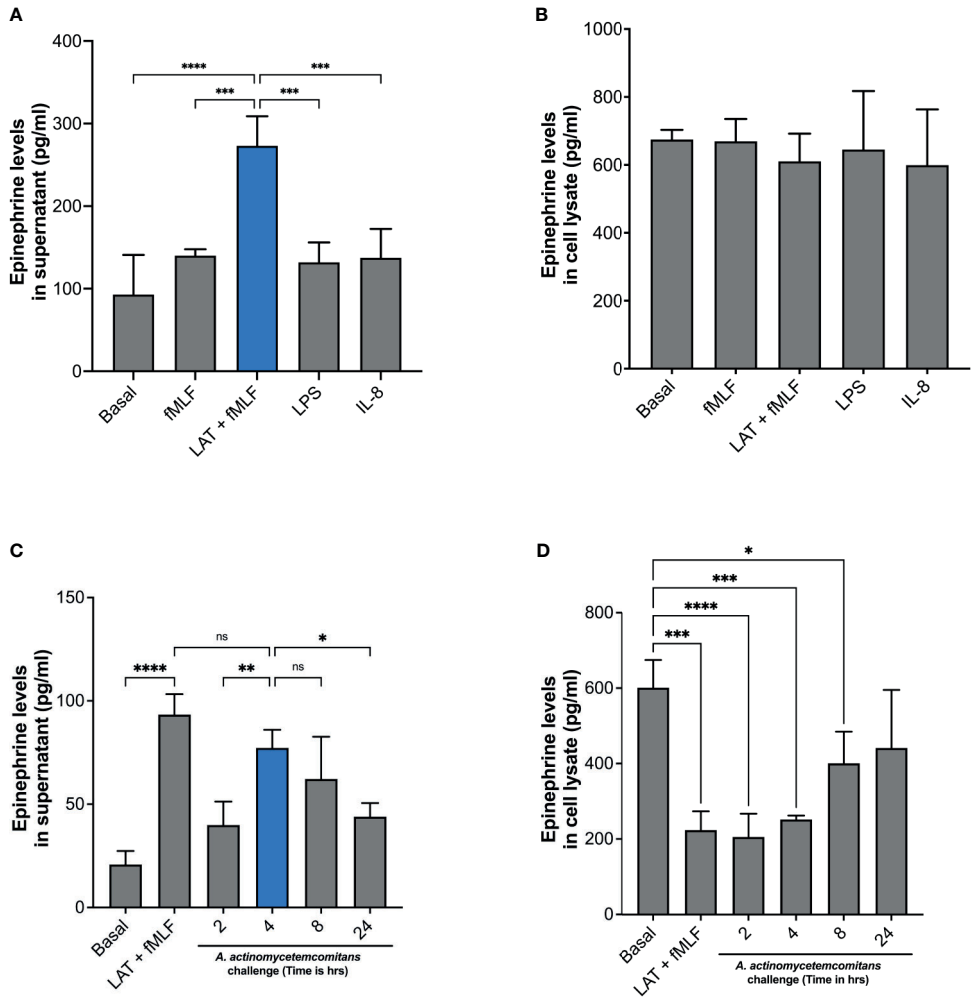



**FIGURE 1** | Human neutrophils release epinephrine upon *A. actinomycetemcomitans* challenge. Human neutrophils ($5 \times 10^6$ cells/ml) were treated with fMLF, Latrunculin A (LAT) + fMLF, LPS, or IL-8 to induce epinephrine release. Epinephrine content was determined in neutrophil supernatant **(A)** and cell lysate **(B)** by ELISA. Human neutrophils ($3 \times 10^6$ cells/ml) were challenged with *A. actinomycetemcomitans* (*Aa*; MOI 50) for 2 h, 4 h, 8 h, and 24 h At the end of each time point, supernatant **(C)** and cell lysates **(D)** were collected and epinephrine content was measured by ELISA. Epinephrine concentration is plotted as the mean ± SD of four independent experiments. Statistical differences among experimental conditions and time points were analyzed by one-way ANOVA **(A, B)** or repeated measures one-way ANOVA **(C, D)**, followed by Tukey's post-test. ns, not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

basal (**Figure 2A**). In contrast, dopamine β-hydroxylase levels decrease when *Aa* is present (**Figure 2B**). The levels of catechol-o-methyltransferase (**Figure 2C**) and monoamine oxidase-A (**Figure 2D**) remained comparable to basal, only peaking at 4 h. Catecholamine synthesis enzyme (tyrosine hydroxylase levels) increase at 8 h and 24 h (**Figure 2A**), whereas inactivation enzymes (**Figures 2C, D**) decrease at the same time points. These results indicate that *Aa* challenge induces *de novo* catecholamine synthesis in human neutrophils.

In the infected subgingival pocket, *Aa* functions in consortium with other bacteria in bacterial communities known as biofilms and Fine et al. showed that *A. actinomycetemcomitans* positive subjects with bone loss had high levels of the oral pathogen *F. alocis* (42). In addition, Wang et al. demonstrated that *F. alocis* accumulation in the oral biofilm was stimulated by the presence of specific strains of *Aa* (43). To examine if the induction of epinephrine release is specific to *Aa*, we performed a time course experiment where we investigated the ability of *F. alocis* to induce epinephrine release in human neutrophils. Human neutrophils were challenged with *F. alocis* at a MOI of 10 at 1 h, 2 h, and 4 h incubation and epinephrine detection was performed as described above. *F. alocis* failed to induce significant release of epinephrine (**Supplementary Figure 3A**). Consistent with this, no significant difference in epinephrine content was detected in cell lysates among experimental conditions (**Supplementary Figure 3B**). In addition, human neutrophils were challenged with LPS up to 24 h and no significant epinephrine release or difference in epinephrine content was observed (**Supplementary Figures 3C, D**).

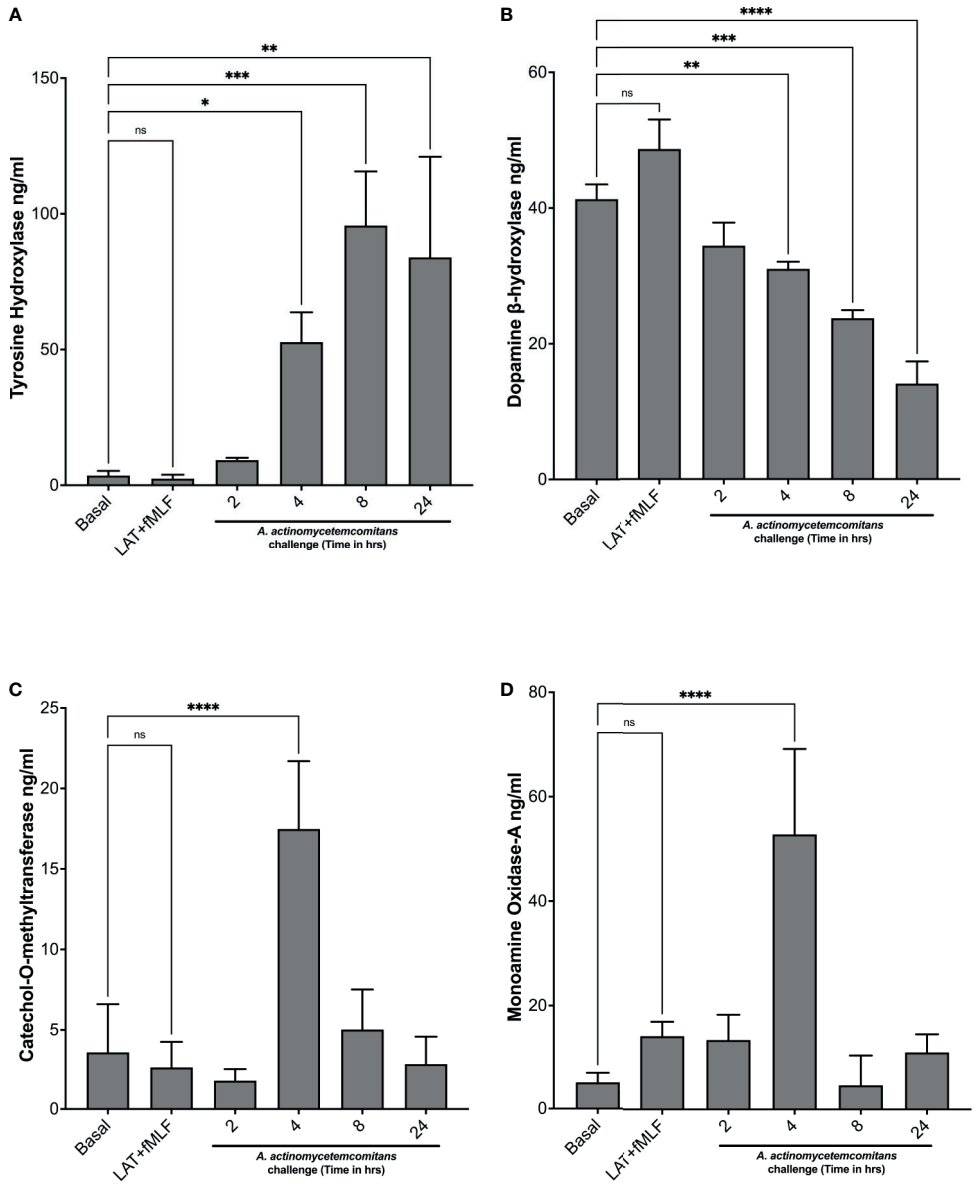



**FIGURE 2** | *A. actinomycetemcomitans* promotes increased levels of enzymes involved in catecholamine metabolism in human neutrophils. Human neutrophils were challenged with *A. actinomycetemcomitans* (MOI 50) for 2 h, 4 h, 8 h and 24 h; cell lysates were collected, and the levels of tyrosine hydroxylase **(A)**, dopamine β-hydroxylase **(B)**, catechol-O-methyltransferase **(C)**, and monoamine oxidase A **(D)** were measured by ELISA. Enzyme levels are plotted as the mean ± SD of three independent experiments. Statistical differences among experimental conditions and time points were analyzed by repeated measures one-way ANOVA, followed by Tukey's post-test. ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

## *A. actinomycetemcomitans* Access Epinephrine by Inducing Exocytosis of Human Neutrophil Granules

In **Figure 1**, we showed that treatment with latrunculin A followed by stimulation with fMLF, which is known to induce the exocytosis of gelatinase and azurophilic granules, induced epinephrine release by human neutrophils, suggesting that epinephrine may be stored in one or more of the neutrophil granules. To demonstrate this, we first determined the ability of *Aa* to induce granule exocytosis. Neutrophils were challenged with *Aa* and granule exocytosis was determined by measuring increase of plasma membrane expression of secretory vesicles, specific granules, and azurophilic granule markers by flow cytometry (see gating strategy and histograms; **Supplementary Figure 4**) and the extracellular release of MMP-9 by ELISA. Stimulation with *Aa* induced significant secretory vesicles mobilization by 15 min, as indicated by increased CD35 expression (**Figure 3A**), which was similar to the exocytosis

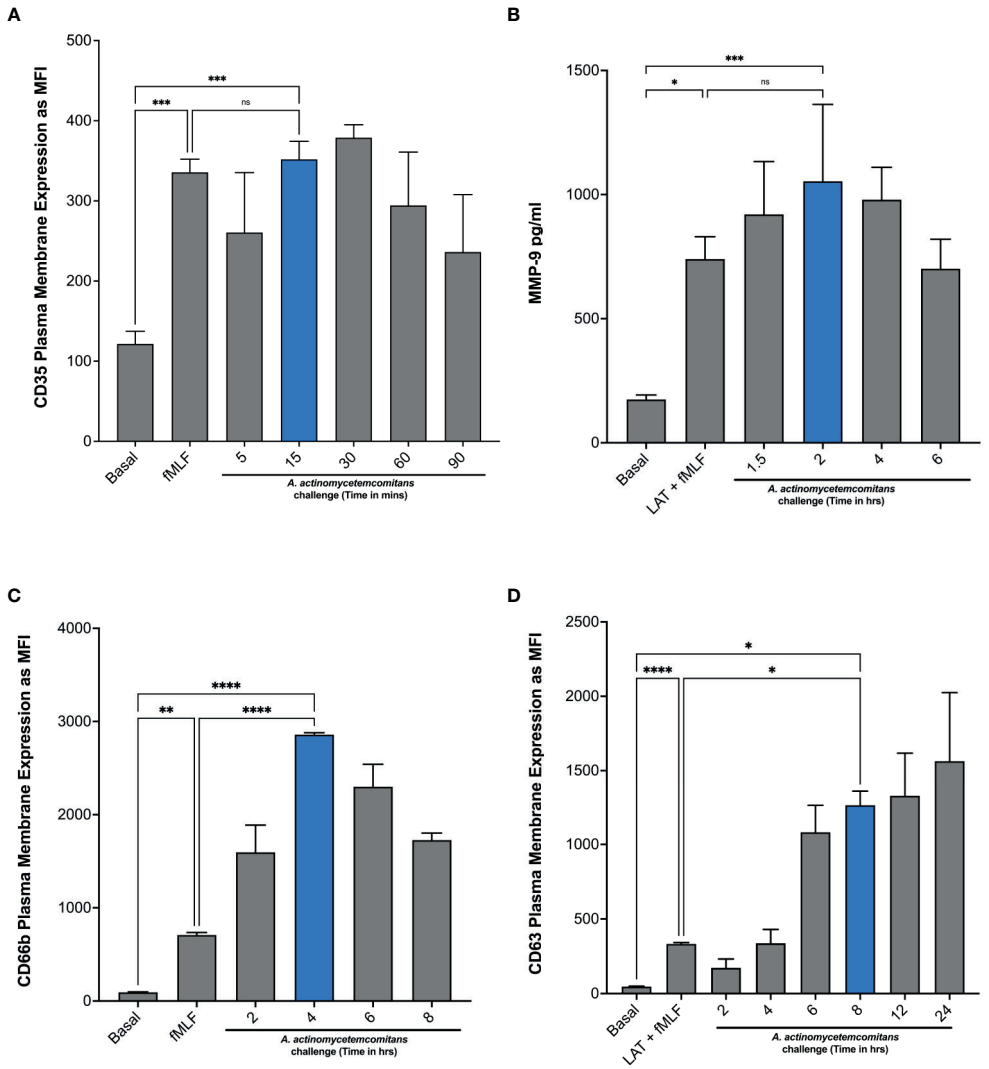



**FIGURE 3** | *A. actinomycetemcomitans* induces exocytosis of all four neutrophil granule subtypes. Induction of neutrophil granule exocytosis was determined by challenging human neutrophils with *A. actinomycetemcomitans* (MOI 50) for 1.5 h, 6 h, 8 h, and 24 h Increased plasma membrane expression of CD35 **(A)**, CD66b **(C)**, and CD63 **(D)** was measured to define secretory vesicles, specific granules, and azurophil granule exocytosis, respectively, by flow cytometry. Basal levels and latrunculin (Lat) + fMLF were used as negative and positive control. Granule markers are plotted as the mean channel florescence intensity (MFI) ± SEM of three independent experiments. For gelatinase granule exocytosis, cell supernatants were collected at basal and following each stimulation, and levels of matrix metallopeptidase 9 (MMP-9) were measured by ELISA **(B).** MMP-9 concentration is plotted as the mean ± SD of three independent experiments. Statistical differences among experimental conditions and time points were analyzed by repeated measures one-way ANOVA, followed by Tukey's post-test. ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

induced by the positive control fMLF. In addition, gelatinase granule exocytosis, measured by ELISA, was significantly induced after 2 h of *Aa* challenge (**Figure 3B**). Specific granule exocytosis, measured by increased CD66b expression, was significantly increased after 4 h of *Aa* challenge (**Figure 3C**). Azurophilic granule exocytosis, measured by increased CD63 expression, was significantly increased starting at 8 h post bacterial challenge (**Figure 3D**). Mobilization of azurophilic granules requires stronger stimulation compared to the other granule subtypes; our results showed that these granules continued to be mobilized by *Aa* increasingly up to 24 h (**Figure 3D**). These results demonstrate that *Aa* challenge induced exocytosis of all four neutrophil granule subtypes.

To further characterize if epinephrine release by neutrophils was caused by *Aa* inducing granule exocytosis, we took advantage of two degranulation inhibitors, TAT-SNAP23, which selectively inhibits secretory vesicles, gelatinase granules, and specific granules (31), and TAT-Syntaxin 4, which blocks exocytosis of all four granule subtypes (40). Human neutrophils were pre-treated with TAT-Syntaxin 4, TAT-SNAP23, or TAT-control peptide fusion proteins, followed by *Aa* challenge at 15 min, 2 h, 4 h, and 8 h. Granule exocytosis was measured by increased

plasma membrane expression of CD35 (secretory vesicles), CD66b (specific granules), and CD63 (azurophilic granules) markers by flow cytometry and the extracellular release of MMP-9 by ELISA. Treatment with TAT-Syntaxin 4 reduced *Aa*-induced mobilization of secretory vesicles (**Figure 4A**), gelatinase granules (**Figure 4B**), specific granules (**Figure 4C**), and azurophilic granules (**Figure 4D**). The *Aa*-induced mobilization of secretory vesicles, gelatinase, and specific granules (**Figures 4A–C**) was significantly reduced by TAT-SNAP23 pre-treatment. As expected, TAT-SNAP23 failed to inhibit *Aa*-induced azurophilic granule exocytosis (**Figure 4D**). Furthermore, cell supernatants were collected at each time point, and granule content release of albumin, lactoferrin, and myeloperoxidase were measured by ELISA. Similar to the results obtained by flow cytometry, challenge of neutrophils with *Aa* for 15 min induced significant release of albumin, which was significantly reduced by

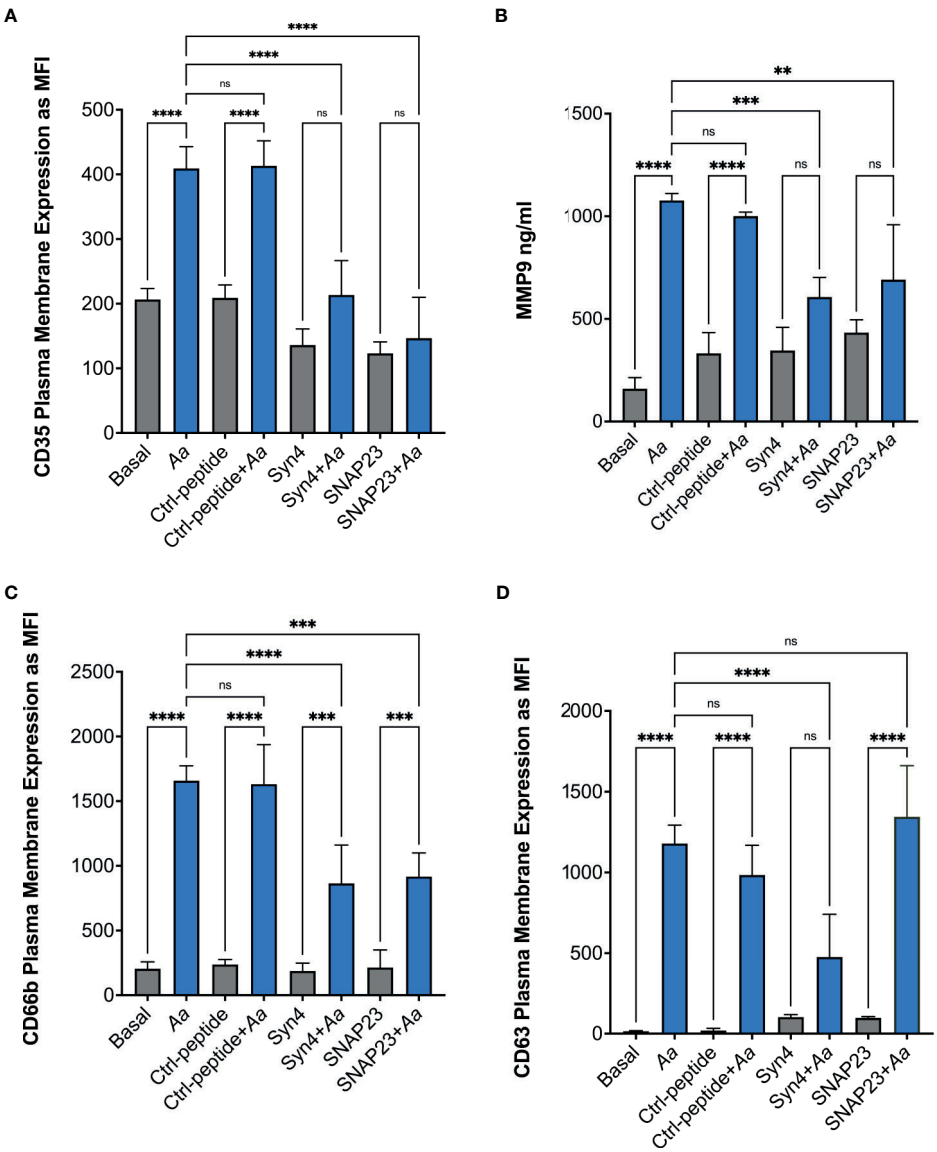



**FIGURE 4** | Pre-treatment with TAT fusion proteins prevented *A. actinomycetemcomitans*-induced neutrophil granule exocytosis. Human neutrophils were unstimulated, challenged with *A. actinomycetemcomitans (Aa)* at MOI 50, or pre-treated with TAT-Syntaxin 4 (Syn4), TAT-SNAP23 (SNAP23), and TAT-Control peptide (Ctrl-peptide) for 15 min followed by *A. actinomycetemcomitans* challenge. The peak time point of *Aa*-induced granule exocytosis was different for each granule subtype. Secretory vesicle peak was at 15 min post-bacterial challenge **(A)**, 2 h for gelatinase granules **(B)**, 4 h for specific granules **(C)**, and 8 h for azurophilic granules **(D)**. Granule markers were measured by flow cytometry and plotted as the mean channel florescence intensity (MFI) ± SEM of four independent experiments **(A, C, D)**. For gelatinase granules, matrix metallopeptidase 9 (MMP-9) was measured from human neutrophil supernatant by ELISA and MMP-9 concentration is plotted as the mean ± SD of four independent experiments **(B)**. Statistical differences among experimental conditions were analyzed by ordinary one-way ANOVA, followed by Tukey's post-test. ns, not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

pre-treatment with TAT-SNAP23 and TAT-Syntaxin 4, but not TAT-control (**Supplementary Figure 5A**). Similarly, *Aa* challenge for 4 h induced release of lactoferrin, and its release was successfully reduced by TAT-SNAP23 and TAT-Syntaxin 4, but not TAT-control (**Supplementary Figure 5B**). As expected, neutrophils challenged with *Aa* for 8 h resulted in significant release of myeloperoxidase, which was inhibited by TAT-Syntaxin 4 but not TAT-SNAP23 or TAT-control (**Supplementary Figure 5C**).

To further characterize which granule subtype might store epinephrine, supernatants were collected to measure epinephrine levels by ELISA. *A. actinomycetemcomitans* challenge of human neutrophils at 15 min showed no significant release of epinephrine similar to basal and TAT-control (**Supplementary Figure 6A**). Moreover, *Aa* challenge at 2 h induced significant release of epinephrine in human neutrophils; pre-treatment of TAT-Syntaxin 4 was able to inhibit epinephrine release but TAT-SNAP23 failed to inhibit release (**Supplementary Figure 6B**). On the other hand, only pre-treatment with TAT-Syntaxin 4, but not TAT-SNAP23, blocked *Aa*-induced epinephrine released after 4 h (**Figure 5A**) and 8 h (**Figure 5B**) of bacterial challenge. Since TAT-SNAP23 does not inhibit azurophilic granule exocytosis, these results demonstrate that epinephrine is stored in azurophilic granules. Furthermore, this shows that epinephrine release by neutrophils is linked to granule exocytosis.

## *A. actinomycetemcomitans* Interacts With Epinephrine in a QseC-Dependent Manner

To determine if *Aa* interacts with epinephrine, increasing CFUs of *Aa* were incubated with a fixed concentration of recombinant epinephrine. Epinephrine interaction with *Aa* increased with increasing *Aa* CFUs (**Figure 6A**). Previous work in our lab demonstrated that QseBC was required for *Aa* biofilm growth and virulence (25) and that the periplasmic domain of QseC was required for activation of the TCS by catecholamines and iron (6). Therefore, we performed epinephrine dose interaction assays using various *Aa* strains deficient for QseC. Interaction of epinephrine was diminished by deletion of the QseC sensor (*ΔqseC)* and in the strain expressing QseC with an in-frame deletion of the periplasmic domain (*qseCΔp)* (**Figure 6B**). The ability of epinephrine to interact with *Aa* was restored when the *qseC* gene was complemented into the QseC deletion mutant (*ΔqseC-comp*) (**Figure 6B**). These results show that *Aa* interaction with epinephrine requires QseC and is dependent on the QseC periplasmic domain.

## Epinephrine From Human Neutrophils Promotes *A. actinomycetemcomitans* Growth and *qseBC* Expression

To determine if the epinephrine levels released by human neutrophils could promote bacterial growth, the supernatants and cell lysates were collected from cells stimulated with latrunculin A and fMLF and used to assess *Aa* growth and expression of the *qseBC* operon. *A. actinomycetemcomitans* was grown anaerobically in CDM alone or supplemented individually or in combination with neutrophil supernatant, neutrophil cell lysate, iron (100 μM), or epinephrine (50 μM). Growth in CDM supplemented with neutrophil lysate or epinephrine alone was comparable to the unsupplemented CDM control (**Figure 7A**). Supplementation with iron, either alone or in combination with epinephrine, resulted in a significant induction of *Aa* growth

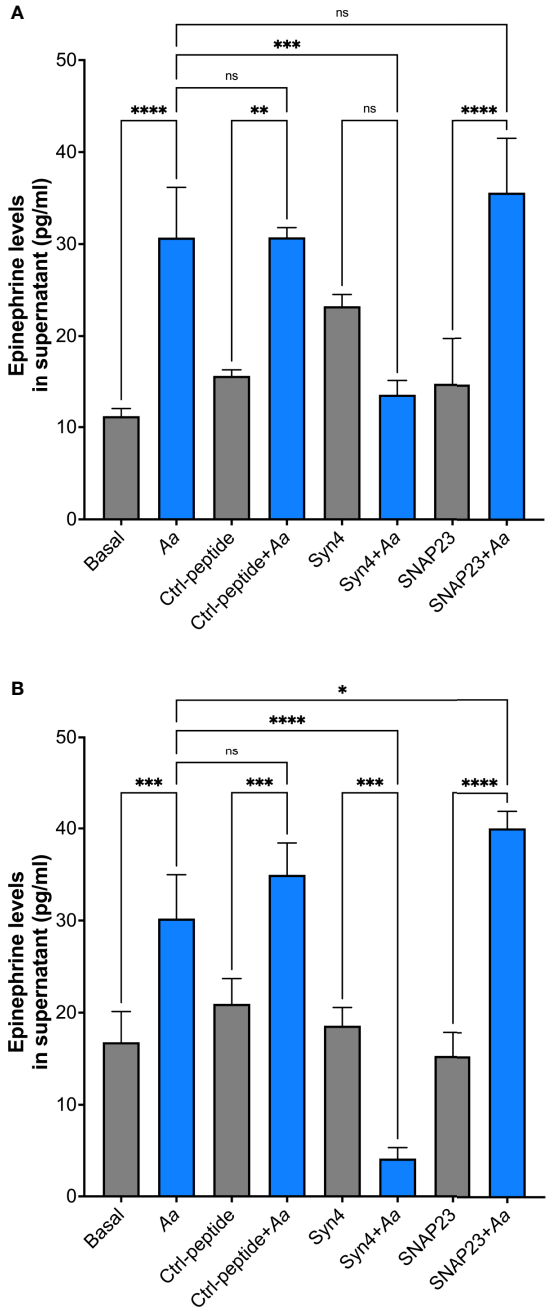



**FIGURE 5** | Human neutrophils store epinephrine in azurophilic granules. Epinephrine storage location was determined by pre-treating human neutrophils with TAT-Syntaxin 4 (Syn4), TAT-SNAP23 (SNAP23), or TAT-Ctrl (Ctrl) for 15 min, followed by a 4-h challenge with *A. actinomycetemcomitans* at MOI 50 **(A)** or 8 h **(B)**. Epinephrine concentrations are plotted as the mean ± SD of three independent experiments. Statistical differences among experimental conditions were analyzed by ordinary one-way ANOVA, followed by Tukey's post-test. ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

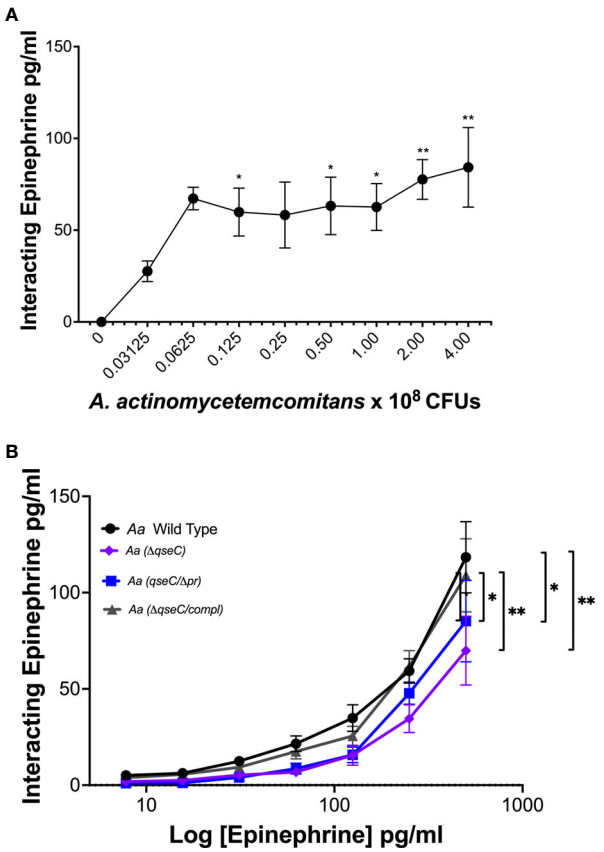



**FIGURE 6** | *A. actinomycetemcomitans* interacts with epinephrine in a QseC-dependent manner. The interaction of epinephrine and *A. actinomycetemcomitans* was determined by incubation of increasing colony-forming units (CFUs) of *A. actinomycetemcomitans* with 250 pg/ml of recombinant epinephrine. Associated epinephrine was plotted against increasing CFUs of *Aa* **(A)**. Requirement of QseC for epinephrine interaction with *A. actinomycetemcomitans* was determined by incubation of the bacterium with increasing concentrations of recombinant epinephrine (0–500 pg/ml) **(B)**. Epinephrine levels were measured by ELISA and plotted as the mean ± SD of five independent experiments. Statistical differences among experimental conditions were analyzed by ordinary one-way ANOVA **(A)** or two-way ANOVA **(B)**, followed by Tukey's post-test. *$p < 0.05$. **$p < 0.01$.

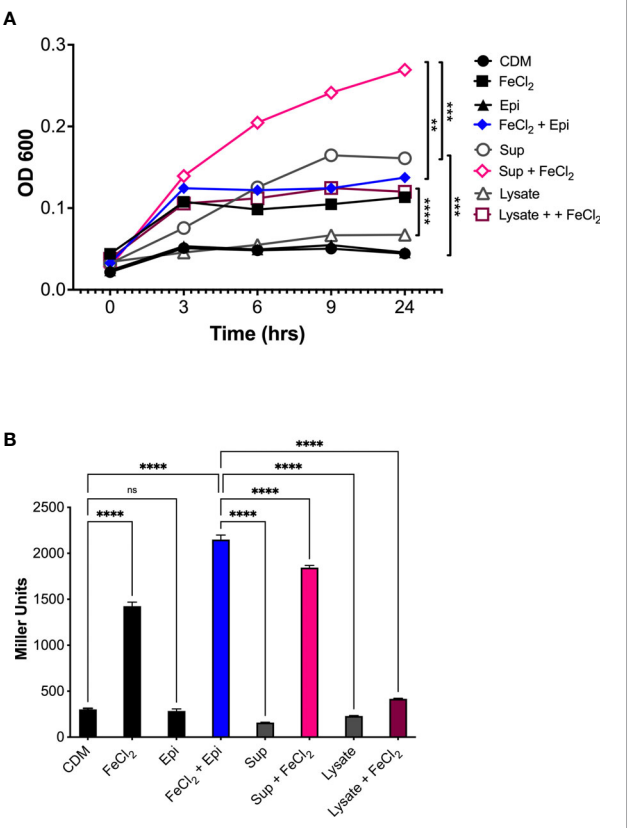



**FIGURE 7** | Host-derived epinephrine promotes *A. actinomycetemcomitans* growth and *qseBC* expression. The supernatants and lysates from human neutrophils stimulated with latrunculin A and fMLF were collected and used as supplement for chemically defined media (CDM). *A. actinomycetemcomitans* was grown under anaerobic conditions in CDM alone or supplemented with human neutrophil supernatant (Sup), cell lysate (Lysate), iron ($FeCl_2$, 100 μM), or Epinephrine (Epi, 50 μM). At designated times, growth was measured by determining the optical density at 600 nm ($OD_{600}$) **(A)**. *A. actinomycetemcomitans* strain (that harbors the *qseBC* promoter-*lacZ* reporter plasmid pDJR29) was grown under anaerobic conditions in CDM alone or supplemented with human neutrophil supernatant (Sup), cell lysate (Lysate), iron ($FeCl_2$, 100 μM), or Epinephrine (Epi, 50 μM). Expression of the *qseBC* operon was determined by measuring β-galactosidase activity after 24 h **(B)**. The $OD_{600}$ measurements for growth were plotted as the mean ± SEM of three independent experiments **(A)**. The expression of the *qseBC* operon is presented in miller units as the mean ± SD of three independent experiments **(B)**. Statistical differences among experimental conditions were analyzed by two-way ANOVA **(A)** or ordinary one-way ANOVA **(B)**, followed by Tukey's post-test. ns, not significant, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

(**Figure 7A**). Furthermore, medium supplemented with neutrophil supernatant alone promoted growth, which was increased when supplemented with iron (**Figure 7A**). On the other hand, neutrophil cell lysate supplemented with iron promoted growth similar to epinephrine + iron or neutrophil supernatant alone (**Figure 7A**). Supplementation with epinephrine, neutrophil supernatant, or lysate alone failed to induce expression of QseBC (**Figure 7B**). QseBC expression was significantly induced by iron and a further significant increase in expression occurred by supplementation with a combination of iron with epinephrine or neutrophil supernatant (**Figure 7B**). Unexpectedly, supplementation with iron and neutrophil lysate suppressed QseBC expression (**Figure 7B**). This experiment was performed under aerobic conditions with similar results (data not shown). Here, we show that *Aa* makes use of neutrophil-derived epinephrine to promote bacterial growth and expression of the QseBC in the anaerobic environment.

## DISCUSSION

The newly emerging field of microbial endocrinology (16, 44) studies the communication or inter-kingdom signaling (45) that has evolved between microorganisms and their hosts. The study

of inter-kingdom signaling includes the study of hormonal communication, where microorganisms respond to the host neurohormones as environmental cues to regulate the expression of genes necessary for virulence and survival (44, 46–49). There are reports of host–pathogen crosstalk involving *Aa* biofilm sequestering and taking up IL-1β (50). Additionally, it has been shown previously that *Aa* expresses a cytokine binding receptor, BilRI, that allows it to benefit from cytokine release to promote biofilm formation and bacterial growth (51, 52). Previous work by our group demonstrated that the presence of catecholamines and iron *in vitro* promoted *Aa* growth and regulated the expression of genes necessary for virulence and survival in the anaerobic environment (6). However, those studies did not determine if similar effects occurred *in vivo*. Based on work by others, it is known that there is a large infiltration of neutrophils at the subgingival pocket during periodontitis (26, 53, 54) and murine neutrophils have been shown to release and synthesize catecholamines when stimulated with LPS (27). Therefore, we considered human neutrophils as a potential source of catecholamines for *Aa*. In our *ex vivo* studies, we showed that *Aa* induces epinephrine release in human neutrophils depleting stored catecholamine levels and indirectly inducing catecholamine synthesis. As a novel finding, we identified azurophilic granules as the storage location of epinephrine in human neutrophils and that *Aa* gains access to epinephrine by inducing granule exocytosis. Finally, we proved that host-derived epinephrine promoted *Aa* growth and QseBC expression under anaerobic conditions.

Marino et al. (28) detected catecholamines in human peripheral blood mononuclear cell medium, but the report failed to determine if these catecholamines were released and, if so, what stimuli induced release. Likewise, catecholamine release has been shown in other immune cells such as rat and human lymphocytes (55, 56). Although there is extensive work done in murine neutrophils, there was no report of human neutrophils releasing catecholamines and identified stimuli. Here, we present for the first time that human neutrophils release epinephrine when stimulated with latrunculin A + fMLF and when challenged with *Aa*, but not by *F. alocis*, which occupies the same oral niche as *Aa* (see below). These observations support previous findings by Parantainen et al. (39), the first to identify endogenous catecholamines in human neutrophils. However, at the time, the source of these catecholamines was not determined or if neutrophils participated in catecholamine synthesis. Later, further evidence of catecholamines present intracellularly in human neutrophils was found and enzymes involved in catecholamine metabolism were detected by high-performance liquid chromatography (HPLC) (57). In our experiments, we observed that the levels of epinephrine in cell lysates increased after 8 h and 24 h of bacterial challenge, suggesting *de novo* catecholamine synthesis by human neutrophils. This was confirmed by the presence of significant levels of tyrosine hydroxylase levels in the presence of *Aa*. In contrast, dopamine β-hydroxylase levels decreased when *Aa* was present. Tyrosine hydroxylase catalyzes the first and rate-limiting step of the catecholamine synthesis pathway, explaining larger amounts of this enzyme. A potential reason for this is that dopamine β-hydroxylase catalyzes dopamine into norepinephrine, and not all dopamine will be catalyzed, explaining the decrease in enzyme levels. We also found significant levels at 4 h of the catecholamine inactivating enzymes catechol-o-methyltransferase and monoamine oxidase-A. This correlates with the increased epinephrine release induced by *Aa* at 4 h; as epinephrine is released, demand for degradation increases. Similarly, as stored epinephrine is depleted, there is demand for catecholamine synthesis at later time points, and inactivation enzymes decrease at this time. A potential limitation of our study is that granule exocytosis experiments were done under aerobic conditions and not under low oxygen or anaerobic conditions. The periodontal pocket is considered a hypoxic environment, with decreasing oxygen levels as the depth of the periodontal pocket increases, becoming increasingly hypoxic during infection due to activation of the NADPH oxidase complex (58, 59). Therefore, it is likely that neutrophils are exposed to hypoxic conditions *in vivo* when recruited to the gingival pocket. Although we did not perform experiments with both *Aa* and neutrophils under anaerobic conditions, preliminary work in our lab with neutrophils challenged with *F. alocis* showed no difference in the lifespan of neutrophils grown aerobically or anaerobically. Additionally, untreated neutrophils under anaerobic conditions resulted in enhanced viability compared to cells grown aerobically. Though we infer that human neutrophils will undergo granule exocytosis under hypoxic conditions, this remains to be tested in the future.

We demonstrated that *Aa* induces the mobilization of secretory vesicles, gelatinase granules, specific granules, and azurophilic granules in human neutrophils. These findings align with previous observations that identified enhanced surface expression of CD63 and CD66b in oral neutrophils, characteristic of neutrophils that are undergoing active granule exocytosis (60, 61). Johansson et al. (62) showed that purified leukotoxin A induces release of granule contents from human neutrophils, and the authors observed increased expression of CD63 and CD66b along with the release of elastase and lactoferrin. In our *ex vivo* model, we obtained similar observations by challenging human neutrophils with a low leukotoxic strain of *Aa* compared to high concentrations of purified leukotoxin. Furthermore, through selective inhibition of granule exocytosis, we identified azurophilic granules as the storage location for epinephrine in human neutrophils. To the best of our knowledge, this observation serves as the first evidence of the storage location of epinephrine in human neutrophils. Human neutrophils are recruited out of circulation by interaction with neutrophil-specific adhesion molecules on the blood vessel wall. This interaction in addition to chemoattractant gradient such as IL-8 promotes the mobilization of secretory vesicles. Once neutrophils cross through the epithelium, they continue to receive signals such as increasing IL-8 gradient potentially released by gingiva epithelial cells and formyl peptides from invading bacteria, resulting in mobilization of gelatinase granules. The primed neutrophil arrives at the infected subgingival pocket where there are overwhelming inflammatory signals that may induce the release of specific and azurophilic granules. Alternatively, these granules

can fuse with vacuoles containing phagocytized bacteria, forming a phagolysosome (63–65). Thus, *Aa* may induce the release of azurophilic granules at the subgingival pocket to gain access to epinephrine and in turn the release of potent antimicrobial protein contents of these granules may contribute to tissue damage and disease progression.

When human neutrophils were challenged with *Aa* for 2 h, we expected to see a high release of epinephrine, due to depleted epinephrine levels in cell lysate. A plausible explanation to the difference in levels of epinephrine released versus stored at 2 h is that some of the epinephrine released could be interacting with *Aa* and therefore no longer in the supernatant. Epinephrine interaction with *Aa* increased with increasing *Aa* CFUs. Furthermore, *Aa* interaction with epinephrine was found to require QseC, in a QseC periplasmic domain-dependent manner. This supports previous findings made by our group that identified catecholamines (norepinephrine and epinephrine) as the signal that is sensed by QseC and that the periplasmic domain of QseC is required for *qseBC* operon expression in the presence of catecholamines and iron (6). It also strengthens the importance of QseC in recognizing catecholamines for growth and virulence of *Aa* (25). The exact details of the interaction of *Aa* with epinephrine remains unclear, but the discussed results make us conclude that the QseC periplasmic domain is required. As for a receptor for epinephrine, when *Aa* is grown in the presence of catecholamines and iron, the expression of the enterobactin operon, which contains a gene for FepA, remains unchanged (6). In *E. coli*, FepA is an outer membrane protein that serves as a receptor for the ferric enterobactin siderophore (66, 67). The uptake of this siderophore into the bacterial cells is TonB-dependent (67, 68). We performed different experiments to determine if FepA was required for *Aa* interaction with epinephrine using a *ΔfepA* mutant *Aa* strain and we found no difference in interaction compared with the wild-type strain (data not shown). Moreover, as an alternative for iron entry into the bacterial cells, there are the ferrous iron transport system (Feo) and periplasmic-binding protein-dependent transport (PBT) (69–72). These are predominant iron transport systems at low oxygen conditions when ferrous iron is stable and is more prevalent than ferric iron (17). In the case the ferric iron is predominant, it will be chelated by ferric iron reductases and then uptake is mediated by ferrous permeases (73, 74). Rhodes et al. (19) demonstrated that *Aa* makes use of inorganic iron to grow under chelated conditions and proposed that *Aa* may acquire iron through the expression of systems which function independently of an outer membrane receptor or TonB-dependent transport. The elucidation of which TonB and receptor independent iron transport system is used by *Aa* will require further investigation.

Under anaerobic conditions, we showed that *Aa* growth in limited media is promoted in the presence of neutrophil supernatant or cell lysate supplemented with iron. Likewise, supplementation with neutrophil supernatant and iron induced expression of QseBC. Interestingly, neutrophil cell lysate alone or supplemented with iron QseBC expression was suppressed. Human neutrophil lysates contain all internal contents of neutrophils, including components such as calprotectin. Calprotectin is a metal binding protein, abundant in neutrophil cytoplasm (75, 76). Another protein that is found in specific granules is the neutrophil gelatinase-associated lipocalin. This protein scavenges iron from siderophores, and it has been shown to remove iron from catecholamines as well (77). These proteins can be sequestering the supplemented iron competing with epinephrine present in lysate, making it unavailable to *Aa* and consequentially suppressing the induction of the QseBC.

In *Aa*-positive subjects, *F. alocis* was detected at high levels (42) and *F. alocis* accumulation in the oral biofilm has been shown to be stimulated by the presence of specific strains of *Aa* (43). Based on our experiments, we determined that *F. alocis* does not induce release of epinephrine in human neutrophils. This result goes in hand with previous findings that *F. alocis* does not induce the exocytosis of azurophilic granules (38, 78). In the case that *F. alocis* is found to be catecholamine-responsive, it will suggest that it may be benefiting from the ability of *Aa* to induce epinephrine release. However, at present, there is no report of *F. alocis* being influenced in some way by catecholamines. *A. actinomycetemcomitans* has also been found to be associated to *Fusobacterium nucleatum*, whose growth is increased by hormones like epinephrine and norepinephrine (21); *Lactobacillus* spp. expresses transporter systems for uptake of catecholamines (79) and other catecholamine-responsive species such as *Prevotella* spp. and *Leptotrichia* (16, 80, 81). Some of these bacteria have been shown to lack the ability to produce siderophores as is the case of *Aa* (82, 83). Therefore, it is likely that these bacteria work as a team to thrive in the subgingival pocket, where the role of *Aa* may be to induce catecholamine (i.e., epinephrine) release by the infiltrating human neutrophils.

In conclusion, our *ex vivo* experiments demonstrated that human neutrophils store and release epinephrine upon stimulation with latrunctin A + fMLF. We show that *Aa* can induce epinephrine release in human neutrophils, consequently depleting epinephrine storage and promoting catecholamine metabolism. Furthermore, one of the most important contributions of this study was the finding of azurophilic granules as the storage location of epinephrine in human neutrophils and that *Aa* gains access to it by inducing granule exocytosis. Additionally, we found that epinephrine interaction with *Aa* depends on QseC expression and requires the QseC periplasmic domain. Finally, human-neutrophil-derived epinephrine supplemented with iron promoted *Aa* growth and QseBC expression under anaerobic conditions. The presented findings supply evidence towards the growing field of microbial endocrinology by contributing to the understanding of the crosstalk between bacteria and the host endocrine system. Furthermore, it expands the knowledge of the role of stress hormones in periodontal disease and potentially other chronic inflammatory diseases.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of Louisville. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

HO performed the experiments and participated in the study design, data analysis and interpretation, and drafting of the manuscript. SU and DD equally participated in the study design, data interpretation, critical revision of the manuscript, and study supervision. DD obtained funding for the study. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.707096/full#supplementary-material

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# Single-Cell RNA Sequencing Identifies New Inflammation-Promoting Cell Subsets in Asian Patients With Chronic Periodontitis

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Periodontitis is a highly prevalent chronic inflammatory disease leading to periodontal tissue breakdown and subsequent tooth loss, in which excessive host immune response accounts for most of the tissue damage and disease progression. Despite of the imperative need to develop host modulation therapy, the inflammatory responses and cell population dynamics which are finely tuned by the pathological microenvironment in periodontitis remained unclear. To investigate the local microenvironment of the inflammatory response in periodontitis, 10 periodontitis patients and 10 healthy volunteers were involved in this study. Single-cell transcriptomic profilings of gingival tissues from two patients and two healthy donors were performed. Histology, immunohistochemistry, and flow cytometry analysis were performed to further validate the identified cell subtypes and their involvement in periodontitis. Based on our single-cell resolution analysis, we identified HLA-DR-expressing endothelial cells and $CXCL13^+$ fibroblasts which are highly associated with immune regulation. We also revealed the involvement of the proinflammatory $NLRP3^+$ macrophages in periodontitis. We further showed the increased cell-cell communication between macrophage and T/B cells in the inflammatory periodontal tissues. Our data generated an intriguing catalog of cell types and interaction networks in the human gingiva and identified new inflammation-promoting cell subtypes involved in chronic periodontitis, which will be helpful in advancing host modulation therapy.

**Keywords: single-cell sequencing, gingiva, periodontitis, inflammatory microenvironment, mucosa**

## BACKGROUND

Periodontitis, a chronic inflammatory lesion of the collective periodontium, is characterized by irreversible and progressive degradation of the periodontal tissue and causes tooth loss and alveolar bone defects. Accumulating evidence has linked periodontitis to some noncommunicable diseases including cardiovascular disease, diabetes, chronic kidney disease, respiratory diseases, and cognitive

disorders (1, 2). Being the sixth most common human disease, severe periodontitis represents a substantial health and socioeconomic burden due to its health impact and high costs of treatment (3). However, current periodontal treatment approaches focusing primarily on biofilm reduction have shown insufficiency to result in clinical improvement and to prevent the relapse of the disease.

It is now well recognized that periodontitis results from dysbiosis and the dysregulation of immune homeostasis (4). Several types of cells are included in the hosts' armamentarium against dysbiosis, epithelial cells, endothelial cells, fibroblast cells, immune cells, and undifferentiated mesenchymal cells (5–7). All these cells precisely orchestrate an appropriate response to the biofilm microorganism and its components. In periodontitis, however, an inappropriate and excessive host response arises, resulting in collateral periodontal tissue damage.

Considering the importance of host immune response in the pathogenesis of periodontitis, it is imperative to develop host modulation therapy. Recently, the diversity of cell subsets has been recognized as a substrate for host modulation strategies. For instance, the Treg-recruiting formulation system has been injected to treat severe experimental periodontitis (8). Agents inducing the inflammatory-to-resolving conversion of macrophages were suggested to arrest periodontitis progression and stimulate bone regeneration (9). It should be noted that the behavior of cells, especially immune cells, is highly regulated as the cells perceive the changes in the microenvironment (10, 11). However, these altered phenotypes finely tuned by the pathological microenvironment in periodontitis remained largely unknown.

Transcriptome analysis based on bulk tissue RNA-seq has provided comprehensive overview of molecular events of the inflammatory response in periodontitis (12–14). However, most studies only focused on the averaged transcriptional signatures on a preselected cell type or crossed all cell types in the whole tissue without the information on the cellular heterogeneity in the periodontal tissue. The advance in single-cell technologies offers an opportunity to obtain the transcriptomes of individual cell types in the human tissues (15, 16). Recent transcriptional profiling of oral mucosa by single-cell RNA sequencing had led to the identification of a stromal-neutrophil axis in tissue immunity (17) and a decrease of epithelial and mesenchymal subpopulations from health to mild oral inflammation (18).

So far, the key inflammation-promoting cell subsets and the interactive networks within the pathological microenvironment in periodontitis remain incompletely understood. Moreover, due to the higher susceptibility of Asians to severe periodontitis (19, 20), transcriptomic data are needed to better understand the host response in Asian patients. Herein, we investigated the inflammatory response within the periodontal microenvironment, based on the transcriptomic profiling of a total of 29,967 single cells of human gingival tissues from two Asian patients with periodontitis and two healthy donors. We identified that HLA-DR-expressing endothelial cells and CXCL13$^+$ fibroblasts were highly associated with immune regulation. In addition, the immune cell proportion changed significantly in the inflammatory environment. The data indicated that the proinflammatory NLRP3$^+$ macrophages play an important role in periodontitis and the increased cell-cell communication between macrophage and T/B cells existed in the inflammatory periodontal tissues. Our findings offer a novel perspective on the periodontal inflammatory microenvironment and serve as a useful resource for developing host modulation therapy whereby adjuncts to mechanical debridement for managing chronic periodontitis.

# METHODS

## Sample Collection and Ethics Approval

Collection of samples was approved by the Ethics Committee of Shanghai 9th People's Hospital in China (SH9H-2019-T158-2). The experiments conformed to the principles of the Helsinki Declaration revised in 2008. A total of 20 individuals were involved in this study, including 10 periodontitis patients and 10 healthy volunteers (**Supplementary Table S1B**). For periodontitis, only individuals with stage III or IV periodontitis according to the new classification of periodontitis (21) were enrolled. For periodontally healthy volunteers, individual with intact periodontium without clinical inflammation were enrolled (22). The inclusion criteria for all participants were as follows: (a) no smoking; (b) no systemic disease; (c) not pregnant or breastfeeding; (d) no medication within the preceding 3 months; and (e) no periodontal therapy within the previous 6 months. All donors gave informed consent prior to participation into the study. Clinical assessment and biopsy sampling were conducted at the Department of Oral Implantology, Shanghai 9th People's Hospital, Shanghai Jiaotong University, China. Healthy samples were collected from healthy volunteers during crown-lengthening procedure. The diseased samples were collected from patients during open-flap debridement. For scRNA-seq, periodontal tissues of two patients with periodontitis and two healthy individuals were collected. The demographic information and clinical parameters of the two groups are shown in **Supplementary Table S1**.

## Histology and Immunohistochemistry

Human periodontal soft tissue biopsies were stained with H&E for morphological evaluation. For fluorescent immunostaining, human periodontal soft tissue biopsies were incubated with primary antibodies diluted in 3% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) overnight at 4°C. Primary antibodies used include decorin (1:50, ab175404, Abcam, Cambridge, UK), osteoglycin (1:50, sc-374463, Santa Cruz Biotechnology, Dallas, TX, USA), HLA-DR (1:50, ab92511, Abcam), CD11b (1:50, ab8878, Abcam), CD3 (1:50, ab135372, Abcam), CD19 (1:50, ab134114, Abcam), CXCL13 (1:100, PA5-47035, Invitrogen, Waltham, MA, USA), and CD31 (1:50, ab9498, Abcam). Next-day samples were incubated in Alexa-fluor 488, 594 Monkey anti-Mouse, Alexa-fluor 647 Monkey anti-Rabbit, or Alexa-fluor 488 Monkey anti-Goat secondary antibodies (Jackson ImmunoResearch, Jackson, PA, USA). Nuclei were counterstained

with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired using Zeiss LSM 880. Samples were evaluated in a blinded fashion at two to three different levels of sectioning according to the staining extent and intensity.

## Preparation of Single-Cell Suspensions

Once the sample was retrieved, it was dissociated and processed for scRNA-seq immediately. Periodontal soft tissue samples were minced into small fragments of less than 1 $mm^3$ by surgical scissors and dissociated into single cells in dissociation solution (2 mg/ml IV collagenase, 2 Units/ml Dispase II in $Ca2^+$- and $Mg2^+$-free HBSS) covered with tinfoil on a shaker (shaking speed of 200 rpm) at 37°C for 60 min; 0.1 μg/mL DNase I was added in the last 10 min. The dissociated tissue was filtered to ensure single-cell suspension using 100 and 40 μm cell strainers (Falcon) successively. Cells were subjected to red blood cell lysis for 10 min and centrifuged and resuspended (500 g, 10 min) twice. After resuspension in defined volumes of PBS + 0.4% BSA, 10 μl of the cell suspension was used for cell counting by an automated cell counter (Thermo Fisher, Waltham, MA, USA) to determine the concentration of live cells. Single-cell samples with final cell viability above 90% and final concentration of 600–1,200 cells/μl were stored on ice until further processing. The whole procedure was performed on ice whenever possible.

## Flow Cytometric Analysis

Single-cell suspensions were washed twice with ice-cold flow cytometry staining (FACS) buffer (2% FBS + 1 mM EDTA in PBS), incubated with blocking buffer (1:100, 564765, BD Bioscience, Franklin Lakes, NJ, USA) for 15 min at 4°C. For cell surface antigen staining, single-cell suspensions were stained with CD45 (1:100, 368511, BioLegend, San Diego, CA, USA) and CD11b (1:100, 101228, BioLegend) in the dark for 1 h on ice and then washed two to three times with FACS buffer. For intracellular cytokine detection, cells were fixed and permeabilized with fixation/permeablization buffer (eBioscience, Waltham, MA, USA) according to the manufacturer's protocol. Cells were then stained with NLRP3 (1:100, IC7578S-100UG, Novus, St. Louis, MO, USA), C1QA (1:100, NB100-64597, Novus), and PRDM1 (1:100, 565276, BD Bioscience) antibodies. Data were acquired within 2 h after staining on an LSR Fortessa (BD Biosciences), and analysis was performed by using FlowJo software (Tree Star, Ashland, OR, USA). Calculations made in bar graphs were generated in GraphPad Prism.

## Single-Cell RNA Sequencing and Read Processing

Single cells from independent periodontal samples were captured in four batches using the 10× chromium system (10× Genomics). The cells were partitioned into Gel Bead-In-Emulsions and barcoded cDNA libraries, then prepared using the Chromium Single Cell 3′ library and Gel Bead Kit v3 (10× Genomics). Single-cell libraries were sequenced in 100 bp paired-end configuration using an Illumina NovaSeq and mapped to the GRCh38 human reference genome using the Cell Ranger toolkit (version 3.0.0). The preliminary data analysis generated a file containing a barcodes table, a genes table, and a gene expression matrix. Next, we obtained an overview website containing a considerable amount of information, such as number of cells, median number of detected genes, sequencing saturation, and sequencing depth.

## Filtering and Normalization of scRNA-seq Data

We installed R (version 3.5.1) and Seurat R package (version 3.1.5) for downstream analysis. First, the substantial background levels of ambient RNA in the single-cell suspension caused problems for subsequent analysis. Thus, we applied SoupX (version 1.4.5) for background correction. Next, for quality control of each matrix, lowly detected genes (<0.1% cells) and cells with a small number of genes (<350 genes) were discarded from the downstream analysis. We the filtered out unhealthy cells that generally have high mitochondrial mRNA loads (>20%) and high ribosome RNA loads (>40%). We found that different cell types expressed different numbers of genes, particularly between immune and nonimmune cells. Thus, we applied a slightly different criteria to remove supposed *ambient RNA contamination* (<1,700 UMI for healthy detected per cell) and potential double droplets (>6,500 genes for healthy and >4,000 genes for periodontitis tissues detected per cell). After the step above, we obtained 10,501 high-quality periodontitis cells (median UMI: 3,544; median 1,203 genes/cell) and 19,977 healthy periodontal cells (median UMI: 16,455; median 3,169 genes/cell).

## Dimension Reduction, Unsupervised Clustering, Annotation, and Visualization

The expression value of each gene was first normalized by TPM/10 and then log-transformed (NormalizeData function in Seurat with default parameters). Using the variation-stabilizing transformation (vst) method, the top 2,000 variable genes were selected in each matrix and were used as input for the "FindIntegrationAnchors" function. The four expression matrices were then integrated with the "IntegrateData" function. The integrated data were dimension reduced with principal component analysis (PCA; top 30 dimensions) first and then further reduced to two dimensions with UMAP which was also used to visualize the clusters. The nearest neighbors were defined among cells with KNN method (FindNeighbors), and cells were then grouped with Louvain algorithm (FindClusters in Seurat, resolution equal to 1.5; PCA:top 25 dimensions). Specifically, we removed one cluster, considered contamination, due to the coexpressed markers of plasma and epithelial cells (data not shown). Finally, we retained a total of 29,967 cells after stringent quality controls for further analysis, with 19,806 (66.09%) from healthy tissues and 10,161 from the periodontitis tissues. Annotation of the clusters was performed by checking known markers for cell types that potentially would exist in the sample, and some clusters were merged as they were annotated as a major cell type. Average expression levels of each subtype marker were calculated by AddModuleScore in Seurat with default parameters. For subclustering, cells from a major cell type were taken as the input. We performed dimension reduction, clustering, and annotation using the same method as described above.

## Detection of Differentially Expressed Genes

To obtain the DEG list of each cluster, only the genes expressed in more than 30% of that cluster were considered, and the expression in all other cells was used as background. For statistical test, we used the default Wilcoxon's test implemented in Seurat. DEGs were defined as genes whose log fold change was over 0.2 compared with the background and with a *q*-value (FDR) smaller than 0.05.

## Gene-Set Enrichment Analysis

We conducted the gene-set enrichment analysis for DEGs of each cluster using clusterProfiler (23), GSVA, and GSEABase packages, with which the enriched GO biological process terms were calculated.

## SCENIC Analysis

SCENIC analysis was conducted as described previously (24). We used the pySCENIC package (version 0.10.3), a lightning-fast python implementation of the SCENIC pipeline. The differentially activated TFs of each subcluster were identified by the Wilcoxon's rank sum test against all the other cells of the same cell type.

## Gene Cluster Associated With M1/M2 and Proliferation Phenotypes

The M1/M2 phenotype of each macrophage cell was defined as the mean expression of gene signatures (23, 25). CCL5, CCR7, CD40, CD86, CXCL9, CXCL10, CXCL11, IDO1, IL1A, IL1B, IL6, IRF1, IRF5, and KYNU were included in M1 macrophage signature. Furthermore, CCL4, CCL13, CCL18, CCL20, CCL22, CD276, CLEC7A, CTSA, CTSB, CTSC, CTSD, FN1, IL4R, IRF4, LYVE1, MMP9, MMP14, MMP19, MSR1, TGFB1, TGFB2, TGFB3, TNFSF8, TNFSF12, VEGFA, VEGFB, and VEGFC are the member of the signature of M2 macrophages. In addition, the proliferation genes include AURKA, BUB1, CCNB1, CCND1, CCNE1, DEK, E2F1, FEN1, FOXM1, H2AFZ, HMGB2, MCM2, MCM3, MCM4, MCM5, MCM6, MKI67, MYBL2, PCNA, PLK1, TOP2A, TYMS, and ZWINT.

## Profiling the Cell-Cell Communication in Healthy and Periodontitis Samples

The cell-cell communication was measured by quantification of ligand-receptor pairs among different cell types. Gene expression matrices and metadata with major cell annotations were used as input for the CellphoneDB or CellChat (26) software. The default CellPhoneDB database and parameters were used. Healthy and periodontitis data were computed separately. The cell-cell network was visualized with circlize (version 0.4.10).

# RESULTS

## Single-Cell Profiling of Human Gingival Tissues in Healthy Donors and With Periodontitis

To generate transcriptome profiles of human gingival tissues, samples from four Asian donors were obtained. Two of the donors were diagnosed with periodontitis (P1 and P2). The other two samples were obtained from healthy volunteers who underwent crown lengthening procedures (Nor1 and Nor2). Tissues were collected fresh, dissected, and digested into single cells (**Figure 1A** and **Supplementary Table S1A**). For each sample, single cells were captured using the droplet-based microfluidic chromium system (10× Genomics). The number of genes expressed differed in the various cell types, particularly between immune and nonimmune cells (**Supplementary Figures S1A, B**).

Considering potential batch effects and background noises among samples, we applied SoupX (27) to correct ambient RNA in the background and merged these data using the CCA method in Seurat (28). The single-cell data are presented in two-dimensional space using a uniform manifold approximation and projection (UMAP) method, and relative cell type abundance analysis within the stromal and immune cell population was performed (**Figures 1B**, **3F**). Clustering analysis cataloged these cells into 10 distinct cell lineages annotated with canonical marker gene expression, thereby corresponding to epithelial cells, stromal cells (endothelial, vascular mural, and fibroblast), and immune cells (T, NK, B, plasma, myeloid, and mast cells) (**Figure 1C**). Differential expressed genes (DEGs) of each cell type were computed and the top 5 DEGs were visualized (**Supplementary Figure S1C**).

Next, we assessed the cell-type-specific expression patterns of genes related to Mendelian disorders (based on OMIM database), which provided insights into the contribution of specific cell types to gingival abnormality (**Figure 1D** and **Supplementary Table S2**). Cell type-specific expression patterns confirmed fibroblast as particularly high expressors of the *C1S* and *C1R* gene, mutated in Ehlers-Danlos syndrome, periodontal type. Two reported gene mutants, *SOS1* (29) and *REST* (30), associated with gingival fibromatosis (GF), were highly expressed in endothelial cells. Additionally, the *CTSC* gene, mutations of which are responsible for aggressive periodontitis in juveniles, was found to be widely expressed in the nonimmune cell population. While in immune cells, the expression of *CTSC* was highly enriched in myeloid cells.

## Local Inflammatory Environment Within Human Gingival Tissue

The gingival epithelium provides the first line of defense against chemical, physical, or microbial challenge by following a regulated scheme of differentiation that results in the integrity of epithelial barrier. We partitioned the epithelial cells into four diverse clusters based on previous reports (31, 32): junctional epithelium (JE1-JE2), basal (BAS1-BAS2), spinous (SPN1-SPN3), and differentiated granular keratinocytes (GRN1-GRN3) (**Figures 2A, B** and **Supplementary Figure S2B**).

We further analyzed the expression of characteristic genes and top 5 DEGs in each cluster (**Figure 2C** and **Supplementary Figure S2C**). JE1 expressed amelogenesis-associated proteins ODAM and ODAPH (C4orf26). Both JE1 and JE2 expressed serum amyloid A family (SAA1 and SAA2). Furthermore, HLA-DRs (HLA-DRB1 and HLA-DRA) presenting extracellular pathogens were found to be highly expressed in JE2 and BAS1. BAS2 exhibited high levels of

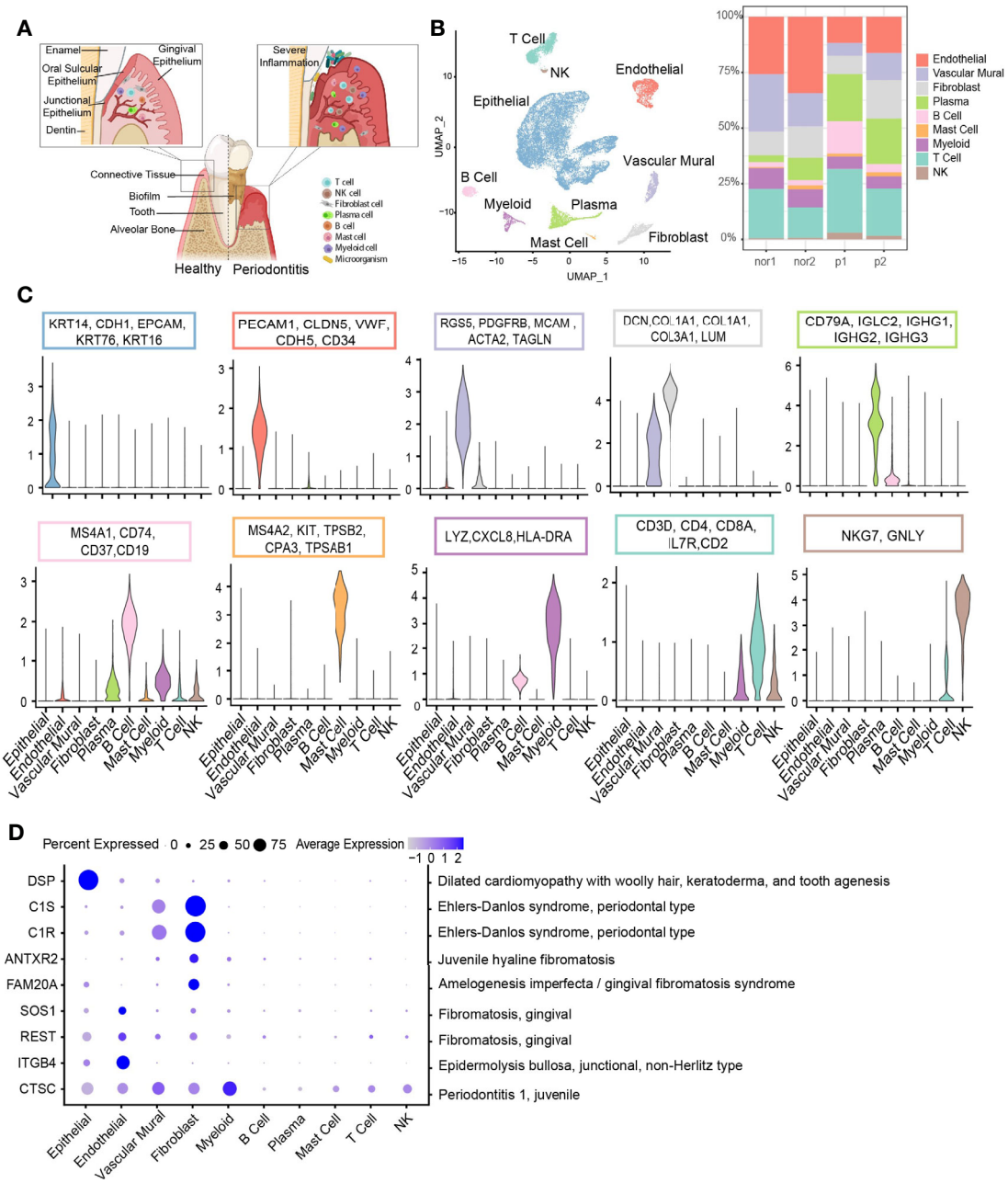


**FIGURE 1** | Overview of the clustering and annotation of the single-cell RNA sequencing data for gingival tissues. **(A)** Schematic of gingival tissues in health (left) and periodontitis (right) analyzed in this study (The graph was created with BioRender.com). **(B)** UMAP representation of major cell types identified by scRNA-seq ($n$ = 4; 29,967 cells; left) and bar plots indicating the percentage of the nonepithelial of subtypes in each donor (Nor1 and Nor2 are healthy donors; P1 and P2 are periodontitis patients; right). **(C)** Violin plots showing the expression scores of selected canonical marker gene sets across all 10 subsets. **(D)** Dot plot depicting gene expression levels and percentage of cells expressing genes associated with periodontal disease according to the OMIM database.

mitosis-related genes, denoting its possible stage at G2/m stages (**Supplementary Figure S2D**). The three spinous clusters were found to express *GSTA4* involved in antioxidative stress (33) and *CYB5A* detoxifying carcinogens from cigarette (34). GRN2 and GRN3 expressed genes associated with the anti-inflammatory response. We also noticed that GRN1-3 and JE1 expressed β-defensins (*DEFB1*) against bacterial challenge (35) and *S100A7/8/9* modulating the inflammatory response (36).

Analysis of the pathway in epithelial subsets was performed by gene-set variation analysis (GSVA) (37) (**Supplementary Figure S2E**), supporting the above inference about the cell function, such as amelogenesis for JE1, DNA replication for

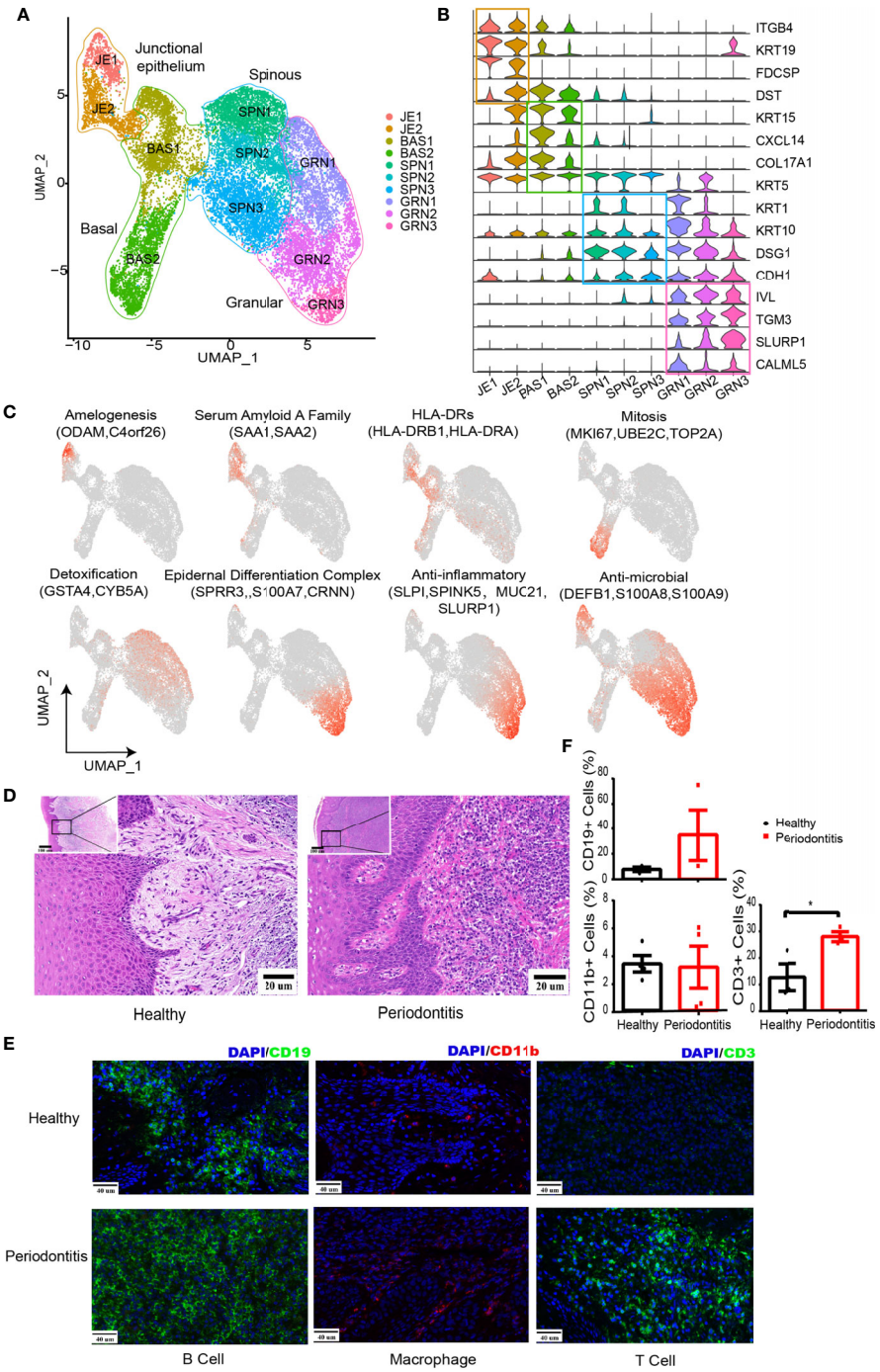


**FIGURE 2** | Changes in the microenvironment within human gingival tissue in periodontitis. **(A)** UMAP representation for human gingival epithelial cells in the combined health ($n$ = 2; 18,012 cells) and periodontitis ($n$ = 2; 502 cells) dataset. **(B)** Violin plots showing expression levels of cluster-defining genes for epithelial subsets. Expression values are normalized and scaled averages. **(C)** UMAP plots displaying functional molecule scores in the epithelial cells of all subpopulations. Red indicates maximum expression, and grey indicates low or no expression of each particular set of genes in log-normalized UMI counts. **(D)** H&E staining of the representative gingival tissue from health (left) and periodontitis (right) sections. Scale bar: 20 μm. **(E)** Representative immunofluorescence (IF) staining for $CD19^+$ B cells (green, one column), $CD11b^+$ macrophages (red, two columns), and $CD3^+$ T cells (green, three columns) with DAPI (blue) in gingival sections of healthy (top) and periodontitis patients (bottom). Images were acquired at ×40 magnification (scale bar: 40 μm). **(F)** Bar graphs demonstrate percentage of $CD19^+$ B cells (left top), $CD11b^+$ macrophages (left bottom), and $CD3^+$ T cells (right bottom) in gingival sections of healthy ($n$ = 4) and periodontitis patients ($n$ = 4) based on immunofluorescence analysis. The number of cells is counted based on five different limited areas on a slide from one sample then the mean was calculated. One dot per individual. Small horizontal lines indicate the mean (± s.d.). *$p \leq 0.05$, as determined by Student's $t$-test.

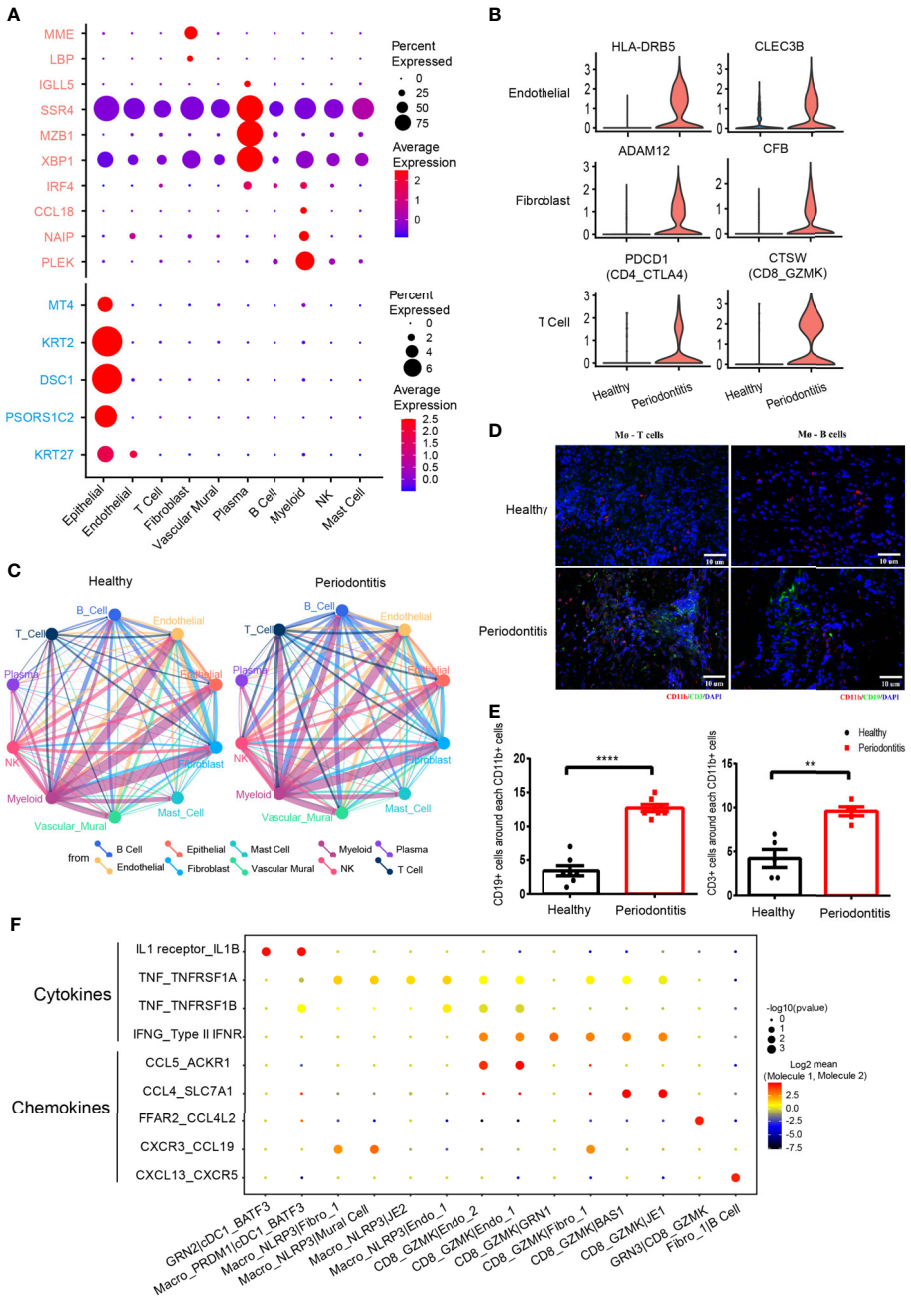


**FIGURE 3** | Differentially expressed genes and cell-cell interactions in health and periodontitis. **(A)** Bubble heatmap showing cellular expression patterns of genes associated with periodontitis based on previous bulk RNA sequencing. Rose stands for reported upregulated genes in periodontitis, and turquoise stands for reported downregulated genes in periodontitis. Dot size indicates fraction of expressing cells, colored according to z-score-normalized expression levels. **(B)** Violin plots showing the expression of specifically increased genes in different cell clusters in periodontitis. The gene expression levels are normalized and transformed as ln (CPM/10). **(C)** Cell-cell interaction network in health and periodontitis. Colors and widths of edges represent number of interaction pairs between cell types. **(D)** Immunofluorescent (IF) staining of T/B cell and macrophages. Red shows the signal of CD11b staining (macrophage marker); green shows the signal of CD3 (T-cell marker) at left and the signal of CD19 (B-cell marker) at right; and blue shows DAPI staining. **(E)** The number of $CD3^+$ cells around each $CD11b^+$ cell at left and the number of $CD19^+$ cells around each $CD11b^+$ cell at right (healthy donors, $n = 5–7$; patients, $n = 5–7$). The number of T/B cells within a limited distance of a macrophage cell is counted then the mean was calculated. One dot per individual. Small horizontal lines indicate the mean (± s.d.). $^{**}p \leq 0.01$, $^{****}P < 0.0001$ as determined by Student's *t*-test. **(F)** Dot plot of the interaction between cytokine/chemokine and their receptors in selected subcell types of periodontitis. Size of spot indicates significance (–log10(*p*-value)). Color indicates expression levels (log2 mean (molecule 1–molecule 2).

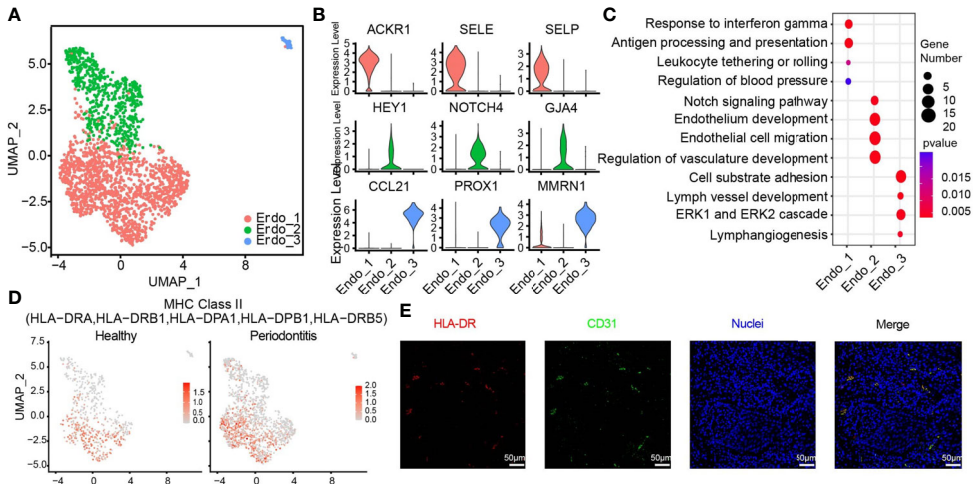


**FIGURE 4** | HLA-DR expressing endothelial subcluster in human gingiva. **(A)** UMAP visualization of endothelial subclusters in the combined health (*n* = 2; 596 cells) and periodontitis (*n* = 2; 1,363 cells) dataset. **(B)** Violin plots showing expression levels of cluster-defining genes for endothelial subsets. Expression values are normalized and scaled averages. **(C)** Dot plots showing the enriched gene ontology biological process terms for each endothelial cluster. **(D)** The expression of HLA-DR was projected on the UMAP plot. Red indicates maximum gene expression, while grey indicates low or no expression. The gene expression levels are normalized and transformed as ln (CPM/10). **(E)** Immunofluorescence assay for the MHC class II marker HLA-DR (red) and the endothelial cell marker PECAM1 (CD31, green) and with DAPI (blue) in the subepithelial region. Scale bar: 50 μm.

BAS2, and keratinocyte differentiation for GRN3. Furthermore, SCENIC (38) was utilized to identify different transcription factors (TFs) underlying the regulation of each epithelial phenotype (**Supplementary Figure S2F**; **Supplementary Table S3**). For instance, RUNX2 might be a potential regulator of the ODAM expression in junctional epithelium.

Compared with the healthy tissue, all of the nonepithelial cell types have their proportion increased in periodontitis, including T and B cells, which is consistent with the inflammation phenotype. Hematoxylin and eosin (H&E) stain showed a dense infiltrate of lymphocytes in periodontitis tissue (**Figure 2D**). The increased infiltration of T and B cells in periodontitis were further confirmed with immunofluorescence analysis (**Figures 2E, F**). Furthermore, we combined our data with a recent published single-cell transcriptome profiling of human gingival tissue (18). A similar distribution with their data of healthy individual and mild periodontitis could be observed, which further confirms the reliability of our data (**Supplementary Figure S3A**).

## Endothelial and Fibroblasts Subclusters Highly Associated With Immune Regulation

To gain more insight into the local inflammatory microenvironment within the gingival tissue, next, we focused on stromal cell clusters.

The 1,959 endothelial cells can be classified into three distinct subpopulations (**Figure 4A** and **Supplementary Figure S3B**). Endo_1 expressed *ACKR1*, *SELE*, and *SELP*. Endo_2 was characterized by *GJA4*, *HEY1*, and *NOTCH4*. We discovered a small group of lymphatic endothelial cells (LECs), named Endo_3, which expressed *PROX1*, *MMRN1*, and *CCL21* (39) (**Figure 4B**). The pathway analysis illustrates that Endo_1 is highly correlated with the immune response, response to interferon gamma, and upregulating adhesion molecules to tether or roll leukocyte. Meanwhile, Endo_1 is also involved in the regulation of blood pressure. In Endo_2, Notch signaling and other molecules contributing to endothelium development and migration were enriched. The enrichment pathways of Endo_3 meet the definition of LECs and are also involved in cell substrate adhesion (**Figure 4C**). We identified an endothelial cell state with high expression level of MHC class II genes such as *HLA-DRA*, *HLA-DRB1*, and *HLA-DPB1*, which was normally found in professional antigen-presenting cells (40). The endothelial cell states in healthy tissue, particularly Endo_1, exhibited lower feature score of MHC class II compared with those in diseased tissues, suggesting the importance of endothelial cells in gingival tissue-specific immunity (**Figure 4D**). Immunofluorescence assays for the MHC class II marker HLA-DR and the endothelial cell marker PECAM1 (CD31) further confirmed the existence of antigen-presenting endothelial cells in gingival tissues of periodontitis (**Figure 4E**).

We identified two subclusters of fibroblasts with multiple differentially expressed genes against each other (**Figure 5A** and **Supplementary Figure S3C**). Fibro_1 was characterized by high expression of *CXCL13*, *IL32*, and *SFRP2*. Fibro_2 expressed higher levels of *OGN*, *PRELP*, and *RUNX2* compared with Fibro_1 (**Figure 5B** and **Supplementary Figure S3D**). We then compared pathway enrichment between two fibroblast clusters (**Figure 5C**). Fibro_1 showed increased immune response pathways. Pathways associated with osteoblast development and bone remodeling are increased in Fibro_2. The IF staining of the periodontitis gingival tissue also identified two fibroblast subpopulations, which is consistent with the seq

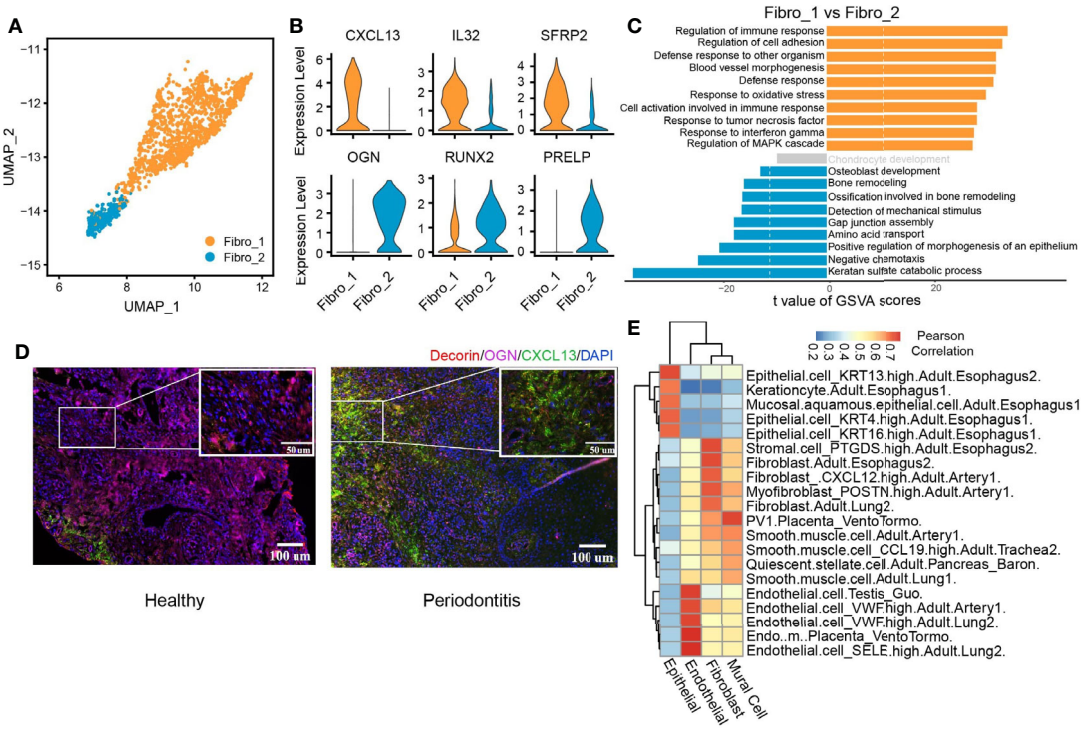


**FIGURE 5** | CXCL13$^+$ fibroblast subcluster associated with immune response. **(A)** UMAP visualization of two fibroblast subclusters in the combined health ($n$ = 2; 244 cells) and periodontitis ($n$ = 2; 1,260 cells) dataset. **(B)** Violin plots showing the expression distribution of selected genes associated with functions in the fibroblast clusters. The gene expression levels are normalized and transformed as ln (CPM/10). **(C)** Differences in pathway activities scored per cell by GSVA between fibro_1 and fibro_2. Shown are $t$ values from a linear model, corrected for fibro_1. **(D)** Immunofluorescent (IF) staining validation of fibroblast subtypes. Red shows the signal of decorin staining (fibroblast marker); green shows the signal of CXCL13 staining; purple shows the signal of osteoglycin (OGN), and blue shows DAPI staining. Scale bar: 100 μm for main images and 50 μm for detail images. **(E)** Application of scHCL analysis for nonimmune cells. Each row represents one cell type in scHCL. Each column represents a cell cluster in our dataset. Pearson's correlation coefficient was used to evaluate cell-type gene expression similarity. Red indicates a high correlation; blue indicates a low correlation.

data (**Supplementary Figure S3E**). Also, the predominant subpopulation converted from OCN$^+$ fibroblasts (Fibro_2) to CXCL13$^+$ (Fibro_1) fibroblasts when the gingival tissue was infiltrated with inflammatory cells in periodontitis (**Figure 5D**). Finally, we utilized scHCL (40) to verify the cluster identification and further explore the similarities between clusters (**Figure 5E**). By calculating Pearson's correlation coefficients, the reliability of the cluster identification was confirmed. Interestingly, both epithelial and fibroblast cells were highly similar to those of esophageal origin, suggesting the effect of food intake on the cellular function of gingival fibroblast and epithelial cells.

## Diverse Immune Cell Subtypes With Hyperinflammatory Response in Periodontitis

We next performed subclustering on myeloid cell types containing 148 cells from healthy donors and 539 cells from patients. We revealed seven subtypes of myeloid cells (**Figure 6A**). Three DC subsets were characterized by low expression of CD14, and three CD14-high expressing clusters were identified as macrophages based on their high expression of CD68, CD163, and MRC1 (24, 41) (**Supplementary Figure S4A**). Plasmacytoid DC (pDC), cDC1, and cDC2 were further distinguished by specific expression of *LILRA4/GZMB/JCHAIN*, *BATF3/CLEC9A/CADM1*, and *CD1C/CLEC10A/FCER1A*. Three clusters of CD14-high macrophages, Macro_PRDM1, Macro_NLRP3, and Macro_C1QA, were distinguished based on the expression of *FCGR2B/PRDM1/HES1*, *NLRP3/IL1B/EREG*, and *C1QA/SEPP1/SPOE*. There is a CD14$^+$ monocyte cluster showing different features with DC and macrophages. All myeloid subtypes could be found in both normal and patient samples (**Figure 6B**). The pDC group highly expressed granzyme B, which has been reported to suppress T-cell expansion (42). Macro_NLRP3 exhibited itself as a proinflammatory phenotype by highly expressing NLRP3,a known inflammasome-mediating macrophage M1 polarization and interleukin (IL)-1β production in inflammatory diseases (43), as well as proinflammatory cytokine gene IL-1B and inflammatory biomarker gene S100A8 (44, 45). Macro_C1QA expressed SEPP1, a potential marker of anti-inflammatory M2 phenotype associated with antioxidant defense in the extracellular space (25, 46). Macro_C1QA also

expressed a set of genes found previously in tumor-associated macrophages (47), including C1QA, APOE, and C1QB, resembling the signature of M2 phenotype (48).

The phenotypes of macrophages were analyzed in depth from angiogenesis and phagocytosis (**Figure 6C**). The gene associated with phagocytosis was highly expressed in Macro_C1QA, while Macro_NLRP3 dominated angiogenesis. Further analysis showed that Macro_PRDM1 simultaneously resembled the signatures of M1 and M2 cells (**Figure 6D**). Flow cytometry profiles also confirmed the three macrophage subpopulations in the gingival tissue and showed an increased proportion of $CD11b^+$ $NLRP3^+$ macrophages in gingival tissue in periodontitis patients compared with that in healthy individuals (**Figure 6E** and **Supplementary Figure S4B**), which suggested that the $CD11b^+$ $NLRP3^+$ subpopulation is highly associated with the development of periodontitis.

Another diverse immune cell cluster is T and NK cells, which were divided into five subtypes (**Figures 6F, G**). CD4_CTLA4 highly express Treg-associated molecules, including *TNFRSF18*, *TNFRSF4*, *CTLA4*, and *FOXP3* (**Figure 6H** and **Supplementary Figure S4C**). CD4_FOS from patients highly expressed immediate-early genes (*FOS*, *JUN*), which may be associated with T-cell activation or the effect of enzymatic digestion (49). Furthermore, CD8 and NK cells express cytotoxic genes, including *GZMK*, *GZMA*, *GNLY*, etc. Notably, an increased expression level of CCR5 or CCR1 ligand *CCL4/CCL4L2/CCL3L3* by CD8 T and NK cells was observed in patients.

## Differentially Expressed Genes and Cell-Cell Interactions in Healthy and Periodontitis Tissues

We investigated the cellular expression patterns of genes related to periodontitis, which were identified from previous bulk RNA sequencing (12–14, 50–53). We noticed that most of the genes upregulated in periodontitis were found to be expressed in plasma and myeloid cells. By contrast, genes downregulated in periodontitis were expressed in epithelial cells (**Figure 6A**). Differential expression analysis within individual cell types was performed (**Figure 6B**). In endothelial cells, HLA class II molecules *HLA-DRB5* and *CLEC3B* associated with extracellular proteolysis were upregulated in periodontitis. In fibroblast, a metalloprotease *ADAM12* and *CFB* involved in activating B cells was increased in disease. We also found *PDCD1* was highly upregulated in the CD4_CTLA4 cluster from periodontitis, which indicates that PD-1 pathway may contribute to the protective effect of Treg in disease stage (54). *CTSW* related to cytotoxic capacity was found to be upregulated in CD8_GZMK in periodontitis. These genes, previously masked in the mean expression data, provided novel insights for the characterization of periodontitis and will be helpful in advancing its therapy.

Periodontitis is a process of inflammation in the gingival tissue, which involves the cross talk of multiple cell types. Here, we used CellPhoneDB (55) to profile the communication among cell types in healthy and periodontitis tissues (**Figure 6C**). In both healthy and periodontitis tissues, myeloid cell and nonimmune cells including epithelial, endothelial, and fibroblast cells showed the strongest interactions. While the overall cell-cell interaction increased in the periodontitis tissue, the interaction between myeloid and other immune cells (T, B, and NK cells) exhibited a significant increase. We showed the top 10 cell-cell interactions increased in patients (**Supplementary Figure S4D**). Myeloid and B cells interact more frequently with other cells. Multicolor IHC staining of periodontal tissues showed the increased cell-cell interactions between macrophage ($CD11b^+$) and T cells ($CD3^+$) and macrophage ($CD11b^+$) and B cells ($CD19^+$) (**Figures 3D, E**). IL-1, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) are important cytokines which are highly involved in the progression of periodontitis (56). We revealed the cell subtype interaction based on the increased expression of these cytokines and their receptors in periodontitis. For instance, M2_cDC1_BATF3 constitutes a major source of IL-β. TNF is mainly derived from Macro_NLRP3 and CD8_GZMK T cells. We noticed that CD8 T cell is the primary source of IFN-γ which interacted with both epithelial and stromal cells. Furthermore, $NLRP3^+$ macrophages and CD8 T cells are recruited through CXCR3_CCRL19, which are associated with Fibro_1 and Mural cell. Notably, we also found that $CXCL13^+$ fibroblast interacts with B cells through CXCL13-CXCR5 axis, further substantiating its role in immune regulation.

# DISCUSSION

Here, we present the transcriptomic profiling of a total of 29,967 single cells of human gingival tissue using the scRNA-seq method. By identifying an intriguing catalog of cell types and their phenotypes, revealing the altered gene expression profiling and cell-cell communication under diseased condition, our data highlight key areas for advances in the biology of periodontitis that will be helpful in the diagnosis and treatment of periodontitis.

Ten major cell types with great diversity and heterogeneity were identified in our dataset. Junctional epithelial forms the direct attachment to the tooth surface and is exposed to the tooth-adherent microbial communities. We identified the junctional epithelial population with high expression level of SAA proteins. Studies have shown SAA protein triggers inflammatory cytokine secretion *via* interacting with TLR2 pathway in human gingival fibroblasts (57) and the SAA-TLR axis plays an important role in the chronicity of periapical inflammation (58). Thus, our result indicated that SAA-TLR axis in junctional epithelial may be closely related to the first immune-defense mechanisms against periodontal microbiota. Potential regulators of junctional epithelial, such as RUNX2, were identified through SCENIC analysis. This result is in consistency with recent studies where a critical role of RUNX2 in maintaining the integrity of the dentogingival junction was found (59, 60).

Cells, especially immune cells, given the cellular plasticity, have been reported to acquire altered phenotype *in situ* upon specific stimuli within the local biochemical and mechanical microenvironment, which is defined by growth factors,

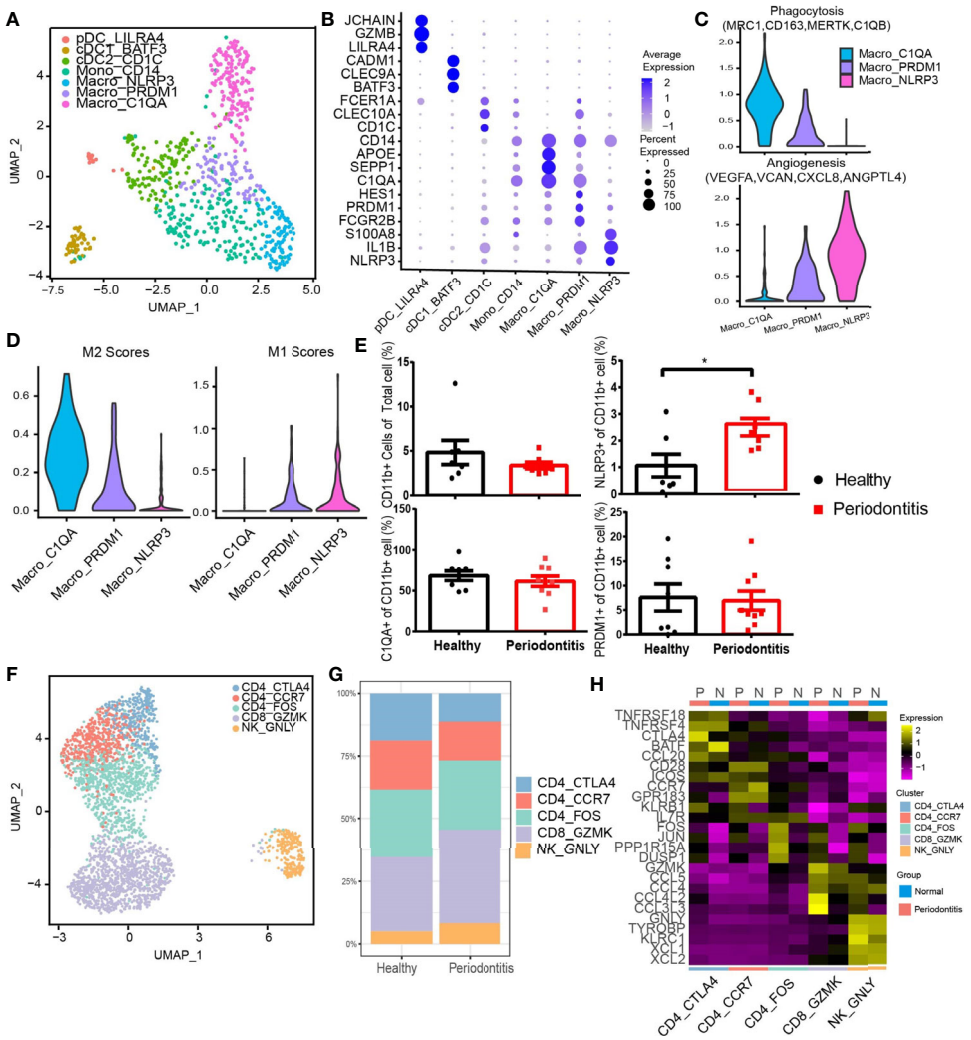


**FIGURE 6** | Diverse immune cell subtypes with hyperinflammatory response in periodontitis. **(A)** UMAP visualization of seven myeloid clusters in the combined health (*n* = 2; 148 cells) and periodontitis (*n* = 2; 539 cells) dataset. **(B)** Bubble heatmap showing marker genes across seven myeloid clusters from **(A)**. Dot size indicates fraction of expressing cells, colored according to z-score-normalized expression levels. **(C)** Violin plots showing the expression of angiogenesis- and phagocytosis-related genes (see *Methods* for details) in three macrophage clusters. The gene expression levels are normalized and transformed as ln (CPM/10). **(D)** Violin plots showing the expression of classically activated macrophages (M1) and alternatively activated (M2) macrophage-related genes (see *Methods* for details) in three macrophage clusters. The gene expression levels are normalized and transformed as ln (CPM/10). **(E)** The relative percentage of $CD11b^+$ cells in total cells and the proportion of $C1QA^+$, $NLRP3^+$, and $PRDM1^+$ subpopulations in $CD11b^+$ cells were analyzed by flow cytometry (healthy donors, *n* = 8 and patients *n* = 8; see **Supplementary Figure S4B** for gating strategy). **(F)** UMAP visualization of five T and NK cell subclusters in the combined health (*n* = 2; 276 cells) and periodontitis (*n* = 2; 2,599 cells) dataset. **(G)** Bar plots indicate the relative proportion of T-cell subsets in each sample (healthy = 2 and periodontitis = 2). **(H)** Heatmap showing the average expression of the top 5 differentially regulated genes for T and NK clusters identified in healthy gingival and periodontitis tissues. *$P < 0.05$ as determined by Student's t-test.

neighboring niche cells, and extracellular matrix (10, 11). Here, in this study, we also found a series of proinflammatory stromal cell subtypes and immune cells that respond to local inflammatory stimuli. We showed the presence of the recently identified antigen-presenting endothelial cells (40) in periodontitis, as revealed by the combined expression of CD31 and MHC class II genes.

Gingival fibroblasts have long been recognized as a heterogeneous population, but the extent of heterogeneity has hitherto remained poorly explored (61). We identified $CXCL13^+$ fibroblast subset potentially involved in immune response, which exhibited increased presence in periodontitis. Indeed, CXCL13 is involved in the pathogenesis of several autoimmune diseases and inflammatory conditions by regulating lymphocyte infiltration within the microenvironment (62). Moreover, $CXCL13^+$ fibroblast subset was also characterized by high expression of IL-32. Recent evidence has shown that IL-32 activates typical cytokine signal pathways of NF-κB and p38 MAPK.

Its expression is closely correlated with proinflammatory cytokines production (TNF-α and IL-1β) and with clinical conditions of periodontitis (63, 64). Furthermore, the enhanced interaction of $CXCL13^+$ fibroblast and B cells through CXCL13-CXCR5 were revealed, implying a potential treatment target in periodontitis.

Heterogeneity of myeloid cells was depicted, and novel phenotypes of macrophage, hitherto considered dichotomous (65), were revealed. Our analysis confirmed that the *in vitro*-characterized M1 and M2 cells do not reproduce the given tissue featured with a distinct local environment (66). The increased proportion of $CD11b^+$ $NLRP3^+$ macrophages in gingival tissue in periodontitis suggested their crucial role in the pathology of periodontitis. Our result also showed an increased level of CCR5 ligand in cytotoxic CD8 T cells of patients, underscoring their role in inflammatory cell recruitment in periodontitis. Chemokine receptor CCR5 is involved in the migration of leukocyte subpopulations throughout experimental periodontitis (67); our result provided further evidence for arresting periodontitis progression with the blockage of CCR1 and CCR5 (68).Taken together, these findings provided new perspectives in the host modulation therapy of periodontitis.

Finally, we further investigated the biology of periodontitis in three directions: profiles of known periodontitis-related genes; comparison between healthy and periodontitis group; and the cell-cell communication alteration between conditions. The increased cell-cell interaction between macrophage and T/B cells in periodontitis highlighted the importance of macrophage in linking the innate and adaptive immune responses and thus in the pathogenesis of periodontal diseases (69, 70). Furthermore, the interaction of $NLRP3^+$ macrophages and structural cells through TNF-TNFRSF1A and CXCR3_CCRL19 offered us a hint of the biological function of $NLRP3^+$ macrophages in periodontitis.

In gingiva, an important soft tissue within the periodontium, the number of epithelial cells is far more than the number of immune cells. Under pathological conditions, cell types are not affected equally in the development of periodontitis. Thus, scRNA-seq becomes the most impartial and effective approach to obtain the transcriptome of each cell type in the gingiva. Our single-cell profiles not only provide an abundance of resources on the inflammatory responses and cell population dynamics within the microenvironment in periodontitis but also offer insights into the biological foundation of periodontal pathogenesis, which potentially serve as the basis of host modulation therapy.

It is worth noting that we enrolled patients with stage III or IV periodontitis according to the new classification scheme proposed at the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. Due to the fact that no evidence of rapid bone loss could be found, all of the patients were diagnosed with moderate rate of progression (grade B). Our results could serve as a community resource with focus on stage III or IV—grade B periodontitis. Admittedly, the complexity of the periodontitis could not be fully grasped, as the gingiva was located in restricted areas and the number of samples that were sequenced was also limited. Nevertheless, given the robustness of scRNA-seq, it is possible to scale up the current study to provide much improved resolution in the future. In addition, further *in vivo* and *in vitro* mechanistic studies are also needed to verify cell-specific functionality and crucial signaling pathway involved in periodontitis pathogenesis and treatment.

## DATA AVAILABILITY STATEMENT

The sequencing data have been deposited in the China National Genebank Database under the accession number (CNP0001395).

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shanghai 9th People's Hospital in China. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

HL and BL conceived the project. S-jQ, Q-rH, YZ, and L-yZ participated in the data analysis. RC and JM advised the data analysis. S-jQ, L-yZ, and R-yC collected the human donor gingiva and performed the phenotyping and dissection. Q-rH, J-jM, and L-yZ prepared the nuclei sample and performed the single-nuclei RNA-seq. S-jQ, L-yZ, and R-yC performed functional validation. S-jQ, Q-rH, H-cL, and BL wrote the manuscript with input from all other authors. All authors proofread the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.711337/full#supplementary-material

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CASE REPORT
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# Case Report: A Case of Hereditary Gingival Fibromatosis With a High Level of Human β Defensins in Gingival Epithelium

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Hereditary gingival fibromatosis [HGF, (MIM 135300)], a rare benign oral condition, has several adverse consequences such as aesthetic changes, malocclusion, speech impediments, and abnormal dentition. However, relatively few studies have addressed the beneficial effects of thick gingival tissues in resisting external stimuli. In this report, we present a unique case of a family affected by HGF that manifests as a 'healthy' gingiva. Human β-defensins (hBDs) are known to play a pivotal role in the clearance and killing of various microbes, and contribute to maintaining a healthy oral environment, which is currently emerging research area. However, the expression pattern and localisation of hBDs in patients with HGF have not yet been reported. hBD-2 and hBD-3 in the pedigree we collected had relatively elevated expression. High hBD levels in the gingival tissue of patients from the family may be beneficial in protecting oral tissue from external stimuli and promoting periodontal regeneration, but their role and the mechanisms underlying HGF need to be clarified.

**Keywords: non-syndromic hereditary gingival fibromatosis, clinical manifestation, human β-defensins, case report, oral defense**

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## INTRODUCTION

HGF is a rare benign oral condition that can present as generalised slow thickening and non-bleeding fibrous enlargement of keratinized gingivae (1). This condition may lead to aesthetic changes, malocclusion, speech impediments, and abnormal dentition (2). However, to the best of our knowledge, none of the studies on patients with HGF reported the beneficial protective effects of thick gingival tissues against external stimuli. Our study describes a unique case of patients in a family affected by HGF, with relatively elevated serum hBD levels, pink and firmly consistent gingivae, alveolar bone thickening to various degrees, and a higher regrowth rate of gingival tissues.

The gingival epithelium is constantly exposed to varying microbial environments and physical and chemical stimuli generated by mastication and ingestion. The function of the epithelium in the host response has been increasingly recognised to serve to form a rigid mechanical barrier against periodontopathogenic bacteria. However, it also protects oral tissue by sensing and initiating the

**Abbreviations:** HGF, Hereditary gingival fibromatosis; NHGF, Non-syndromic Hereditary gingival fibromatosis; hBDs, human β- defensins; hBD-2, human β-defensin-2; hBD-3, human β-defensin-3.

innate immune response, including secretion of various pattern recognition receptors and antimicrobial host defence peptides (3). Antimicrobial peptides are significant multifunctional modules in the natural defence system (3). Human β-defensin-2 and -3 (hBD2 and hBD3) are two representative microbial peptides that are the members of the defensin family.

Considering the relatively healthy oral condition of these patients, we speculated that certain genic mutations might serve beneficial for protecting the host from dental plaque when a series of toxic factors are produced by bacteria penetrating their thick epithelial wall, and the generally thickened gingival tissue associated with this pedigree may be a desirable consequence of the battle between antimicrobial peptides and bacterial invasion. Further studies are warranted to elucidate the unique gingival features. Therefore, the difference in the expression of hBD-2 and hBD-3 between HGF and normal gingival epithelium was chosen as the target of this study to investigate the chemical barrier provided by hBDs.

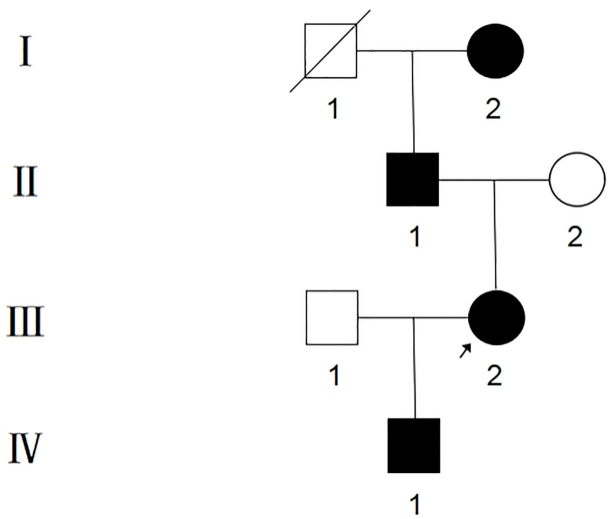


**FIGURE 1** | Pedigree of Chinese family with NHGF. Affected individuals are indicated by blackened symbols. Circles and squares denote female and male members respectively, and a slash through a symbol denotes a deceased individual. The proband is indicated by the arrow.

## CASE PRESENTATION

The proband, III-2, female, 34 years old, complained about gingival hyperplasia affected appearance and eating in the Stomatological Hospital of Zhengzhou University. His father and son had similar symptoms. Gingivectomy was performed at the ages of 15, 18, 20, and 25 because of gingival hyperplasia. All individuals were classified as affected or unaffected based on clinical features, family history, and histopathological manifestations, as described by Hart et al. (**Figure 1**). NHGF, which affects both sexes, is inherited through autosomal dominance. The family members with NHGF manifested a relatively 'healthy' gingiva, and their periodontal biotype was classified as a thick flat type on clinical examination (2, 4). Patients among the family members generally had fair oral hygiene, and even those who were first-generation presented firmly consistent gingivae, which consisted of dense fibrous connective tissue that felt tough and tuberculous on palpation. Furthermore, we found that the alveolar bone of these patients was significantly thickened during the gingivectomy (**Figure 2**). The first-generation I-2 aged 91 years showed relatively fair oral hygiene and had pink enlarged gingiva with marked and abundant stippling covering almost the entire crown. II-1, the father of the proband aged 63 years, exhibited significant hyperplasia of the posterior dental area and had teeth that were partially or entirely engulfed by fibrotic tissue (**Figure 3A**). Marked, abundant stippling was observed in the intraoral image of the proband (**Figure 3B**). IV-1, son of the proband, who was 5 years old, exhibited extensive hyperplasia of the entire gingival margin, papilla, and attached gingiva of both the maxillary and mandibular dentition (**Figure 3C**). A strikingly distinctive facial appearance was also observed in this pedigree, comprising both hypertrichosis as previously reported and abnormal changes in the nose and mouth. All affected individuals manifested a coarsened facial appearance, marked nasal and upper lip protrusion, and an open bite. They had flat nasal bridges, broad noses, and bow mouths. Their hair and eyebrows were bushy, whereas their weights and heights were all within the normal range and did not manifest any intellectual disability.

The gingival tissue HE staining of the patients in the pedigree was characterised by hypocellular and hypovascular dense fibrous connective tissue covered by an integrated stratified squamous epithelium. In addition, prominent deposition of collagen fibres underlying the gingival epithelium with acanthosis and extended long slender rete ridges were observed

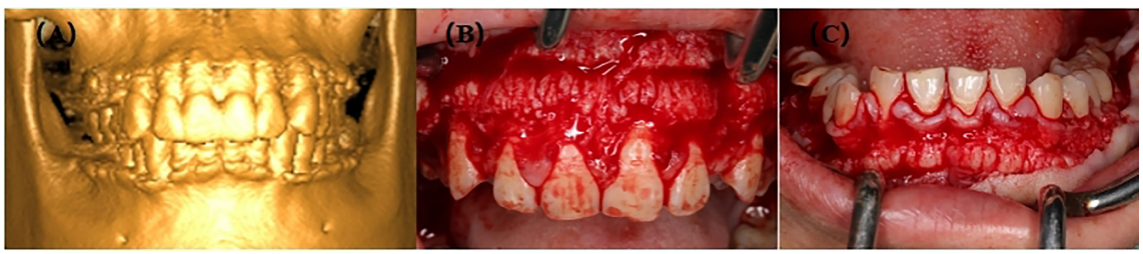


**FIGURE 2** | **(A)** 3D image of III-2; **(B)** Preoperative picture of maxilla from III-2; **(C)** Preoperative picture of mandible from III-2.

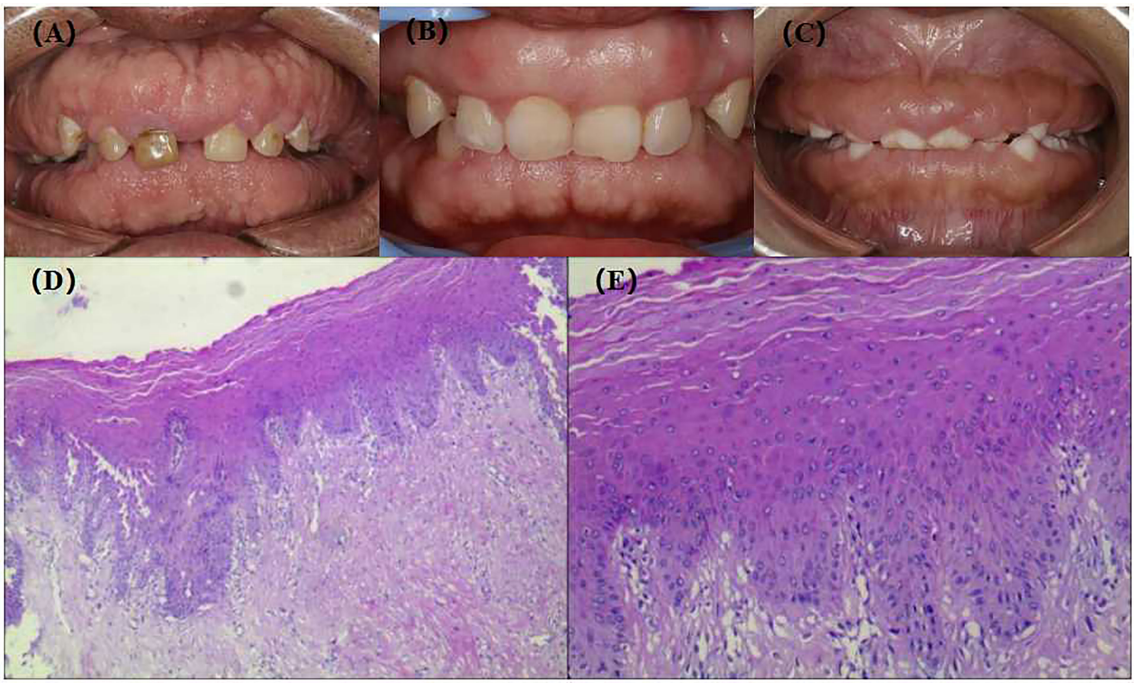


**FIGURE 3** | Extra-oral images, intra-oral images and HE staining of gingival tissue of patients with NHGF. **(A)** II-1, 63 years old; **(B)** III-2, 35 years old; **(C)** IV-1, 5 years old; **(D)** HE staining of gingival tissue III-2(40×); **(E)** HE staining of gingival tissue III-2(200×).

(**Figures 3D, E**), which is consistent with the histological characteristics of gingival fibromatosis.

The immunohistochemistry showed that hBD-2 was mainly confined to the cytoplasm, whereas hBD-3 was detected in the cell nuclei and cytoplasm (**Figures 4C, I** arrows). In addition, hBD-2 staining was intense, brownish-red, in the cytoplasm of tissue sections from the NHGF group (**Figures 4A–C**). In contrast, weak staining was observed in the control group sections, which were slightly yellow (**Figures 4D–F**). The hBD-3 staining was significant, brown, in both the nuclei and cytoplasm of sections from the NHGF group, whereas those of the control group were blue (**Figures 4G–L**). Furthermore, hBD-2 and hBD-3 were generally expressed in all epithelial layers of the NHGF group sections but were not detected in the underlying connective tissue layer. In addition to the corneum, granulosum, and spinosum, basal cells were clearly stained, and the expression density gradually increased from the corneum to the basal layer. The strongly stained areas were distributed in bands or sheets, indicating that hBD-2 and hBD-3 positively expressed in the basal layer of the gingival epithelium tissue from the pedigree.

We treated the patients with basic periodontal treatment, full-mouth alveolar bone repair, and gingivoplasty. The wound was irradiated by laser healing mode for 15 min after the operation, and periodontal maintenance treatment lasted throughout the treatment cycle. The gingival tissue condition of the patients was greatly improved, the dentition was neat, the occlusion was stable, the shape was beautiful, and the patients' self-confidence was greatly improved.

## DISCUSSION

HGF usually presents as an isolated incidence of gingival hyperplasia or occasionally appears as an oral manifestation of certain syndromes. This article describes the unique clinical characteristics of NHGF, which is a rare benign oral condition characterised by a progressive increase in keratinized gingiva showing an autosomal dominant inheritance or, infrequently, an autosomal recessive inheritance (5). Presently, the clinical diagnosis of NHGF is still based on histopathological examination. Although highly recurrent characteristic hyperplastic gingiva can cause severe functional and aesthetic problems, the gingival tissue from the pedigree we collected might show better resistance to detrimental external stimuli. Recently, a series of innate host defence molecules, such as antimicrobial host defence peptides in the human gingiva, have emerged as a research focus, and have proven their indispensable roles in maintaining periodontal health. Antimicrobial host defence peptides contribute to establish a state of 'controlled' immuno-inflammatory surveillance by depolarizing and disrupting microbial cell membrane integrity (6). Because of their polymorphous nature and paramount significance in the natural host defence, these molecules have been termed 'host defence peptides'. The first antimicrobial peptides identified in the oral epithelial tissue were hBDs, which show prominent specific activity against various microbes, and have recently attracted considerable attention (3). These peptides stimulate local dendritic cells and recruit T-cells into the nearby gingival epithelium to link innate and acquired immunological responses (7, 8).

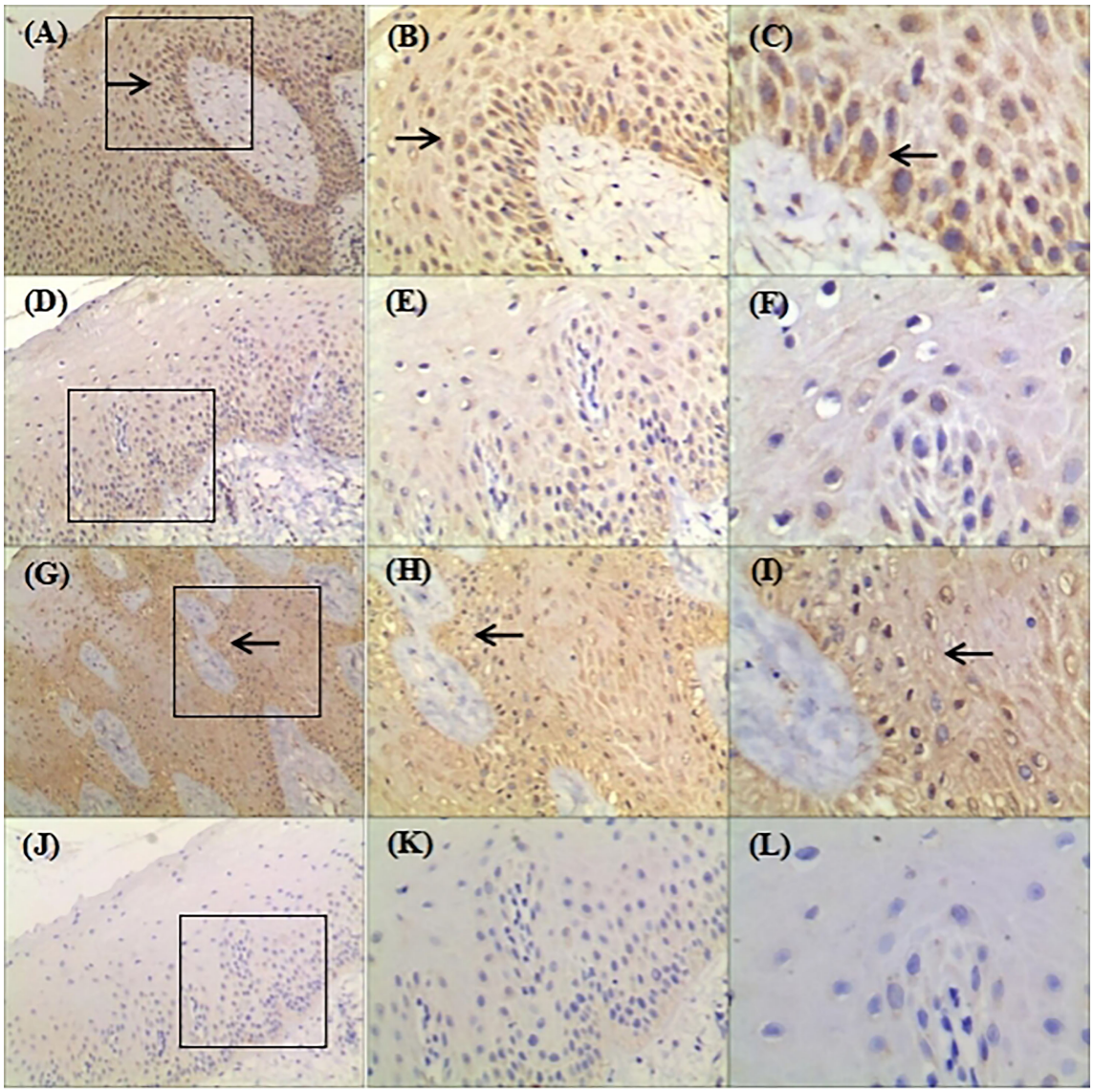


**FIGURE 4** | Representative images showing expression of hBD-2 and hBD-3 in gingival tissue samples from the family (original magnification ×100, ×200, ×400). **(A–F)**: Gingival samples were stained with rabbit polyclonal IgG antibody to hBD-2 (1:300) using standard IHC protocol; Circled areas in **(A)** was amplified in **(B)** and **(C)**; **(D)** was amplified in **(E)** and **(F)**; **(A–C)** were derived from patient with NHGF; **(D–F)** were derived from a healthy control gingival biopsy; **(C)** hBD-2 was detected in the cytoplasm; Arrows indicate positive staining. **(G–L)**: Gingival samples were stained with rabbit polyclonal IgG antibody to hBD-3 (1:400) using standard IHC protocol; Circled areas in **(G)** was amplified in **(H, I)**; **(J)** was amplified in **(K, L)**; **(G–I)** were derived from patient with NHGF; **(J–L)** were derived from a healthy control gingival biopsy; **(I)** hBD-3 was detected in the nuclei and cytoplasm. Arrows indicate positive staining.

The current research focus has shifted from antimicrobial capabilities to effects on the immunoregulation of congenital and acquired immune responses (9). For example, defensins interact with numerous inflammatory factors, regulate epithelial cell proliferation, participate in periodontal regeneration, promote wound healing, induce or curb pro-inflammatory cytokines, facilitate or inhibit angiogenesis, enhance chemokine production, promote chemotaxis of diverse leukocytes, mediate degranulation of mast cells, and regulate the host cell gene expression (10–13). More specifically, hBD-2 activates the antigen presentation activity of dendritic cells and stimulates the production of interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-22 production. Furthermore, it serves as a surveillance factor by inhibiting IL-17 production *via* suppression of cytokine signalling 3 (SOCS3) (3). In addition to being a chemotactic factor for macrophages, monocytes, and mast cells, hBD-3 is a chemokine for immature dendritic cells and CD45 RA+/CD4+ T lymphocytes, which reside in the oral mucosa and play essential roles in immunity. With the exception of periodontal tissue inflammation and injury, pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, and IL-17 can modulate

systemic diseases (14–16). hBD-3 effectively inhibits TNF-α and IL-6 accumulation to induce potential anti-inflammatory properties, leading to inflammation resolution (10, 17). With increasing available information about the beneficial effects of hBDs in humans, it is important to study the functions of these defensins in periodontal immunoregulation and fibrous tissue regeneration in more detail. hBD-3 can be used to improve root surface biocompatibility and promote periodontal ligament fibroblast attachment and proliferation (11). Coincidentally, we observed that the regrowth rate of the gingival tissues from patients with NHGF pedigree was higher than that of normal gingiva after gingivectomy, which is consistent with the results of previous investigations. Moreover, hBD-3 can interact with the host defence and inflammation mechanisms in tissue reconstruction in articular cartilage. Specifically, it participates in remodelling articular cartilage tissue by increasing the secretion of cartilage-degrading matrix metalloproteinases and reducing the production of endogenous regulatory factors, such as tissue inhibiting factors of metalloproteinases 1 and 2 (18). hBDs may facilitate tissue regeneration, which is important for the recovery from gingival recession or periodontal surgery, further investigations should aim to determine the beneficial effects of hBD-associated NHGF. Provided that it proves to be beneficial to the physiological development of periodontal tissue, we will continue to monitor this family to explore the potential key role of NHGF in periodontal immunoregulation and fibrous tissue regeneration.

Previous research on the biochemical pathogenesis of NHGF was primarily focused on connective tissue cells of patients with NHGF, while ignoring the pathological features of the epithelium and the interaction between gingival epithelia and underlying fibroblasts (19–21). hBD-2 and hBD-3 were mainly distributed in the basal layer of the pedigree investigated in our study (**Figures 4A, B, G, H** arrows). The basal layer is an interfacial surface with the lower lamina propria, which contains blood vessels and contributes to the formation of the gingival epithelium by providing nutrients and potentially impacts the recognition of signals from the body. Although junctional epithelium frequently causes inflammation, in contrast to the basal layer, hBDs have not been detected (22). The cells of the junctional epithelium are comparatively undifferentiated, which indicates that the expression of hBDs in the oral stratified squamous epithelium depends on the normal differentiation of epithelial cells (23). Specific interactions between the gingival epithelium and the underlying lamina propria may drive the development of HGF.

In the HGF family we studied, the expression of hBDs in gingival epithelium was statistically higher, and their oral health status was relatively healthy, suggesting that hBDs may be beneficial in regulating host responses to oral pathogen challenges to maintain homeostasis of the oral environment. We could not determine the relationship between elevated expression of hBDs and gingival thickening at present because of the insufficient number of samples. We speculate that hBD-2 and hBD-3 could be potential facilitators of communication between the gingival epithelium and underlying lamina propria without compromising the host.

## PATIENT'S PERSPECTIVE

These patients did not have any intellectual disability or other systemic diseases, presenting only a clinically NHGF phenotype. They plan to continue gingival cosmetic repair to achieve greater confidence.

## DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University, China (2021-KY-0039). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

DC conceived the idea of the study and the experimental design. GG performed the immunohistochemistry, image analysis, and data analysis, and contributed to the preparation of the manuscript. QT and AH contributed to the conception and design of the experiments and contributed reagents and analysis tools. RY and FS prepared the figures and tables. All authors reviewed the paper.

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## SUPPLEMENTARY MATERIAL

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# Quercetin Preserves Oral Cavity Health by Mitigating Inflammation and Microbial Dysbiosis

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Failure to attenuate inflammation coupled with consequent microbiota changes drives the development of bone-destructive periodontitis. Quercetin, a plant-derived polyphenolic flavonoid, has been linked with health benefits in both humans and animals. Using a systematic approach, we investigated the effect of orally delivered Quercetin on host inflammatory response, oral microbial composition and periodontal disease phenotype. *In vivo*, quercetin supplementation diminished gingival cytokine expression, inflammatory cell infiltrate and alveolar bone loss. Microbiome analyses revealed a healthier oral microbial composition in Quercetin-treated versus vehicle-treated group characterized by reduction in the number of pathogenic species including *Enterococcus, Neisseria* and *Pseudomonas* and increase in the number of non-pathogenic *Streptococcus* sp. and bacterial diversity. *In vitro*, Quercetin diminished inflammatory cytokine production through modulating NF-κB:A20 axis in human macrophages following challenge with oral bacteria and TLR agonists. Collectively, our findings reveal that Quercetin supplement instigates a balanced periodontal tissue homeostasis through limiting inflammation and fostering an oral cavity microenvironment conducive of symbiotic microbiota associated with health. This proof of concept study provides key evidence for translational studies to improve overall health.

**Keywords: flavonoids, periodontal diseases, IL-6, TNF, A20, microbiome, toll-like receptor (TLR), NF-κB**

## INTRODUCTION

A symbiotic consortium between microbiome, microbiome- associated molecular patterns (MAMPs) and innate sensors such as Toll like receptors (TLR) are responsible for a regulated immune response and sustained periodontal tissue homeostasis. Ligand recognition by TLRs initiates an array of signaling cascades including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), one of the archetypical drivers of the host and adaptive immune response in periodontitis (1–3). The subsequent release of cytokines and chemokines is necessary for priming host immunity and maintaining periodontal tissue integrity. To avoid excessive inflammatory

responses, host cells are equipped with negative-regulatory mechanisms to control TLR-mediated inflammatory responses and restore immune system balance. Ubiquitination is one of the most prominent regulator of signaling downstream of TLRs (4, 5). Ubiquitination is a highly dynamic, enzymatically-catalyzed posttranslational modification, which regulates cellular and immune functions through distinct polyubiquitin signals (6). To date, ubiquitination and ubiquitin-editing molecules have been characterized as important regulators of NF-κB in the pathophysiology of numerous chronic inflammatory conditions (7, 8). Ubiquitin-editing enzyme, A20, also known as TNF-α inducible protein 3 (TNFAIP3) has emerged as a critical gatekeeper of immune homeostasis in various systemic conditions, including periodontitis, through its ability to limit inflammation (9–12). If one or more of these regulatory mechanisms go awry and the initial immune response fails to terminate timely, the constant influx of inflammatory mediators creates a local microenvironment favorable for the growth of gram-negative, inflammogenic oral bacteria resulting in microbial dysbiosis (11, 13–16). The subsequent oral landscape promotes a continuous rhythmic process in which inflammation and dysbiosis collectively drive periodontal tissue destruction in susceptible individuals and lead to periodontal disease, one of the most common chronic diseases worldwide. Further, persistent chronic localized inflammation is associated with multiple systemic complications (1, 17–19). Therefore, the development of targeted strategies which regulate inflammation is critical for the maintenance of periodontal tissue homeostasis and overall health.

The discovery of natural compounds targeting the host immune responses offer promise to sustain health and improve clinical outcomes. Quercetin, a natural plant-derived dietary polyphenol, possesses high safety profile and extensive beneficial properties including potent antioxidant, anti-inflammatory, anti-cancer, antiviral, anti-hypertensive and anti-aging drug effects (20–26). Quercetin has been used to improve the disease outcomes in several disorders including rheumatoid arthritis, neuroinflammation and gastrointestinal disorders (27–30). Similarly, emerging evidence also indicates the oral-protective properties of Quercetin. Periodontal disease progression is driven by deregulated inflammation and a dysbiotic microbiota underlying the importance of evaluating the ability of therapeutic compounds to modulate both the host tissue responses and the microbiome. Yet, there are still no studies which assessed the effect of Quercetin supplementation on inflammation and the oral microbiome simultaneously in the course of periodontitis.

We report here the results of first systematic investigation which assessed the effect of oral delivery of Quercetin during the course of periodontitis through monitoring changes in inflammation, microbiome and alveolar bone loss. To accomplish this goal, periodontitis was induced in mice using ligatures to observe changes in the microbiota and the host without the addition of exogenous pathogens (31, 32). Using this *in vivo* model of periodontitis, we were able to show for the first time that oral delivery of Quercetin facilitates a sustained periodontal tissue homeostasis and mitigates the disease through modulating inflammatory response and oral microbial composition. Corroborating *in vivo* data, Quercetin diminished cytokine production through its effect on NF-κB:A20 signaling axis in human derived macrophage like cells exposed to lipopolysaccharide (LPS) and periodontal bacteria: *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*. These preclinical studies reveal proof-of-concept evidence identifying Quercetin as a promising natural based therapeutic to restore periodontal-host and -microbiome tissue homeostasis.

# MATERIALS AND METHODS

## Animals

10-12-week-old male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All mice were maintained in a specific pathogen free (SPF) environment and housed in the same room. Mice were provided with water and a standard laboratory diet ad libitum. They were supplied with hardwood chips as bedding and housed in a temperature-controlled, air-conditioned room on a 12-hr light-dark cycle. For each independent experiment, 10 mice were assigned to the Quercetin treated group, while 5 mice were assigned to the vehicle treated group. All the mice were cohoused for two weeks to standardize microbial communities prior to the start of experiments (33). On day 1 of the study, mice were randomly assigned to experimental groups by husbandry staff with no involvement in the study design. Thereafter, mice were housed in separate cages based on experimental groups. Cages were changed daily to prevent Quercetin redosing by coprophagy and prevent feces accumulation and limit coprophagy (34, 35). Mice either received twice daily oral administration of Quercetin (40mg/kg/twice per day; Cayman Chemical: Item No.10005169) or an equal amount of phosphate buffered saline (PBS: Life Technologies) for 5 days. Body surface area was used to determine the concentration of Quercetin to be used in the mice. By using body surface area, a 40mg/kg dosage in mice would equate to 195mg of Quercetin for a 60kg human (36). Quercetin was solubilized in an ethanol-water mixture to enhance its absorption (37). A one-inch straight stainless-steel oral gavage needle with a round-ball stainless steel tip was placed at the end of a 1mL syringe and it was used for oral administration. Quercetin and vehicle were administered in small, 50µl, doses to make sure all the compound was swallowed before the introduction of the remaining medication. The total dosing volume was 300 µl of either Quercetin or vehicle two times daily. Periodontal inflammation and bone loss were induced by ligature placement on day 6 of the experimental timeline. Quercetin-treated and vehicle-treated were anesthetized intraperitoneally with a 200 µl mixture of ketamine (10mg/mL) and xylazine. Black braided 5-0 threads were placed at the left side of the maxilla interdentally between the first and second molars, while the right sides remained un-ligated as controls. Twice daily oral administration of Quercetin or vehicle treatment resumed on day 7 and mice were euthanized

one-week post-ligation. Oral swabs of teeth and gingival tissue were taken before the induction of periodontitis on day 1 and day 6 and final swabs were taken at the completion of the study on day 13. The gingival tissues around all maxillary molars, without including palatal or buccal mucosa, were carefully excised using a new sterile #15 scalpel blade under 3.5x magnification by a trained periodontist using Dental Loupes (38–41). The mice were excluded from the study if the underlying bone was damaged during excision of the gingival tissues or the ligatures were lost. Maxillary jaws and gingival tissues were assessed for bone loss and inflammatory mediators and cellular infiltrate, respectively (42–44). The Institutional Animal Care and Use Committee of Virginia Commonwealth University approved all procedures and experiments were carried out following their guidelines.

## Microbes

The bacterial strains were *Porphyromonas gingivalis* (strain ATCC 33277) and *Fusobacterium nucleatum* (strain ATCC 25586) and grown anaerobically (5% $CO_2$, 10% $H_2$, 85% $N_2$) at 37°C. *P. gingivalis* was maintained in Brain Heart Infusion broth supplemented with 0.05% Yeast Extract, 5g/mL Hemin, 0.5g/mL Vitamin K and 0.1% cysteine. *F. nucleatum* was grown in Brain Heart Infusion broth containing 5 mg/L Hemin, 0.5 mg/L menadione, 1g/L cysteine, 5g/L Yeast Extract, 0.001% *N*-acetyl muramic acid, and 5% fetal bovine serum. The bacterial suspensions were killed by heating at 80°C for 10 minutes. Sterility of killed bacteria was confirmed by enumeration of CFU on an agar plate.

## Cell Culture

The human monocytic leukemia cell line (THP-1; ATCC TiB-202) were grown in in RPMI 1640 (Life Technologies, Cat# 11875093) media supplemented with 10mM HEPES (Sigma, Cat# 1101100250), 1mM sodium pyruvate (Fisher Scientific, Cat# 11360070), 4500 mg/L glucose (Fisher Scientific, Cat# D16-500), 0.05 mM mercaptoethanol (Sigma, Cat# M6250-100ML) 10% fetal bovine serum, and 1% penicillin/streptomycin (Invitrogen, Cat# 15140122) at 37°C in a humidified 5% $CO_2$ atmosphere. The cells were maintained in a logarithmic phase of growth ($2 \times 10^5$ to $8 \times 10^5$) by passage every 3 to 4 days and were subsequently used for *in vitro* stimulation assays.

## Microscopic Computed Tomography Analysis

Maxillae were dissected after euthanasia and fixed in 10% neutral-buffered formalin for at least 24 hours prior to imaging and scanned with a desktop micro-CT system (Brüker Skyscan 1173, Skyscan NV, Kontich, Belgium) at a resolution of 1,120 × 1,120 pixels (image pixel size of 15.82 μm) over 180°,80 kV voltage, 80 μA current and 250 ms exposure time. Five x-ray projections were acquired every 0.2° and averaged. A standard Feldkamp reconstruction was done using NRecon software (Brüker) with a beam-hardening correction of 15% and a Gaussian-smoothing kernel of 1. Histomorphometric analysis was performed with the DataViewer MicroCT visualization Software (Brüker, Kontich, Belgium) with a 1,092 × 1,092 pixel size in all three spatial dimensions and setting the sagittal plane parallel to the X-ray beam axis. The amount of linear alveolar bone present was calculated and measured with DataViewer software using sagittal images measuring the distance from the cemental-enamel junction (CEJ) to the alveolar bone crest (ABC). Histogram settings were set to 255 to measure the CEJ-ABC junction distance and also to distinguish between hard tissues (i.e. alveolar bone) and enamel from the soft tissues. Linear measurements were taken (in millimeters) from the CEJ to the ABC in the interdental region between the first and second molars (M1-M2) or the second and third molars (M2-M3). All scans were reoriented with DataViewer to the same position for bone loss evaluation such that the CEJ and the root apex next to the measurement appeared in the micro-CT slice that was to be analyzed and standardize measurements according to previously established methods (44–51). Specifically, all images were oriented so that the CEJ of the first and second molar were parallel to the horizontal axis and the CEJ and root apex appeared in the same slice generating a standardized region of interest with set anatomical limits. A total of four CEJ-ABC measurements were made from the sagittal view in DataViewer. The average of the four linear measurements from each mouse was used for the subsequent analysis. Measurements were made by a blinded-investigator and analysis was performed three times for each mouse with similar results. Data are presented from 5 independent experiments from a total of 37 Quercetin-treated mice and 21 vehicle-treated control mice. One-Way ANOVA analysis using GraphPad Prism software from GraphPad Software Inc (La Jolla, CA, USA) was performed to compare the means of the 4 independent groups (Control+Vehicle, Ligature+Vehicle, Control+Quercetin, Ligature+Quercetin). 3D representative images were obtained using CTVox 3D visualization software (Brüker). Linear measurements were not made using this software as Brüker's 3D visualization software solely offers 3D model creation, viewing and flexible control of Micro-CT scans (52–54).

## Histological Study of Mouse Gingival Tissues

Gingival tissues from Quercetin-treated and vehicle-treated control mice were removed using previously established protocols with slight modifications (44, 55). Using a #15 scalpel blade and 3.5x magnification Dental Loupes, a trained periodontitis excised the gingival tissues surrounding the three maxillary molars on the ligated or control side of the maxilla without including buccal and palatal tissues and damaging underlying tissues or bone (38–41). Tissues were fixed in 4% formaldehyde and sent to the Virginia Commonwealth University Cancer Mouse Models Core Laboratory where they were processed to paraffin. Briefly, fixed tissues were processed under a vacuum using the automated Tissue Tek Tissue Processor VIP (Sakura Finetek, Torrance, CA). The processor dehydrated the tissue using gradual increases in concentrations of ethanol from 70% to 100%, then was cleared with Citrisol

(National Diagnostics) finishing with 4 changes of Paraplast Plus (VWR) paraffin wax at 60 degrees. Processed and paraffin embedded samples are then individually placed into a stainless steel mold containing molten Paraplast Plus with an embedding ring and allowed to harden on a cryo plate for 20 minutes before removing from mold. Using a standard rotary microtome, 5μm thick sections are floated on a 40-degree C water bath and then mounted onto positive charged slides and set on end to dry at room temperature. Progressive H&E staining of paraffin embedded sections is performed using the automated Agilent Dako CoverStrainer slide processing system. The instrument process involves baking, dewaxing, hydrating and staining through to the dehydrated, coverslipped and dried slide. Hematoxylin, Eosin and Bluing Buffer and mounting medium are purchased from Agilent Technologies as validated ready-to-use reagents for the Dako CoverStrainer. Histology images were acquired using the Q-Color 5 imaging system from Olympus Microscopy with a 10× magnification objective lens. Quantification of numbers of nucleated (hematoxylin-positive) in the gingiva was performed by a blinded examiner using CellSens software at 20× magnification. Counting was performed using the "Count and Measure" tool and the "Manual Threshold" option to choose an initial nucleated cell for reference. Once the cell was selected, subsequent cells were automatically selected until all nucleated cells in the connective tissue were highlighted. Data were reported as area stained (square micrometers) in each field of view. Nine regions/fields of view were analyzed per ligated side, and six regions/fields of view were analyzed per control side, per mouse, respectively. A minimum of 7 mice were examined per treatment group and data are representative of three independent analyses.

## Quantitative Real-Time Polymerase Chain Reaction

Gingival tissues from Quercetin-treated and vehicle-treated control mice were isolated and harvested using previously established protocols with slight modifications and as described in the previous sections (44, 55). Total RNA was extracted from gingival tissues using the RNeasy kit (Qiagen, Cat# 74136) and genomic DNA (gDNA) eliminator spin columns. The RNA concentrations were determined with NanoDrop. For each gingival side of each mouse approximately 1800 ng- 7500 ng of total RNA was isolated. Samples were never pooled of mice receiving the same treatment. 800 ng of total RNA was used for cDNA synthesis with High Capacity cDNA Reverse Transcription Kit, following the users' manual (Applied Biosystems, Cat# 4368814). Primers were obtained by Invitrogen. Sequences for primers used were as follows: mIL-6 forward: 5′-TCTATACCACTTCACAAGTC GGA-3′, mIL-6 reverse: 5′-GAAT TGCCATTGCACAACTCTTT-3′; mTNF forward: 5′-CTGAACTTCGGGGTGAT CGG -3′, mTNF reverse: 5′-GGCTTGTCACTCGAATTTTGAGA-3′, TNFAIP3/A20 (mouse) forward: 5′-AGGTCGGTGTGAACGGATTTG -3′, and reverse: 5′-GGACAGTTGGGTGTCTCACATT-3′; TNFAIP3/A20 (human) forward: 5'-TTGTCCTCAGTTTC GGGAGAT-3' and reverse: 5'-ACTTCTCGACACCAG TTGACTT-3'. Results were normalized with respect to the values obtained for the housekeeping gene GAPDH. ΔCt was calculated by subtracting the Ct value of the housekeeping gene (GAPDH) from the target gene Ct value. ΔΔCt was calculated by subtracting the control group target gene ΔCt from the experimental group target gene ΔCt. Relative mRNA expression was calculated by $2^{-\Delta\Delta Ct}$. Data are presented as averages of 3 independent experiments from 26 Quercetin-treated mice and 11 vehicle-treated control mice.

## Microbial Sample Processing and Library Preparation

Bacteria were obtained by oral swabbing of the teeth and gingival surface with Ultra-Fine polystyrene swab (Puritan Medical Products) and placed in DNA/RNA Shield (Zymo Research, Irvine, CA). Samples were set to ZymoBIOMICS for Targeted Metagenomic Sequencing. DNA Extraction was performed using ZymoBIOMICS-96 MagBeadDNA Kit (Zymo Research, Irvine, CA). The DNA samples were prepared for targeted sequencing with the *Quick*-16S NGS Library Prep Kit (Zymo Research, Irvine, CA). The primer sets used were *Quick*-16S Primer Set V3-V4 (Zymo Research, Irvine, CA). These primers were custom-designed by Zymo Research to provide the best coverage of the 16S gene while maintaining high sensitivity. The sequencing library was prepared using an innovative library preparation process in which PCR reactions were performed in real-time PCR machines to control cycles and therefore limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned up with the Select-a-Size DNA Clean & Concentrator (Zymo Research, Irvine, CA) then quantified with TapeStation (Agilent Technologies, Santa Clara, CA) and Qubit (Thermo Fisher Scientific, Waltham, WA). The ZymoBiomics Microbial Community Standard (Zymo Research, Irvine, CA) was used as a positive control for each DNA extraction and each targeted library preparation. Negative controls (blank extraction control, blank library preparation control) were included to assess the level of bioburden carried by the wet-lab process.

## Sequencing Data Analysis

The final library was sequenced on Illumina MiSeq with a v3 reagent kit (600 cycles). The sequencing was performed with >10% PhiX spike-in. The quality control, feature table construction, diversity and taxonomic analysis were performed in QIIME2. The t-Distributed Stochastic Neighbor Embedding (t-SNE) plot was created by Rtsne package and the differential abundance was tested by ALDEx2 package in R. The raw data with quality scores lower than 25 was trimmed in a quality control step. Alpha diversity was performed by testing the observed OTU, evenness and Shannon index. The significance of alpha diversity was analyzed using Kruskal–Wallis test and corrected using the Benjamini–Hochberg procedure with a false discovery rate of 5%. Beta diversity metrics was computed using Bray-Curtis distance, following by PERMANOVA statistical analysis and corrected using the Benjamini–Hochberg

procedure with a false discovery rate of 5%. PERMANOVA is a multivariate ANOVA with permutations. It is meant to test differences between groups with a lot of variables and with permutations to avoid possible biases. Statistical differences reveal that the distribution and abundances of experimental groups are different (56). The ordination plot was generated by performing dimensionality reduction using t-SNE. Silva-132-99-nb-classifier was used to assign taxonomy to the OTUs, following by differential abundance analysis using LEfSe (57). The abundance of taxa was determined by dividing the hits of a specific taxon by the total hits of all taxa in the sample. Our analyses identified a total of 163 species which were present in levels greater than 0.1% in more than 5% of total samples. These bacteria were included for differential abundance analysis. Sequencing was performed in each individual sample once. Additionally, the OTUs were also assigned to taxonomy using HOMD 16s rRNA database, following by the same steps for differential abundance analysis.

## *In Vitro* Stimulation Assays

THP-1 cells were suspended at a density of $3 \times 10^6$ in 6-well plates and treated with 25ng/mL of phorbol 12-myristate 13-acetate (PMA: Sigma, Cat# P1585) overnight to differentiate into macrophages (43, 58, 59) The cells were then treated with Quercetin (5μg/mL) dissolved in ethanol for 2 hours prior to stimulation with bacteria and toll-like receptor agonists. The cells were challenged with heat-killed *P.gingivalis* (multiplicity of infection [MOI] of 1:100), *F.nucleatum* (MOI 1:50), *P. gingivalis* lipopolysaccharide (LPS) (10μg/mL) (*In vivo*gen, Cat# tlrl-ppglps), Pam3Cys-Ser-(Lys)4 (Pam3CSK4) (10ng/mL) (*In vivo*gen, Cat# tlrl-pms), and CpG oligonucleotide (ODN) 2006 (100μg/mL)(*In vivo*gen, Cat# tlrl-2006-5) for up to 24 hours. Inflammatory cytokine levels (TNF and IL-6) were determined in cell-free culture supernatants using ELISA (TNF ELISA kit: Thermo Fisher, Cat# 88-7346-22 and IL-6 ELISA kit: Thermo Fisher, Cat# 88-7066-22). For *in vitro* assays evaluating A20 mRNA levels, the cells were challenged with heat-killed *F.nucleatum* (multiplicity of infection [MOI] of 1:10), and Pam3Cys-Ser-(Lys)4 (Pam3CSK4) (10ng/mL)(*In vivo*gen, Cat# tlrl-pms) for up to 6 hours. Total RNA was isolated using RNeasy plus Mini Kit by QiaCube (Qiagen) and 800ng of total RNA was used for cDNA synthesis and subsequent qRT-PCR. All *in vitro* studies were performed with at least three sets of independent experiments with a minimum of 3 replicates per time point/stimulation.

## Immunofluorescence Staining

THP-1 cells ($3x10^4$) were grown on 8-chamber polystyrene vessel tissue culture treated glass slide (BD Falcon, USA) and pretreated with 25ng/ml PMA to differentiate to macrophages overnight. The cells were then treated with Quercetin (5μg/mL) dissolved in ethanol for 2 hours prior to stimulation with bacteria and toll-like receptor agonists. The cells were stimulated with heat-killed *P.gingivalis* (multiplicity of infection [MOI] of 1:100), and *F.nucleatum* (MOI 1:50), for up to 60 minutes. Following treatment, cells were washed in cold PBS and then fixed in 4% paraformaldehyde. The cell membrane was then permeabilized in buffer containing 0.3% Triton-100 and 0.1% NaN3 in PBS for 30min at RT. Nonspecific binding sites were blocked by incubation with 5% BSA, 0.1% NaN3 and 0.3% triton-X in PBS in a humidity chamber for 1h at RT or overnight at 4°C. Cells were incubated with anti-NF-κBp65 antibody (ProteinTech, 1:100) overnight at 4°C. After incubation, cells were rinsed twice for 5 min in PBS with gentle shaking and incubated with Alexa 488 goat anti-rabbit polyclonal antibody (Invitrogen, 1:250) for 1h at RT. Cells were washed 6 times in PBS with gentle shaking. Coverslips were mounted with antifade mounting medium with DAPI (Vectashield Vibrance) and kept under dark conditions at RT. The images were collected by Nikon confocal laser microscope at a magnification of 630X and zoom 2 to assess nuclear localization of NF-κB. For each group a minimum of 4 images were collected. Three sets of independent experiments were conducted with a minimum of 120 cells analyzed.

## Statistical Analysis

Statistical analysis of microbial samples is given in the Sequencing Data Analysis section above. Statistical analysis for all other experiments was performed using GraphPad Prism software from GraphPad Software Inc (La Jolla, CA, USA). All data were expressed as the mean ± the standard deviation. The difference between two groups was established by the unpaired *t* test with Mann-Whitney correction. Multiple group comparisons were performed by one-way ANOVA with Tukey's *post hoc* test to identify differences between specific groups. A value of $p<0.05$ was considered to be statistically significant. The number of animals examined per group and number of times the experiments were carried out are given above and in the Figure Legends.

# RESULTS

## Quercetin Diminishes Gingival Inflammation and Alveolar Bone Loss in Experimental Periodontitis

The efficacy of Quercetin to modulate the immune responses and periodontal disease phenotype was assessed using the murine ligature-induced periodontitis model. Quercetin was administered orally at a dosage (40mg/kg/twice daily) comparable to humans a week before ligations and continued throughout the experimental period as described (**Figure 1A**). As anticipated, alveolar bone loss was observed in both groups following ligations (**Figures 1B, C**). Supporting a protective effect, the linear bone loss in the distance from the cementoenamel junction and alveolar bone crest was reduced in mice which received oral Quercetin supplement compared to the control group (**Figure 1D**). Periodontal bone loss typically mirrors the degree of inflammation present, therefore we further assessed immune cell infiltration in the dissected gingival tissues using H&E staining. In corroboration with the bone loss data, ligated sites displayed increased inflammatory cell infiltration in both experimental and control groups whereas the immune cell

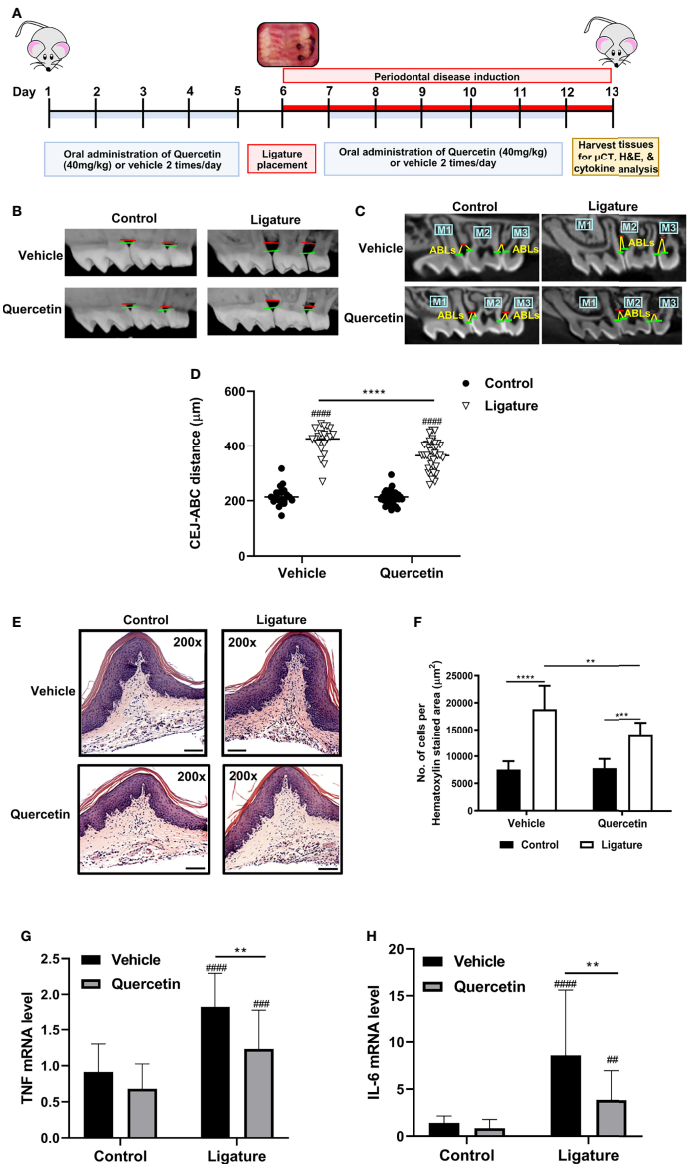


**FIGURE 1** | Quercetin Treatment Decreases Periodontal Inflammation and Bone Loss. **(A)** Schematic presentation demonstrating the experimental design system. In male C57BL/6J mice, silk 5-0 ligatures were placed on the left side of the maxilla interdentally between first and second molars, while the right sides were left unligated as controls. Mice were treated with Quercetin by oral gavage or vehicle matched control six days prior to ligature placement and maintained with the same treatment regimen one-week post-ligation. Mice were euthanized and periodontal tissues were examined. **(B)** Representative images of 3D micro-CT reconstructions of periodontal bone in Quercetin treated and vehicle treated mice. The distance between horizontal lines represents the distance between cemental-enamel junction and alveolar bone crest. **(C)** Representative two-dimensional images of sagittal slice views of Quercetin-treated and vehicle-treated mice. Linear measurements were taken of the alveolar bone loss (ABL) in the interdental space from the cemental-enamel junction to alveolar bone crest. **(D)** Distance between the cemental-enamel junction and alveolar bone crest was analyzed and plotted to determine periodontal bone loss (n=37 mice for Quercetin treatment, n=21 mice for vehicle treatment). The average of 4 CEJ-ABC measurements were calculated per mouse. Individual mouse datapoints are plotted. Averages and standard deviations are shown. ****$p \leq 0.0001$. $^{\#\#\#\#}$Ligated gingival tissues versus corresponding experimental controls ($p \leq 0.0001$). **(E)** Representative images from H&E stained histological sections from gingival tissues derived from ligated and control periodontium in Quercetin-treated and vehicle-treated mice. **(F)** Quantitative analysis of inflammatory cell infiltrate in the periodontium. Positive cells were counted per $\mu m^2$ gingival connective tissue. Data are representative of three independent experiments, with a minimum of seven mice analyzed in each treatment group per experiment. Averages and standard deviations are shown. **$p \leq 0.01$, ****$p \leq 0.0001$. **(G, H)** Gingival tissues derived from ligated and control periodontium in Quercetin treated and vehicle treated mice were digested and applied to RNA preparation. The mRNA levels of mTNF **(G)**, mIL-6 **(H)** were determined with qRT-PCR and the relative mRNA expression levels were plotted (n=11 for vehicle treatment, n=26 for Quercetin treatment). Averages and standard deviations are shown. **$p \leq 0.01$, ***$p<0.001$, ****$p \leq 0.0001$. $^{\#\#\#\#}$Ligated gingival tissues versus corresponding experimental controls ($p \leq 0.0001$). $^{\#\#\#}$Ligated gingival tissues versus corresponding experimental controls ($p \leq 0.001$). $^{\#\#}$Ligated gingival tissues versus corresponding experimental controls ($p \leq 0.01$).

infiltrate in the ligated tissues of Quercetin-supplemented mice was significantly reduced compared to that of the mice in the vehicle group (**Figures 1E, F**). We subsequently determined gingival tissue cytokine expression to further elucidate the effect of Quercetin on the inflammatory response in the oral mucosa. As expected, induction of periodontitis led to the increased TNF and IL-6 expression in the gingival tissues. Substantiating the efficacy of oral delivery of Quercetin and anti-inflammatory action, the gingival tissues dissected from Quercetin-supplemented mice displayed significantly diminished cytokine expression profiles compared with their vehicle-treated counterparts (**Figures 1G, H**).

## Quercetin Mitigates Oral Microbial Dysbiosis in Experimental Periodontitis

To characterize Quercetin effects on the temporal dynamics of inflammation-induced microbial dysbiosis within the oral cavity, changes in the oral microbiota were assessed upon treatment with Quercetin or vehicle matched control before and after periodontitis onset as described in **Figure 2A**. Oral bacteria were collected at the baseline of the study (Day 1), time of ligature placement (Day 6), and after the disease onset (Day 13). Before the onset of periodontitis (Days 1 and 6), the oral microbiota of each treatment group displayed increased alpha diversity which is consistent with healthy microbiota compared to those collected following disease establishment (Day 13) (**Figures 2B, C**). After periodontitis onset, the Evenness index was decreased compared to healthy group indicating few taxonomic groups dominate during the disease (**Figure 2B**). Similar results were obtained with the Shannon index, reflecting less community richness and evenness in the oral microbiota of animals driven by experimental periodontitis (**Figure 2C**). Confirming the efficacy of oral Quercetin delivery, after periodontitis onset, Quercetin-supplemented mice exhibited significantly higher Evenness and Shannon indices relative to the vehicle-treated group (**Figures 2B, C**). These results suggest that Quercetin supplement aids to maintain the diversity of the oral microbiome. The taxonomic compositions of each treatment group were also distinguished by dissimilarity (**Figures 2D, E**). Through visualization of clusters using the t-SNE algorithm, two-dimensional ordination demonstrated similarity of the oral microbiome between the treatment groups prior to periodontitis induction (**Figure 2D**). However, after periodontitis onset, treatment groups were well-separated indicating heterogeneity between the oral microbial composition of Quercetin and vehicle treated mice (**Figure 2D**). Variation in community composition among treatment groups was also characterized by Bray-Curtis measures of taxonomic distance (**Figure 2E**). As expected, significance was only observed in the microbial composition between Quercetin and vehicle-treated mice after the induction of periodontitis (**Figure 2E**). Corroborating our alpha and beta diversity analyses, the mice from both treatment groups displayed similar taxonomic profiles before the development of periodontitis (**Figures 3A, B**). However, after periodontitis onset, there were several species-, genus-, and family- level differences between bacterial communities in Quercetin-treated and vehicle-treated mice (**Figure 3C**). Mice which received Quercetin had increased levels of *Streptococcus*, which is often associated with a symbiotic microbiome (**Figure 3D**). Additionally, mice which received vehicle treatment, displayed increased levels of several bacteria including *Enterococcus, Neisseria* and *Pseudomonas*, which are often associated with periodontitis or other inflammatory conditions (**Figure 3D**). Furthermore, when using HOMD reference database specific for the oral microbiome, Quercetin-treated mice displayed increases in *Streptococcus sanguinis* and *Streptococcus parasanguinis*, known commensal bacteria in the oral cavity (**Supplementary Figure 1**).

## Quercetin Maintains Periodontal Tissue Homeostasis Through its Effect on NF-κB: A20 Axis and Cytokine Production

The interaction between host immune cells and the oral microbiome, and subsequent crosstalk between innate signaling pathways play a critical role in determining periodontal disease outcome. Macrophages are one of the key cells responding to microbial insult in periodontitis pathogenesis. We therefore sought to determine the effect of Quercetin on TLR signaling and cytokine responses in human macrophages challenged with periodontal bacteria and various TLR agonists. *P. gingivalis* and *F. nucleatum* were included as representative organisms as they possess unique virulence factors favoring microbial dysbiosis and periodontal tissue destruction and are frequently associated with numerous systemic complications (60–63). Briefly, macrophages were treated with Quercetin for 2 hours prior to challenge with bacteria or TLR agonist, LPS, and cytokine response was assessed post-infection. Corroborating its anti-inflammatory action, Quercetin treatment significantly reduced TNF and IL-6 production in human macrophages following infection with *P. gingivalis* (**Figure 4A** and **Supplementary Figure 2A**) and *F. nucleatum* (**Figure 4B** and **Supplementary Figure 2B**). Cytokine levels remained comparable in Quercetin-treated unstimulated control macrophages compared to vehicle-treated unstimulated control cells, suggesting that Quercetin functions to mitigate inflammation downstream of TLR signaling. To further characterize the effect of Quercetin on inflammatory response, macrophages were treated with Quercetin or vehicle and challenged with TLR agonists ([*P. gingivalis* LPS for TLR4], [Pam3CSK4 for TLR2], and [CpG oligonucleotide 2006 [ODN] for TLR9]) for up to 24 hours (**Figures 4C–E** and **Supplementary Figures 2C-E**). As expected, quercetin treated cells produced significantly less cytokine in response to *P. gingivalis* LPS (**Figure 4C** and **Supplementary Figure 2C**), Pam3CSK4 (**Figure 4D** and **Supplementary Figure 2D**) and ODN2006 (**Figure 4E** and **Supplementary Figure 2E**) compared to vehicle-treated group.

We then sought to determine the mechanisms that Quercetin can exert its effect on periodontal inflammation and define downstream signaling pathways. The TLR : NF-κB:A20 signaling axis is one of the critical pathways governing inflammation in the oral mucosa through modulating cytokine gene expression. We first assessed NF-κB nuclear translocation, a

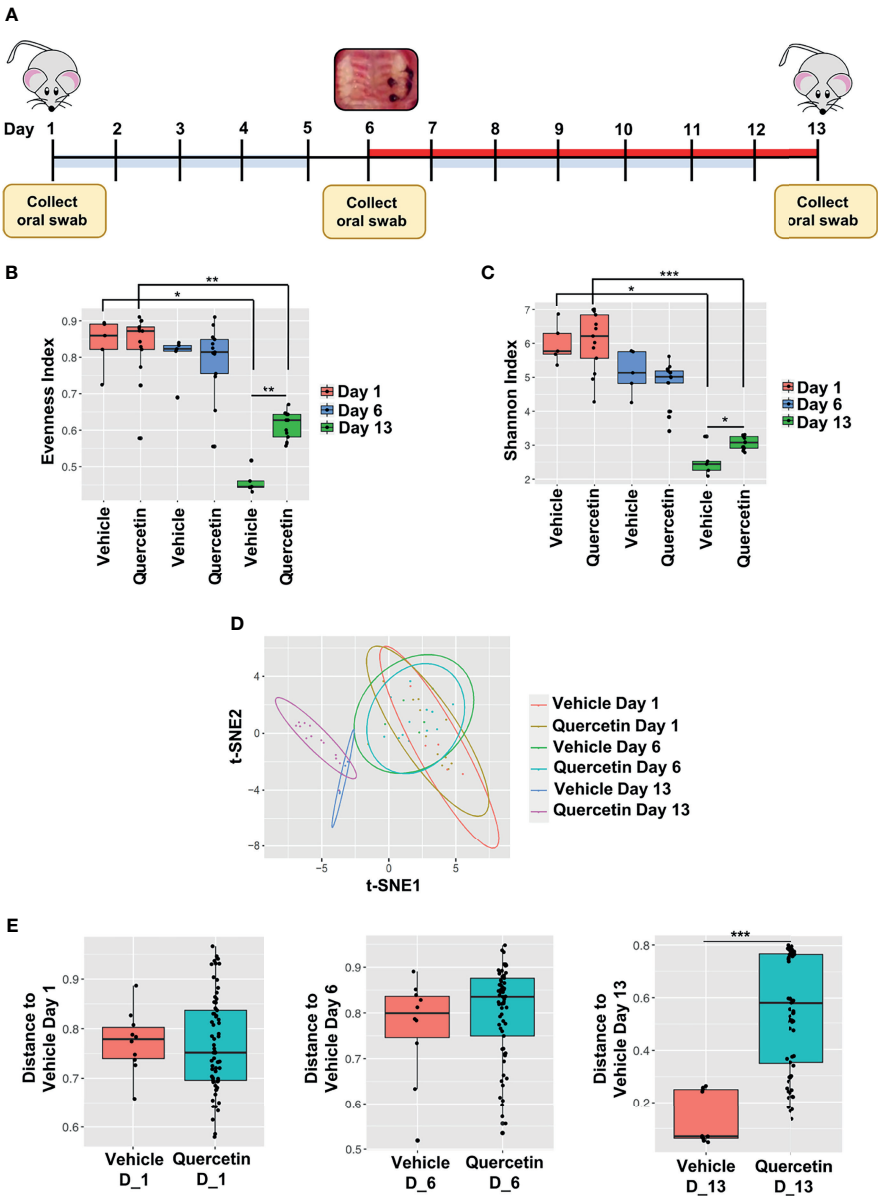


**FIGURE 2** | Quercetin Treatment Increases Bacterial Diversity in Mice with Experimental Periodontitis. **(A)** Schematic diagram illustrating the periodontal induction and treatment experimental design and timeline of oral swab collection for subsequent 16S rRNA sequencing microbiome analysis. **(B, C)** Alpha diversity was assessed in the oral microbiota of 5 vehicle- and 13 Quercetin-treated mice before and after the induction of periodontitis and the Evenness **(B)** and Shannon index **(C)** of vehicle- and Quercetin-treated mice on Day 1, 6 and 13 are shown. *p ≤ 0.05, **p ≤ 0.01. **(D, E)** Dissimilarity was assessed in the oral microbiota of 5 vehicle- and 13 Quercetin-treated mice before and after periodontitis induction and the ordination is shown by the t-Distributed Stochastic Neighbor Embedding (t-SNE) plot **(D)**. The Bray-Curtis dissimilarity between vehicle- and Quercetin-treated mice in Days 1 (left), 6 (middle) and 13 (right) are shown **(E)**. ***p ≤ 0.001.

hallmark of NF-κB activation, in vehicle- and Quercetin-treated human macrophages using immunofluorescence and confocal imaging. Our results showed increased NF-κB translocation in vehicle-treated macrophages upon infection with *F. nucleatum* (**Figures 5A, B**) and *P. gingivalis* (**Figures 5C, D**) compared to Quercetin-treated cells indicating that Quercetin regulates oral bacteria-induced inflammatory cytokine response through its effect on NF-κB signaling pathway. The ubiquitin-editing enzyme, A20, is an inducible and broadly expressed cytoplasmic protein that inhibits TNF- and TLR-induced NF-κB activity and previous studies suggested its modulation by Quercetin treatment (9, 10, 64, 65). To determine the effect of Quercetin on A20 expression in the oral mucosa, we next examined A20 mRNA levels in response to Quercetin treatment in human macrophage-like cells challenged with *F. nucleatum*- and Pam3CSK4 and noted increased A20 expression in cells treated with Quercetin compared to those which received vehicle (**Figure 6A**). Further, we also assessed the effect of

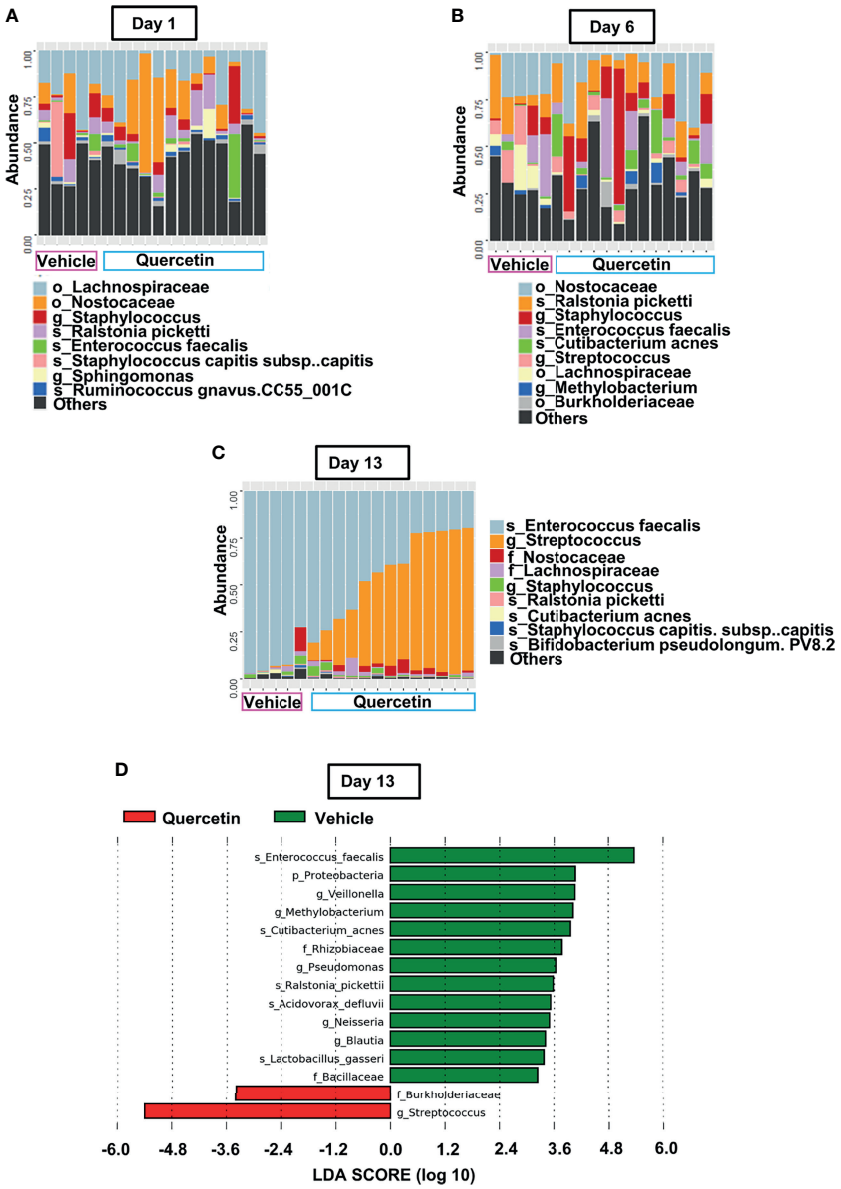


**FIGURE 3** | Quercetin Treatment Alters Microbial Composition Induced by Periodontitis. **(A–C)** Prominent bacterial taxa in 5 vehicle- and 13 Quercetin-treated mice at Day 1 **(A)**, Day 6 **(B)** and Day 13 **(C)**. **(D)** Significantly altered taxa in Quercetin treated compared to vehicle treated mice after periodontitis onset (Day 13) as determined by linear discriminant analysis effect size (LDA) scores using Silva-132 classifier.

Quercetin on gingival tissue A20 expression *in vivo*. As expected, there was an upregulation of A20 mRNA in the ligated gingival tissues of vehicle treated mice, indicating enhanced inflammation in these tissues (**Figure 6B**). Consistent with the improved disease phenotype, the ligated tissues of Quercetin treated mice displayed similar A20 mRNA levels compared to the control tissues (**Figure 6B**). Overall, these results support *in vivo* data and confirm the anti-inflammatory effect of Quercetin as it applies to host-microbiome interactions in the oral cavity. It is also of note that consistent with the proven clinical safety profile of Quercetin, we observed no effect on cell viability or structure throughout the experiments which indicates that decreased cytokine production was not due to undesirable effects of Quercetin on macrophages.

## DISCUSSION

Despite many advances in the field, periodontal diseases continue to be one of the most common inflammatory conditions worldwide posing a significant overall health problem and financial burden (66, 67). There is an urgent need to develop effective, safe, cheap and practical preventive approaches to facilitate the maintenance of a balanced host-

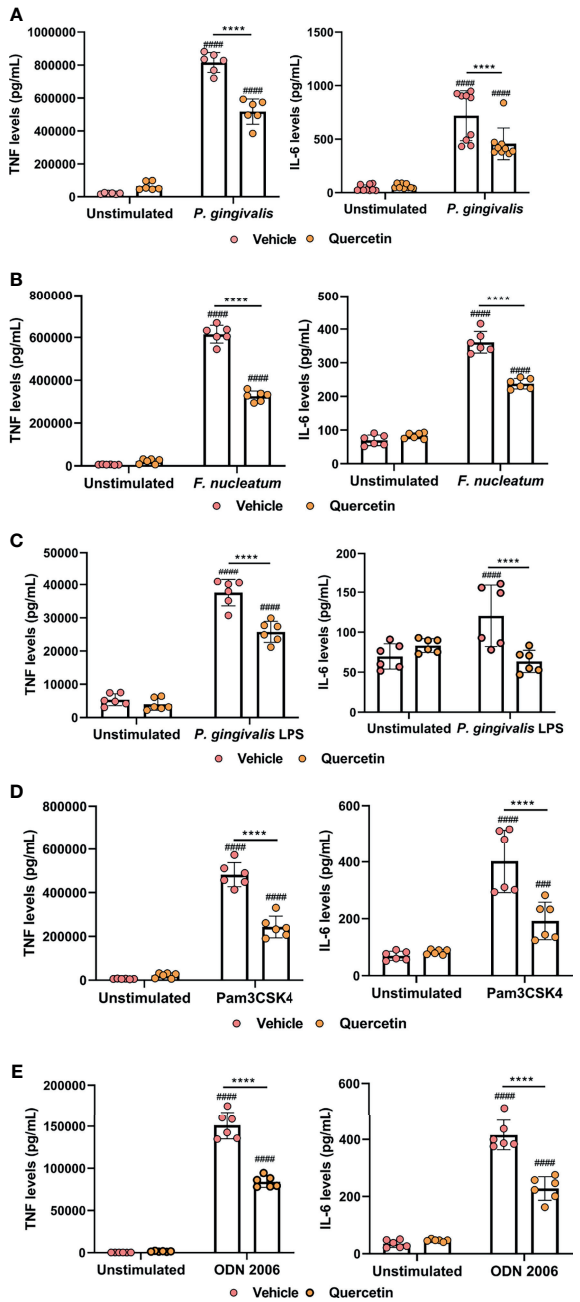


**FIGURE 4** | Quercetin diminishes cytokine production in human macrophages challenged with oral bacteria and toll-like receptor agonists. THP-1 macrophages treated with Quercetin (5μg/mL) or vehicle matched control were infected with oral bacteria or toll-like receptor agonists for up to 24 hours. **(A–E)** IL-6 and TNF levels in the supernatants were determined with ELISA. ELISA was conducted on **(A)** macrophages infected with *P. gingivalis* (100 MOI), **(B)** macrophages infected with *F. nucleatum* (50 MOI), **(C)** macrophages infected with *P. gingivalis* LPS (10μg/mL), **(D)** macrophages infected with Pam3CSK4 (10ng/mL) and **(E)** macrophages infected with ODN 2006 (100μg/mL). Each experiment was performed at minimum three times independently and averages and standard deviations are shown. $^{****}p \leq 0.0001$. $^{\#\#\#\#}$Unstimulated control cells versus corresponding stimulated cells ($p \leq 0.0001$). $^{\#\#\#}$Unstimulated control cells versus corresponding stimulated cells ($p \leq 0.001$).

microbiome interactions and prevent the progression of the disease. This is especially important for susceptible populations such as aging, diabetics, and immunocompromised, who exhibit severe forms of the diseases (68–71). In this investigation, we report key data for future translational studies which show that oral Quercetin usage can improve periodontal disease outcomes through its effect on host inflammatory response and oral microflora (**Figure 7**). Specifically, we noted significantly improved disease phenotype as measured by decreased alveolar bone loss, inflammatory cell infiltrate, and gingival tissue cytokine expression in Quercetin-treated versus vehicle-treated mice. We further confirmed the mechanism of anti-inflammatory action of Quercetin using an *in vitro* disease model and revealed that Quercetin can diminish inflammatory response to periodontal bacteria and in human macrophage-like cells through its effect on NF-κB signaling pathway.

The oral cavity is in constant exposure to microbial and physical insult and therefore it is crucial to identify novel ways to mitigate the effect of these stressors to sustain periodontal tissue homeostasis and prevent disease progression. The current paradigm for the treatment of periodontal disease supports development of novel strategies which target key molecular pathways to limit prolong inflammation and facilitate timely resolution (72). Recently, research efforts focus on harnessing the beneficial properties of natural products and mediators in periodontal inflammation as safe and cost-effective therapeutics (31). In general, the studies which investigate the efficacy of immune modulatory reagents administer compounds after the disease induction phase to evaluate therapeutic effects and mostly use local injections as the delivery method to achieve maximum concentration at the lesion site (31, 34, 73). In this study we introduced Quercetin through an oral route prior to the onset of the disease in an attempt to enhance absorption into periodontal tissues and establish a sustained effect (74, 75). In addition, using this study design we were able to evaluate the effect of this natural compound both in health and disease states. Indeed, Quercetin had no impact on oral microbial composition in health as shown by the lack of differences in the microbiome between Day 1 and Day 6 of the study whereas it improved dysbiosis during the course of the disease (Day 13). These results further confirm the safety profile, efficacy and health benefits of Quercetin in the oral cavity. Our results concur with previous studies which delivered Quercetin through subcutaneous and intragastric delivery to halt periodontitis and reveal for the first time the effectiveness of oral delivery which is a more clinically plausible and practical application for supplemental use in periodontal practice (76–78). In fact, after just 2 weeks of Quercetin supplementation, mice exhibited decreased alveolar bone loss compared to vehicle-treated mice. Most recently, Quercetin was shown to prevent oxidative stress-induced injury of periodontal ligament cells and reduce alveolar bone loss in periodontitis after intragastric delivery (78). Similar beneficial effects of Quercetin have been consistently noted in other immune and inflammatory conditions which share common pathophysiological features with periodontitis (28–30, 79–82). *In vitro* studies involving human and mouse cells as well as *in vivo* mouse models have detailed the role of Quercetin in

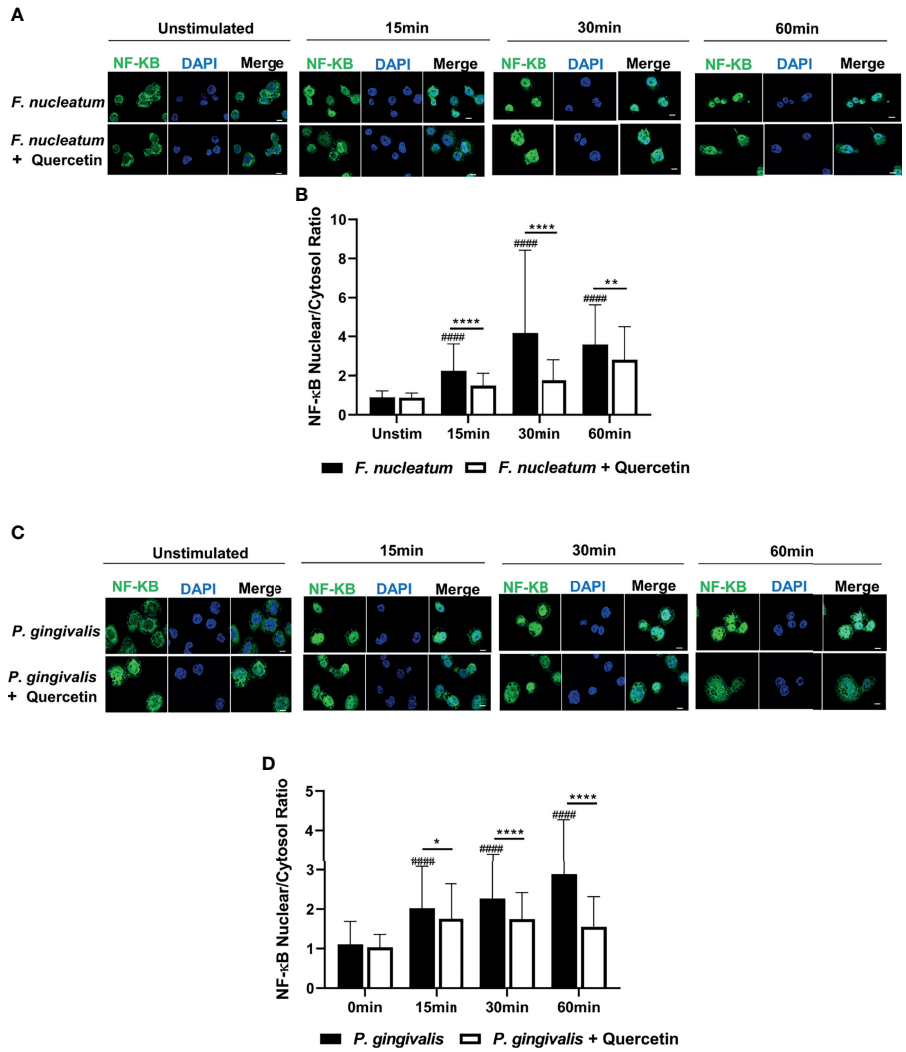


**FIGURE 5** | Quercetin decreases NF-κB nuclear translocation upon LPS-and oral bacteria-induced inflammation in human macrophages. THP-1 macrophages treated with Quercetin (5μg/mL) or vehicle matched control were infected with LPS or oral bacteria for indicated times. NF-κB was stained with NF-κB antibody followed by Alexa-Fluor488 (green) and the nucleus was stained with DAPI (blue). Images were captured with Nikon Confocal laser microscope. **(A)** Representative images of vehicle- and Quercetin-treated cells infected with *F. nucleatum* (25 MOI) **(C)** and *P. gingivalis* (50MOI) and the quantitative analysis of nuclear to cytosolic ratio of NF-κB was shown, respectively **(B, D)**. Scale bars=10μM. Data are representative of three independent experiments and averages and standard deviations are shown. *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001. ####Unstimulated control cells versus corresponding stimulated cells (p ≤ 0.0001).

protecting against neuroinflammation by inhibiting nitric oxide production and neuronal apoptosis and prevent inflammation-related neuronal injury and neurodegeneration (83–86). It has been reported to improve disease phenotype in collagen- and zymosan-induced arthritis in mice by decreasing inflammatory cytokine levels, cartilage and bone destruction, and synovial inflammation as well (87, 88). During experimental allergic inflammation in mice, orally administered Quercetin significantly reduced cytokine levels in bronchoalveolar lavage fluid and mucus production in the lungs (89). Similarly, Quercetin supplementation has been demonstrated to decrease susceptibility for the development of asthma, bronchial hyper-reactivity and chronic obstructive pulmonary diseases in human and mouse studies (90–93). Quercetin also has been reported to improve diabetes-related complications in *in vivo* mouse and rat studies by interfering with the innate signaling pathways such as TLRs and peroxisome proliferator-activated receptor ɣ (PPARɣ) and inhibiting the activity of nuclear factor κ light-chain enhancer of activated B cells (NF-κB) which subsequently reduces the levels of TNF and CRP (94–96). As therapies targeting the modulation of the host immune response continue to offer promise alleviating adverse clinical outcomes, these results collectively suggest that Quercetin supplementation may be effective to sustain health at both oral and distant sites. In light of our current findings and accumulating evidence supporting the benefits of Quercetin in health and disease,

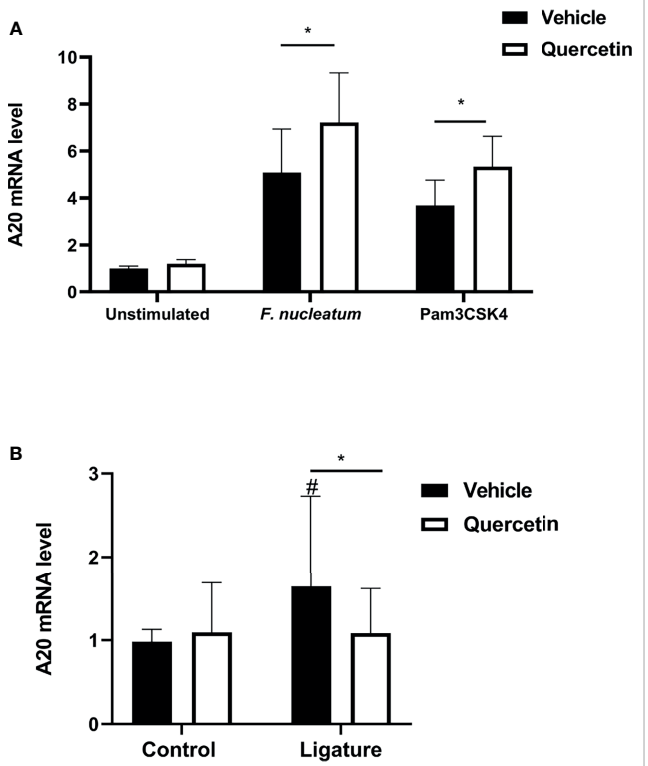


**FIGURE 6** | Quercetin modulates A20 mRNA levels in pre-clinical periodontitis disease models. **(A)** THP-1 macrophages treated with Quercetin (5μg/mL) or vehicle matched control were infected with oral bacteria (*F.nucleatum* [10 MOI]) or toll-like receptor agonist (Pam3CSK4 [10ng/mL]) for up to 6 hours and applied to RNA preparation. The mRNA levels of mA20 were determined with qRT-PCR and the relative mRNA expression levels were plotted. Each experiment was performed three times independently and averages and standard deviations are shown. *$p \leq 0.05$. **(B)** Gingival tissues derived from ligated and control periodontium in Quercetin treated and vehicle treated mice were digested and applied to RNA preparation. The mRNA levels of mA20 were determined with qRT-PCR and the relative mRNA expression levels were plotted (n=11 for vehicle treatment, n=26 for Quercetin treatment). Averages and standard deviations are shown. *$p \leq 0.05$. #Ligated gingival tissues versus corresponding experimental controls.

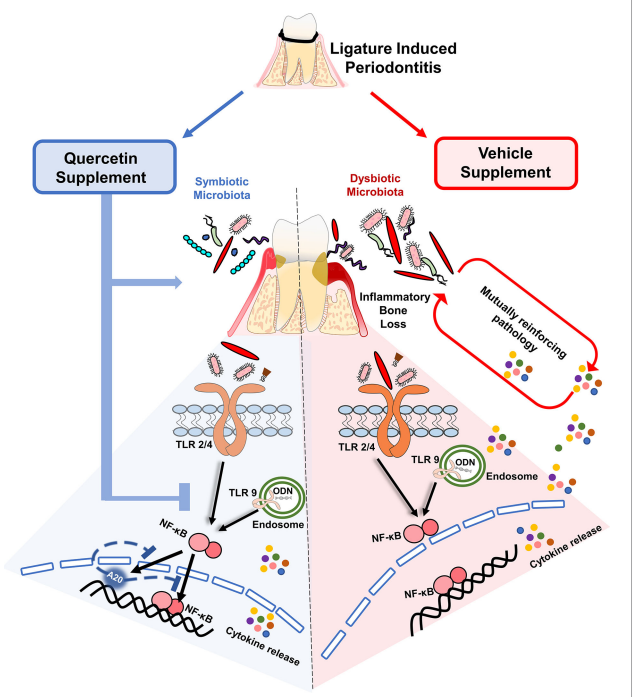


**FIGURE 7** | Quercetin supplement alleviates the progression of periodontitis by aiding in the preservation of host- and microbiome-homeostasis. A self-perpetuating pathogenic cycle of deregulated inflammation, exacerbated cytokine release, and bacterial dysbiosis fuels tissue destruction and the subsequent development of periodontitis in response to ligature placement. Quercetin, a ubiquitous polyphenolic flavonoid, promotes health through various biological mechanisms. Upon periodontitis induction, Quercetin supplement reduced gingival inflammation, alveolar bone loss and limits bacterial dysbiosis. At the cellular level, periodontal tissue homeostasis requires the stringent regulation of inflammatory pathways mainly driven by the activation of innate sensors, such as TLRs. Downstream of TLR activation, Quercetin supplement decreased cytokine production by regulating NF-κB activation and possibly modulating A20 expression, in response to oral bacteria and TLR agonist challenge. Collectively, the regulation of inflammation with Quercetin supplement has a significant impact on gingival inflammatory gene expression and bacterial composition that is coincident with ameliorating disease progression.

future studies are warranted to develop local delivery methods and assess the efficacy using different concentrations in the management of periodontal diseases in a more targeted fashion before and after the onset of the disease (31). Considering the established associations between the severity of periodontitis and numerous systemic conditions including diabetes, it would be also of interest to determine whether Quercetin supplementation can improve periodontal clinical parameters in these susceptible patient cohorts as well.

In the oral cavity, the initiation of inflammation is largely driven by engagement of the microbiome and microbiome associated molecular patterns with TLRs and the subsequent activation of NF-κB and inflammatory mediator production. Hitherto, there was a lack of knowledge about the mechanistic role of Quercetin in the oral cavity at the cell-microbiome interface. Previous studies have investigated the potent anti-inflammatory effects of Quercetin in human gingival fibroblasts, a cell type predominating in gingival connective tissue. Quercetin was shown to inhibit the inflammatory response to *P. gingivalis* LPS in human gingival fibroblasts *via* suppressing the NF-κB signaling pathway (97). In early periodontal lesions, macrophages also play a critical role in the immune landscape. Macrophages counteract pressures from a diversity of stimuli, including the oral microbiome, which can drive their polarization into distinct inflammatory or resolution states (98, 99). Therefore, understanding how therapeutic agents drive these responses will be critical for elucidating determinants of successful resolution in periodontitis. In this study, our results indicated that Quercetin functions as a regulator of inflammation through modulating NF-κB and cytokine production in macrophages upon oral microbial infection. These findings are consistent with previous mechanistic

studies which revealed that Quercetin attenuates inflammation through interfering with multiple signaling pathways including NF-κB, mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase/Akt (PI3K/Akt), signal transducer and activator of transcription proteins (STAT), inflammasome complex and regulates cellular functions including apoptosis, cellular senescence, and autophagy in several models of diseases and cell types (100–108). Notably, the beneficial role of Quercetin has also been extensively studied in cancer, and the potent anti-inflammatory and anti-tumor functions have been documented in breast cancer and liver cancer through its ability to inhibit NF-κB signaling cascade (109, 110). One of the key negative regulators of NF-κB activation is the ubiquitination enzyme, A20 (9–11, 111). In several inflammatory diseases, including periodontal disease, A20 plays role as one of the key regulatory agents (43, 44, 64). Recent evidence suggests that Quercetin can diminish inflammation through its effect on A20 in cystic fibrosis cells (65). Similarly, we were able to show the induction of A20 in response to Quercetin treatment in *F. nucleatum*- and Pam3CSK4-treated human macrophage-like cells. This observation is consistent with the previous reports and our current data demonstrating reduced NF-κB activity following Quercetin treatment *in vitro* and suggests that Quercetin can regulate inflammation through the NF-κB/A20 signaling axis. Further, we also assessed the effect of Quercetin on gingival tissue A20 expression *in vivo*. A20 is a downstream regulator of NF-κB and its levels are closely regulated by the level of inflammation. As expected, there was increased A20 expression in the ligated sites of vehicle treated mice compared to unligated sites at Day 13 which indicate increased levels of inflammation. In contrast, ligated sites of Quercetin treated mice displayed similar level of A20 expression as the healthy (unligated) sites which provides further evidence that Quercetin treatment alleviates inflammatory response in the periodontal tissues. Recent studies have also suggested Quercetin's mode of action is mediated by several factors including transcriptional, posttranscriptional and posttranslational regulation through direct DNA binding, microRNA regulation, and modulation of epigenetic machinery such as DNA methyltransferase and histone deacetylase activities (112–115). In the current study, *in vivo* disease model was originally designed to assess the effect of Quercetin on periodontitis phenotype and gingival biopsies were harvested at the end of the experimental period. Endogenous regulators of inflammation are dynamic and their levels are tightly regulated in the tissues to sustain tissue homeostasis. To fully characterize the mechanism of action of Quercetin on different signaling pathways and monitor dynamic changes *in vivo*, future studies are warranted using biological specimens obtained at multiple time points during the initiation, progression and resolution phases of the disease. Overall, considering how genetic and epigenetic alterations affect the progression of periodontitis and associated conditions, new insights into mechanisms by which Quercetin influences cellular functions can pave the way to understanding and regulating its action in targeted therapies in humans.

The hallmark of advanced periodontal lesion is impaired host-microbiome homeostasis which is characterized by prolonged inflammation, decreased polymicrobial diversity and further tissue damage (13–15). In addition to determining the effect of Quercetin on periodontal inflammation, we also monitored changes in oral microbiome composition before and after disease induction. As expected, there was decreased microbial diversity in all groups of mice with periodontitis compared to healthy groups. When comparisons were performed among periodontitis groups, mice receiving Quercetin supplements displayed increased bacterial diversity compared to the vehicle treated group. Consistently, species specific analyses revealed an increase in commensal flora, *S. sanguinis* and *S. parasanguinis*, after periodontitis onset in Quercetin treated group versus vehicle group (116). These results suggest that Quercetin treatment may support a periodontal tissue microenvironment which consists of a microbial composition associated with health. Further supporting this notion, vehicle-treated mice with periodontitis exhibited increases in *Enterococcus*, *Blautia* and *Lactobacillus*, which are all concomitant with periodontitis and periodontitis-related complications including diabetes (31, 34). Collectively, these results indicate that Quercetin treatment likely drives resilience against inflammophilic bacterial infection and was more effective at enriching beneficial oral taxa compared to vehicle treatment. Our results are also consistent with the studies reporting similar findings in the gut. Quercetin was shown to increase gut microbiome diversity and reduce the populations of *Fusobacterium* and *Enterococcus* in mice with inflammatory bowel disease protecting them against *C. rodentium*-induced colitis (80). These observations may have significant implications as *F. nucleatum* has emerged as an opportunistic pathogen in both oral and extraoral tissues and is associated with several disease outcomes (60, 62, 117). Remarkably, the therapeutic potential of Quercetin in remodeling gut microbiota has also been indicated in metabolic disorders such as obesity, nonalcoholic fatty liver disease, atherosclerosis, and diabetes-related sequelae such as diabetic peripheral neuropathy (118–121). While there is still no consensus whether Quercetin acts as an antimicrobial agent, it is likely that Quercetin-induced microbial changes are due to its effect on reducing inflammation (77). In fact, using a murine model of periodontitis induced by *Aggregatibacter actinomycetemcomitans* infection, subcutaneous treatment of Quercetin reduced bone loss and inflammatory cytokine production without affecting bacterial load on the last day of the experiment (77). Our study reveals that Quercetin does not appear to reshape the microbiome in states of health, as there were no observed differences between the microbiome in Day 1 and Day 6 of Quercetin- and vehicle-treated mice. Only after the induction of periodontitis were there differences in the microbiota, possibly due to Quercetin primarily modulating inflammatory pathways. Considering the diversity of the oral microbiome, it will certainly be crucial to determine how Quercetin can affect bacterial growth and virulence.

In summary, our data explicitly revealed that oral delivery of Quercetin helps sustain periodontal tissue health through mitigating inflammation and promoting a symbiotic microbial

community composition (**Figure 7**). Due to its high tolerability and safety profile, it may prove as an effective, low-cost and long-term supplement in chronic disease populations, including those who are susceptible for periodontal diseases (21, 122–126).

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI; PRJNA776284.

## ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of Virginia Commonwealth University.

## AUTHOR CONTRIBUTIONS

SS contributed to the conception of the experiments. SS, EM, and SH contributed to the design of the experiments. EM, SH, X-JX, YL, MJ, and CB performed experiments. SS, EM, X-JX, YL, MJ, and BZ analyzed results. SS and EM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.774273/full#supplementary-material

**Supplementary Figure 1 |** Quercetin Treatment Alters Oral Microbial Composition Induced by Periodontitis. Significantly altered taxa in Quercetin treated compared to vehicle treated mice after periodontitis onset (Day 13) as determined by linear discriminant analysis effect size (LDA) scores using HOMD 16S rRNA database.

**Supplementary Figure 2 |** Quercetin diminishes cytokine production in human macrophages challenged with oral bacteria and toll-like receptor agonists. THP-1 macrophages treated with Quercetin (5μg/mL) or vehicle matched control were infected with oral bacteria or toll-like receptor agonists for up to 12 hours. **(A–E)** IL-6 and TNF levels in the supernatants were determined with ELISA. ELISA was conducted on **(A)** macrophages infected with P. gingivalis (100 MOI), **(B)** macrophages infected with F. nucleatum (50 MOI), **(C)** macrophages infected with P. gingivalis LPS (10μg/mL), **(D)** macrophages infected with Pam3CSK4 (10ng/mL) and **(E)** macrophages infected with ODN 2006 (100μg/mL). Each experiment was performed three times independently and averages and standard deviations are shown. ****p<0.0001. ####Unstimulated control cells *versus* corresponding stimulated cells (p<0.0001). #Unstimulated control cells *versus* corresponding stimulated cells (p<0.05).

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# SARS-CoV-2 Specific IgG Antibodies Persist Over a 12-Month Period in Oral Mucosal Fluid Collected From Previously Infected Individuals

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**Background:** Developing an understanding of the antibody response, seroprevalence, and seroconversion from natural infection and vaccination against SARS-CoV-2 will give way to a critical epidemiological tool to predict reinfection rates, identify vulnerable communities, and manage future viral outbreaks. To monitor the antibody response on a larger scale, we need an inexpensive, less invasive, and high throughput method.

**Methods:** Here we investigate the use of oral mucosal fluids from individuals recovered from SARS-CoV-2 infection to monitor antibody response and persistence over a 12-month period. For this cohort study, enzyme-linked immunosorbent assays (ELISAs) were used to quantify anti-Spike(S) protein IgG antibodies in participants who had prior SARS-CoV-2 infection and regularly (every 2-4 weeks) provided both serum and oral fluid mucosal fluid samples for longitudinal antibody titer analysis.

**Results:** In our study cohort (n=42) with 17 males and 25 females with an average age of 45.6 +/- 19.3 years, we observed no significant change in oral mucosal fluid IgG levels across the time course of antibody monitoring. In oral mucosal fluids, all the participants who initially had detectable antibodies continued to have detectable antibodies throughout the study.

**Conclusions:** Based on the results presented here, we have shown that oral mucosal fluid-based assays are an effective, less invasive tool for monitoring seroprevalence and seroconversion, which offers an alternative to serum-based assays for understanding the protective ability conferred by the adaptive immune response from viral infection and vaccination against future reinfections.

**Keywords: SARS-CoV-2 antibodies, oral mucosal fluid immunity, SARS-CoV-2 immunology, antibody monitoring, ELISAs**

## INTRODUCTION

As of August 2021, the novel coronavirus, SARS-CoV-2, has had a detrimental global impact with over 200 million reported cases, 4.4 million lives lost, and economic calamities worldwide (1). Technological breakthroughs in vaccine development and mass vaccinations in countries like the United States and Israel are proving effective for case management and mitigation of its impacts (2). Despite the early successful efforts in controlling SARS-CoV-2 infection, the viral variants have remained within the population with a likelihood of developing into an endemic disease. Additional research is needed to understand seroprevalence, seroconversion, the persistence of antibody against the virus, the antibody titers in naturally infected *versus* vaccinated population, and the clinical implications related to immunity offered.

Long-term humoral immunity is mediated by various classes of antibodies. The trajectories of the development and decay of commonly described antibodies IgA, IgM and IgG, experience independent peaks and only overlap during early periods (less than one month post exposure). The concentrations of IgM and IgA antibodies diminish too quickly to conduct long-term studies, typically within a month of infection (3, 4). However, IgG concentrations, specifically for SARS-CoV-2, remain high and stable even after several months (5) and seem to correlate with concentrations of neutralizing antibody titers (6). For these reasons, IgG is an extremely valuable biomarker for tracking long-term immune responses.

Humoral immune response monitoring *via* antibody titer levels using automated, high-throughput ELISAs offers an accurate, and scalable method to survey the prevalence of antibodies in a population. Current serum-based ELISAs have several limitations including invasiveness of specimen collection, higher cost, required assistance of a health care worker, and advanced sample processing. To effectively monitor seroconversion and seroprevalence within a population, an effective, and non-invasive method for antibody detection is required. Oral-fluid based assays could act as proxy to serum-based assays, as they have been successfully used to detect or monitor antibody levels for other clinical conditions such as HIV infection, Hepatitis C, Measles, and Rubella (7–9). To that end, OraSure Technologies® has developed an oral specimen collection device (OSCD) and a total antibody ELISA for use with oral mucosal fluid collected from this device. An earlier study from our group showed that it is possible to quantify the antibody titers from oral mucosal fluids collected by the OSCD (10). It should be noted that, at present, the relationship between concentrations of antibody and possible immune protection is not well understood.

From the perspective of monitoring long-term humoral immunity, the question of how long we can expect antibodies against the novel SARS-CoV-2 virus to persist both in serum and oral mucosal fluid remains. Thus, we have designed and conducted a longitudinal clinical study to further understand the relationship between SARS-CoV-2 infection and the persistence and change in IgG antibody titers over time to advance our understanding and its potential implication for long-term immunity. Here, we collected, analyzed, and quantified SARS-CoV-2 IgG in oral mucosal fluids and serum of individuals at various timepoints over a period of one year and focus on the persistence of IgG antibody levels in oral mucosal fluids.

## MATERIALS AND METHODS

### Study Design

Participation was offered to subjects who were 18 years of age and tested negative or positive for COVID-19 *via* PCR test on oral swab specimens at a Curative site in Los Angeles County. Enrollment aimed for 240 participants with at least 1/3 negative, to be used as controls, and 2/3 positive by PCR, including 30% asymptomatic positive participants. Once enrolled in the study, participants may unenroll at any time for any reason. Participants were compensated $50 per visit and began the study approximately 14 days after symptom-offset to ensure the safety of the phlebotomists. Collections were scheduled once every 7 days for the first month, twice a month for the following month, and once a month thereafter. The actual participant adherence followed as 10-14 days between each appointment for the first six collections.

Previous studies have indicated that the highest SARS-CoV-2 viral load occurs two days before the onset of symptoms (11), thus SARS-CoV-2 IgG, hereafter IgG, dynamics were analyzed in relation to days post-symptom onset (PSO). Oral mucosal fluid sample availability for this study was limited due to preliminary assay optimization and lower sample volume compared to serum. Due to this limitation, we were not able to obtain IgG concentrations for every time point for all participants.

### Oral Mucosal Fluid IgG Longitudinal Study Cohort

For the current study, we included individuals who met the following criteria: 1) were originally confirmed SARS-CoV-2 positive by PCR assay; 2) had at least three oral mucosal fluid samples available for testing throughout the study period of approximately 12-months; 3) reported a symptom onset date; and 4) reported specific symptoms that they experienced. Symptomology of participants following infection was recorded at the time of first sample collection. Participants reported various flu-like symptoms, including fever, cough, chills, shortness of breath, chest pain/tightness, diarrhea, stuffy nose, sore throat, muscle pain, headache, weakness, as well as other SARS-CoV-2 specific symptoms including loss of taste and smell.

### Oral Mucosal Fluid IgG Post-Vaccination

A subset of the participants received COVID-19 vaccination (Pfizer or Moderna) toward the end of the study which provided us a means to understand vaccine induced IgG response in previously infected individuals.

### Human Oral Mucosal Fluid and Serum Sample Collection

Oral mucosal fluid samples were self-collected using the OraSure Technologies oral specimen collection device (OSCD) (OraSure®

Technologies, Bethlehem, PA). The collection was supervised by the same phlebotomist that performed the serum collection, the tip was broken off the collection device and the device were then placed into a secondary tube. For sample processing at the laboratory, the secondary tubes were centrifuged at 800 RCF for 15 minutes. When finished, the collection device was disposed of in a biohazard bag and the solution eluted from the collection pad was aliquoted into labeled microcentrifuge tubes and stored at -80°C until assay.

For serological sample collection, participants undergo a standard venipuncture procedure. Licensed phlebotomists sampled approximately 10 mL of blood using a 21G straight needle with a safety cap (BD; Franklin Lakes, NJ) into two Serum Separation Transport tubes (BD; Franklin Lakes, NJ). Once collected, the sample remained at ambient temperature for 60 minutes to coagulate and was then centrifuged at 1000 RCF for 15 minutes. Samples were then placed on ice until delivered to the laboratory site where the serum was aliquoted and stored at -80°C until assay.

## PCR Self-Sampling

The RT-PCR SARS-CoV-2 test (FDA-EUA 137809) is healthcare worker-observed, self-administered and proceeds according to previously reported guidelines (12). Briefly, participants cough hard three times while shielding their cough *via* mask and/or coughing into the crook of their elbow and then proceed to swab, in the following order, the inside of their cheeks, along the top and bottom gums, under the tongue, and finally on the tongue, to gather enough saliva.

## Oral Mucosal Fluid-Based Antibody Tests

The OraSure Technologies SARS-CoV-2 Total Antibody saliva-based assay (OraSure® Technologies, Bethlehem, PA) binds antibodies that target both S1 and S2 subunits of the SARS-CoV-2 spike protein, the mediator in cell entry and infection. The OraSure assay requires the use of oral mucosal fluid collected from the OSCD which contains a preservative. The automated assay sequence, using the Dynex DSX Automated ELISA system (Dynex Technologies; Chantilly, VA), is as follows: a) Prior to sample loading, 25uL of a diluent solution is first added to each well followed by 100 µL of sample; b) The sample is then incubated for one hour at ambient temperature. The DSX houses a plate washer that washes each strip individually using a time delay function adjusted for the time it takes to add the samples to the wells. Each well was washed 6 times with 300 µL of wash buffer; c) 100 µL of the peroxidase-conjugated rabbit anti-human IgG (H+L) enzyme conjugate was added to each well and incubated for one hour at ambient temperature; d) Following another six well washes, 100 µL of enzyme substrate was added to each well and allowed to incubate for 30 minutes. The final addition of 100 µL of stopping solution quenches the reaction; e) Absorbance values of the samples on the plate are then read on the DSX plate reader both at 450nm and 620nm. The final absorbance signal represents IgG levels in the sample, adjusted for background (620nm). All oral mucosal fluid samples were run in duplicate and the average of the absorbances were taken for analysis.

## Quantification of IgG Titers in Oral Mucosal Fluids

The antibody assay was performed according to the manufacturer's instructions. The OraSure kit includes a calibrator, negative control, and a positive control that was added to every plate. All the samples and runs were qualified as per manufacturer's recommendation. The ratio of the absorbance of each sample to the calibrator average was taken and compared to the manufacturer's suggested cutoff values. The manufacturer recommended cutoff values for determination of samples were <0.8 for the negative samples and >1.0 for the positive samples. Samples in the range of 0.8 to 1.0 were considered equivocal but were still utilized in the quantification portion of the study.

Relative quantification of antibody titer was performed using a S1-specific monoclonal IgG antibody as a reference antibody, which had no known cross-reactivity to the S2 domain of the spike protein. Additionally, we did not observe cross reactivity of IgA and IgM antibodies (monoclonal) in our IgG quantification assay. The standard curve was used to calculate the IgG antibody concentration in specimens from absorbance values at 450/630 nm from the ELISA assay. Specimens with antibody titer levels exceeding the range of the standard curve were diluted in a sample dilution buffer and re-ran. The Limit of Detection (LOD) was 1 ng/mL and the Limit of Quantification (LOQ) was 1.5 ng/mL.

## Quantification of IgG Titers in Serum

EuroImmun (Catalog #: EI2606-9601 G, Lubeck, Germany) SARS-CoV-2 IgG ELISA targeting the S1 subunit of the spike protein was performed according to a published protocol (13) on the Thunderbolt automated instrument (Gold Standard Diagnostics (GSD); Davis, CA). Briefly, sera were diluted 1:101 in each well with buffer and then incubated at 37°C for 1 hour. Sample wells were washed three times, then conjugate was added and incubated at 37°C for 30 minutes. After another wash step as previously described, the substrate was added and incubated at ambient temperature for 30 minutes. Stopping solution was then added and the absorbance of sample wells was measured immediately at both 450 nm and 600 nm. The reading at 600 nm was automatically subtracted as noise during output report generation. All samples were run in duplicate, and the data was exported and analyzed according to established protocol (13) to obtain a ratio (Equation 1) for the adjusted optical density of the sample well. This ELISA kit itself was developed specifically for detection of SARS-CoV-2 IgG antibodies specific to the S1 subunit of the spike protein in serum. The quantification method was developed using a monoclonal IgG1 antibody specific for the S1 subunit of the SARS-CoV-2 spike protein from (Catalog#:NR-52392, BEI Resource, Manassas, VA) The stock concentration is 1.04 mg/mL and a standard curve with five dilutions was created at 3.1, 6.25, 12.5, 25, and 30 ng/mL.

$$Ratio = \frac{OD\ of\ the\ control\ or\ clinical\ sample}{OD\ of\ the\ calibrator\ average}$$

Equation 1. Antibody detection is qualified by the ratio, determined by dividing the OD of the control or sample by the averaged OD of the test kit calibrator.

## Data Analysis

All statistical analysis, and plots were generated using GraphPad Prism Software (GraphPad Prism, San Diego, USA). Correlation, ANOVA, t-test analyses were done on datasets using built-in functions of the Prism software. ANOVA and paired t-tests were chosen to compare and identify significance in changes in antibody concentration over time in individual participants.

## Ethical Approval

The study was initially approved by The UCLA Institutional Review Board (UCLA IRB) (IRB#20-000703). The UCLA IRB waived the requirement for signed informed consent for the research under 45 CFR 46.117(c) (2). The study team complied with all UCLA and Advarra IRB policies and procedures, as well as with all applicable Federal, State, and local laws regarding the protection of human subjects in research as stated in the approved IRB (Pro00045766).

## RESULTS

From April 2020 to May 2021 a total of 339 participants were enrolled. During the study, 136 participants were unenrolled due to incomplete sample return. 42 PCR positive participants who experienced symptoms following infection were selected for this analysis. Of the 42 participants, 17 individuals (40.5%) identified as male and 25 (59.5%) identified as female (**Figure 1A** and **Table 1**). The average age of participants was 45.5 +/- 19.3 years and age bins for categorization and analysis were selected based on previous studies (14). During the time of collection, 7 individuals (16.6%) were younger than 25 years of age, 11 (26.2%) were between the ages of 25 and 40, and 24 individuals (57.2%) were older than 40 years of age (**Figure 1B** and **Table 1**).

IgG antibody response to SARS-CoV-2 infection from post symptom onset (PSO) was quantified in oral fluid specimens over a period of 12 months. The average IgG titers remained relatively stable over a period of nearly one year (**Figure 2A**). A paired t-test comparison of IgG concentrations in oral mucosal fluid samples collected in the first 2 months following symptom onset (51.32 +/- 116.3 ng/mL) and after 6 months post symptom onset (25.17 +/- 25.99 ng/mL) did not find a statistically significant decline ($p = 0.1915$) (**Figure 2B**). Antibodies remained detectable in all participants for up to 12 months following symptom onset, and the average concentration of the most recent timepoint (365 +/- 30 days) for all individuals was 9.75 +/- 13.04 ng/mL.

While our primary focus was to characterize the persistence of IgG in oral mucosal fluid samples over a period of one year, a small number (n=13) of the study participants received vaccination during the study (**Table 2**). These participant specimens collected after vaccination were excluded from the longitudinal and correlation analyses. We looked at the individuals' mean IgG antibody concentration at the first time point (Vaccine T0, 626.9 +/- 559.1 ng/mL) post vaccination and found that it was higher than the maximum ($COVID_{Max,}$ 108.8 +/- 214.2 ng/mL) observed endogenous IgG concentration (**Figures 3A, B**).

Similar analyses (longitudinal trend and quantification) for antibody titers in serum can be found in the supplementary information (**Supplementary Figures 1–3**). The average IgG concentration found in the serum (11,601 +/- 21,227 ng/mL) of all participants throughout the study was higher than the average concentration found in oral mucosal fluids (29 +/- 33 ng/mL). A comparative analysis between serum and oral fluid seroconversion reveals consistent trends between the two specimen mediums (**Figure 4A**). IgG concentrations in the oral mucosal fluid positively correlated with the response in the serum of participants ($R^2 = 0.64$, $p < 0.0001$).

Three individuals (S2, S29, S37) did not have a detectable antibody concentration in early oral mucosal fluid sample

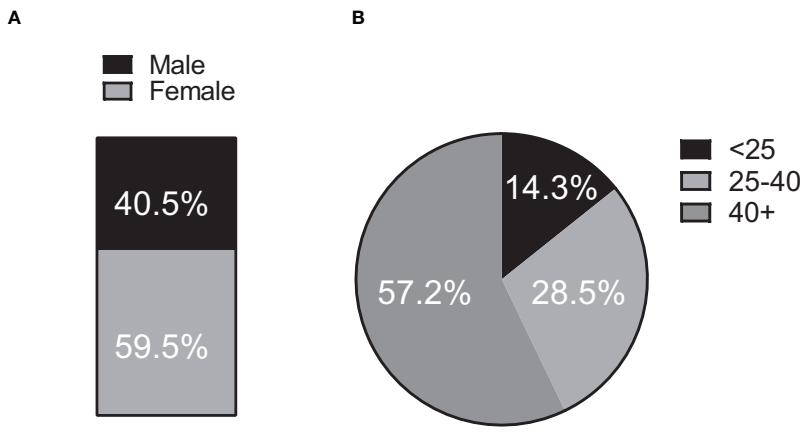


**FIGURE 1** | Population demographics. 42 (n = 42) individuals contributed oral mucosal fluid samples at timepoints spanning over 12-months for the clinical study. **(A)** Sex representation. 17 of 42 participants (40.5%) identified as male and 25 (59.5%) as female. **(B)** Age representation. Seven (7) individuals (16.6%) were younger than 25 years of age at the time of sample collection, 11 (26.2%) were between the ages of 25 and 40 and 24 individuals (57.2%) were older than 40 years of age.

**TABLE 1** | Summary of oral mucosal fluid and serum samples collected from each participant, including their age and sex.

| Participant | Age | Sex | Sample Type | Timepoints(Total) |
|---|---|---|---|---|
| S1 | 58 | F | Serum | 10 |
| S2 | 19 | F | Serum | 10 |
| | | | Oral Fluid | 7 |
| S3 | 62 | F | Serum | 10 |
| S4 | 25 | F | Serum | 9 |
| | | | Oral Fluid | 6 |
| S5 | 59 | M | Serum | 9 |
| S6 | 54 | M | Serum | 10 |
| S7 | 50 | F | Serum | 9 |
| | | | Oral Fluid | 6 |
| S8 | 25 | F | Serum | 9 |
| S9 | 22 | M | Serum | 8 |
| S10 | 27 | F | Serum | 10 |
| | | | Oral Fluid | 6 |
| S11 | 58 | F | Serum | 9 |
| | | | Oral Fluid | 5 |
| S12 | 40 | M | Serum | 10 |
| | | | Oral Fluid | 9 |
| S13 | 43 | F | Serum | 8 |
| | | | Oral Fluid | 4 |
| S14 | 27 | F | Serum | 5 |
| | | | Oral Fluid | 5 |
| S15 | 22 | F | Serum | 9 |
| S16 | 22 | F | Serum | 9 |
| | | | Oral Fluid | 8 |
| S17 | 37 | F | Serum | 8 |
| S18 | 43 | F | Serum | 9 |
| | | | Oral Fluid | 10 |
| S19 | 31 | M | Serum | 8 |
| S20 | 56 | F | Serum | 10 |
| | | | Oral Fluid | 7 |
| S21 | 48 | M | Oral Fluid | 7 |
| S22 | 25 | F | Serum | 10 |
| | | | Oral Fluid | 5 |
| **Participant** | **Age** | **Sex** | **Sample Type** | **Timepoints (Total)** |
| S23 | 24 | F | Serum | 9 |
| S24 | 36 | M | Serum | 10 |
| S25 | 41 | F | Serum | 9 |
| | | | Oral Fluid | 8 |
| S26 | 23 | M | Serum | 10 |
| | | | Oral Fluid | 6 |
| S27 | 37 | M | Serum | 9 |
| | | | Oral Fluid | 3 |
| S28 | 42 | M | Oral Fluid | 7 |
| S29 | 49 | F | Serum | 11 |
| | | | Oral Fluid | 11 |
| S30 | 30 | M | Oral Fluid | 9 |
| S31 | 22 | M | Oral Fluid | 10 |
| S32 | 36 | F | Serum | 10 |
| | | | Oral Fluid | 8 |
| S33 | 42 | M | Serum | 10 |
| | | | Oral Fluid | 9 |
| S34 | 22 | M | Serum | 10 |
| | | | Oral Fluid | 8 |
| S35 | 57 | F | Serum | 9 |
| | | | Oral Fluid | 9 |
| S36 | 50 | F | Serum | 9 |
| | | | Oral Fluid | 8 |
| S37 | 39 | M | Serum | 10 |
| S38 | 30 | M | Serum | 10 |
| | | | Oral Fluid | 5 |
| S39 | 22 | M | Serum | 10 |
| | | | Oral Fluid | 8 |
| S40 | 22 | F | Oral Fluid | 9 |
| S41 | 25 | F | Oral Fluid | 8 |
| S42 | 26 | F | Oral Fluid | 5 |
| S43 | 62 | F | Oral Fluid | 8 |
| S44 | 66 | M | Oral Fluid | 7 |
| S45 | 66 | F | Oral Fluid | 9 |
| S46 | 78 | M | Oral Fluid | 7 |
| S47 | 69 | F | Oral Fluid | 7 |
| **Participant** | **Age** | **Sex** | **Sample Type** | **Timepoints (Total)** |
| S48 | 75 | F | Oral Fluid | 8 |
| S49 | 67 | F | Oral Fluid | 7 |
| S50 | 69 | F | Oral Fluid | 7 |
| S51 | 69 | M | Oral Fluid | 6 |
| S52 | 74 | M | Oral Fluid | 8 |
| S53 | 70 | F | Oral Fluid | 6 |
| S54 | 71 | M | Oral Fluid | 6 |
| S55 | 69 | M | Oral Fluid | 7 |

collections but developed a detectable concentration later in the study period (**Figure 4B**).

Interestingly, it was observed that these three individuals (S2, S29, S37) had lower concentrations of antibodies in serum (802, 1,000, and 500 ng/mL respectively) than the average observed IgG levels in other individuals (13629 +/- 22,564 ng/mL). Based on this small sample subset, we report that the oral mucosal fluid assay has a sensitivity of 87.5% at early time points. In another independent study (data not shown), we tested 60 paired serum and oral mucosal fluids samples from a cohort who were negative to SARS-CoV-2 infection and found the assay specificity to be 100%.

## DISCUSSION

In this study, we have successfully monitored the persistence of IgG antibodies in serum and oral mucosal fluid specimens collected up to 12 months post symptom onset of SARS-CoV-2 infection. Furthermore, we developed a less invasive method to perform relative quantification of IgG against the S1 and S2 subunits in oral mucosal fluids that yields comparable results to the relative quantification of the S1 subunit of SARS-CoV-2 in serum. This is one of the first studies to longitudinally track and quantify antibody titers over a period of one year in response to SARS-CoV-2 infection using oral mucosal fluids with a serum-based assay as a comparator.

The change in antibody titer over the time course of the study was not found to be statistically significant in oral mucosal fluids, though we did observe an IgG titer asymptote around 6 months post-symptom onset. Our findings agree with two other recent studies showing the persistence of IgG in saliva specimens 60- and 115-days post-symptom onset (15, 16). In all specimens and sample types in the present study, antibodies that were originally detectable remained persistent throughout the timespan of the study (12-months), an encouraging finding which is consistent with other reports (5, 17, 18).

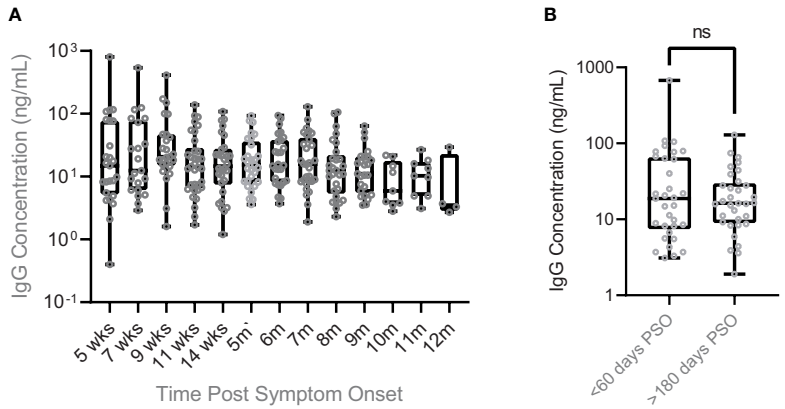


**FIGURE 2** | Persistence of SARS CoV-2 IgG antibody levels in oral mucosal fluids of COVID infected individuals. **(A)** SARS-CoV-2 IgG antibody concentrations were maintained for up to a year post symptom onset. Individuals who received vaccination during the time of this clinical study had their samples following vaccination omitted from this plot. The average antibody concentration was 62.64 +/- 159.3 ng/mL after 5 weeks PSO and 9.750 +/- 13.04 ng/mL after 12 months PSO. **(B)** A comparison of SARS-CoV-2 antibody concentration in the early days post symptom onset (<60 days) to the later days (>180 days) finds nonsignificant change in concentration over time. The average concentration of early SARS-CoV-2 IgG antibodies collected fewer than 60 days post symptom onset was 51.32 +/- 116.3 ng/mL, while after more than 180 days post symptom onset the average concentration dropped to 25.17 +/- 25.99 ng/mL. A paired two-sample t-test comparing these means found the decrease to be non-significant (p = 0.1915). ns, not significant.

**TABLE 2** | Summary of total oral mucosal fluid collections from the vaccinated participants of the study.

| Participant | Age | Sex | Vaccine Manufacturer | Time Elapsed from 1st dose | Time points (Total) |
|---|---|---|---|---|---|
| S16 | 22 | F | N/A | 8 days | 9 |
| S35 | 56 | F | Pfizer | 9 days | 10 |
| S42 | 26 | F | Pfizer | 0 days | 7 |
| S44 | 66 | M | Pfizer | 6 days | 8 |
| S46 | 78 | M | Moderna | 32 days | 8 |
| S47 | 69 | F | Moderna | 18 days | 8 |
| S48 | 75 | F | Moderna | 34 days | 10 |
| S49 | 67 | F | Moderna | 37 days | 9 |
| S50 | 69 | F | Moderna | 28 days | 8 |
| S52 | 74 | M | Moderna | 44 days | 9 |
| S53 | 70 | F | Pfizer | 30 days | 7 |
| S54 | 71 | M | Pfizer | 30 days | 7 |
| S55 | 69 | M | Moderna | 39 days | 9 |

*Vaccine manufacturer, time elapsed from their first dose to their maximum IgG concentration, as well as sex and age are described.*
*NA, not applicable.*

In a comparative analysis performed on a subset of our study cohort who received vaccination prior to the conclusion of the study, we found antibody concentration post-vaccination to be six-fold higher when compared to the mean maximum IgG concentration associated with natural infection. This result agrees with another research group which analyzed antibody response to Pfizer/BioNTech vaccination in serum in previously infected as well as COVID naïve healthcare workers (19). A recent work found anti-SARS-CoV-2 RBD IgG and neutralizing antibodies to be 10-100-fold higher following vaccination in 6 previously infected healthcare workers (20). In addition, they found that IgG titers in previously infected healthcare workers were similar at 21 days after their first vaccination and 7 days after their second vaccination. Another study found similar patterns of anti-SARS-CoV-2 spike protein IgG in a larger study cohort of healthcare workers from a medical center in Southern California (21). This suggests that previously infected individuals may only need one vaccine dose to confer adequate IgG titer compared to both doses in COVID naïve individuals, which may provide a strategy to maximize vaccine supply.

Though vaccination means that we can no longer quantify endogenous immunity following infection in those individuals, it is extremely reassuring to know that vaccination confers boosted antibody production. As more and more people become vaccinated, we are using the lessons learned from this study to monitor the persistence of antibodies in oral mucosal fluids against the Moderna or Pfizer vaccine in an ongoing year long clinical trial. Our early results are encouraging as we observe an increasing antibody titer in vaccinated participants consistent with what we observed in this cohort (10).

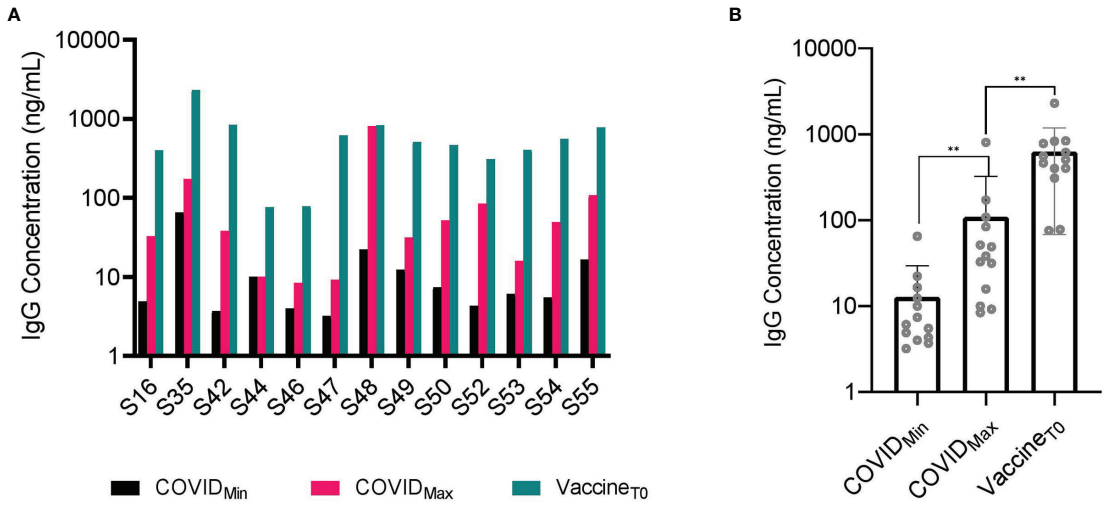


**FIGURE 3** | Vaccinated individuals experienced a significantly higher concentration of SARS-CoV-2 IgG antibodies in oral mucosal fluid than that prompted by viral infection. **(A)** SARS-CoV-2 IgG antibody concentration in fourteen (n = 14) individuals who received vaccination following infection. All 14 individuals experienced a significant (p-value < 0.001) increase in SARS-CoV-2 IgG antibody concentration following vaccination by paired two sample t-test. **(B)** A comparison between minimum (COVID_Min) and maximum antibody (COVID_Max) concentration post infection with antibody concentration post vaccination (Vaccine T0) reveals a statistically significant increase by paired two-sample t-test (p-value <0.001). Even compared to the highest concentration of post infection SARS-CoV-2 antibodies in these individuals at an average concentration (107 +/-206 ng/mL), the average concentration of post vaccination SARS-CoV-2 antibodies (626 +/- 537 ng/mL) was significantly higher. **p-value < 0.001.

While our study provides critical insight into the persistence of IgG antibodies in both serum and oral mucosal fluid over a 12-month period, we believe our study size may be a major limitation. In earlier research, oral fluids and oral mucosal fluids proved to be a reliable specimen to monitor antibody response to various pathogens, including HIV and Hepatitis C. Sensitivity and specificity of the oral fluid-based assays were as follows: HIV [(98% & 98%) (22) and (99.3% & 99.8%) (13)], and Hep C [(89.9% & 100%) (23) and (98% & 98%) (24)]. Among oral fluids based assays, passive drool assays were less sensitive and specific than oral mucosal based assays such as OraSure. At some instances, oral mucosal fluids based assays could be less sensitive and specific compared to serum based assays. However, upon optimizing the assays for a specific pathogen, oral fluids provide remarkable flexibility in ease of collection, handling, cost, and importantly an improved sensitivity and specificity. While our SARS-CoV-2 antibody sensitivity is at 87.5% and specificity is at 100%, a smaller sample size is a source of concern. Further research is needed to validate the sensitivity and specificity parameters in a larger cohort.

Importantly, the relationship between the titers of S1 and S2 subunits of spike protein specific IgG antibodies and neutralization antibody titers need further research. As this understanding will enable us to use the S1/S2 antibody ELISA assay as a surrogate assay for complex virus neutralization assays. A recent report found a strong positive correlation between both anti-spike ectodomain and anti-RBD IgG titers and SARS-CoV-2 virus neutralization titers using convalescent plasma of 68 COVID-19 patients (25). This finding is consistent with other reports which found positive correlations between SARS-CoV-2 viral neutralization and S-RBD-specific IgG (26) as well as anti-S ELISA titers (27–29), warranting investigation of neutralization capacity for our current study cohort. Understanding the roles of S1/S2 antibodies as well as neutralization antibodies in a larger population will help us determine the role of these antibodies, and concentrations needed to confer long-term immunity against viral infection and reinfections.

Further research is needed on a larger sample set to help clarify these questions and to determine the correlation between IgG levels measured in this assay and the neutralizing antibody levels in oral mucosal fluids. Additionally, we were unable to evaluate the impact of other immune responses on protection against SARS-CoV-2 reinfection. It is known that T-cells play an important role in long term immune protection and therefore has been recently investigated in relation to SARS-CoV-2. A recent report found detectable IFN-γ secreting T cell responses in 71% (42/59) of recovered COVID-19 patients enrolled in their study (30). Similarly, SARS-CoV-2 specific $CD8^+$ T cell responses have been shown to be important determinants of immune protection for individuals, including those with mild infection (25, 31, 32).

## CONCLUSION

Our current study provides critical insight into understanding the persistence of IgG antibodies in both serum and oral mucosal fluid over a 12-month period. However, an extended study

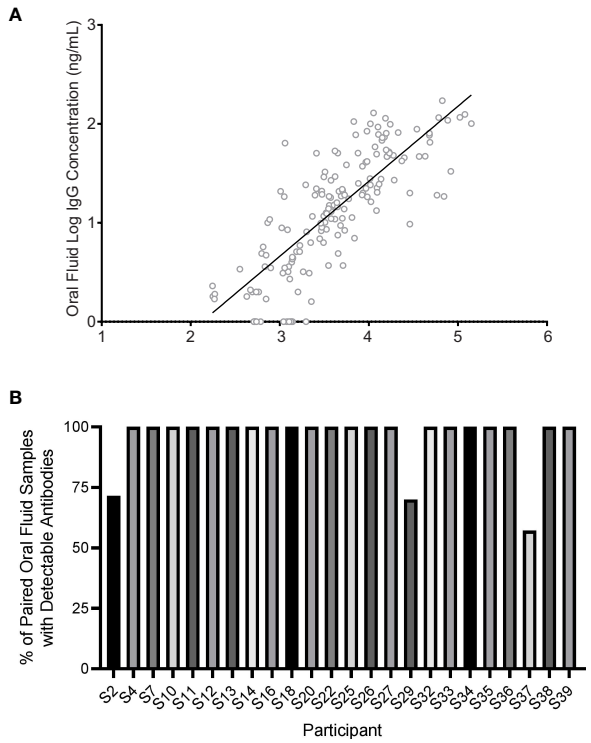


**FIGURE 4** | SARS-CoV-2 IgG in oral mucosal fluids of participants (n = 24) correlated with that of their serum. **(A)** Oral mucosal fluid IgG increased with serum IgG in the participants ($R^2$ = 0.64, p < 0.0001). Each data point represents a single timepoint collection for both serum and oral mucosal fluids for a specific participant. The regression line is shown as the solid line. **(B)** Correlation between antibody detection in participants with paired serum and oral fluid specimens was typically high. On an individual basis, three individuals with detectable SARS-CoV-2 antibodies in serum (S2, S29, and S37) developed detectable oral mucosal fluid antibodies and maintained detectable antibody levels later in the time course.

period and a larger study cohort are needed to understand the following: a) long term persistence of antibodies in serum and oral mucosal fluids, b) characterize the difference in immune response to natural infection and vaccination, and c) understand the role of T-cells in individuals with and without antibody response. While vaccines provide much needed hope for ending this pandemic, full closure will be aided with an understanding of how long immunity persists and how frequently vaccination is needed. To accomplish this public health objective, we believe understanding the relationship between antibody levels and neutralization capacity to offer immune protection is critical. Through the establishment of a consistent, inexpensive, and non-invasive method for detection and relative quantification of SARS-CoV-2 antibodies in oral mucosal fluid specimens, communities will be provided an effective tool for making public health decisions to manage small outbreaks. Effective monitoring will enable identification and tracking of new variants *via* sequencing and the evaluation of antibody efficacy to neutralize the virus. Enforcement and availability of these tools will allow communities to better provide resources for treating infected individuals, reduce the spread of the virus, and return to pre-pandemic normalcy.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The study was initially approved by The UCLA Institutional Review Board (UCLA IRB) (IRB#20-000703). The UCLA IRB waived the requirement for signed informed consent for the research under 45 CFR 46.117(c) (2). The study team complied with all UCLA and Advarra IRB policies and procedures, as well as with all applicable Federal, State, and local laws regarding the protection of human subjects in research as stated in the approved IRB (Pro00045766). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

PC and AA designed and ran experiments, analyzed and interpreted data, and drafted the manuscript. MM designed the study protocol, designed experiments, analyzed and interpreted data, and drafted the manuscript. ND and MS analyzed and interpreted data, ran the experiments, and drafted the manuscript. RL, CB, and JC ran the experiments and organized data for processing. FT and VS conceptualized and designed the study protocol, designed experiments, and edited the manuscript. AI oversaw and designed experiments, oversaw data collection and analysis, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.777858/full#supplementary-material

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# Saliva and Serum Cytokine Profiles During Oral Ulceration in Behçet's Disease

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Behçet's disease (BD) is a chronic, multi-systemic disorder of unknown aetiology typified by recurrent oral and genital mucocutaneous lesions, uveitis and vasculitis. Innate and adaptive immune system dysregulation has been implicated in pathogenesis with alterations in serum cytokine profiles. Few studies have investigated salivary cytokines in BD, despite more than 90% of BD patients first presenting with oral ulceration. The aim of this pilot study was twofold; firstly to investigate whether cytokine levels in matched serum and saliva samples show a differential profile in BD (with and without oral ulcers), recurrent aphthous stomatitis (RAS) and healthy controls (HCs), and secondly, to explore if any differential profiles in serum and/or saliva could provide a panel of cytokines with diagnostic and therapeutic potential for BD. Concentrations of 12 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFN-γ, TNF-α, TNF-β) were measured using the Human Th1/Th2 11-Plex FlowCytomix™ kit with IL-17A, in BD (N=20), RAS (N=6) and HCs (N=10). A differential range of cytokines was detected in serum and saliva with the majority of cytokine levels higher in saliva. The most prevalent salivary cytokines were IL-1β, IL-2, IL-8, IL-10 and TNF-α present in all samples in contrast to serum where the most prevalent cytokine detected was IL-8 (91.9%). The least abundant cytokine was IFN-γ in both saliva (43.2%) and serum (2.7%). After normalizing saliva for protein content, BD patients with oral ulcers (BD-MA) had significantly higher levels of salivary IL-1β ($p=0.01$), IL-8 ($p=0.02$), TNF-α ($p=0.004$) and IL-6 ($p=0.01$) than HCs. Notably, BD patients without oral ulcers (BD-MQ) also had significantly higher salivary IL-1β, IL-8 and TNF-α ($p \leq 0.05$) than HCs. During relapsed (BD-RE) and quiet (BD-Q) systemic episodes, salivary IL-β and TNF-α were also significantly increased with IL-8 significantly higher only in BD-Q ($p=0.02$). BD oral ulcers signify a potential reactivation of systemic inflammation. Identifying cytokines released during asymptomatic episodes and oral ulceration might lead to targeted drug therapy to prevent recurrent oral ulcers and possible disease relapse. This is the first study to report salivary cytokine levels in BD. The detectable levels suggests cytokine profiling of BD saliva may provide an alternative, less invasive, sensitive procedure for frequent monitoring of disease activity and progression.

**Keywords: Behçet's disease, cytokines, saliva, ulceration, oral mucosa, immune profiling, inflammation, FlowCytoMix**

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## INTRODUCTION

Behçet's disease (BD) is a chronic, multisystemic, recurrent vasculitis disease of unknown aetiology (1–3). Typified by early presentation with recurrent oral ulceration, patients may go on to present with genital ulcers, cutaneous lesions including erythema nodosum and folliculitis, joint involvement and life threatening vasculitis (4) as well as central nervous system disease such as meningo-encephalitis and neuro-psychiatric symptoms, although peripheral nerve/muscle damage is rare (5). Uveitis is also common and can lead to sight loss (6–10).

The disease is difficult to diagnose and in the absence of a definitive laboratory test, a differential clinical scoring system remains the only method to ensure correct diagnosis. First developed by the International Study Group for BD (11) it has since been ratified in an international 27 country survey (12). A definitive diagnosis may take many years to establish with concomitant impact on the quality of life of patients (13, 14).

Aetiopathogenesis remains unknown, but a common consensus suggests it may be triggered by an infection or an environmental stimulus in a genetically susceptible host (15, 16). Attempts to classify BD as an autoimmune, auto-inflammatory or a spondyloarthropathy fail as BD patients frequently exhibit features common to all these conditions (17, 18).

Infectious aetiology theories have indeed provided evidence of reactivity to Herpes Simplex (8), *Streptococcus sanguis* (19) and microbial heat-shock proteins (HSP) causing cross reactivity reactions with self-proteins as possible triggers of BD (20, 21). Cross reactivity of microbial HSP with self-proteins has been implicated in both BD and RAS but with very different outcomes (22). The ability to detect and respond to infection may also be impaired in BD where unusual splice variants of TLR2 and TLR4 suggest a defect in the crosstalk between innate and adaptive immune responses, and where significant reductions in the response to cognate agonists of TLR1/2 heterodimers have been observed in buccal cells (23).

Genetic susceptibility has also been investigated and strong associations with some HLA genes such as HLA-B51, a splice variant of HLA-B5, has been long established for multiple populations (8, 24–27). Several other studies showed links between BD and MHC class I chain related genes (MIC-A and MIC-B), also suggesting these antigens as candidates for genetic susceptibility as they are expressed on fibroblasts, gastric epithelium and endothelium cells and act as ligands for the NKG2D activating NK receptor found on both gamma delta ($\gamma\delta$) T cells and $CD8^+\alpha\beta$ T cells. Both of which have been found to have roles in BD pathogenesis, while NK cells have been found to be depleted in the circulation of BD patients (28–30).

More recently genome wide association studies (GWAS) have confirmed the association with HLA-B51 but have also pointed to associations with IL-10 variants, and variants lying between the IL-23 receptor (IL-23R) and IL-12 receptor (IL-12R$\beta$2) as well as IL-12A genes (31, 32). IL-10 is a potent suppressor of inflammation while IL-23 is a pro-inflammatory cytokine that stimulates T helper cell proliferation and increases the production of inflammatory cytokines such as IL-1, IL-6, IL-17 and TNF-$\alpha$. These genes are engaged in both innate and adaptive immune response communication networks through cytokine signalling and support a hypothesis of immune dysregulation in BD (25, 27). Hyperfunctional neutrophils are characteristic of BD leading to an overactive neutrophil response (33, 34). Various neutrophil priming and activating factors are up-regulated in BD e.g. IL-8, TNF-$\alpha$ (35) and IL-1$\beta$ (36). Furthermore, significantly increased levels of neutrophil elastase in plasma (37) and saliva (38) have been detected in both quiescent and active symptomatic BD. More recently a cell atlas of the oral mucosa has suggested a strong neutrophil/stromal cell regulatory interaction controlling tissue immunity (39).

One of the key immunological features of BD is alteration of blood cytokine levels (40, 41). Early work suggested that BD displayed a Th1 profile (42–44), and more recently Th1/Th17 cytokine polarisation of $CD4^+$ T cells and increased IFN-$\gamma$, TNF-$\alpha$, IL-8 and IL-17 levels have been correlated with BD activity (27, 44–46). However, cytokine production is often transient and tightly regulated, due to their high biological activity and the network within which they are up- or down-regulated according to need. Elevated cytokine levels in body fluids reflect activation of pathways associated with inflammation or disease progression in many disorders and as such offer a window into treatment regimens and/or disease progression monitoring (47, 48). The triggers for cytokine induction are not well understood and/or controversial and the master controllers for induction include an array of transcription factors able to cross activate or inhibit cytokine production. The Suppressor of Cytokine signalling (SOCS) proteins negatively regulate the JAK-STAT pathway of cytokine induction and have been found to be differentially expressed in BD patients. Comparisons made between expression of SOCS 1 and 3 mRNA in BD, RAS and HC showed an upregulation of these molecules in BD. Furthermore, there was a differential expression between buccal mucosal cells and peripheral blood cells which suggested that cytokines are differently regulated in the mucosa compared with the peripheral blood cells (monocytes and neutrophils) (49, 50).

While there have been many studies reporting differences in serum cytokine levels there have been no studies investigating levels in saliva. This is especially surprising considering between 85-100% of BD patients first present with oral ulceration which precedes the onset of all other symptoms and continue to erupt episodically (51). The oral microbial environment has been implicated in the pathogenesis of BD (16, 52) with patients experiencing new onset oral ulceration following dental and periodontal treatment (53, 54). Studies, including our own, have concluded that the oral health of BD patients is impaired (14, 55–57) while clinical treatment of oral symptoms results in better outcomes of systemic disease (38). Furthermore, the importance of dental and periodontal treatment for controlling oral ulceration activity and systemic disease has been reported (54) and highlighted in several studies, however its importance in the preventative management of BD has remained largely unrecognised (58).

BD and recurrent aphthous stomatitis (RAS) patients share nearly indistinguishable histological features of episodic oral ulceration (59), presenting with minor, major or herpetiform

lesions. In BD, ulcers are more frequent and slower to heal sometimes with scarring not seen in RAS. Their oral biopsies are mainly described as non-specific with large infiltrations of neutrophils, some macrophages and mast cells (18, 60, 61). Gamma-delta (γδ) T cells may also be present which are rarely seen in normal oral mucosa (62, 63). Both BD and RAS ulcers are also positive for CD4 and CD8T cells as well as Th-1 cytokines such as IL-12, IFN-γ and TNF-α. However, Th-2 cytokines such as IL-4 have only been detected in BD ulcers (64). In the past, ELISA was the "Gold Standard" for cytokine analysis but in more recent times the development of multiplex assays has marked a step change in the ability to detect and measure multiple analytes in a variety of samples including serum, saliva, synovial and vitreous fluids (65–67). Furthermore, multiplex analysis allows the simultaneous measurement of many different proteins in smaller sample volumes and offers the ability to investigate multiple inflammatory networks in a single clinical sample (67).

Cytokines involved in pathogenesis of BD can be categorized into proinflammatory cytokines and chemokines, Th1 type, Th2 type and Th17 type cytokines. Therefore, we decided to measure 12 inflammatory cytokines and chemokines, representative of these categories, namely, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70. IL-17A, IFN-γ, TNF-α and TNF-β in matched saliva and serum from BD, RAS, and HC. Firstly the aim was to investigate whether the levels and types of inflammatory cytokines measured in saliva reflect those in serum; and secondly, to characterize the cytokine profile of BD and RAS patients with and without oral ulcerations and/or systemic manifestations. For BD patients whose vasculitis makes drawing blood for routine tests a considerable challenge, saliva is more readily accessible and a less invasive window into their disease activity and progression.

# MATERIALS AND METHODS

## Patients and Healthy Controls

Behçet's Disease (BD) patients were recruited from outpatients attending the Behçet's Centre of Excellence clinics at the Royal London Hospital, Bart's and The London NHS trust that had been previously diagnosed according to the International Study Group for Behçet's Disease (1990) with disease activity recorded using the ISBD activity form 2006. Individuals with recurrent aphthous stomatitis (RAS) were allocated as a disease control group and healthy controls were from self-identified healthy volunteers (HC). Matched serum and saliva samples were collected on the same day from 20 BD patients (7 males (M):13 females (F), mean age 38 ± 10.4 years, 10 HCs (5M:5F, mean age 34.7 ± 11.1 years) and RAS disease controls (matched RAS N=6, 4M:2F, mean age 38 ± 16. One RAS saliva and one RAS serum sample were from different patients. For RAS serum, 4M:3F, mean age 39.3 ± 15. For RAS saliva, 5M:2F, mean age 36.7 ± 15 years). The demographics for the patients and controls are described in **Tables 1A** and **B**, respectively. For this pilot study, if one or more oral ulcers were present during specimen collection, then the patient was deemed mouth active (BD-MA). If there was no mouth ulcer present, patients were recorded as mouth quiet (BD-MQ). BD patients were also assessed on their systemic activity during clinical attendance and deemed systemic active, or having relapsed, if they had at least three symptoms characteristic of BD according to the ISG (1990). The presence of activity in three or more clinical sites was considered as disease relapsed (BD-RE) and if not, disease quiet (BD-Q). BD medications are shown in **Table 1A**. Patients taking biologics were excluded from the study. The study was approved by the local research ethics committee (REC number P/03/122) and written informed consent was obtained from all patients and HC.

## Serum Collection and Processing

Blood was collected into Vacutainers® (Becton, Dickinson Co. UK) allowing coagulation in order to derive fibrinogen-free serum. Vacutainers® were centrifuged at 3300 x g for 6 minutes (min) at room temperature (RT). Serum was aliquoted and immediately stored at -80°C and thawed immediately prior to cytokine analysis.

## Collection and Processing of Saliva Samples

Patients and HC volunteers rinsed with 5 ml of water prior to collection of unstimulated whole saliva and then asked to expectorate over a maximum period of 5 minutes into a 20 ml sterile universal tube that was then immediately placed on ice. From clinic, saliva samples were transferred to the laboratory, centrifuged at 4°C for 15 minutes at 3500 x g in order to remove cellular debris and saliva supernatants were then aliquoted and immediately stored at -80°C. Aliquots were thawed immediately prior to protein measurement or cytokine analysis. Total protein concentration was determined using the 2-D Quant Kit (GE Healthcare) as indicated by the manufacturer.

## Measuring Cytokines and Chemokines in Matched Saliva and Serum: FlowCytomix™ Multiplex Assay

The Human Th1/Th2 11-Plex FlowCytomix™ kit plus one extra cytokine, IL-17A (from eBioscience® formerly Bender MedSystems®) was used to simultaneously measure a panel of 12 inflammatory cytokines and chemokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-17A, IFN-γ, TNF-α and TNF-β) in undiluted, unstimulated saliva and matched undiluted serum from BD, RAS, and HC participants according to the manufacturer's instructions. The limits and range of detection of the panel of cytokines provided by the manufacturer are shown in **Table 2**. A BD FACS Canto™ II flow cytometry instrument was used for the data collection. Analyte concentrations were calculated against the standard curves using the FlowCytomix™ Pro 3.0 Software.

## Statistical Analysis

GraphPad Prism version 7.04 was used for analysis. Median cytokine measurements in each group were compared to one another using the Mann Whitney U, non-parametric statistical test (2 tailed). Significance is indicated by exact p values shown in

figures and tables. Spearman's Rho with two tailed significance was used for correlation analysis.

# RESULTS

## Clinical and Demographic Characteristics

Of the 20 BD patients, investigated 65% were female and 35% male. BD relapse was diagnosed in 35% of the patients. The most frequent clinical symptoms at the time of sampling, were joint involvement and oral ulcers (45%) followed by genital ulcers (20%). Treatment information was available at the time of saliva and blood collection for all 20 BD samples; 17 patients were on immunosuppressive treatment (85%) with only three patients (15%) not receiving any medication (**Table 1A**).

## Comparison of Matched Saliva and Serum Levels in BD and RAS Patient Groups

The levels of Th1 cytokines, (IL-1β, IL-2, IL-12p70, IFN-γ, TNF-α and TNF-β), Th2 cytokines, (IL-4, IL-5, IL-6 and IL-10), chemokine IL-8 (CXCL8) and Th17 (IL-17A), were quantified in 36 matched serum and saliva samples from BD patients (**Table 1A**), HCs, and RAS (**Table 1B**).

Out of the 12 cytokines measured in saliva, the most prevalent were IL-1β, IL-2, IL-8, IL-10 and TNF-α being present in all 37 (100%) samples in contrast to serum where the most prevalent cytokine detected was IL-8, present in 94.4% of samples. The least abundant cytokine was IFN-γ in both saliva and serum, present in 45.9% and 2.7% of samples (**Figure 1**, **Table 2**), respectively.

**TABLE 1A** | Behçet's Disease Patients demographics for saliva and serum analysis.

| Demographic and Clinical Features of Behcet's Patients (N=20) | | |
|---|---|---|
| **Mean Age ±SD, Years** | **37.3±9.25** | |
| **Gender** | **Number (N)** | **%** |
| Male | 7 | 35 |
| Female | 13 | 65 |
| Clinical Features | | |
| Oral Ulcers | 9 | 45 |
| Genital Ulcers | 4 | 20 |
| Eye Lesions | 3 | 15 |
| Skin Lesions | 3 | 15 |
| Joint Involvement | 9 | 45 |
| Erythema Nodosum | 2 | 10 |
| Vascular Involvement | 1 | 5 |
| CNS | 2 | 10 |
| Relapsed | 7 | 35 |
| Treatment | | |
| Prednisolone (PRED) | 4 | 20 |
| Azathioprine (AZA) | 2 | 10 |
| Colchicine (COLC) | 3 | 15 |
| PRED + AZA | 2 | 10 |
| PRED + COLC | 1 | 5 |
| PRED + COLC + AZA | 3 | 15 |
| AZA + COLC | 1 | 5 |
| Cyclosporine A + COLC | 1 | 5 |
| No Treatment | 3 | 15 |

Overall, when comparing cytokine values for the BD, RAS, and HC groups, there was a stark contrast in the prevalence of saliva cytokines compared to serum (**Figure 1**). This was also apparent when comparing the differences between BD and HC group median concentrations (ΔCytokine pg/ml) (**Figure 2** and **Supplementary Table S1**). For example IL-1β was measured in all BD saliva samples (median range 727.4 – 2383 pg/ml) (**Table 3**) but only in 19.4% of BD serum samples and with a lower median concentration (median range 0.01-36.8 pg/ml). This is the most marked increase followed by IL-8. In addition, An increase IL-6, TNF-α and TNF-β levels in BD saliva was evident with little or no change in serum levels relative to HC. IL-2 was higher in BD serum but lower in saliva, while IL-4, IL-5 and IL-12p70 were all lower in saliva with no marked changes to serum levels (**Supplementary Table S1**). IL-17A showed a small increase in saliva concentration compared with serum, whereas IL-10 levels were also low but similar in both saliva and serum when compared to HCs (**Figure 2**, **Table 3** and **Supplementary Table S1**).

In RAS saliva and serum, IL-1β, IL-8, and TNF-β were also higher with no change in serum levels for IL-1β and TNF-β, however IL-8 serum levels were lower in RAS relative to HC. IL-2 levels were higher in RAS serum and decreased in saliva. Similarly, in BD saliva, IL-2, IL-4 and IL-5 were also decreased in RAS saliva. Conversely, IL-12p70 was increased in RAS saliva whereas in BD saliva, IL-12p70 was decreased relative to HCs.

Comparison of BD and RAS group median Δ cytokine levels also highlighted some key differences between these patient groups (**Figure 2**). For instance, IL-8 and IL-10 are increased in both BD saliva (ΔIL-8 = 178.2pg/ml, ΔIL-10 = 20.35pg/ml) and serum (ΔIL-8 = 17.6 pg/ml, ΔIL-10 = 11.9 pg/ml) whereas in RAS patients, IL-8 and IL-10 levels are higher only in saliva (ΔIL-8 = 575 pg/ml, ΔIL-10 = 40.3pg/ml) (**Supplementary Table S1**).

## Comparing Th1, Th2, and Th-17 Cytokine Levels in Serum and Saliva

Out of all the saliva Th1 cytokines, IL-1β and IL-8 were detected at the highest levels across all patient groups (**Table 3** and **Table S2**). IL-8 levels were higher in saliva than serum with the group median levels of salivary IL-8 in BD (MA and MQ) and RAS (MA and MQ: data not shown, RAS-ALL **Supplementary Table S2**) all higher than HCs however in serum, only BD IL-8 levels were higher (**Table 3**). IL-1β levels were the highest in saliva with both BD and RAS levels higher than HCs. Of all groups, BD-MA, had the greatest concentration however, this did not reach significance. In contrast, no IL-1β was detected in HC serum, with very low levels of IL-1β in RAS and only five of the 19 BD patients having measurable levels. It was interesting to note that the serum cytokine concentration range was markedly reduced compared to saliva; however, these differences did not reach significance among all the study groups. Maximum values obtained in a serum from a BD-MA sample was 1367 pg/ml (**Figure 1A**) whereas in saliva the range for all the groups was up to 4000 pg/ml with only one HC outlier sample giving a value of more than 8000 pg/ml (**Figure 1C**).

IL-2 was readily detected in all saliva samples but in only half of serum samples. Serum and saliva cytokine levels of Th1 cytokines

**TABLE 1B** | Demographics for Healthy Control (HC) and Recurrent Aphthous Stomatitis (RAS) study groups.

| Demographics | Healthy Controls (N=10) | | Recurrent Aphthous Stomatitis (RAS) N=7 (6*) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | SERUM | | SALIVA | | MATCHED* | |
| Mean Age ± SD, Years | 34.7±11.1 | | 39.3±15 | | 36.7±15 | | 38±16 | |
| Gender | N | % | N | % | N | % | N | % |
| Male | 5 | 50 | 4 | 57.1 | 5 | 71.4 | 4 | 66.7 |
| Female | 5 | 50 | 3 | 42.9 | 2 | 28.6 | 2 | 33.3 |

*Analysis was carried out on matched serum and saliva from all healthy control samples, however in the RAS cohort, *matched saliva and serum were collected on the same day for N=6 patients. One RAS serum and saliva sample were from different patients which is represented above in the different mean ages and gender numbers in the RAS serum and saliva patient cohorts.*

IL-12p70 and IFN-γ did not reach any significant differences among all the study groups (**Table 3** and **Supplementary Figures S1, S2** respectively). Saliva IFN-γ was detected in only 16 out of 37 samples and was the least abundant cytokine in saliva.

Amongst the Th2 cytokine measurements for saliva (**Table 3**, **Supplementary Figure S2**), the patient groups had medians below 100 pg/ml. HC, BD-MQ, and BD-MA saliva had a similar IL-4 median concentration. In serum, IL-4 was detected in only 31.6% of BD samples and in one RAS and HC sample (**Table 2**). Similarly, IL-5 was detected in almost all saliva samples but only 25% of serum samples across all groups (**Table 2**). IL-5 HC median level of 85.9 pg/ml in saliva was higher than BD-ALL, BD-MQ and RAS 67.7, 60.8 pg/ml and 62.8 pg/ml respectively, however this did not reach significance (**Table 3** and **Supplementary Figure S2**).

IL-6 was detected in 13.8% serum samples but found in 75.7% of saliva samples across the cohort (**Table 2**). Median IL-6 concentrations were higher in BD and RAS patients compared to HCs, however only IL-6 reached a significant increase in BD-MA saliva compared with HC (p=0.04) (**Table 3**). Further analysis revealed that IL-6 levels were also significantly higher in the saliva of BD patients with ulcers (BD-MA) than those without (BD-MQ, p=0.03) (**Supplementary Figure S2**).

The data obtained for IL-17A showed that while it was detected in only a few serum samples, it was present in almost all saliva samples tested across all the groups however there was no significant differences (**Tables 2**, **3**).

## Cytokine Levels and Medications

Since most of the BD patients in this study were receiving immunosuppressive therapies (85%) at the time of saliva and serum collection, it was important to investigate if the treatment regimen affected cytokine concentrations. Therefore we compared saliva and serum samples (data not shown) from patients on single therapy (ST) [i.e. prednisolone (PRED) or azathioprine (AZA) or colchicine (COLC)-only medication (N=9)], or a combination of different therapies (CT) that included combinations of PRED, AZA, COLC and/or Cyclosporin A (CY-A) (N=8) with patients not on any medication (N=3). No medications were reported for RAS patients and HC groups.

All Th2 salivary cytokine levels were higher in BD patients receiving no treatment. IL-17A, IL-2, IL-12p70, IFN-γ, TNF-α and TNF-β were also higher whereas IL-1β and IL-8 levels were more than 2-fold lower in the BD patients not receiving any medication (**Table 4A**) but this did not reach statistical

**TABLE 2** | Number of samples with detectable cytokine levels in BD, RAS and HC matched saliva and serum as measured by the FlowCytomix assay.

| | FlowCytoMix® detection limits pg/ml | | Number of positive samples | | | | Number of positive samples | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Saliva | | | | Serum | | | |
| | Lower | Upper | BD N=20 | RAS N=7 | HC N=10 | % | BD N=19* | RAS N=7** | HC N=10 | % |
| IL-1β | 4.2 | 20,000 | 20/20 | 7/7 | 10/10 | 100 | 5/19 | 2/7 | 0/10 | 19.4 |
| IL-2 | 16.4 | 20,000 | 20/20 | 7/7 | 10/10 | 100 | 11/19 | 4/7 | 5/10 | 55.6 |
| IL-4 | 20.8 | 20,000 | 18/20 | 7/7 | 9/10 | 91.9 | 6/19 | 1/7 | 1/10 | 22.2 |
| IL-5 | 1.6 | 20,000 | 19/20 | 7/7 | 10/10 | 97.3 | 5/19 | 2/7 | 2/10 | 25 |
| IL-6 | 1.2 | 20,000 | 14/20 | 6/7 | 8/10 | 75.7 | 3/19 | 1/7 | 1/10 | 13.8 |
| IL-8 | 0.5 | 10,000 | 20/20 | 7/7 | 10/10 | 100 | 19/19 | 6/7 | 9/10 | 94.4 |
| IL-10 | 1.9 | 20,000 | 20/20 | 7/7 | 10/10 | 100 | 10/19 | 1/7 | 3/10 | 38.9 |
| IL-12p70 | 1.5 | 20,000 | 20/20 | 6/7 | 10/10 | 97.3 | 4/19 | 0/7 | 2/10 | 16.7 |
| IL-17A | 2.5 | 10,000 | 18/20 | 7/7 | 8/10 | 89.2 | 3/19 | 1/7 | 3/10 | 19.4 |
| IFN-γ | 1.6 | 20,000 | 8/20 | 4/7 | 4/10 | 43.2 | 1/19 | 0/7 | 0/10 | 2.7 |
| TNF-α | 3.2 | 20,000 | 20/20 | 7/7 | 10/10 | 100 | 16/19 | 3/7 | 7/10 | 72.2 |
| TNF-β | 2.4 | 20,000 | 18/20 | 7/7 | 10/10 | 94.6 | 4/19 | 1/7 | 4/10 | 25 |

*The lower and upper detection limits of the FlowCytomix assay are shown. Overall % prevalence for each cytokine in saliva (N=37) and serum (N=36).*
**Denotes that one BD-MQ serum sample was excluded from the analysis due to an erroneous flow cytometer reading (BD N=19*). Therefore, *prevalence (%) out of 36 for serum samples.*
***RAS cohort, matched saliva and serum was collected on the same day for N=6 patients. One RAS serum and saliva sample are each from different patients.*

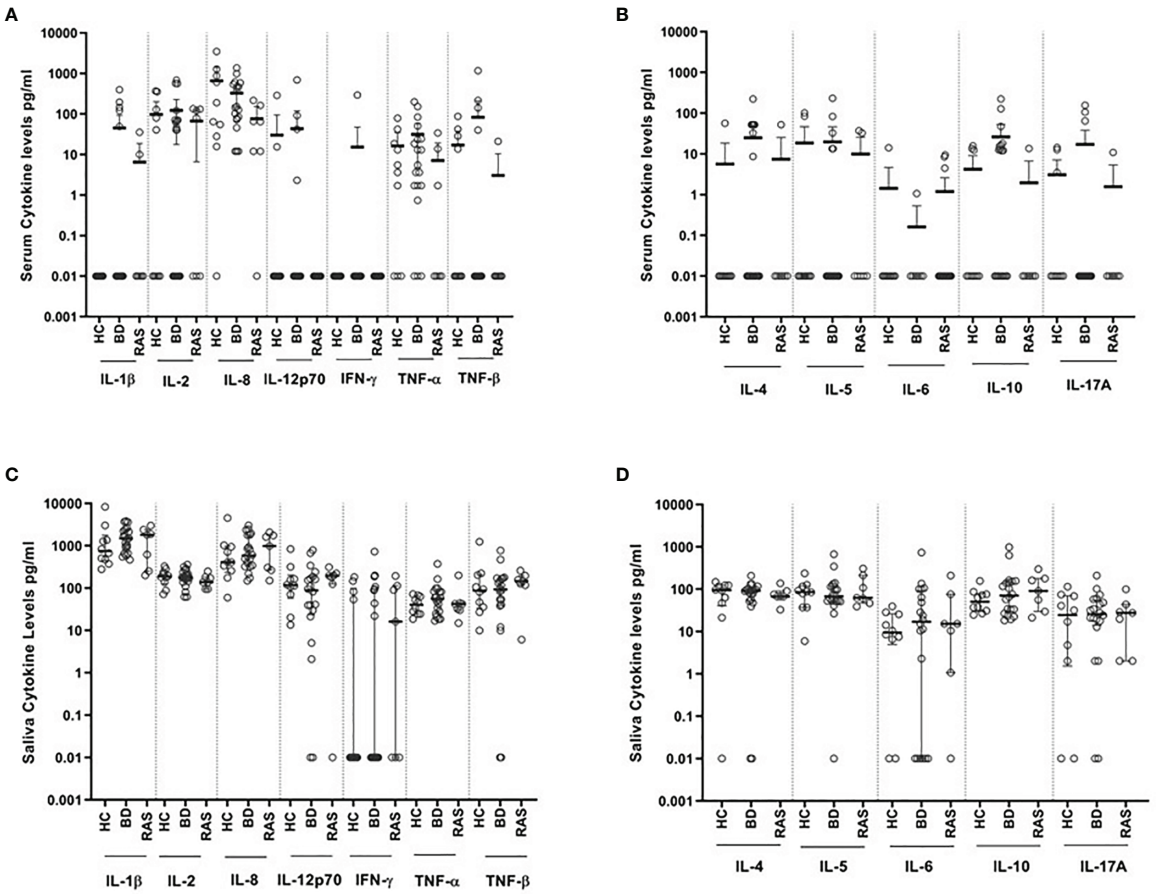


**FIGURE 1** | Levels of cytokines (pg/ml) detected in saliva and serum from BD (N=20), RAS (N=7) and HC (N=10). **(A)** Serum Th1 cytokines and IL-8 chemokine, **(B)** Serum Th2 cytokines and IL-17A, **(C)** Saliva Th1 cytokines and IL-8 chemokine, **(D)** Saliva Th2 cytokines and IL-17A.

significance. Next, we wanted to investigate whether COLC and AZA alone or in combination affected salivary cytokine levels since we have previously reported that BD patients treated with both these medications have significantly lower neutrophil elastase levels (38) during episodes of oral ulceration. The general trend was that patients on neither COLC or AZA had slightly higher IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-17A, IFN-γ, TNF-α and β saliva concentrations than BD patients on both AZA and COLC treatment however this was not significant (**Table 4B**). In contrast, IL-8 and IL-10 levels were lower in patients on neither medication, 572.8 pg/ml and 56.9 pg/ml as compared to patients on both COLC and AZA, 835.6 pg/ml and 70.8 pg/ml respectively. Of note, IFN-γ the least detectable cytokine in saliva, was still quantifiable in 43.2% of BD saliva samples and had a higher median in BD patients not receiving COLC, AZA or a combination of these medications (**Table 4B**).

## Normalised Salivary Cytokine Levels

Total protein concentrations were measured in all saliva samples from BD, RAS and HCs and found to be significantly lower in BD patients than in HCs (p=0.02) (**Figure 3**). This prompted us to review the measured cytokine levels and normalise them to the total protein concentration. Following normalisation of the data, IL-1β, IL-6, IL-8, IL-10 and TNF-α concentrations were all significantly higher in BD than in HCs with only IL-10 concentrations significantly higher in RAS than in HCs (p=0.04) (**Figure 4** and **Table 5**). To determine if normalised cytokine saliva levels differed with disease activity, we investigated the levels of all the cytokines in BD-RE and BD-Q patients and found that IL-1β and TNF-α concentrations were significantly higher in both BD-RE and BD-Q when compared to HC saliva (**Figure 5**). IL-8 was also significantly higher in BD-Q than HCs (p=0.02). Further analysis of the treatment regimen with the normalised saliva data revealed that salivary concentrations of IL-10 (p=0.03) and IL-12p70 (p=0.03) were significantly lower in patients taking both COLC and AZA than BD patients treated with only AZA. IL-1β concentrations were also lower in BD patients taking both medications, however these levels did not reach significance (p=0.06) (**Supplementary Figure S3**).

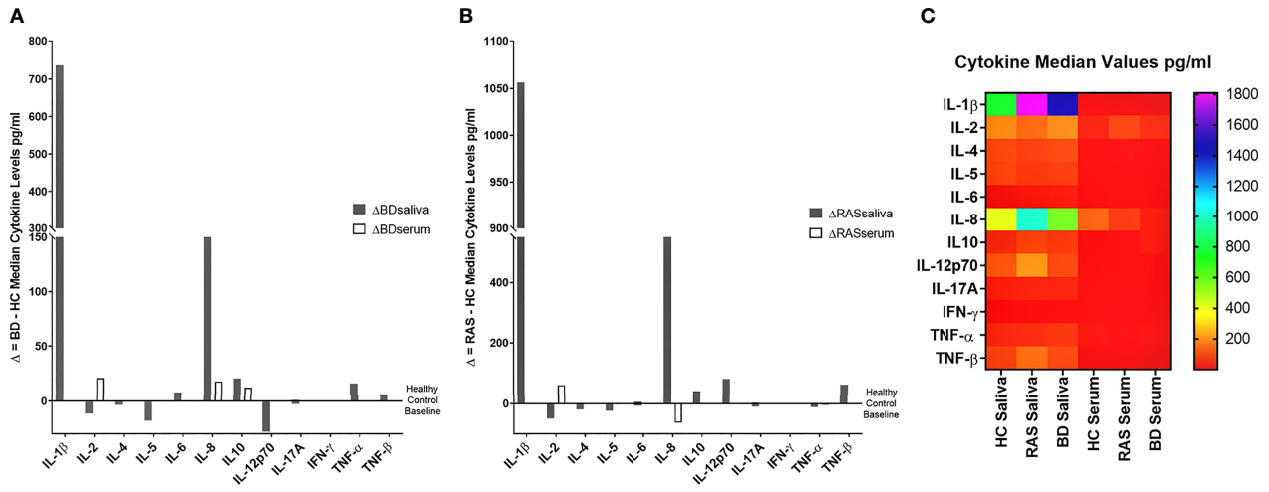


**FIGURE 2** | Group median cytokine differences between **(A)** BD and HC and **(B)** RAS and HC. For each cytokine, the following was calculated: Group Median BD cytokine concentration (pg/ml) - Group Median HC cytokine concentration (pg/ml) or Group Median RAS cytokine concentration (pg/ml) - Group Median HC cytokine concentration (pg/ml) to reveal the delta (Δ), or difference, of the disease group cytokine levels and the HC. **(C)** Heat map shows differential expression of 12 cytokines in saliva and serum.

## Correlations Between Normalised Salivary Cytokine Levels Across BD Patients

To determine if there was a relationship between the different cytokines in saliva, we used Spearman's correlation analysis. Correlation analyses between the different cytokines and normalised BD saliva samples showed that IL-12p70, IL-10, TNF-β and TNF-α concentrations positively correlated with IL-5 ($p<0.0001$) (**Figures 6A, C, E, I**). IL-12p70 and TNF-β also highly correlated with each other $r=0.9$ ($p<0.0001$) (**Figure 6F**) IL-12p70 and IL-10 ($r=0.95$, $P<0.0001$) (**Figure 6B**). Additional correlations were found between other BD salivary cytokines and these are summarised in **Table 6**.

# DISCUSSION

This pilot study investigated the use of FlowCytomix™ multiplex assays to measure multiple cytokines in matched serum and saliva samples to explore their differential expression and potential as diagnostic or activity markers for BD. Recurrent ulceration is a primary symptom of BD and precedes systemic systems implying that oral ulceration is a key event in this disease. Numerous studies have reported differences in systemic cytokines in BD serum, but only a handful of studies investigating inflammatory markers in BD saliva have been carried out (68–71) including our own recent work (38). Saliva is a highly dynamic fluid that has the potential to reveal the on-going pathology of BD. The potential of saliva as a diagnostic tool for oral and systemic disease has been an exciting development in clinical medicine in recent years (72–75).

Cytokines have the capacity to act in an autocrine, paracrine and endocrine manner so that their effects can be both local and distal with considerable variations in biodynamic range (76–79), (**Supplementary Figure S4**). Our study suggests that the range of cytokines in serum and saliva are very different with Th1, Th2 and IL-17A cytokines more abundant in saliva than in serum across all groups. After normalizing the protein content, saliva samples provided valuable information on the state of oral immunity in all groups as well as key inflammatory markers in BD patients during dormant oral symptoms.

ELISA has been the "Gold Standard" cytokine detection method with commercial assays varying in detection limits. A BD study conducted by Zouboulis, et al., reported that up to 51% of their serum samples were below detectable limits of their ELISA assay (10 ng/ml) (80). Using the FlowCytomix™ array, we had an improved yield with 100% of BD saliva samples detected above the lower limit and only two out of the 36 serum samples below detection. Other analyte targets in serum were not as readily detected. Excluding IL-8 and TNF-α (**Table 2**), the majority of undiluted serum samples were below detectable limits. Using the same small volume (25 µl) with saliva revealed better cytokine detection than serum. Saliva had an average of only 3.3 out of 37 saliva samples (9%) per analyte below the assay detectable levels, the exception being salivary IFN-γ which had 21 saliva samples below the 1.6 pg/ml assay detection limit (**Table 2**).

## IL-8 and IL-1β

IL-1β and IL-8 concentrations were elevated in BD and RAS saliva compared to HCs, however, in serum, IL-1β levels were very low, and IL-8 concentrations were higher only in BD patient serum. IL-8 has been one of the main serum chemokines studied in BD and has been detected in significantly high concentrations

**TABLE 3 |** Concentrations of cytokines in serum and saliva of patients presenting with oral ulceration (Mouth Active (BD-MA)) or without (Mouth Quiet (BD-MQ)) oral ulceration involvement.

| Cytokines | Group | Serum† | | Saliva | | p-values* |
|---|---|---|---|---|---|---|
| | | Median | IQR | Median | IQR | |
| IL-1β | HC | 0.01 | 0.01, 0.01 | 752.4 | 447.1, 1775 | |
| | BD-MQ | 0.01 | 0.01, 48.8 | 1168 | 933.7, 2233 | 0.22 |
| | BD-MA | 0.01 | 0.01, 85.2 | 1512 | 602.6, 3068 | 0.24 |
| | BD-ALL | 0.01 | 0.01, 36.8 | 1489 | 727.4, 2383 | 0.15 |
| IL-2 | HC | 20.3 | 0.01, 185.1 | 187.8 | 125.4, 249.8 | |
| | BD-MQ | 0.01 | 0.01, 175.6 | 190.6 | 138.1, 238 | 0.85 |
| | BD-MA | 66.9 | 20.3, 96.8 | 173.6 | 137.7, 246.3 | 0.68 |
| | BD-ALL | 40.6 | 0.01, 71.3 | 176.4 | 139.6, 232.4 | 0.72 |
| IL-4 | HC | 0.01 | 0.01, 0.01 | 94.8 | 40.5, 124.7 | |
| | BD-MQ | 0.01 | 0.01, 32.7 | 82.4 | 47, 116.6 | 0.74 |
| | BD-MA | 0.01 | 0.01, 51.9 | 93.1 | 67.6, 101.7 | 0.95 |
| | BD-ALL | 0.01 | 0.01, 51.9 | 91.4 | 53, 102.5 | 0.80 |
| IL-5 | HC | 0.01 | 0.01, 21.2 | 85.9 | 36.9, 119.1 | |
| | BD-MQ | 0.01 | 0.01, 8.2 | 60.8 | 50.8, 114.2 | 0.59 |
| | BD-MA | 0.01 | 0.01, 13.5 | 95.2 | 46.9, 126.3 | 0.65 |
| | BD-ALL | 0.01 | 0.01, 13.5 | 67.8 | 51.3, 117.5 | 0.94 |
| IL-6 | HC | 0.01 | 0.01, 0.01 | 9.5 | 4.9, 26.3 | |
| | BD-MQ | 0.01 | 0.01, 2.1 | 10.4 | 0, 18.9 | 0.69 |
| | BD-MA | 0.01 | 0.01, 0.01 | 63.9 | 13, 103.2 | **0.04** |
| | BD-ALL | 0.01 | 0.01, 0.01 | 17.1 | 0.01, 91.3 | 0.41 |
| IL-8 | HC | 127.4 | 25.1, 975.5 | 410.3 | 237.5, 966.9 | |
| | BD-MQ | 135.8 | 65.9, 476.8 | 572.8 | 342.4, 1787 | 0.55 |
| | BD-MA | 363.9 | 44.5, 601.5 | 604.3 | 325.4, 1881 | 0.54 |
| | BD-ALL | 145 | 72.6, 535 | 588.5 | 329.6, 1804 | 0.47 |
| IL-10 | HC | 0.01 | 0.01, 12.8 | 50.5 | 29.8, 78.4 | |
| | BD-MQ | 6 | 0.01, 41.4 | 75.7 | 26.9, 142.9 | 0.59 |
| | BD-MA | 13 | 0.01, 17.3 | 65.9 | 28, 160 | 0.67 |
| | BD-ALL | 11.9 | 0.01, 16.5 | 70.8 | 28.54 | 0.57 |
| Il-12p70 | HC | 0.01 | 0.01, 3.9 | 116.5 | 57.8, 203.7 | |
| | BD-MQ | 0.01 | 0.01, 0.6 | 82 | 5, 161.4 | 0.39 |
| | BD-MA | 0.01 | 0.01, 21.1 | 94.8 | 34.7, 214.3 | 0.84 |
| | BD-ALL | 0.01 | 0.01, 0.01 | 88.4 | 23.4, 182.5 | 0.50 |
| IL-17A | HC | 0.01 | 0.01, 5.8 | 24.4 | 1.5, 70 | |
| | BD-MQ | 0.01 | 0.01, 16.1 | 27.2 | 12.7, 48 | 0.74 |
| | BD-MA | 0.01 | 0.01, 0.01 | 21 | 10.7, 59.2 | 0.86 |
| | BD-ALL | 0.01 | 0.01, 0.01 | 25.7 | 14, 55.2 | 0.75 |
| IFN-γ | HC | 0.01 | 0.01, 0.01 | 0.01 | 0.01, 98.8 | |
| | BD-MQ | 0.01 | 0.01, 0.01 | 0.01 | 0.01, 89.9 | 1 |
| | BD-MA | 0.01 | 0.01, 0.01 | 0.01 | 0.01, 147.8 | 0.75 |
| | BD-ALL | 0.01 | 0.01, 0.01 | 0.01 | 0.01, 93.5 | 0.86 |
| TNF-α | HC | 0.01 | 4.6, 24.6 | 40.2 | 24.4, 64.5 | |
| | BD-MQ | 1.3 | 23.9, 100.9 | 42.2 | 18.7, 81.5 | 0.96 |
| | BD-MA | 1.2 | 3.6, 13 | 59.0 | 40.4, 123.9 | 0.10 |
| | BD-ALL | 5.5 | 1.7, 24.4 | 55.8 | 25.4, 83.3 | 0.36 |
| TNF-β | HC | 0.01 | 0.01, 32.1 | 87.4 | 33.3, 209 | |
| | BD-MQ | 0.01 | 0.01, 35.6 | 53.9 | 38.3, 170.5 | 0.69 |
| | BD-MA | 0.01 | 0.01, 20.1 | 114.8 | 47.4, 163.2 | 0.74 |
| | BD-ALL | 0.01 | 0.01, 0.01 | 92.9 | 38.8, 169.5 | 0.96 |

*†Serum concentrations showed no significant differences between groups.*

**p-value when compared with HC saliva. Significant values in bold.*

*Serum HC N=10, BD-MQ N=10, BD-MA N=9, BD-ALL N=19. Non-normalized saliva HC N=10, BD-MQ N=11, BD-MA N=9, BD-ALL N=20. Median concentrations with interquartile ranges (IQR) are shown. Cytokines below the lower levels limits of detection were considered not detectable and therefore arbitrarily assigned concentrations of 0.01 pg/ml. p values are given for comparisons between HC and BD subgroups.*

compared to HC but not consistently between active and inactive episodes of disease (81). BD patients with active vascular clinical presentation had a 4-5-fold increase of IL-8 over those without any vascular symptoms, while IL-8 concentrations in inactive BD patients with a history of vasculitis were still 2-fold higher than inactive BD with no vascular association (82). An increase in IL-8 mRNA has been shown to be directly associated with BD serum-treated macrophages from HC *in vitro* (83) and although there was a diverse source of IL-8 from various cells (including neutrophils, monocytes, macrophages, endothelial and epithelial cells), lymphocytes were considered to be the major contributors (84).

**TABLE 4A** | Saliva cytokine concentrations from Behçet's Disease (BD) patients on Single treatment (ST), Combined Treatment (CT) or No Medication (NO).

| Cytokines pg/ml | Single Therapy (ST) N=9 | Combined Therapy (CT) N=8 | No Medication (NO) N=3 | Ratio ST/NO | Ratio CT/NO |
|---|---|---|---|---|---|
| IL-1β | **2433** (759.2-3665) | **1317** (958.6-2057) | **658.7** (546.5-1667) | 3.7 | 2.0 |
| IL-2 | **173.6** (167.1-206) | **167.4** (94.2-296.9) | **276.9** (109.1-2867) | 0.6 | 0.6 |
| IL-4 | **82.4** (51.7-96.5) | **94.8** (58.6-128.6) | **95.56** (38.9-131.1) | 0.9 | 1.0 |
| IL-5 | **60.8** (51.8-86) | **77** (51.3-134.2) | **118.5** (26.6-670) | 0.5 | 0.7 |
| IL-6 | **18.9** (1.2-103) | **13.3** (0.01-43.5) | **23.7** (0.01-136.6) | 0.8 | 0.6 |
| IL-8 | **604.3** (372.3-2160) | **654.3** (285.5-910) | **325.4** (148.9-1787) | 1.9 | 2.0 |
| IL-10 | **56.9** (24.7-147.7) | **70.8** (25.9-129.4) | **162.6** (33.6-983.1) | 0.4 | 0.4 |
| IL-12p70 | **82** (19.7-178) | **67.8** (9.1-149.2) | **241.5** (29.9-786.8) | 0.3 | 0.3 |
| IL-17A | **27.2** (30.8-38.7) | **15.3** (0.5-67.3) | **57.6** (2-210.9) | 0.5 | 0.3 |
| IFN-γ | **0.01** (0.01-97.7) | **0.01** (0.01-141.6) | **43.3** (0.01-93.5) | 0 | 0 |
| TNF-α | **59** (35.2-82.7) | **46.6** (20.1-94.1) | **52.6** (20.6-97.1) | 1.1 | 0.9 |
| TNF-β | **87.3** (39.3-131.1) | **93** (13.5-170.5) | **179** (10-773.2) | 0.5 | 0.5 |

*Table shows median concentrations in bold and interquartile range (IQR).*

IL-8 is a potent chemokine eliciting a strong immune response to a variety of stimuli, recruiting neutrophils, activating leukocytes in response to bacterial antigens and enhancing adherence of circulating leukocytes to endothelial cells during inflammation (81). Neutrophils and lymphocytes express the IL-8 receptor (85) as do monocytes and NK cells (86). Interestingly IL-8 was increased in RAS saliva but not serum, suggesting that while the ulcers may have similar morphologies to those of BD, RAS remains a local condition without the systemic manifestations associated with BD.

IL-1β is recognised as a "master cytokine" in inflammation, inducing other cytokines and acting as the main mediator coordinating attacks on invading microbes (87, 88) or responses to injury. Produced mainly by blood monocytes, but also from macrophages, dendritic cells, and neutrophils, it increases fever and hypotension. Increased levels of IL-1β has been shown to simultaneous increase in IL-8. IL-1β has been investigated in BD, but not to the extent of IL-8 or TNF-α. A significant rise of serum IL-1β has been detected in BD active and inactive patients compared to HC (89). Importantly, IL-8 has also been suggested as an inducer of matrix metalloproteinase which may have a role in ulcer development (90).

During oral ulceration, higher levels of IL-8 and IL-1β reflect mucosal damage and recruitment of inflammatory cells into the wound. Interestingly, in this study both BD-MQ and RAS-MQ (RAS data not included) had higher levels of salivary IL-8 and IL-1β to that of HCs, suggesting their continued presence during non-ulcer periods may reflect the mucosal instability of these patient groups. Furthermore, salivary IL-1β tended to be higher in quiet, systemically inactive, BD-Q patients than in relapsed patients. We have recently described high levels of neutrophil elastase in BD saliva and have proposed a working model of BD oral environment which involves neutrophil recruitment and IL-8 and IL-1β secretion (38).

In ELISA based assays comparing sera from patients with or without oral ulceration, IL-8 was significantly increased in individuals with oral ulcers (80, 82, 91). Importantly, our study also found that IL-8 concentrations were higher in serum of orally active BD patients compared to HCs and confirms that these levels are also high in orally active saliva, thereby indicating that the local high IL-8 mucosal response is mirrored in systemically higher IL-8 levels in the serum of orally active patients.

**TABLE 4B** | Saliva cytokine concentrations from Behçet's Disease (BD) patients taking colchicine (COLC) and/or Azathioprine (AZA).

| Cytokines pg/ml | COLC N=5 | AZA N=4 | COLC+AZA N=4 | Neither N=7 |
|---|---|---|---|---|
| IL-1β | **1466** (755.2-3030) | **1340** (998.4-2053) | **1421** (752.4-2057) | **1667** (546.5-3703) |
| IL-2 | **173.6** (156-260.6) | **176.9** (81.2-232.4) | **135.1** (66.6-317.9) | **179.3** (166.4-276.9) |
| IL-4 | **89.6** (78.7-112.9) | **77.6** (13-113.3) | **87.7** (19.7-179.7) | **93.1** (47-100) |
| IL-5 | **52.8** (51.8-203) | **86** (62.8-110) | **73** (12.7-129.4) | **74.8** (40.9-134) |
| IL-6 | **0.01** (0.01-82.1) | **18.4** (2.6-86.6) | **13.3** (2.9-40.2) | **23.7** (2.3-136.6) |
| IL-8 | **494.8** (310.6-2089) | **544.7** (378.1-2009) | **835.6** (333.6-910) | **572.8** (325.4-1953) |
| IL-10 | **33.6** (25-386.3) | **127.6** (39.31-156.2) | **70.8** (42.2-122.6) | **56.9** (22.4-162.6) |
| IL-12p70 | **40.8** (0.01-506.5) | **157.7** (54.4-180.6) | **49.9** (2.8-108.2) | **82** (39.5-241.5) |
| IL-17A | **31.8** (16.9-72.6) | **36.1** (6-57.7) | **9.9** (0.5-59.7) | **27.2** (19.4-57.6) |
| IFN-γ | **0.01** (0.01-138.8) | **0.01** (0.01-80.1) | **0.01** (0.01-149.6) | **22** (0.01-93.5) |
| TNF-α | **63.4** (33.3-122.8) | **51.3** (18.7-94.7) | **46.6** (21.1-75.9) | **52.6** (28.3-97.1) |
| TNF-β | **53.9** (26.1-321.5) | **101.1** (62.3-157.6) | **66** (0.01-157.7) | **98.5** (38.3-179) |

*Bold the median values in the 3 columns and interquartile range (IQR) in BD patients identified as taking COLC or AZA, both or neither AZA or COLC. COLC refers only to the exclusion of AZA and vice-versa, however this does not exclude other medications that the patient may have been taking.*

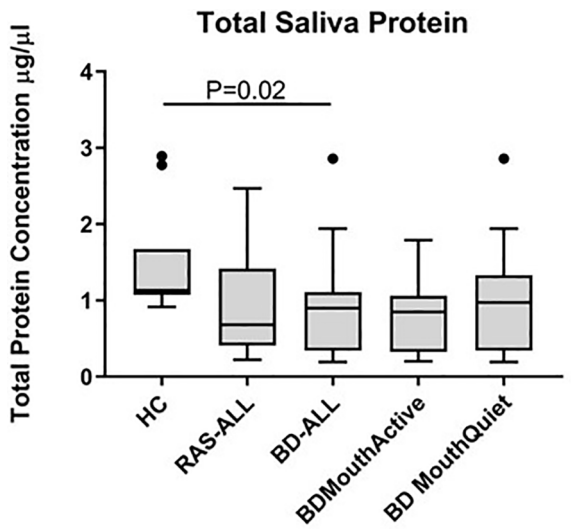


**FIGURE 3** | Total protein concentrations in saliva of healthy controls (HC), Behçet's Disease (BD) and recurrent aphthous stomatitis (RAS) patients. The Tukey box plots show levels in BD-ALL (N=20), RAS-ALL (N=7) and HC (N=10). The BD patients were further grouped into patients with oral ulceration, BD Mouth Active (N=9), and patients with no oral ulceration, BD Mouth Quiet (N=11). Mann-Whitney U test, significance based on two tailed 95% confidence interval (CI). Exact P values are indicated.

IL-8 is also produced by gingival epithelial cells in response to oral microbiota *in vitro* (92) and by nicotine (93), while a recent review has referenced data showing upregulation of IL-8 in oral diseases such as Oral Lichen Planus and periimplantitis with contradictory results in some studies of oral Leukoplakia (75). IL-8 can exist as a monomer, dimer or as a mixture of both and has profound effects on chemokine receptors CXCR1 and CXCR2. These molecules are important in neutrophil recruitment and activation and the monomeric form of IL-8 is thought to bind to glycosaminoglycans, which are present in the oral mucosal pellicle (94, 95).

The ratio of salivary IL-8 and IL-1β (average ratio of 1:2) showed a strong predictability that IL-1β would be nearly twice as high than IL-8 in 89% of the samples. Together, they showed a correlation in the collective saliva samples from all groups. However, serum samples did not adhere to this ratio. Furthermore, in normalised BD saliva samples, IL-1β and IL-8 showed a strong correlation ($r=0.78$, $p<0.0001$). While IL-8 has been suggested as a serological marker for assessing BD activity (91), our data would suggest that IL-8 alone is not specific enough since both RAS and BD patients had high levels of this chemokine. However, IL-8 paired with IL-1β measurements in saliva, could improve the validity of pre-empting an active ulcer episode. The IL-8 and IL-1β correlation demonstrates the reliability of saliva samples to provide accurate data for the potential monitoring of inflammatory markers.

## IFNγ

IFN-γ was originally recognised as the main constituent of the Th1 response (96, 97). It is produced by cells of both the innate and acquired immune systems, namely NK cells and T-cells. IFN-γ targets B cells, macrophages, and endothelial cells activating the expression of Class II MHC molecules on the surface of these antigen presenting cells (98). Although BD is often recognised as a Th1 dominant response (44), there are studies that have conflicted with the classic generation of increased IFN-γ. For instance, IFN-γ was measured in serum of BD active patients and was found to be lower than BD quiet, RAS, and HC (99). In our study, only 16 out of 37 saliva samples and one out of 36 serum samples had detectable IFN-γ levels. The trend was for the IFN-γ IQR (interquartile range) to be higher in the saliva from BD patients with active ulcers, which illustrated that only when the oral mucosa is damaged does the classic IFN-γ drive forward the Th1 response. Frassanito, et al, also found increased circulating IFN-γ from only symptomatic BD patients when compared to BD quiet and HCs (44).

## TNF-α

TNF-α is a rapidly released cytokine that can act as an alarm against foreign or stress stimuli (100). Mainly produced by monocytes and macrophages it can also be released from T-cells, neutrophils, mast cells, endothelial cells and keratinocytes (101–103). In this study, saliva TNF-α was significantly increased in both BD patients with and without oral ulcers. For those with oral ulcers, TNF-α likely plays a beneficial role in recruiting immune cells to battle pathogens associated with the wound. However, an increased presence of TNF-α in the mouth of BD patients without any ulcers could lead to an influx of neutrophils which as we have previously proposed can lead to mucosal instability following an exacerbated inflammatory cycle (38).

Although RAS-MA patients had the highest TNF-α median concentration in saliva (data not shown), due to the small sample size comparisons were not able to be elucidated. More samples are required from the RAS patient group for a more accurate assessment since it has been shown to be highly expressed in RAS oral lesions (61). Interestingly, the highest median TNF-α levels were measured in BD-MQ and not BD-MA serum. This suggests that TNF-α may be involved in stimulating a systemic response in BD. TNF-α has been one of the most intensively investigated cytokines in BD (41). BD patients with active uveitis have been shown to have significantly higher levels of TNF-α than those without any eye involvement for 3 months (104) and anti-TNF-α therapy (such as Infliximab) has been helpful in controlling active episodes of uveitis in BD (105) as well as oral and genital ulceration (106). For RAS patients, an *in vitro* study has shown that TNF-α was higher in unstimulated peripheral blood monocytes from RAS patients with ulcers than in HCs (107). Collectively, the data indicates that TNF-α has an important role in BD and RAS pathologies.

## IL-2 and IL-12p70

IL-12p70, another pro-inflammatory cytokine which is known to drive the Th1 immune response by inducing IFN-γ and IL-2 (108), was found to be increased in RAS saliva. Although this did not lead to higher levels of IL-2, IFN-γ was higher in RAS saliva (IQR 0.01-113.7pg/ml) than BD and HCs. In all BD patients,

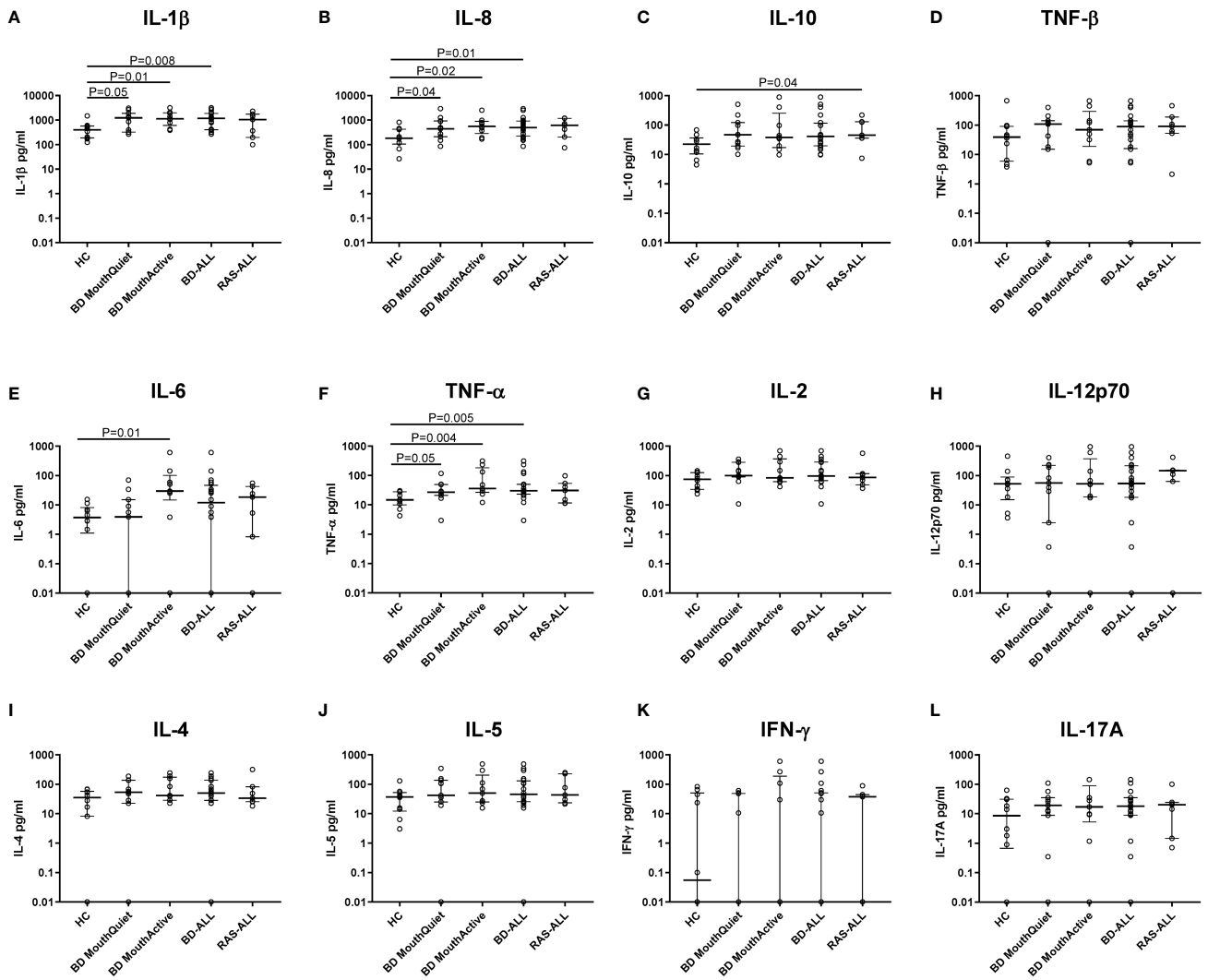


**FIGURE 4** | Saliva cytokine levels normalised to total protein differentially expressed in BD and RAS patients as compared to HCs. Plots show the cytokine concentrations (pg/ml) from BD-ALL (N=20), RAS-ALL (N=7) and HC (N=10). The BD patients were further grouped into patients with oral ulceration, BD Mouth Active (N=9), and patients with no oral ulceration, BD Mouth Quiet (N=11). **(A)** IL-1β, **(B)** IL-8, **(C)** IL-10, **(D)** TNF-β, **(E)** IL-6, **(F)** TNF-α, **(G)** IL-2, **(H)** IL-12p70, **(I)** IL-4, **(J)** IL-5, **(K)** IFN-γ, **(L)** IL-17A. The median and interquartile range are shown (Median ± IQR). Mann-Whitney U test, significance based on two tailed 95% confidence interval (CI). Exact P values are indicated.

normalised IL-12p70 also showed a strong correlation with TNF-β in saliva (r=0.9, p<0.0001), suggesting their simultaneous presence is worthy of further investigation for potential synergistic activity. In *ex-vivo* studies of inflamed gut mucosa IL-12 and TNF-α could be induced by stimulating the cells with Human HSP-70 (109) a molecule that has been implicated in BD pathogenesis.

Elevated IL-2 levels have been reported in sera from BD patients with uveitis compared with non-uveitis patients (110) and in a mixed BD cohort with active disease but where only 6/44 patients had uveitis compared with healthy controls (111). In our study, serum samples from RAS-MA (n=2) had higher levels of IL-2 compared to RAS-MQ patients, however, since the sample size is small this warrants further investigation. Similarly, high median levels were also observed in BD-MA serum possibly reflecting the ongoing inflammatory process during oral ulceration. In saliva, however, similar IL-2 levels were detected in all groups. This indicates that IL-2 detected in saliva was not necessarily affected by oral ulceration in our cohort. IL-2 can also be influenced by IL-6. IL-6 can promote T helper and cytotoxic T-cell proliferation by increasing their IL-2 secretion (112, 113).

## IL-6 and IL-17

IL-6 is produced by T-cells, B cells, and macrophages and can stimulate the proliferation of B cells and immunoglobulins as well as differentiate T lymphocytes (114, 115). Naïve T-cells can be stimulated by IL-6 and transforming growth factor beta to become IL-17A secreting Th17-type cells (116). An increased

**TABLE 5 |** Concentrations of cytokines in normalized saliva of BD patients presenting with oral ulceration, Mouth Active (BD-MA), or without oral ulceration, Mouth Quiet (BD-MQ) compared to HC.

| Cytokines | Group | Normalized Saliva | | p-values* |
|---|---|---|---|---|
| | | Median | IQR | |
| IL-1β | HC | 399.3 | 187.5, 571 | |
| | BD-MQ | 1239 | 324.8, 1887 | **0.05** |
| | BD-MA | 1126 | 605.8, 1978 | **0.01** |
| | BD-ALL | 1182 | 403.4, 1883 | **0.008** |
| IL-2 | HC | 74.3 | 34, 126 | |
| | BD-MQ | 99.1 | 90.4, 283.4 | 0.11 |
| | BD-MA | 84.0 | 62.1, 372 | 0.18 |
| | BD-ALL | 96.6 | 67.2, 289.4 | 0.08 |
| IL-4 | HC | 35.2 | 8.1, 57.4 | |
| | BD-MQ | 53.6 | 22.4, 136.1 | 0.31 |
| | BD-MA | 41.4 | 28.7, 173.2 | 0.13 |
| | BD-ALL | 50.6 | 28.3, 138.1 | 0.14 |
| IL-5 | HC | 36.7 | 12.2, 52.3 | |
| | BD-MQ | 41.8 | 25.1, 136 | 0.25 |
| | BD-MA | 50.1 | 25, 205 | 0.25 |
| | BD-ALL | 46 | 25.5, 130 | 0.17 |
| IL-6 | HC | 3.7 | 1.1, 8.1 | |
| | BD-MQ | 3.9 | 0.01, 15.1 | 0.92 |
| | BD-MA | 29.6 | 14.7, 101.1 | **0.01** |
| | BD-ALL | 12.1 | 0.01, 46.5 | 0.20 |
| IL-8 | HC | 182.3 | 104.8, 426 | |
| | BD-MQ | 442.5 | 210.4, 925.6 | **0.04** |
| | BD-MA | 551.1 | 282.6, 893.2 | **0.02** |
| | BD-ALL | 493 | 226.2, 916.9 | **0.01** |
| IL-10 | HC | 22.5 | 10.5, 36.8 | |
| | BD-MQ | 46.6 | 19.6, 122.2 | 0.10 |
| | BD-MA | 38 | 17.3, 253.2 | 0.11 |
| | BD-ALL | 41 | 19.6, 116.3 | 0.06 |
| IL-12p70 | HC | 52.3 | 15.2, 89.3 | |
| | BD-MQ | 55.7 | 2.5, 223.8 | 0.92 |
| | BD-MA | 52.3 | 18.8, 374.2 | 0.80 |
| | BD-ALL | 54.0 | 18.2, 215.9 | 0.80 |
| IL-17A | HC | 8.5 | 0.7, 31.1 | |
| | BD-MQ | 19.0 | 8.8, 35.1 | 0.43 |
| | BD-MA | 17.0 | 5.2, 89.9 | 0.40 |
| | BD-ALL | 18.0 | 8.9, 35.0 | 0.32 |
| IFN-γ | HC | 0.06 | 0.01, 50.3 | |
| | BD-MQ | 0.01 | 0.01, 48.4 | 0.58 |
| | BD-MA | 0.01 | 0.01, 188.7 | 0.70 |
| | BD-ALL | 0.01 | 0.01, 50.7 | 0.90 |
| TNF-α | HC | 14.7 | 9.9, 27.8 | |
| | BD-MQ | 27.1 | 21.1, 49.3 | **0.05** |
| | BD-MA | 36.1 | 26.6, 182.8 | **0.004** |
| | BD-ALL | 30.4 | 23.2, 50.0 | **0.005** |
| TNF-β | HC | 39.3 | 6.0, 91.7 | |
| | BD-MQ | 109.0 | 15.1, 141.4 | 0.46 |
| | BD-MA | 69.5 | 19.0, 295.4 | 0.32 |
| | BD-ALL | 89.2 | 15.9, 141.2 | 0.30 |

**p-value when compared with HC saliva. Significant values are in bold.*

*Normalized saliva HC (N=10), BD-MQ (N=11), BD-MA (N=9). Median concentrations with interquartile ranges (IQR) are shown. Cytokines below the lower levels limits of detection were considered not detectable and therefore arbitrarily assigned concentrations of 0.01 pg/ml.*

level of IL-17A has been detected in BD serum (117) and the IL-23/IL-17 pathway is seen as crucial in several autoimmune diseases such as rheumatoid arthritis that had previously been regarded at Th1 related disease (118, 119). In addition to CD4+ Th17 cells, IL-17A is produced by γδ T cells, NK cells (120), and is secreted by CD3+ (CD4-/CD8-) cells infiltrating salivary glands in Sjögrens syndrome (121) and in the plasma from those with systemic lupus erythematosus (122). In response to extracellular pathogens, IL-17 can induce the production of IL-8 thus recruiting neutrophils and contributing to inflammation. However when the release of IL-17A is exacerbated, this can also lead to the destructive tissue pathologies of inflammatory and autoimmune diseases (123). IL-17A was incorporated into our multiplex assay panel because it has been shown to recruit

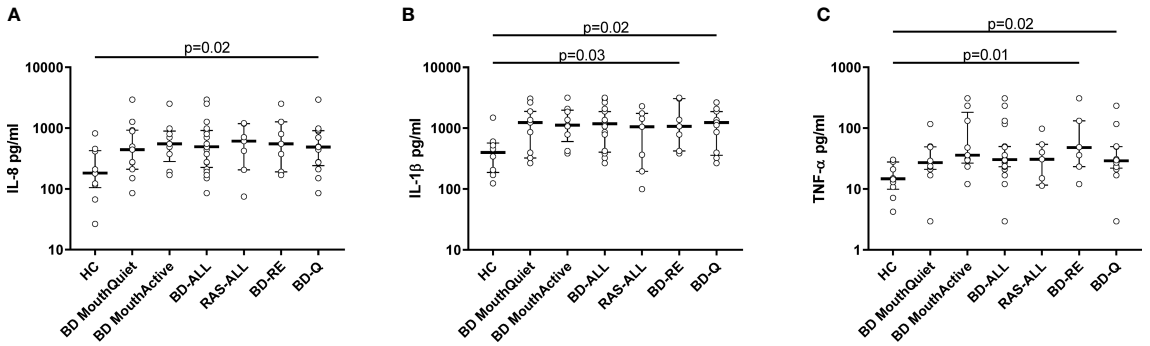


**FIGURE 5** | Disease Activity. Normalised cytokine levels differentially expressed in saliva from BD and RAS patients as compared to HC. Graphs show the concentrations of IL-8 **(A)**, IL-1β **(B)** and TNF-α **(C)** expressed in pg/ml from BD-ALL (N=20), RAS-ALL (N=7) and HC (N=10). The BD patients were further grouped into disease activity relapsed patients BD-RE (N=7) and disease activity quiet patients BD-Q (N=13). Data is presented as median ± IQR. Significant differences were determined by two tailed Mann-Whitney U test with 95% confidence interval (CI). Exact p values are indicated.

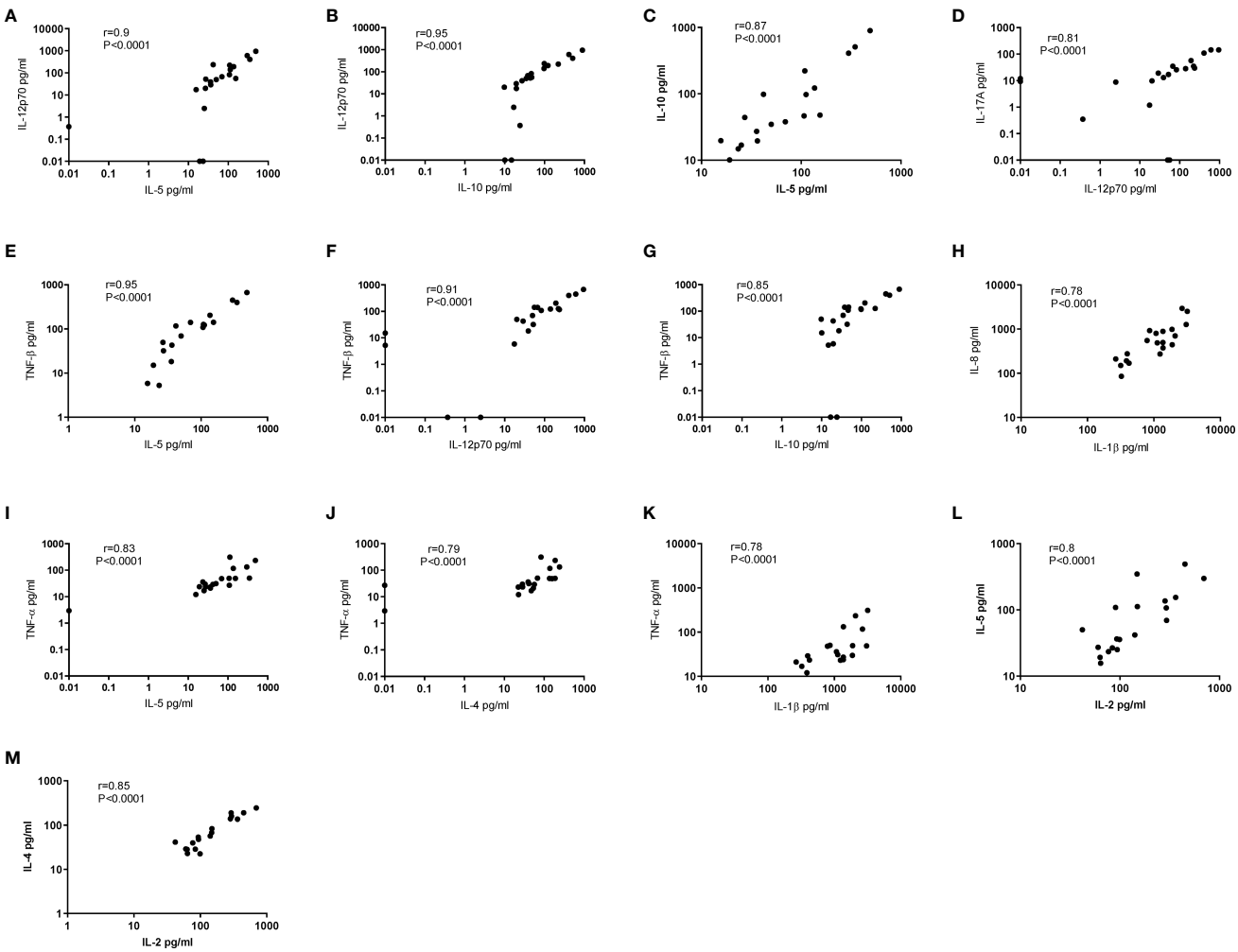


**FIGURE 6** | Correlation coefficients between 12 cytokines in Saliva. Correlation of normalised salivary cytokine concentrations in BD saliva (N=20). Spearman correlation was determined, r and p values with 95% CI. **(A)** IL-12p70 vs IL-5, **(B)** IL-12p70 vs IL-10, **(C)** IL-10 vs IL-5, **(D)** IL-17A vs IL-12p70, **(E)** TNF-β vs IL-5, **(F)** TNF-β vs vs IL-12p70, **(G)** TNF-β vs IL-10, **(H)** IL-8 vs IL-1β, **(I)** TNF-α vs IL-5, **(J)** TNF-α vs IL-4, **(K)** TNF-α vs IL-1β, **(L)** IL-5 vs IL-2, **(M)** IL-4 vs IL-2.

**TABLE 6** | Correlation matrix of 12 cytokines in normalised saliva.

| | IL-1β | IL-2 | IL-4 | IL-5 | IL-6 | IL-8 | IL-10 | IL-12p70 | IL-17A | IFN-γ | TNF-α |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IL-1β | | | | | | | | | | | |
| IL-2 | 0.54135 | | | | | | | | | | |
| IL-4 | 0.43400 | **0.8522** | | | | | | | | | |
| IL-5 | 0.61654 | **0.8015** | 0.74539 | | | | | | | | |
| IL-6 | 0.16382 | 0.37641 | 0.19781 | 0.18059 | | | | | | | |
| IL-8 | **0.77744** | 0.38346 | 0.35276 | 0.51278 | 0.01753 | | | | | | |
| IL-10 | 0.41654 | 0.63759 | 0.55961 | **0.87068** | 0.08382 | 0.25564 | | | | | |
| IL-12p70 | 0.44152 | 0.71606 | 0.64409 | **0.90034** | 0.07203 | 0.29109 | **0.94998** | | | | |
| IL-17A | 0.28808 | 0.6273 | 0.54251 | 0.68447 | 0.03087 | 0.26326 | 0.7296 | **0.80963** | | | |
| IFN-γ | 0.08486 | 0.39462 | 0.38967 | 0.46845 | 0.08428 | -0.0178 | 0.52107 | 0.58154 | 0.58069 | | |
| TNF-α | **0.77594** | 0.73083 | **0.79353** | **0.83308** | 0.0061 | 0.73684 | 0.65263 | 0.70177 | 0.59195 | 0.37425 | |
| TNF-β | 0.60098 | 0.77849 | 0.72573 | **0.95374** | 0.17303 | 0.56337 | **0.84919** | **0.90971** | 0.73288 | 0.42533 | 0.82287 |

*Correlation Matrix of BD normalised saliva concentrations. Spearman's correlation was calculated and r values are shown; those plotted in* ***Figure 6*** *are in bold.*

neutrophils whose proteases can cause detrimental damage to the mucosa (124). IL-17A has not previously been measured in saliva of BD or RAS patients, but has been investigated in chronic periodontitis in which it was significantly lower than HC (125). There is conflicting ELISA data in studies of IL-17 in BD. Chi, et al., failed to detect IL-17A in BD sera (117), while Shen, et al., reported serum IL-17 levels were significantly higher in BD serum compared to HCs and in BD patients with uveitis than those without (126). Saedeghi, et al., measured serum IL-17 levels in BD but found no significant differences between patients with and without uveitis (110). We also found IL-17 difficult to detect in serum (18.9% of samples above lower limits of detection over all groups). However, in saliva 89.2% of samples were above the lower limits of detection. This may reflect the sensitivity of the assay methods as Gholijani, et al., using a multiplex cytokine assay system, reported similar IL-17 levels to our study, however, they found significant differences between BD and healthy control sera, possibly reflecting the larger sample size (n=44 for BD and HC) and recent diagnosis (111).

Serum IL-6 was detected in only five samples across the cohort. In contrast, salivary IL-6 was significantly higher in BD-MA compared to HCs and BD-MA and BD-MQ although, normalised IL-6 salivary levels were significantly higher only in BD-MA, again, this was not reflected in IL-17A saliva levels. Low serum IL-6 in BD patients but high individual IL-17A measurements could be due to IL-17A production by a non-classical Th17 type cell such as γδ T cells or NK cells (120) which does not depend on IL-6 stimulation. However, these cytokine measurements require a larger samples size to confirm that IL-17A is independent of IL-6 in this system. IL-6 has previously been undetectable in BD and HC plasma using ELISA with detection limits of 18 pg/ml (114). The IL-6 detection limit for our FlowCytomix array was 1.2 pg/ml, and although evidence of the presence of IL-6 was measured in serum, our data coincides with that of other previous reports which state that serum IL-6 was absent or at particularly low levels in BD and HC. However, in another study IL-6 was significantly increased in BD patients compared to RAS and HC Using a similar multiplex bead assay to our own, many of their samples were below the level of detection. Yet, IL-6 was still described by Curnow, et al, as one of the most abundant inflammatory cytokines amongst a panel of 10 detected in serum (127). In our study, the majority of serum samples had no detectable IL-6; however, it was detected in most saliva samples, showing the highest levels in the BD group with active ulcers. Since a decreased production of IL-6 in the oral mucosa is thought to drive rapid oral mucosal healing (128) its presence in BD-MA could perpetuate the ulcer. The results of our data do not support the previous hypothesis that IL-6 has a prominent role to play in systemic inflammation in BD but it does suggest that oral mucosa inflammatory cells release IL-6 in response to oral ulceration. Early work on the influence of IL-6 in periodontitis (129) suggested it might be protective. It has also been suggested that mastication can up regulate Th17 cells in inflamed gingival mucosa (130).

## IL-4 and IL-5

IL-4 and IL-5 are Th2-type cytokines that stimulate B cells to proliferate and mature. IL-5 also promotes maturation of eosinophils. IL-4 is considered a critical element for driving the development of Th2 immune response (131). In our study, IL-4 levels were detectable in only a few serum samples (mainly BD-MQ) but in almost all saliva samples. However, while RAS levels were lower than BD and HCs there was no significant differences between patient groups and HCs, therefore, IL-4 may be a more prominent force driving the production of antibodies in the mouth other than systemically driving a Th2 response. This is possibly a strategy for maintaining levels of antibodies such as SIgA which has important heterotypic functions, such as viral agglutination, over and above antigenic specificity, in oral mucosal tissue that is constantly turning over and where fluids from salivary gland and gingival crevicular fluid are constantly replenished, compared to the closed circulatory system.

## IL-10

IL-10 is also a key immunoregulatory cytokine produced by almost all leukocytes, and Th17 type cells and has previously been found to promote would healing (132, 133). IL-10 has been found in high levels in active BD patient serum (99) but has not significantly increased in highly active BD compared to inactive or mild disease (134). IL-5 and IL-10 showed a strong correlation in normalised BD saliva samples. Both of these Th2-type cytokines can promote B cell proliferation and maturation

leading towards a Th2 response. While IL-10 can help to regulate the Th1/Th2 balance it also inhibits T-cells from making IFN-γ which would explain the low levels in our cohort (135).

## Medication

The effect of medication on salivary cytokines was difficult to unravel with few significant differences between the treatments and the small sample size, however, it was noted that the combination of COLC+AZA showed lower median levels of both a pro-inflammatory cytokine (IL-12p70) and an anti-inflammatory cytokine (IL-10) to a significant extent. Normalised IL-1β salivary levels were also lower with the combination treatment however, failed to reach significance. It is rare to find an individual with BD or who is being investigated for BD that is treatment naïve. Therefore, it is difficult to assess a baseline cytokine profile not influenced by immunoregulatory medications. For this pilot study, the findings are a first look at the influence of pro-inflammatory cytokines in saliva in this cohort.

## Saliva as a Biomarker for Diagnosis and Disease Progression

Other systemic diseases in which oral lesions arise such as systemic lupus erythematosus (SLE) and oral cancer have also had their saliva cytokines investigated. In Marques, et al's study, they found an increase in IFN-γ, IL-10, IL-17, IL-1β, IL-6, and IL-4 in SLE patients without periodontal disease compared to HC (136). Also in a meta-analysis, IL-8, IL-1β, TNF-α, IL-6, and IL-1α were found to be increased in oral cancer compared to HC and leukoplakia and were suggested as cancer biomarkers (137) In our study, the significant differences between HC and BD-MQ is an important finding to be able to differentiate the levels of cytokines present in "normal" saliva content and orally asymptomatic BD patients saliva. Increased detection of IL-8, IL-1β, and TNF-α may not be specific to BD for diagnostics, but can still be useful to monitor the level of oral inflammation in order to deter further ulcerations, and potentially, accompanying systemic manifestations.

Homeostatic regulation in the oral cavity and systemically involves a pattern of cytokine expression which may appear to be nonspecific. However, recent findings by ter Horst et al. and Li et al. suggest a complex pattern which is more individual than expected (138, 139). Ter Horst et al. (2021) also suggest a seasonality in some responses (140), and anecdotally this has been observed in BD patients with worsening symptoms during autumn. Age related changes have also been cited by these studies and IL-6 associated quantitative trait loci were identified. Key cytokines in their studies were IL-18 and IL-22 which were not included in our panel. However, it is our hypothesis that that the balance of various cytokines in HC, RAS, BD-MA and BD-MQ might be quite different and provide a panel that might aid differential diagnosis of this difficult disease. Inclusion of IL-15, IL-22 and IL-18, or indeed other cytokines, in future multiplex studies might be more revealing in future expanded studies.

Salivary cytokine concentrations were normalised to total protein after we observed that the volume collected differed between patients over the same collection time and some patients found it more difficult to produce saliva than others. The difficulty that some patients may have in producing saliva may be related to their medication regime and may not be as a direct effect of BD. Indeed, we found that the total protein concentration varied between patients and that in BD this was significantly lower than in HC. Furthermore, in the mouth there is a constant turnover of saliva that is lost through swallowing, whereas in serum the protein concentration was assumed to be fairly constant with clinical reference values at between 60-80g/L (141).

It is worth bearing in mind that serum and plasma samples are derived from a closed system while saliva is the pool of materials directly secreted by the salivary glands but with the potential for contributions from serum exudates from the gingival margins or indeed secretions from cells within the mucosal layers. The oral tissues are also covered in a "Mucosal Pellicle" which is known to be an active environment (95, 142) where heterotypic associations between signalling molecules and receptors has the potential for enhancing signalling cascades.

It should also be noted that the surface epithelium of the oral mucosa is in a constant state of loss and regeneration and the stability/integrity of the mucosa is dependent on a strict homeostasis. With that in mind we are cognisant that the fluid in the oral cavity, "saliva", is a highly dynamic fluid that has the potential to reveal the ongoing pathology of this disease. The potential of saliva as a diagnostic tool for oral and systemic disease has been an exciting development in clinical medicine in recent years (72–75).

We are not able to say whether there are receptors in the mouth for all the cytokines we measured as well as what the half-life of these cytokines and chemokines is in saliva and how sensitive these cytokines are to the dynamic pH changes in saliva. Nor, indeed if they are in monomeric, dimeric or in heterotypic associations which may make their detection more difficult. However, as more studies of oral diseases and other conditions are carried out using saliva, we feel that our approach to investigating a panel of cytokines that might define or predict disease progression using a non-invasive sample is of significant value in support of patients with BD and other inflammatory diseases (143).

## Limitations of Study

BD is a rare disease and therefore presents challenges when designing an investigative study. This pilot study consisted of 20 BD patients, 7 RAS patients, and 10 HC. Of this small cohort, only 15% of the BD patients were treatment naïve at the time of sampling. Ideally, all samples would be from untreated patients, however we have tried to limit the cohort to drugs with similar actions and have excluded patients who were taking biologics. For future studies, newly diagnosed patients who have not started medication should also be compared with those on established anti-inflammatory medications. Also, a power calculation should be carried out to establish the minimum number of patients and controls for a more robust statistical analysis. A larger RAS cohort, with an equal number of individuals with and without ulcers, would also provide meaningful disease control data especially when analysing saliva samples. The following criteria should be taken into account for BD and RAS patients: ulcer size, duration, and number present at the time of sampling, years since diagnosis/

symptoms, HLA-B51 status and/or geographic origins. For all patients and HCs: smoker status, alcohol consumption, periodontal/caries status and oral hygiene habits.

### Summary

Oral ulceration is the most common characteristic of BD and is often predictive of the onset of systemically active disease. This pilot study used FlowCytomix™ multiplex assays to measure multiple cytokines in matched serum and saliva samples to explore their differential expression and potential as diagnostic or activity markers for BD.

The elevated saliva cytokines IL-8, IL-1β, and TNF-α in BD patients without oral ulcers could be included in a potential cytokine panel to assist in the differential diagnosis, predicting the reoccurrence of ulcers or systemic relapse, and monitoring treatments. There are clear benefits for using saliva over serum for the detection of cytokines and multiplex assays lend themselves to screening with limited sample volumes. Normalisation of saliva samples to total protein was found to be key in standardising this highly dynamic specimen.

Our study highlighted that IL-1β, IL-8, TNF-α, IL-6 and IL-10 saliva cytokines play a role perpetuating BD oral inflammation and should be incorporated in further investigations. IL-10 and IL-6 would also be of interest in dissecting the immune dysregulation in RAS. Although the IL-17A measurements were unexpectedly low, the IL-17/IL-23/IL-22/IL-6 axis might benefit from further elucidation in a larger cohort. In a disease such as BD where the spectrum of manifestations requires highly individualised and patient specific interventions, these methodologies are of considerable value to support frequent monitoring of the episodic disease and potentially predict and prevent relapse.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the City and East London (P/03/122). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

TN, MH, LB, and EH-P contributed to conception, design, data acquisition and analysis. LB, TN, and EH-P drafted and critically revised the manuscript. MH critically revised the manuscript. FF helped with the data analysis and interpretation as well as critically reviewed the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.724900/full#supplementary-material

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REVIEW
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# Innate Phagocyte Polarization in the Oral Cavity

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The oral cavity is a complex environment constantly exposed to antigens from food and the oral microbiota. Innate immune cells play an essential role in maintaining health and homeostasis in the oral environment. However, these cells also play a significant role in disease progression. This review will focus on two innate phagocytes in the oral cavity: macrophages and neutrophils, and examine their roles during homeostasis and disease development, with a focus on periodontal disease and cancer. Macrophages have a well-known ability to polarize and be activated towards a variety of phenotypes. Several studies have found that macrophages' polarization changes can play an essential role in maintaining health in the oral cavity and contribute to disease. Recent data also finds that neutrophils display phenotypic heterogeneity in the oral cavity. In both cases, we focus on what is known about how these cellular changes alter these immune cells' interactions with the oral microbiota, including how such changes can lead to worsening, rather than improving, disease states.

**Keywords: macrophage, neutrophil, inflammation, cellular polarization, periodontal disease, oral cancer**

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## INTRODUCTION

The oral cavity is the main gateway into the human body, leading to the respiratory and gastrointestinal tracts. It has a wide variety of microbial niches, and has the second most abundant microbiota after the gastrointestinal tract, consisting of ~800 bacterial species categorized into six major phyla (1). In this context, similarly to other mucosal barriers, the local oral immune system needs to find a balance of coexisting with the commensal microbiota while responding appropriately to pathogens (2). The crosstalk between microbiota and the innate immune system is essential to maintaining this host-microbe homeostasis, with the commensal microbiota itself playing a crucial role in regulating immune homeostasis (2–4). Indeed, the oral cavity is a unique mucosal environment where immune cells must be able to recognize and eliminate pathogens while maintaining tolerance to food antigens and the resident microbiota. Oral immunity is composed of a diverse and dynamic network of interactions with both innate and adaptive immunity components contributing to the maintenance, integrity, and host protection of oral tissues. However, as innate immunity is the first line of defense, it plays a pivotal role in both protecting the host and maintaining homeostasis (5, 6). While the saliva and gingival crevicular fluid (GCF) contain host immune molecules that can respond rapidly to protect the periodontium and the oral hard tissues against pathogens such as antimicrobial peptides, complement, and secretory

IgA (7), these defense mechanisms only provide short-term protection and have a limited specificity (8). The oral mucosa resident and transmigrating immunologically active innate immune cells, including macrophages and neutrophils, also play important roles in maintaining effective immune surveillance.

The importance of innate professional phagocytes in maintaining a healthy and mature immune system is revealed upon a change in 'ideal' functional inflammatory immune cell infiltrate: such changes lead to degradation in the health of the periodontal tissues (4). For example, a lack of neutrophil infiltration into the oral cavity (neutropenia) leads to an increase in periodontal disease (9) while an overabundance and dysregulation of neutrophils during periodontal disease causes host tissue damage (10). Similarly, a reduction in macrophage numbers during aging contributes to an increase in periodontal disease (11), while macrophages themselves also contribute to the alveolar bone resorption seen during *P. gingivalis* induced periodontal disease (12). Recent data suggests part of this finely tuned balance of phagocytes is likely due to phenotypic variance within these cell types. Macrophages are responsible for not only host defense, but also have important tissue repair and homeostatic roles (13) and possess a spectrum of phenotypes with different responses during host and microbial interactions (14); (15). Neutrophil phenotypes during infection, inflammation and cancer are also being recognized (16–19), including in response to periodontal disease (20–23). This review will examine the current knowledge of the role polarization, or phenotypic changes, in macrophages and neutrophils is thought to play in the oral environment, especially during the development of periodontal disease.

## MACROPHAGE FUNCTIONS IN THE ORAL ENVIRONMENT

Macrophages are located in the lamina propria below the epithelium and are among the first innate cells to interact with microorganisms and microbial products, and so are an important cell type under both homeostatic and disease conditions in the oral cavity. Under physiological conditions macrophages are important for cell turnover and maintenance of the extracellular milieu (24), while also being required to recognize, internalize and kill microbes in order to clear infections (25). The recognition of microbes by macrophages also results in production of proinflammatory cytokines, which contribute to inflammation initiation (26). Moreover, macrophages can act as antigen-presenting cells (APCs), collaborating with the early development of acquired immunity (27).

Under homeostatic conditions bone marrow derived monocytes enter tissue and differentiate into tissue specialized macrophages, including Langerhans cells (LCs), in the extracellular matrix of the mucosa (5, 28). Resident oral mucosal macrophages exhibit an array of diverse functions depending on different factors they encounter in their environment, including tissue architecture and microbiota (4, 29). A small portion of oral Langerhans cells that originate from monocytes are a more specialized subset of tissue resident macrophages (30, 31). Even within the same organ, macrophages can occupy different niches and have different specialized functions (28). Interestingly, recent research suggests that during tissue injury caused by myocardial infarction, stroke, and sepsis local proliferation of macrophages dominate over macrophage recruitment (32).

Oral macrophages are important for bridging the innate and adaptive immune response. Macrophages and oral Langerhans cells express high levels of MHCII and CD80/CD86, ingest particulate antigen and can present it to T cells (29, 31, 33, 34). Interestingly, oral LCs, unlike other tissue macrophages and similarly to dendritic cells, can migrate to lymph nodes to present antigen to T cells (30). Oral macrophages are also able to prime systemic immunity, as has been shown through systemic protection after sublingual vaccine administration and systemic antibody response to periodontal pathogens (4). In addition, when macrophages are depleted and mice infected intraorally with the periodontal pathogen *Porphomonas gingivalis*, specific antibody and cytokine responses are decreased, indicating the importance of macrophages in the adaptive immune response to oral bacteria (12).

## MACROPHAGE POLARIZATION IN THE ORAL ENVIRONMENT

Macrophages can polarize to a variety of phenotypes ranging from alternatively polarized M2 macrophages to classically polarized M1 macrophages*in vitro*, which is well described in the literature (6, 28, 35, 36). In general, the M1 phenotype promotes a Th1 response and vigorous microbicidal and tumoricidal activity. In contrast, an M2 phenotype helps parasite clearance, dampens inflammation, promotes tissue remodeling, tumor progression, and possesses immune-regulatory functions (37). In reality, *in vivo* macrophages do not exist on dipoles as a population, but in a continuation of activation. The macrophage phenotype is plastic and in disease and in health there can be wide variety of multiple phenotypes present spanning from strict M1 to strict M2 and anywhere in between (38–40). Throughout this review *in vivo* macrophages will be referred to as M1-like or M2-like for this reason.

In the oral cavity, as with elsewhere in the body, predominate M1-like activation is generally associated with inflammatory diseases and predominate M2-like activation is associated with cancer (**Figure 1**) (6, 36). Indeed, dysregulation of the M1/M2 balance can lead to the progression of the inflammatory response and malignant oral diseases such as oral lichen planus and oral squamous cell carcinoma (SCC). M1-like macrophages can aid the progression of oral lichen planus and potentially induce malignant transformation. Conversely, M2-like macrophages [often termed tumor associated macrophages (TAMs)] aid SCC progression and favors an immunosuppressive tumor microenvironment (41). This is a general and well-known phenomenon of macrophages in many tumors, not just those in the oral cavity, with recent thorough reviews and so won't be

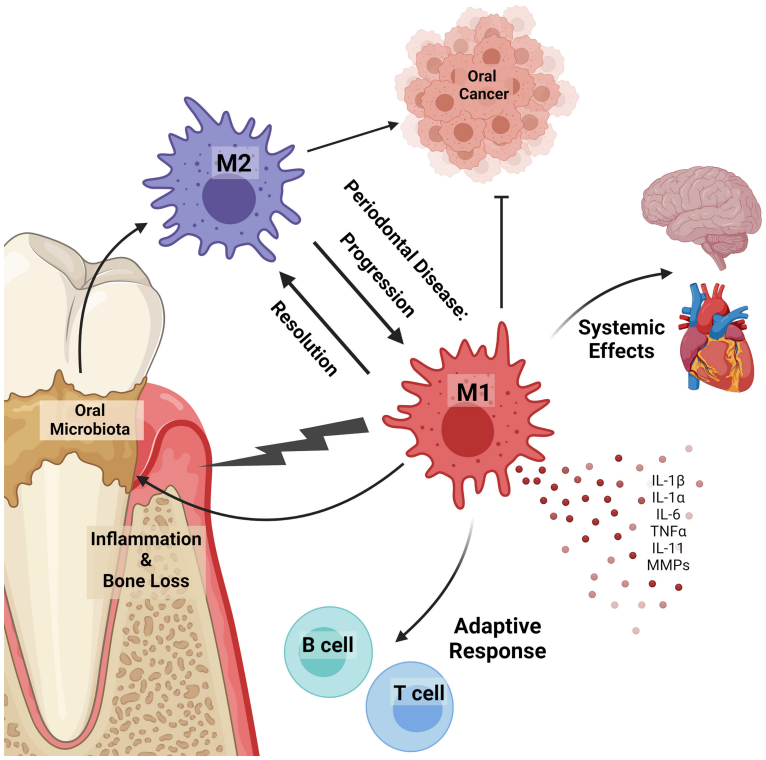


**FIGURE 1** | Macrophages in the oral cavity. Macrophages can polarize in response to oral microbiota and in disease. Inflammatory (M1) macrophages promote inflammation, alveolar bone loss, disease progression, microbiota dysbiosis, and prevent tumor development. Alternatively activated (M2) macrophages contribute to cancer progression and resolution of inflammatory diseases. Macrophages in the oral cavity are linked to systemic diseases, including those in the heart and brain. Inflammatory macrophages release pro-inflammatory cytokines (IL-1β, IL-1α, IL-6, TNFα, IL-11) and matrix metalloproteases (MMPs) that contribute to inflammation and alveolar bone loss. Under homeostatic and disease conditions oral macrophages work to bridge the innate and adaptive immune response by expressing antigens to B and T lymphocytes.

discussed further here (40, 42). Macrophage polarization can be driven by different bacterial species, microbial components, and host immune mediators (38, 43). For example, in murine macrophages a differential M1 or M2 profile occurs in response to representative Gram-negative or Gram-positive gastrointestinal bacteria, including probiotic strains, pathogens, commensals, and strains of food origin (44). In the oral environment, endotoxin and other bacterial products present in sterilized saliva polarize macrophages to an M1-like phenotype, with increased production of pro-inflammatory cytokines (45). Specific oral strains can also elicit unique responses, with data showing oral commensals generally elicit an M2-like phenotype while oral pathogens elicit a more M1-like phenotype (46). Furthermore, a switch from an predominantly M2-like phenotype to a predominantly M1-like phenotype is a potential mechanism for the advancement of periodontal disease (42). Studies using germ-free and specific pathogen free mice have also shed light on the ability of the oral microbiota to differentially modify phagocytes and their responses *in vivo*. For example, there is a significant decrease in IL-1β, an inflammatory cytokine mainly produced by macrophages, in germ free (GF) mice compare to specific pathogen free (SPF) mice (47), and oral Langerhans cells are significantly reduced in GF mice compared to SPF mice, but after microbial recolonization their numbers were restored (34).

## MACROPHAGES IN PERIODONTAL DISEASE

Periodontal disease is a progressive inflammatory disease that results from dysbiosis in the microbiota and an overreactive immune response (48, 49). It is a complex disease, resulting from a myriad of different factors including genetics, environment, and microbes, with the microbial load from health to disease increasing from $10^2$ bacteria in health to $10^8$ bacteria in periodontitis (47). The importance of macrophages in the development and progression of periodontal disease has been shown through depletion of macrophages in a mouse model of periodontal disease, resulting in reduced *P. gingivalis* induced bone resorption (12). Although depletion of macrophages prevents bone resorption, it can also impairs bone regeneration (50). Data suggests aberrant expression of macrophage genes may affect their activation state and expression of signaling molecules, thereby contributing to disease progression (51).

Indeed, many factors released by macrophages can be involved in periodontitis-associated alveolar bone loss. For example, inflammatory cytokines produced in high levels by macrophages including IL-1β, IL-1α, TNFα and IL-6 can activate osteoclasts making it likely that they play an important role in disease-induced bone resorption (26), and IL-1β is known to increase in human groups in association with an increased ratio of inflammatory macrophages over alternatively activated macrophages (52). In addition to cytokines, macrophages release a number of matrix metalloproteinases that are involved in degradation of the extracellular matrix and are important proteases involved in the progression of periodontal disease (53). Macrophage cytokines TNFα, IL-1, and IL-6 stimulate MMPs, all of which are expressed at higher levels in diseased periodontal tissue (54–56), with again some MMPs being linked to an increased M1/M2 ratio during disease (52). Together this shows the important role that macrophages and the myriad of factors they express can play in the destructive properties of periodontal disease.

As described above, macrophages can polarize to a broad range of phenotypes. On one end of this spectrum lays M1 macrophages, which generally have more inflammatory and microbicidal characteristics. Macrophages can polarize to an M1 phenotype through stimulation with IFNγ and TLR ligand interaction, as well as in response to periodontopathic bacteria. Macrophages stimulated with *P. gingivalis* or *P. gingivalis* LPS generally polarize to an M1-like inflammatory phenotype as shown by increased levels of pro-inflammatory cytokines and M1 specific surface markers (46, 57, 58). Mice infected with *P. gingivalis* also show increased levels of M1-like macrophages compared to M2-like macrophages (12). *Aggregetibacter actinomycetecometans*, another pathogen highly associated with periodontal disease, has also been shown to polarize macrophages to an M1-like phenotype (46, 59).

Periodontal disease is an inflammatory disease, so logically inflammatory macrophages (i.e., M1 macrophages) would infiltrate the periodontal tissue during disease: in recent years many studies have shown that this is indeed the case. A human experimental gingivitis study from Topoll and co-workers in 1989 was one of the first to show a decrease in anti-inflammatory macrophages and an increase in inflammatory macrophages (60). Now there have been multiple human studies showing increases of inflammatory M1-like macrophages in periodontal disease in comparison to healthy controls (42, 52, 61). These M1-like macrophages contribute to the inflammatory environment, promoting dysbiosis of the microbial community and periodontal disease progression (10). Macrophage interactions with normally commensal oral bacteria can also change, as seen with the increased, rather than decreased, survival of *Streptococcus gordonii* within IFNγ/LPS stimulated macrophages (62). Animal studies have also found that if the inflammatory response, especially by macrophages, is treated then progression of periodontal disease can be inhibited. One promising treatment is with the anti-inflammatory agent Resolvin-E1, which can resolve inflammation and regenerate bone and soft tissue to a healthy state (63). Such studies with anti-inflammatories have further illuminated periodontal disease as an inflammatory disease. Importantly, if alternatively activated, M2, macrophages are stimulated *in vivo* or injected into animal models of periodontal disease they promote healing and dampen osteoclast activity, thereby reducing bone resorption (50, 64, 65). On the other hand, *P. gingivalis* may promote the activation of macrophages into M2-like TAMs when combined with an OSCC microenvironment that can induce and promote OSCC growth (66).

## SYSTEMIC EFFECTS OF ORAL MACROPHAGES

There has been a recent increase in research focusing on the systemic implications of periodontal disease (48, 67–70). It is known that oral microbes can be found in extraoral locations after gaining access to the circulation, and periodontal disease-associated microbes have been found in multiple extra-oral sites including atherosclerotic plaques (71, 72) and the brain, showing they also can cross the blood-brain barrier (73, 74). Moreover, *P. gingivalis* appears capable of invading and converting myeloid-derived dendritic cells (mDCs) to an atherogenic phenotype in humans with chronic periodontitis (75). Along this line, the uptake of low-density lipoprotein (LDL) by transmigrated macrophages is enhanced in the presence of bacteria leading to accelerated foam cell formation and atherogenesis (48).

In addition to extra-oral bacteria affecting macrophages and systemic disease, the elevated systemic inflammation associated with periodontitis may have multiple systemic complications. For example, periodontal bacteria increase systemic IL-6 levels, driving the expansion of osteoclast precursors (OCPs) which can traffic to sites of bone resorption and differentiate into mature osteoclasts (76), suggesting that changes in the bone marrow may link periodontitis to other bone loss disorders, such as rheumatoid arthritis (77). Additionally, as individuals age there is increased inflammation in a nominally resting state and linked to this there is an increase in primarily M1 macrophage activation with age in nonhuman primates (78). Aging can enrich the gingival environment in anaerobic species leading to a dysregulated and persistent immunoinflammatory response (79). In this context, increases in prevalence and severity of periodontal disease have long been associated with aging (79). Recent evidence finds this long-associated age-related increase in periodontal disease is dependent, at least in part, on these age-related changes in macrophage activation towards an M1-like phenotype (11).

## NEUTROPHIL PHYSIOLOGY

Neutrophils are the most abundant circulating leukocyte, which are among the first cells to respond to bacterial infections and pro-inflammatory signals. Neutrophils are produced in the bone marrow with 1 to $2\times10^{11}$ neutrophils normally generated per day

in an adult human (80). Mature neutrophils can be found in the bone marrow as the bone marrow reserve, circulating through the blood, or in tissues as resident neutrophils (81).

After production, mature neutrophils will remain in the bone marrow for about 5 days, forming the bone marrow reserve from which neutrophils can be rapidly mobilized in case of infection (82, 83). Once neutrophils cross the bone marrow sinusoidal endothelium (about $10^9$ neutrophils/kg body weight exit the bone marrow daily in humans) they enter the sinusoids and eventually migrate out into the general circulation (84, 85). Some of these neutrophils migrate into tissues, including through mucosal membranes and areas requiring constant immune surveillance, such as the oral cavity.

In human blood, neutrophils are generally believed to have a short half-life of about 8 hours, according to experiments in which neutrophils were labelled *ex vivo* and then evaluated *in vivo* (86). However, a study that labeled cells *in vivo via* deuterium-labelled water reported a surprising lifespan for circulatory neutrophils in humans of up to 5.4 days (87). Yet this paper's methods received some criticism as orally administrated deuterium-labelled water would likely label bone marrow neutrophils as well as circulating neutrophils, therefore skewing their supposed longevity in blood (88). Another group proposes that the slow kinetics of labeled cells reported in Pillay 2010 may be due to the slow production of neutrophils in the bone marrow, rather than a long half-life in the blood (18, 89, 90).

These dynamic cells perform a wide range of protective functions, including chemotaxis toward stimuli, extravasation from vasculature and antimicrobial actions through phagocytosis, granule release, reactive oxygen species (ROS) production, and NETosis (91, 92). While neutrophils have traditionally been thought of as stable differentiated cells, evidence now demonstrates they are dynamic cells able to change their characteristics and behavior throughout their lifespan or in differing environments. Their potential responses can vary widely and change according to local signals released during acute or chronic inflammatory conditions, injury, infection, cancer, and autoimmunity. This plasticity has led to increasing interest in understanding their functional phenotypic heterogeneity, similar to other immune cell lineages (93).

## NEUTROPHILS IN THE ORAL ENVIRONMENT

In the oral cavity, neutrophils have been identified in periodontal tissues, gingival crevicular fluid, and within dental biofilms (94–96). Neutrophils transmigrate through oral mucosa and comprise the majority of innate immune cells recruited to the gingival crevice, composing greater than 90% of the crevicular cells (97–101). Phenotypic differences between oral neutrophils in health and with location-specific differences are becoming better understood. Compared with circulating neutrophils, oral neutrophils present site-specific gene expression profiles in healthy individuals (102). Human studies have found that generally, oral tissue-resident neutrophils are in a later stage of their life cycle when compared to circulatory neutrophils and present a higher state of activation when compared to circulatory neutrophils, showing higher expression of activation markers CD11b, CD63 and CD66b, as well as higher constitutive ROS levels (103). In periodontal health a spectrum of neutrophil populations have been reported in humans, as characterized by two distinct subtypes of oral neutrophils; a 'parainflammatory 1' population which is similar to naïve blood neutrophils and a 'parainflammatory 2' population which is the more activated phenotype, possibly being in a primed state and may be crucial for response with the symbiotic biofilm of health (22). Another subset of oral neutrophils has also been identified that present with a significant increase in T cell receptor (TCR) expression compared with circulating neutrophils, and these cells show markedly increased recruitment to sites of inflammation. While the exact role of TCR expression in oral neutrophils is unknown, this supports a role for oral neutrophils in crosstalk between the innate and adaptive immune system in the oral cavity (**Figure 2**) (102).

## NEUTROPHILS IN ORAL CANCER

Myeloid cells can promote tumor progression directly *via* immune suppression or indirectly *via* production of angiogenic factors, matrix-degrading enzymes, or growth factors. The most well characterized of these cells are TAMs that have properties of alternatively activated macrophages, or M2-like macrophages as described above (104). In recent years there has been increasing evidence showing phenotypic and functional plasticity in neutrophils, particularly in oral cancers (105). Like TAMs, these tumor-associated neutrophils (TANs) have differential states of activation/differentiation (104) TANs can polarize to anti-tumorigenic (N1) or pro-tumorigenic (N2) phenotypes. It is important to note that *in vivo* neutrophils, like macrophages, exist on a spectrum of activation and so in reality 'N1' and 'N2' are better understood as ends of a spectrum rather than discrete categories. *In vivo* animal studies show this change is largely directed by TGF–β, which skews differentiation toward the N2 phenotype. *In vitro* studies on human blood and tumor samples have found that N1 TANs express higher levels of immune activating cytokines and chemokines, lower levels of arginase, and have a heightened ability to kill tumor cells *in vitro*. N1 TANs express higher level of CCL3, ICAM-1, and TRAIL. N2 TANs support tumor growth by producing angiogenic factors and matrix-degrading enzymes and suppress the antitumor immune response. They are characterized by increased expression of gene markers, such as MMP9, VEGF-A, and BV8, as well as the decreased expression of CCL3, ICAM-1, and TRAIL (106, 107). Knowledge of TAN and circulating neutrophils in the context of head and neck cancer (HNC) of the oral cavity is somewhat limited, and even less is known about the oral neutrophils which populate the environment closely associated with evolving HNC. These oral neutrophils represent a unique population with functional and phenotypic proprieties distinct from other compartments such as the tumor or mucosal tissue (108). Localization of the oral neutrophils and neutrophil-derived products along with the tumor-derived microenvironment rich is

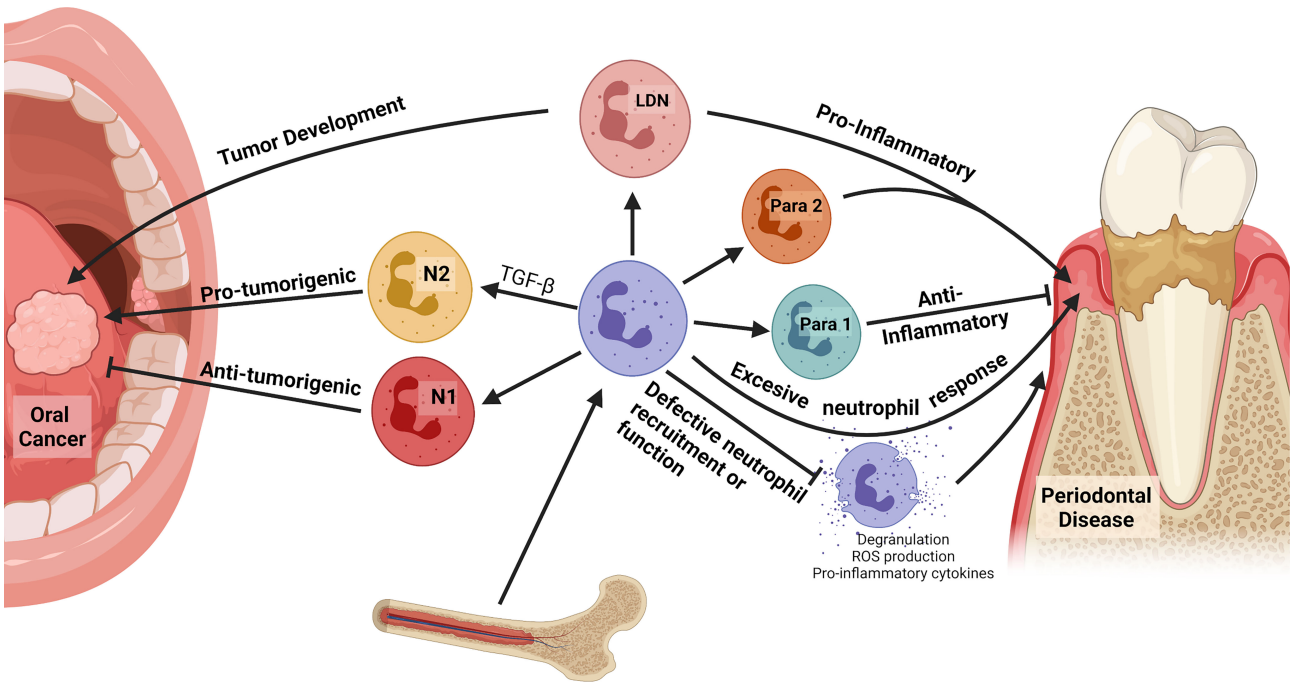


**FIGURE 2** | Neutrophils in the oral cavity. Neutrophils can polarize in response to oral microbiota and in disease, advancing or inhibiting disease progression. In oral cancers, N1 tumor-associated neutrophils (TANs) demonstrate anti-tumorigenic behaviors while N2 TANs are pro-tumorigenic, producing angiogenic factors and suppressing the antitumor immune response. Differentiation to the N1 or N2 phenotype is largely driven by TGF–β, which skews differentiation toward the N2 phenotype. Low-density neutrophils (LDNs) correlate with disease progression. They preferentially propagate in cancer and have a T-cell suppressive function, while proinflammatory LDNs are found in cases of autoimmunity. In addition, there is a subset of oral neutrophils that present with a significant increase in T cell receptor (TCR) expression and are recruited at high rates to sites of inflammation; however, their exact function is unknown. Increased neutrophil recruitment can contribute to the inflammation and alveolar bone loss characteristic of periodontal disease *via* production of ROS, pro-inflammatory cytokines, and degranulation. Furthermore, individuals with defective neutrophil recruitment or function due to genetic abnormalities are more susceptible to severe periodontal disease.

growth factors and cytokines may influence tumor development and differential neutrophil plasticity. For example, oral neutrophils from patients with untreated oral cancer demonstrate distinct functional properties with lower chemotactic ability, superoxide production and reduced killing of microbes (109). Understanding phenotypic differences and properties of oral neutrophils in oral cancer provides a relevant measure of local response in the tumor environment with high prognostic value.

Neutrophil granule changes have also been demonstrated to reflect differential phenotypes as during some inflammatory states or other disease conditions, there exists a subset population of neutrophils which co-sediment with peripheral blood mononuclear cells (PBMCs) due to their unique granularity compared to normal mature neutrophils known as low-density neutrophils (LDNs) (110). LDN numbers correlate with pathological conditions such as rheumatoid arthritis, lupus and cancers including potentially in the early phase of oral squamous cell carcinoma development (111–113). Their overall function is dependent on inflammatory stimuli. For example, a proinflammatory LDN phenotype has been described *in vivo* animal studies for LDNs in autoimmunity and infection while in cancer LDNs primarily have a T cell suppressive function and are often referred to as myeloid-derived suppressor cells (111, 114).

## NEUTROPHILS IN PERIODONTAL DISEASE AND INFLAMMATION

The association between periodontal disease and neutrophil presence is well established. The human oral cavity has a constant bacterial presence that is kept under control by equally constant immune surveillance. Neutrophils comprise the majority of innate immune cells recruited to the gingival crevice and tissue to maintain physiological health, while documented increases in the number of oral neutrophils during periodontal disease which correlates with clinical severity in response to the dysbiotic biofilm and inflammatory changes (98, 101, 102, 115). A lack of neutrophil infiltration into the oral cavity or defects of neutrophil function lead to an increase in periodontal disease (9). Congenital defects in neutrophil development or egress from the bone marrow resulting in significant neutropenia are linked to severe periodontal disease (116). Likewise, defective extravasation of neutrophils into tissue and impaired immune response are also linked to increased periodontal disease. For example, Leukocyte adhesion deficiency are a group of congenital disorders in which neutrophils have defective expression or function of adhesion molecules involved in attachment and migration through the vascular endothelium, resulting in neutrophilia with few neutrophils in peripheral

tissues leading to subsequent recurrent infections including severe periodontal disease (117). On the other hand, increased neutrophil recruitment in attempt to control the dysbiotic biofilm characteristic of periodontal disease or dysregulation of appropriate trafficking and resolution of the neutrophil response contributes to much of the tissue damage in periodontal disease (10). Many of the oral microbes abundant in the dysbiotic community of periodontal disease are able to disarm or impair local neutrophil responses, also rendering these cells ineffective in the gingival tissues (118, 119).

A variety of studies have suggested that neutrophils during periodontal disease are heterogenous populations which likely respond to local microenvironment and microbe cues. Classical studies have revealed that circulating neutrophils from patients with periodontal disease have impaired chemotactic ability, responsiveness and directionality along with changes in surface expression of CD11b or CD16 (120–122), which suggest potential of functional neutrophil subsets. Recent detailed comparative analysis of oral neutrophil surface markers has further revealed distinct upregulation of specific markers during periodontitis characterized by a pro-inflammatory signature along with a functionally activated phenotype with elevated degranulation, phagocytosis, ROS production and NET formation (22, 103, 123). Studies on blood and oral rinses from healthy and chronic periodontitis patients have revealed differential expression of neutrophil surface markers in different biological compartments (124) and during transmigration (125), however ongoing studies are required to identify definitively if observed molecular changes and phenotypic features in oral neutrophils during disease represent active subset switching in response to stimuli or a true differentiated population.

Despite appropriate clinical therapy, a subset of periodontitis patients do not respond effectively and present with continuing disease progression and clinical attachment loss that does not correlate with plaque levels, microbiology assessments, and treatment compliance. Such patients are diagnosed with refractory periodontitis (RP) (126). A unique hyperactive oral neutrophil phenotype characterized by increased potential for ROS production has been identified in these RP patients. These cells were found to produce ROS at a level approaching the maximal capability of the cells (127). While appropriate ROS production is crucial for effective bacterial killing, excessive ROS can contribute to periodontal attachment loss by damaging the extracellular connective tissue (128).

While characteristic neutrophil surface marker signatures have been demonstrated, a distinct neutrophil-specific marker has been reported to be associated with periodontal disease. CD177, also called neutrophil antigen B1 (NB1) or human neutrophil antigen 2a (HNA-2a), is a glycosylphosphatidylinositol (GPI)-linked glycoprotein expressed on the plasma membrane and in granule membranes of neutrophils (129). A high proportion of CD177-expressing neutrophils have also been found in the gingival crevicular fluid (GCF) of periodontitis patients. The proportion of CD177-expressing neutrophils in circulation varies between individuals with a relatively stable bimodal, or occasionally trimodal, expression pattern (130, 131). In humans, the proportion of CD177-expressing neutrophils ranges from 0% and 100% of circulating neutrophils. CD177 can interact with PECAM-1 (expressed on endothelial cells, platelets, monocytes, and granulocytes), which is a key player in neutrophil migration from bloodstream to tissue (132). The proportions of $CD177^+$ neutrophils is higher in GCF from periodontitis patients, as compared to blood from the same donor and this accumulation of $CD177^+$ neutrophils in inflammatory exudate was not seen in two different models of aseptic inflammation, suggesting that this is a periodontitis specific phenotype. Furthermore, the $CD177^+$ neutrophil subtype does not accumulate in the GCF of healthy subjects (133).

As periodontal disease is considered an inflammatory condition with systemic associations, neutrophil phenotypes related to inflammation are likely relevant to health in the oral cavity as well as systemically at distant sites. Recent work has demonstrated that oral inflammation occurring during human experimental gingivitis or ligature induced models of murine periodontitis has systemic effects to produce an exacerbated immune response at a secondary site during a secondary insult (134). This supports a larger role of oral inflammation and particularly neutrophil-derived changes broadly throughout the body.

## SUMMARY

There is an abundance of work highlighting the importance of phagocytic cells, including macrophages and neutrophils, under conditions of health and disease in the oral environment. Both of these cell types play specialized roles in part by polarizing to a variety of phenotypes to alter their phenotypic responses in health and disease. As described in this review, a key role for such polarization in the context of oral diseases, including periodontal disease and oral cancer is well documented. However, while there is increased understanding of the roles for innate immune cell polarization in the oral environment in recent years, their remains a lack of insight into molecular mechanisms fueling the responses of these phagocytes. Future characterization of the plasticity of innate immune cells will provide important information to decipher their detailed roles in driving pathogenic conditions in the oral cavity.

## AUTHOR CONTRIBUTIONS

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